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**Evaluation of Glutamatergic Treatment in
Reducing Nicotine Seeking Behavior in Rats**

Thesis submitted for the degree of **Doctor of Philosophy**

The Open University

Discipline of Life and Biomolecular Science

Affiliated Research Centre:

Mario Negri Institute for Pharmacological Research

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List of abbreviations

5-HT = Serotonin

ABS = Abstinence

ACh = Acetylcholine

AChE = Acetylcholinesterase

aCSF = Artificial CSF

AGS3 = Activator of G protein 3

AMPA = α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

Amy = Amygdala

ANOVA = Analysis of variance

APA = American psychiatric association

ATP = Adenosine triphosphate

BBB = Blood brain barrier

CaMKII = Ca^{2+} /calmodulin-dependent protein kinase II

CET = Cue exposure therapy

CI-AMPA = Ca^{2+} impermeable AMPAR

C_{max} = Peak plasma concentration

CNS = Central nervous system

CP-AMPA = Ca^{2+} permeable AMPAR

CPG = (*S*)-4-carboxyphenylglycine

CPP = Conditioned place preference

CS = Conditioned stimulus

CSF = Cerebral spinal fluid

DA = Dopamine

DCS = D-cycloserine

DMS = Diagnostic and Statistical Manual of Mental Disorders

DLS = Dorsolateral striatum

EPSCs = Excitatory postsynaptic currents

EPSP = Excitatory postsynaptic potential

ERK = Ras-extracellular signal regulated kinase

FDA = Food and Drug Administration

FR = Fix ratio

GABA = γ -Aminobutyric acid

GDS = Global Drug Survey

GLAST = Glial glutamate and aspartate transporter

GLT-1 = Glial glutamate transporter 1

GLU = Glutamate

GSH = Glutathione

GSSG = Glutathione disulfide

Hipp = Hippocampus

HIV = Immune deficiency virus

HPLC = High-performance liquid chromatography

IL = Infralimbic cortex

i.p. = Intra peritoneal

i.v. = Intra venous

Ig = Immunoglobulins

iGluRs = Ionotropic glutamate receptors

KARs = Kainate receptors

LP EXT = Lever press extinction

LTD = Long-term depression

LTP = Long-term potentiation

mGluRs = Metabotropic glutamate receptors

mPFC = Medial prefrontal cortex

MSN = Medium spiny neurons
N-AC = N-acetylcysteine
Nacc = Nucleus accumbens
nAChRs = Nicotinic acetylcholine receptors
NMDARs = N-Methyl-D-aspartate receptors
NPS = New psychoactive substances
NRT = Nicotine replace therapy
OPA = o-phthaldialdehyde
PFC = Prefrontal cortex
PKC = Phosphokinase C
PLC = Prelimbic Cortex
s.c. = Sub cutaneous
S^d = Discriminative stimulus
SEM = Standard error of the mean
SUDs = Substance use disorders
System Xc- = Cystine-glutamate exchanger
t_{1/2} = Half-life
Tmax = Time to Cmax
TNF = Tumor necrosis factor
US = Unconditioned stimulus
vGluT = Vesicular glutamate transporter
VP = Ventral pallidum
VTA = Ventral tegmental area
WHO = World Health Organization

Abstract

Although pharmacotherapy and psychosocial support can help smokers to quit, the high relapse rates indicate a high unmet need for more effective treatment. Recent studies have highlighted changes in glutamate (GLU) homeostasis in the circuitry from the prefrontal cortex (PFC) to the nucleus accumbens (Nacc) as vital in the reinstatement of drug-seeking behavior. Restoring basal concentrations of extracellular GLU, thereby increasing tonic activation of the presynaptic group II metabotropic GLU receptors (mGluR2/3) by a single injection of N-acetylcysteine (N-AC) prevented cues-induced cocaine- and heroin-seeking behavior in rats.

Although nicotine-associated cues reinstate drug-seeking by acting on GLU transmission in the Nacc it is still not clear whether N-AC can inhibit cue-induced reinstatement in abstinent rats after nicotine self-administration. It is also not clear whether chronic N-AC treatment could elicit an enduring reduction in cue-induced reinstatement.

Rats were trained to associate discriminative stimuli (S^D s) with intravenous nicotine vs. saline in two-lever operant cages. Reinforced response was followed by cue (CSs). Re-exposure to nicotine S^D/CS^+ , but not saline S^D/CS^- , revived responding at the previously reinforced lever. A single dose of N-AC (100 mg/kg i.p.) increased extracellular GLU in the Nacc only in rats with an history of nicotine self-administration. Moreover, the same dose of N-AC induced a short-term reduction of cue-induced nicotine-seeking behavior that was completely prevented by pre-treatment with the selective mGluR2/3 antagonist LY341495 (1 mg/kg i.p.). Chronic treatment with N-AC (100 mg/kg) during 14 days of cues exposure therapy, induced long-lasting anti-relapse activity that was still present 50 days after the end of the treatment. To investigate the molecular mechanism in the Nacc underlying chronic N-AC effect, rats were killed at different time points. Western blot analysis revealed that 7 days after treatment, chronic N-AC restored the expression of proteins crucial for GLU homeostasis, while 51 days after treatment the expression of mGluR2 was increased only in chronic N-AC treated rats.

Overall, the results of the Thesis suggest a potential therapeutic use of N-AC for cue-controlled nicotine-seeking behavior.

Chapter One - Introduction

1.1 Drug addiction

Addiction is a chronic disease that induces dysfunctions of several brain circuits including those involved in reward, motivation and memory. It is characterized by the inability to effectively abstain, the impairment in the behavioral control over drug seeking and taking and dysfunction of emotional responses. Although the main focus of the present thesis is drug addiction, it is important to consider that the term addiction is not confined to a few “dangerous” molecules, but it is deeply embedded in our society and includes those for normal substance like food, activity like gambling, emotional patterns like thrill-seeking behavior and procrastination (Andreou and White 2010). Indeed, certain behaviors similarly to drug of abuse, can produce rewards and when this leads to diminished control over the behavior despite adverse consequences, the behavior itself can become addictive.

A technical definition of addiction seeks to frame exactly the severity of the disease. The reference manual of psychiatry (the *Diagnostic and Statistical Manual of Mental Disorders* (DMS) has been evolved over time to try to address this issue. In 1980, the DMS-III defined addiction as tolerance to the drug (i.e. the effects decrease over time and an increasing dose is needed to get the same effect) and/or withdrawal symptoms when drug assumption is stopped. Subsequently in the DSM-IV (1994) drug abuse and drug addiction were identified as two conceptually different diseases. In the DMS-IV, among the criteria for drug addiction, tolerance and withdrawal symptoms remained but they were no longer necessary criteria and five behavioral symptoms were added. These behavioral symptoms can be grouped in three main categories: 1) difficulty in limiting drug use and drug-seeking, 2) apparent strong motivation for the drug, 3) continued use despite negative consequences. The latest edition of the DMS, the DMS-V (2013), defines drug addiction as substance use disorders (SUDs), and indicates 11 criteria by introducing the concept of different severities of SUDs (Table 1.1.1). A score of 2-3 criteria is classified as mild SUDs, 4-5 criteria is moderate, and more than 6 is severe. In the DSM-V, the term *addiction* has become synonymous with the classification of severe substance-use disorder.

DMS-V has also introduced pathological gambling under substance-related and addictive disorders. Moreover, internet gambling disorder was identified in Section III of the DSM-V as an area for future research and it is the next most likely candidate to join pathologic gambling as a behavioral addiction (Jorgenson et al. 2016).

Table 1.1.1 The essential features of a substance use

Impaired control over substance use	Taking the substance in large amount over a long period of time.
	Multiple unsuccessful efforts to decrease or discontinue substance use.
	Spending a great deal of time obtaining and using the substance.
	Intense desire or urge for the drug (craving).
Social impairment	Failure to fulfil major role obligations at work, school, or home.
	Continue substance use despite negative effects.
	Give up to important social, occupational, or recreational activities because of substance use.
Risky use of the substance	Recurrent use of the substance in situations in which it is physically hazardous
	Use of the substance despite knowledge of having physical or psychological problem caused by the substance.
Pharmacological criteria	Tolerance to drug effect.
	Withdrawal symptoms.

1.1.1 Global use of psychoactive substances

Addiction, especially addiction to nicotine and alcohol, is a serious public health problem, leading to a very large number of early deaths.

As reported by the World Health Organization (WHO, 2012), the extent of worldwide use of psychoactive substances is estimated at 2 billion alcohol users, 1.3 billion smokers and 250 million illicit drug users. Among illegal drugs, cannabis is the most commonly used (129-190 million people), followed by amphetamine, cocaine, ecstasy and opioids (Figure 1.1.1). Moreover, a new trend in the use of dangerous new psychoactive substances (NPS) such as synthetic cannabinoids and amphetamine-like stimulants, has been emerging in the last years.

The use of substance of abuse has also economic drawbacks. The WHO estimated that 0.7% of the global burden of disease in 2004 was due to cocaine and opioid use, with the social cost

of illicit substances being about 2%. Illicit substance use is predominantly a male activity, much more than cigarette smoking and alcohol consumption (WHO report 2016). Substance use, either legal or illegal, is also more prevalent among young people (WHO report 2016).

The level of consumption of legal substances (tobacco and alcohol) is declining in developed countries but is increasing in developing countries. World trends in the use of illicit drugs are more difficult to estimate. Cocaine and ecstasy use clearly increased during the 1990s and seems to be still increasing in certain environments (WHO, 2012).

Top 20 Drugs – Last 12 Months – Whole Sample (N=78,819)

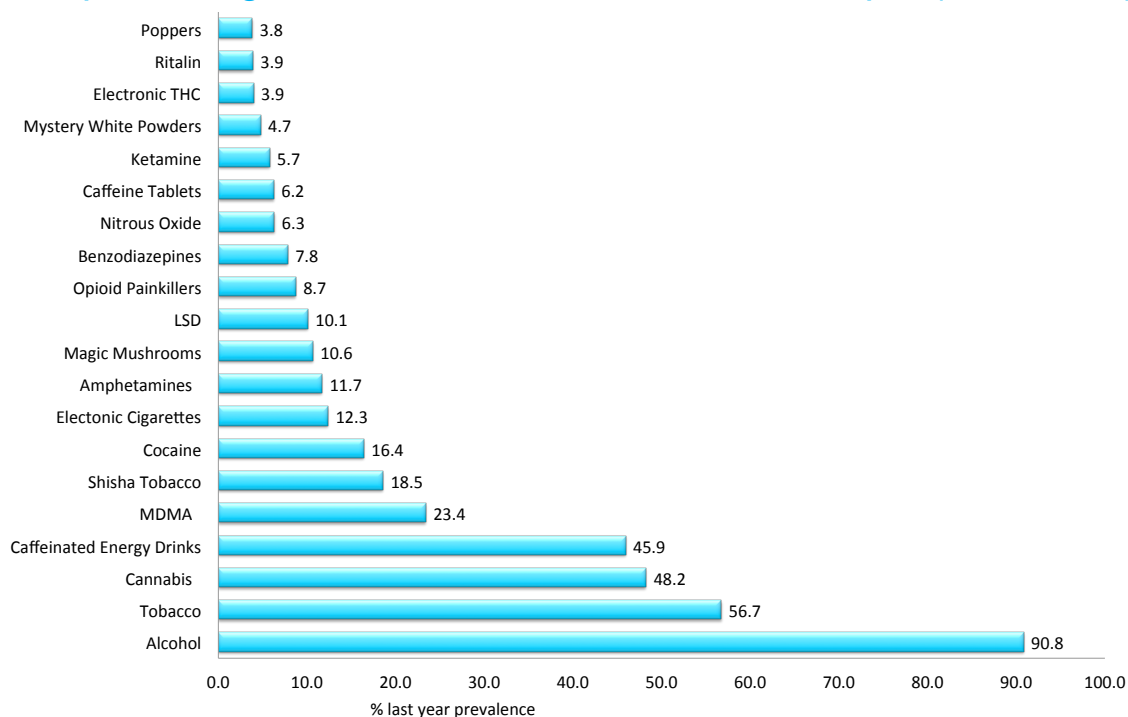


Figure 1.1.1 12-month prevalence of top 20 drugs use. 2014, Global Drug Survey (GDS)

Tobacco epidemic (Figure 1.1.2) remains the leading global cause of preventable disease, killing around 6 million people a year. More than 5 millions of those deaths are the result of direct tobacco use while more than 600,000 is the result of non-smokers being exposed to second-hand smoke. Nearly 80% of the smokers worldwide live in low- and middle-income countries, where the burden of tobacco-related illness and death is heaviest (WHO 2016). Smoking-related illness costs billions of dollars each year, imposing a heavy economic toll on countries, both in terms of direct medical care for adults and lost productivity (WHO 2016).

An important characteristic of SUDs is an underlying change in brain circuits that may persist beyond detoxification, particularly in individuals with severe disorders and although more than 80% of smokers wish to quit, only 35% of them try and less than 5% succeed in staying abstinent (APA 2013). The fact that lung cancer was confirmed to be caused by cigarette smoking over 50 years ago, and since then several other diseases have been added to the list of diseases caused by smoking, this does not seem to be enough to motivate people to give up smoking.

1.1.2 The addiction cycle

Drug addiction is considered a form of aberrant behavioral plasticity that develops gradually with repeated exposure to abused drug (Figure 1.1.3). Several are the reasons – frequently of social origin – that can induce an individual to take drugs. In the early stages of substance use, subjects come into contact with a drug with dependence-producing effects as a result of curiosity, peer pressure, social marketing factors. Some people use psychoactive

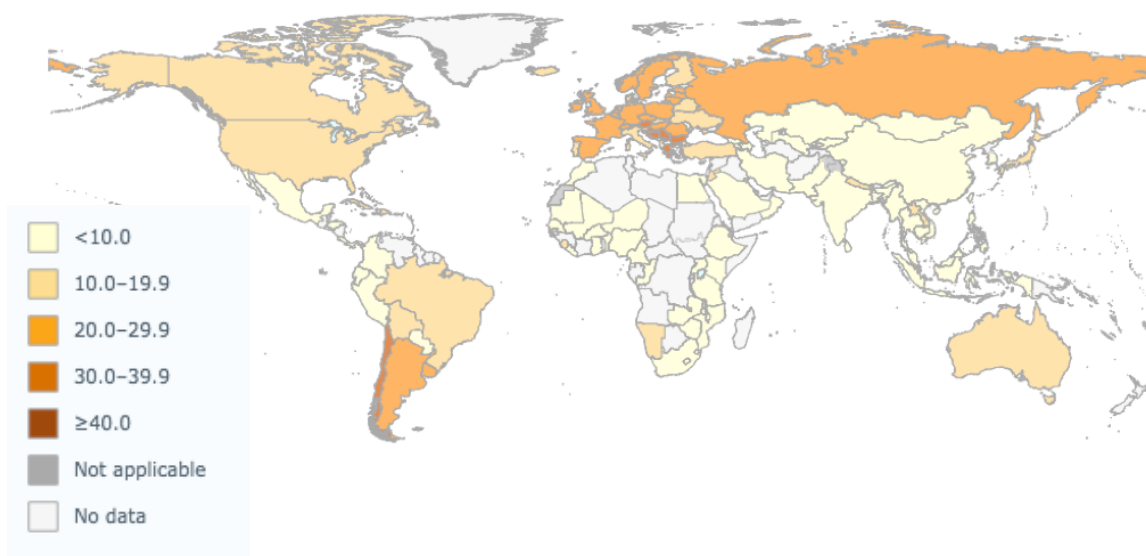


Figure 1.1.2. Prevalence of tobacco use among adults and adolescents aged ≥ 15 , year 2012.

Adapted from WHO web site (<http://gamapserv.who.int/mapLibrary/app/searchResults.aspx>)

substances because they expect benefits from their use, whether by pleasure or by the avoidance of pain. The rewarding properties of drugs do not necessarily consist of sheer sensation of pleasure like the “high” or the “rush” typical of amphetamine and heroin or inhaled “crack” (cocaine base) but can take milder forms of “hedonic” state, such as relief of tension, reduction

of fatigue, increased arousal, improved performance or reduction of borderline psychiatric pathologies (such as anxiety and depression). However, in spite of the real or apparent benefits, the use of some psychoactive substances also carries with it the potential for harm, whether in the short term (such as death due to overdose) or long term (drug dependence and addiction). Until the use of these substances remains at a recreational level (sporadic intermittent use) it usually produces just minimal problems. In some subject, however, the rewarding properties of psychoactive drugs together with the individual's own biological make-up and environmental background may facilitate further exposure to the drug (*regular drug use*). When these drugs are used repeatedly by vulnerable subjects (Kendler et al. 2000) molecular changes in the brain (Nestler 2002) promote continued drug taking that becomes increasingly difficult for the individual to control (Berke and Hyman 2000) (Figure 1.2).

Only a minority of people that use drugs ultimately become addicts. Many factors contribute to this susceptibility, including genetic predisposition as well as social environmental and developmental factors (Volkow et al. 2012; Demers et al. 2014). It has been estimated that 25-35% of individuals that ever self-administered opiates or nicotine and 5-10% who ever self-administered alcohol or cocaine progress to addiction (Kreek and Vocchi 2002).

Addictive drugs are both rewarding (interpreted by the brain as intrinsically positive) and reinforcing (behaviors associated with such drugs tend to be repeated) (White 1989). The continuous assumption of drugs of abuse can produce 1) *tolerance* and/or 2) *sensitization*, 3) *dependence*, 4) *addiction*, 5) *withdrawal syndrome* and 6) *craving*. *Tolerance* is defined as a decrease in the drug effect after a constant dose, or a need for increased dosage to maintain a stable effect.

Sensitization is an enhancement of some drug responses. This phenomenon has been well documented in laboratory animals by evaluating the increase in the locomotor response after psychostimulants administration (Spanagel 1995).

Repeated exposure to psychoactive substances induce drug *dependence*, which refers to an adapted state of cells, circuits and organ systems that occurs in response to excessive drug effect. When unmasked by drug cessation, this adapted state can result in the production of cognitive, emotional or physical *withdrawal symptoms*. However, not all abused drugs induce a clear physical

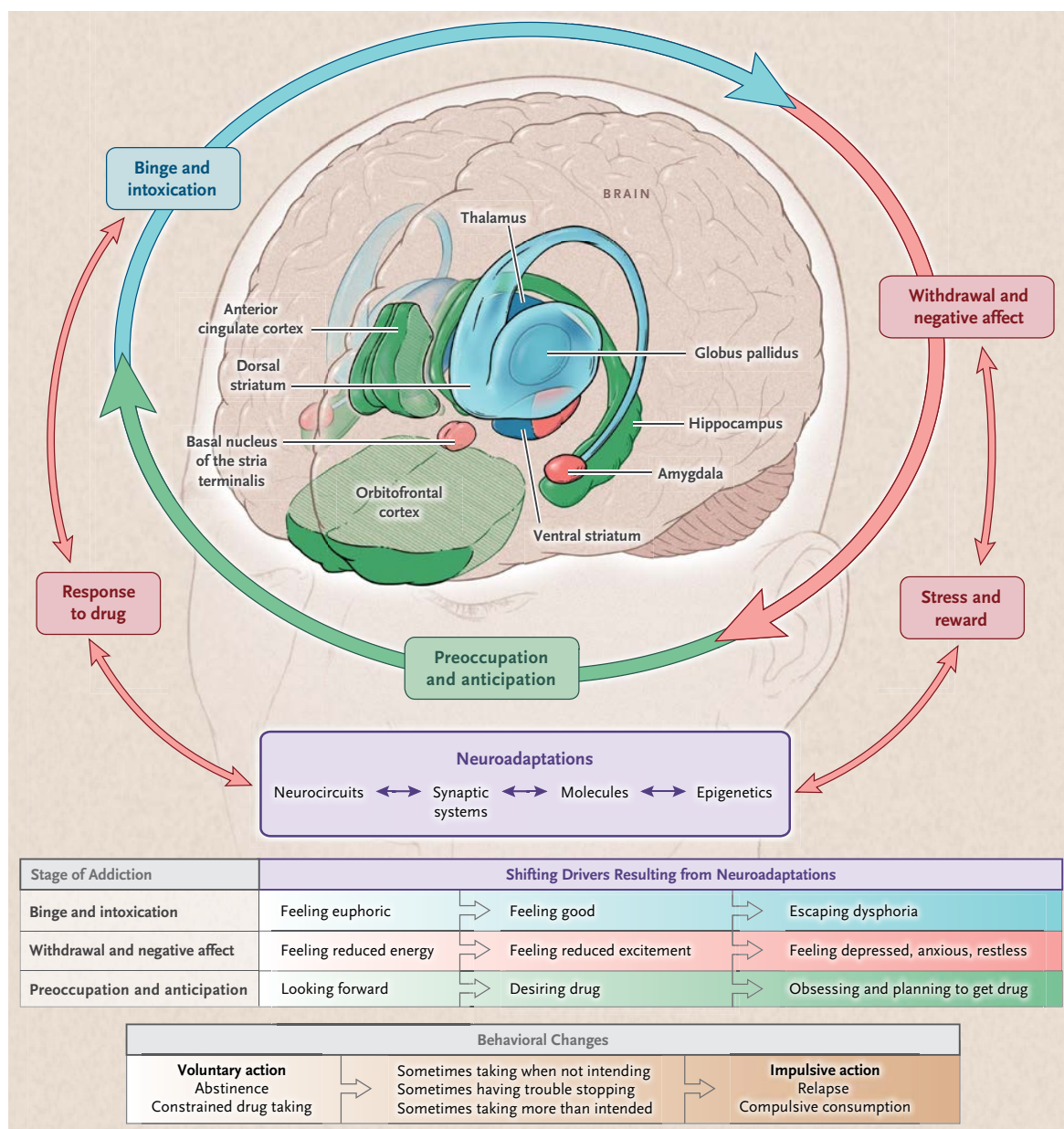


Figure 1.1.3. Addiction cycle. During intoxication, drug-induced activation of the brain’s reward regions (in blue) is enhanced by conditioned cues in areas of increased sensitization (in green). During withdrawal, the activation of brain regions involved in emotions (in pink) results in negative mood and enhanced sensitivity to stress. The strong desire for the drug triggers relapse and reinitiates the cycle of addiction. The compromised neurocircuitry reflects the disruption of the dopaminergic and glutamatergic systems and the stress-control systems of the brain. Adapted from Volkow (2016).

dependence. For example, ethanol, barbiturates and opiates produce physical dependence while cocaine does not result in the severe symptoms that characterize opioid withdrawal.

A common feature of the continuous use of all abused drugs is *addiction* that can be defined as a compulsion to take a drug despite negative consequences. Once *addiction* has taken hold, it tends to follow a chronic course, in which periods of abstinence are followed by relapse to active drug taking (O'Brien et al. 1998). During this period subjects experience the driving power of a new sensation: *craving*, a powerful, often uncontrollable desire for the drugs. Independently from the abuse substance, it has been estimated that more than 80% of individuals relapse to addiction during early or protracted abstinence while less than 20% sustain a protracted abstinence (Kreek and Vocci 2002).

The rewarding properties of drugs, at least as we understand them from their comparison with conventional rewards, do not fully explain the behavioral abnormalities associated with their use. In fact, in the context of dependence, it is important to remember that over a life span, many people experiment with a variety of dependence-producing drugs, but most do not become dependent. A complex interplay of psychological, neurological and personal factors is thought to be responsible for the compulsive pattern of drug-seeking and taking behavior that takes place at the expense of most other activities in addicted people.

1.1.3 Drug craving

The most difficult aspect of addiction treatment is the high risk of relapse; individuals can be abstinent for months or even years but are still susceptible to craving that can stimulate renewed drug-seeking and taking (O'Brien 2005). Drug craving, defined as “the desire to experience the effect(s) of a previously experienced psychoactive substance” is a cardinal feature of drug addiction and is clinically significant because of its potential link to relapse (Markou et al. 1993). Addicted patients describe craving as a powerful “must-have” that drive them to relapse to drug use (Childress et al. 1988).

The difficulties in finding an efficacious pharmacological treatment in preventing the relapse in drug abuse may be due to the heterogeneity of the neurobiological mechanisms underlying the desire to self-administer the drug.

It has been found (O'Brien et al. 1988) that multiple factors may influence relapse.

❖ **Psychiatric factors:**

- Depression
- Anxiety
- Other psychiatric disorders

❖ **Social factors**

- Peer pressure
- Family/employment problems

❖ **Protracted abstinence**

❖ **Conditioned responses**

A successful treatment program should be able to address all the factors thought to influence relapse.

Psychiatric disorders can be approached by means of specific pharmacotherapy, psychotherapy, or a combination of these modalities. Social factors can be influenced by vocational counselling, family therapy, and behavioral treatment.

Drugs of abuse tend to be used repeatedly under similar conditions so that the environment including sights, smell, sounds, and paraphernalia provides conditioned stimuli that can elicit conditioned response even before the drug is taken (preoccupation and anticipation phase of the addiction cycle). Conditioned responses are established during the course of drug using, which may involve thousands of injections or inhalations of the drug over a period of years. Very strong and durable conditioned responses can be established, and these responses are not affected by usual treatments such as detoxification and rehabilitation programs (Volkow et al.

2016). Often patients return home after a period of brief treatment feeling well and confident that they will not resume drug use. They are usually surprised to suddenly feel craving, withdrawal, or even “high” when they encounter people or places associated with their prior drug use. The changes produced by the conditioned stimuli may be limited to subjective effects or may also include physiological changes that can be recorded in animals with biochemical or electrophysiological techniques and in humans with advanced imaging techniques (Ehrman et al. 1992). Conditioned responses have received relatively little systematic attention from a therapeutic perspective. The phenomenon of conditioning is well known in the laboratory as exemplified by Pavlov’s dog. The sight of food caused the dog to salivate, and when food and the sound of a bell were presented together several times, the bell acquired the ability to trigger salivation in the dog, in the absence of any food (Pavlov 1927). Also some classes of drugs can produce conditioned responses in animals (Wiker and Pscor 1967). Conditioned responses are also present in humans who have used abused drugs, and it appears that nicotine is particularly potent in producing conditioned responses (Benowitz 2010).

It is thought that dependence on psychoactive substances could be the results of a complex interaction of the physiological effects of drugs on the brain, and particularly on brain areas associated with motivational and emotion, combined with “learning” about the relationship between drugs and drug-related cues, all of which have a biological basis (Hyman and Malenka 2001). It has been hypothesized that these learning processes are critically dependent upon the same motivational and emotional systems in the brain that are used by psychoactive substances: the mesocorticolimbic system (Hyman and Malenka 2001).

1.2 Animal models of drug addiction

Several animal models have been proposed to study the different aspects of drug addiction (briefly summarized in this Section). However, for the aims of this Thesis my discussion will focus on the operant self-administration and the reinstatement procedure. In general animal models can be viewed as experimental preparations developed for the purpose of studying phenomena found in human. Thus animal models are constructed to evaluate selected parts of the SUDs (Markou et al. 1993).

Conditioned place preference (CPP) CPP measures the rewarding/aversive properties of drugs (i.e. it is a form of Pavlovian conditioning) where an initial neutral stimulus (i.e. environmental cue) is repeatedly paired with the unconditioned stimulus (US). During conditioning, rodents receive non-contingent administrations of drug and saline in two distinct chambers of the apparatus. Testing consists of free access to both the saline-paired and the drug-paired chambers, and often a third, unpaired (neutral) area. A drug is considered to be rewarding if the animal spent more time in the drug-paired chamber, and aversive if the drug-paired chamber is avoided (Tzschentke 1998).

Psychomotor sensitization Repeated intermittent treatment with stimulants, nicotine or opiates sensitizes animals to the locomotor (increased over sessions) activating effects of these drugs. Following a period of withdrawal, animals receive a drug injection, and behavioral responses are greater in rodents that have received prior drug treatment compared to animals that are receiving the drug for the first time. Psychomotor sensitization can be used as a behavioral read-out of an underlying neural sensitization in addiction circuits (Wise and Leeb 1993; Robinson and Berridge 2000).

1.2.1 Operant intravenous self-administration

A more sophisticated animal model termed self-administration is based on response-dependent administration of a drug of abuse. Rodents learn that performing an operant response such as pressing a lever or a nose poke will produce a drug infusion (Schuster and

Thompson 1969). The self-administration procedure can be used to model different aspect of the human situation like the *acquisition*, *the maintenance*, *the abstinence* and the *relapse*.

Based on the operant notion of reinforcement introduced by Skinner (1938), the reinforcing effect of a drug is, by definition, the one that increases the probability that the animal will perform a task in order to self-administer the drug again. Thus, animals will make an operant response (generally lever pressing in an operant conditioning chamber) (Figure 1.2.1) in order to receive an i.v. infusion of several different classes of psychoactive drugs, including: psychomotor stimulants, opiates, nicotine and alcohol. These drugs that are readily self-administered in animals have high abuse potential in humans (Collins et al. 1984). Not all drugs abused by human are self-administered by experimental animals (e.g. hallucinogens), however all the drugs self-administered by experimental animals are abused by humans. The number and pattern of responding during i.v. self-administration studies in animals are determined by a schedule of reinforcement imposed by the experimenter. Drug availability and delivery are typically signaled by an environmental stimulus (i.e. visual and/or auditive).

The rodent drug self-administration model has both reliability and predictive validity. The dependent variable, the number of infusions obtained or the rate of responding during a session provides a reliable measure of the motivation to obtain drugs or, in an alternate framework, in demonstrating that drugs function as powerful reinforcers. Responding maintained by drugs as reinforcers is stable across sessions and can be altered predictably by appropriate manipulations (e.g. modifying the unit dose of the drug or by pharmacological manipulation). Intravenous drug self-administration also has predictive validity. Drugs having high reinforcement potential in experimental animals have reinforcing effects in human as measured by both operant and subjective report (Lamb et al. 1991).

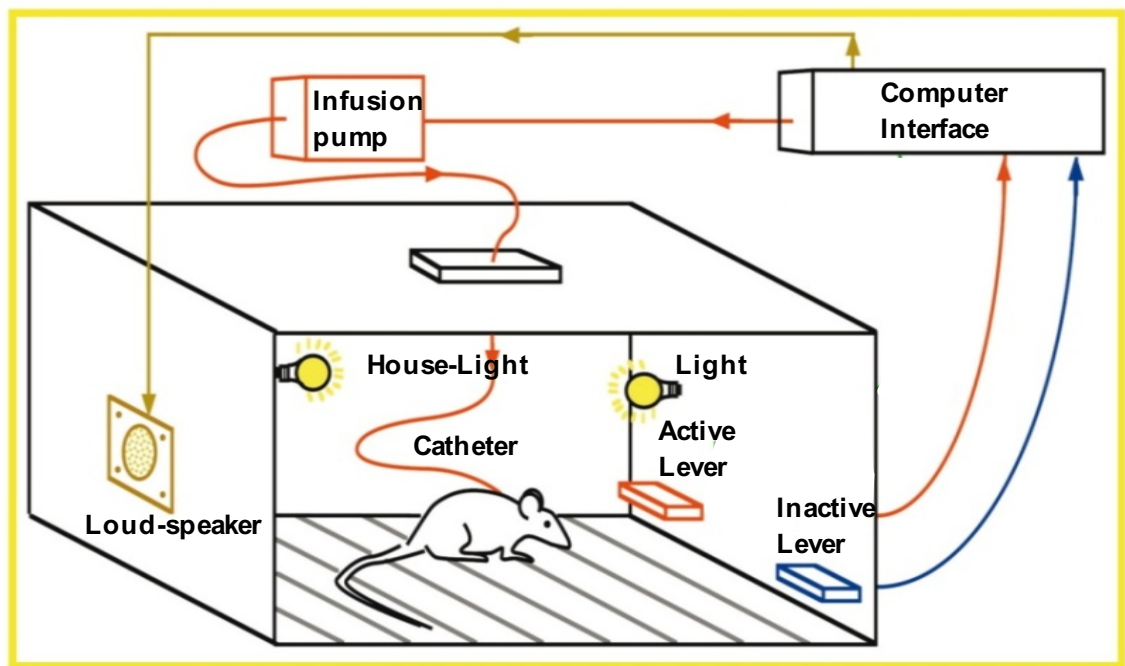


Figure 1.2.1 Self-administration chamber. This type of cage is commonly used for drug self-administration procedure. When the animal presses the active lever an infusion pump delivers an infusion of the drug through a catheter implanted in the jugular vein. Different environmental cues can be available during the session or during drug-infusion (i.e. light or noise cues).

Effect of unit dose on nicotine self-administration

In rats the reinforcing effect of nicotine was first reported by Clark (1969) when he showed that rats were self-administering a nicotine solution either by drinking or through an implanted catheter. Nevertheless, studies of intravenous self-administration of nicotine have been less successful in inducing high levels of drug intake. This can be explained by the fact that many factors like body weight and the duration of the self-administration period may influence the rate of nicotine self-injection (Lang et al. 1977). By using a short access to nicotine self-administration, Corrigan and Coen (1989) showed that it was possible to maintain several weeks of operant self-administration. Moreover, responding maintained by nicotine was clearly dose-dependent (Figure 1.2.2) with an optimal unit doses between 0.01-0.03 mg/kg.

Typically, acquisition of nicotine self-administration is preceded by a food training period, in which hungry rats learn to press a lever in order to receive food. Only recently the impact of

the food training on later nicotine self-administration was evaluated (Garcia et al. 2014). This study concluded that lower nicotine unit dose (0.03 mg/kg) can be used in the absence of food training to study influences on acquisition that more closely model the initial phases of human smoking.

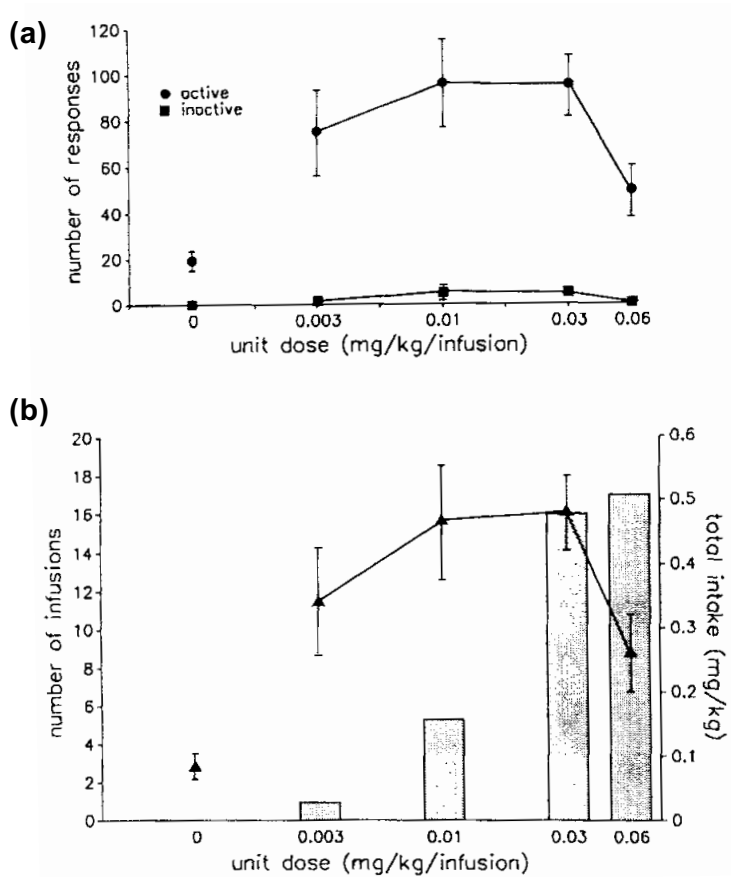


Figure 1.2.2. Dose-response curves for nicotine self-administration showing the number of responses on the active and inactive levers (a) and the number of infusions (b). The *bar graphs* superimposed in the bottom panel show the total session intake of nicotine at each unit dose. Note the wide range in this latter variable across unit dose, and that it tends to reach a maximum at the highest unit doses. (Corrigal et al. 1989).

Operant self-administration: potential pitfalls

Self-administration of a drug can vary as a function of the dose available, species or strain tested and the duration of self-administration sessions. It is also influenced by the availability of alternate reinforcers, the presence or absence of environmental stimuli that signal drug infusion, post-reinforcement interval, and prior history of the subject (Macenski and Meisch 1998). Because self-administration typically results in an inverted U-shaped curve (Figure 1.2.2), both leftward and right ward shifts in the dose effect function will decrease self-administration of some unit doses but simultaneously increase self-administration of other doses (Figure 1.2.2). The interpretation of downward shift in the dose-effect function also is sometimes problematic. Therefore, construction of a full dose-effect function is essential in self-administration studies.

Since a given pre-treatment may decrease self-administration by having nonspecific effects (i.e. sedation/hyperactivity), the influence of a pre-treatment on non-drug reinforcers should also be assessed.

1.2.2 Animal models of reinstatement

In the learning literature, reinstatement refers to the resumption of a previously extinguished conditioned response after acute non-contingent exposure to the US (Catania 1992). The phenomenon of reinstatement of learned behaviors after extinction was originally described in the early classical and operant conditioning studies of Pavlov (1927) and Skinner (1938). For example, Pavlov described the “priming effect” of the re-exposure to the US after extinction in dogs trained in a classical conditioning study, and wrote that “the restoration of an extinguished reflex is greatly accelerated by a fresh application of the US” (Pavlov 1927). Using an operant paradigm, Skinner and others reported reinstatement of lever pressing in rats after extinction training by non-contingent presentation of food or water (Skinner 1938).

These observations using primary reinforcers were then transferred to drugs of abuse. Reinstatement typically refers to the resumption of extinguished lever-pressing behavior after non-contingent exposure to drug or non-drug stimuli (Stewart and Wise 1992). The initial studies on reinstatement of amphetamine or cocaine-seeking by drug-priming were conducted on monkeys by Stretch and Gerber in the early 1970s (Stretch and Gerber 1973). Goldberg, Shuster and colleagues also showed that stimuli paired with morphine or cocaine injections in monkeys reinstated drug-taking behavior after the lever-pressing behavior is extinguished in their absence (Goldberg et al. 1981). Subsequently, Davis and Smith (1976) were among the first to demonstrate the role of conditioned reinforcers in a rodent reinstatement model of self-administration by pairing a neutral stimulus with i.v. morphine or amphetamine. They showed that conditioned stimuli could readily reinstate responding following extinction periods. Furthermore, drug infusions previously ineffective in reestablishing responding became effective when responding resulted in the presentation of stimuli associated with previous drug

injections (Davis and Smith 1976). Subsequent studies by de Wit and Stewart (1981, 1983) demonstrated that a tone previously paired with drug infusions facilitated responding in animals who had undergone within-session extinction trials.

After these pioneering experiments, several laboratories have adopted this animal model, termed extinction-reinstatement, to study factors that underlie relapse drug-seeking induced by exposure to self-administered drug, drug-associated cues, and stressors. The use of this procedure has become increasingly popular, as the number of studies that used the extinction-reinstatement paradigm in animals has increased consistently over recent years.

1.2.3 Extinction-reinstatement paradigm

Reinstatement of drug-seeking in laboratory animals has been primarily studied in rats with a history of drug self-administration (Shaham et al. 2003). All the procedural variations start with “self-administration training” that continues until the rats reach a stable drug-taking behavior (typically less than 15-20% variation from the mean number of infusions over several consecutive sessions). This phase is followed by “extinction training”, in which the self-injected drug is replaced with saline or in which the infusion pump is disconnected: this phase continues until the rats reach a predetermined extinction criterion (i.e. 20% or less responding during the last extinction session as compared to the first extinction session). After extinction of the drug-reinforced behavior, the ability of acute exposure to drugs (i.e. drug priming) or non-drug stimuli to reinstate drug-seeking is determined under extinction conditions (Stretch et al. 1971). There are two main dependent variables during tests for reinstatement: non-reinforced responses on a lever that previously delivered the drug, the active lever; and responses on a lever not associated with drug infusions, the inactive lever. Responses on the active lever are interpreted to reflect reinstatement of drug-seeking. Inactive lever responses are typically interpreted to reflect non-specific activity, but they may also reflect response-generalization (Shaham et al. 2003). The main difference between the various procedures is the time interval between the

presentation of the various phases. Thus “between-session”, “within-session” and “between-within-session” variations of the reinstatement model have been proposed (Shaham et al. 2003).

In the “between-session” procedure, training for drug self-administration, extinction of the drug-reinforced behavior and tests for reinstatement are conducted during sequential daily sessions (Stretch et al. 1971). The advantage of the between-session model is that it mimics somewhat the human situation of relapse to drugs at times that are beyond the acute withdrawal phase. In the “within-session” procedure, tests for reinstatement are carried out in daily sessions consisting of 1-2 h of drug self-administration, followed by 3-4 h of extinction of the drug-reinforced behavior, and then after responding has ceased, a test for reinstatement (de Wit and Stewart 1981). The advantage of this method is that rats can be repeatedly tested for reinstatement after priming injections, demonstrating that neither tolerance nor sensitization is evident after drug priming (de Wit and Stewart 1981). The limitations of the within-session method are that it does not simulate long-term relapse in humans, and the rats are not “truly” drug-free at the time of testing.

In the “between-within” variation, rats are initially trained for drug self-administration. Subsequently, extinction of the drug-reinforced behavior and tests for reinstatement are conducted on the same day after many days of drug withdrawal (Tran-Nguyen et al. 1998). The advantage of this method is that it can be used to study the relationship between the duration of the drug withdrawal period and the reinstatement of drug-seeking. At present, however, it is not clear whether this method is suitable for repeated testing in the same animals. Thus, different groups of rats, tested at each withdrawal period are needed for a clear interpretation of the data.

As for human condition drug priming, stress and drug-associated cues are demonstrated to induce drug-seeking behavior in experimental animals. Thus, three general experimental paradigms have been used for reinstatement of operant responding for drugs of abuse. One established approach is the use of non-contingent (“priming”) injections of drugs to reinstate self-administration (Davis and Smith 1976; de Wit and Stewart 1981). This paradigm has been

found to produce a robust degree of reinstatement and is arguably a good model for pharmacologically induced relapse in addiction (Spealman et al. 1999). A second paradigm employs the use of various external stressors to induce reinstatement of drug-seeking behavior (Shaham and Stewart 1995). This paradigm provides a model for the study of stress activation of craving states as evidenced from clinical studies (Sinha et al. 2000). Recent theoretical models of drug dependence as a state of chronic alteration of brain reward systems have emphasized the crucial role of negative stimuli in the development and perpetuation of drug addiction (Koob and Le Moal 2001), supporting the usefulness of the stress-induced reinstatement model in understanding causal factors of relapse. The third general model (and the focus of my Thesis) is the conditioned-cue model of reinstatement. This paradigm possesses good predictive and face validity for modelling the activation of craving state by conditioned environmental stimuli in drug-addicts.

1.2.4 Drug-associated cue-induced reinstatement

In their initial study, Davis and Smith (1976) trained rats to press a lever for i.v. injections of morphine; each injection was paired by a buzzer presentation (a discrete conditioned stimulus, CS). Lever pressing for morphine was then extinguished by replacing drug infusions with saline in the absence of the CS. During testing, lever presses resulted in response-contingent presentations of the CS, and rats increased their lever-pressing behavior. In contrast non-contingent presentation of discrete CSs have a minimal effect on cocaine-seeking following extinction of lever pressing (de Wit and Stewart 1981; Fuchs et al. 1998). It appears that two features are important for obtaining a reliable effect of discrete drug CS on reinstatement (Grimm and See 2000). First, a compound (i.e. tone + light) cue is more effective in inducing reinstatement than a simple tone or light (See et al. 1999). Second, the drug cues should be presented contingently, or contingently and non-contingently at regular interval, during tests for reinstatement (See et al. 1999; Grimm and See 2000).

Several laboratories have investigated reinstatement induced by different types of drug-associated cues. These include “discrete cues” that are paired with drug infusions during self-administration training (See et al. 1999); “discriminative cues” that, following discrimination training, become predictors of drug availability during the acquisition of the drug self-administration (Ettenberg et al. 1996; Weiss et al. 2000); and “contextual cues” or diffuse “background” stimuli (e.g. operant chamber fan, time of session) that become associated with drug injections, or with the availability and the effects of the self-administered drug (Crombag and Shaham 2002).

Nicotine-associated cue-induced reinstatement

Several factors have been associated with relapse to cigarette smoking. These include exposure to smoking cues, stress, negative affect, and withdrawal symptoms (Kassel et al. 2003). In particular, environmental cues associated with nicotine taking are capable of sustaining nicotine-seeking behavior even in the absence of a pharmacological effect of the drug (Caggiula et al. 2001). The increase in salience for the environmental cues associated with nicotine assumption was demonstrated both in humans (Balfour et al. 2000) and in animal models (Goldberg et al. 1981; Corrigall and Coen 1989) of nicotine addiction. These cues are capable of maintaining significant levels of operant behavior for extended periods of time and of reinstating the behavior after lever press extinction (Caggiula et al. 2001). LeSage et al. (2004) were among the first to demonstrate that CS reinstated extinguished nicotine self-administration. Moreover, they found that nicotine-paired stimuli were more effective than nicotine priming in reinstating nicotine self-administration.

In preclinical settings, reintroduction of nicotine-associated cues after lever press extinction resulted in drug-seeking behavior evaluated as the number of active lever presses on the previous nicotine-paired lever in the absence of nicotine (Cohen et al. 2005; Liu et al. 2006; Liu et al. 2010). More recently Cervo et al. have also shown that reintroduction of a discriminative stimulus predictive of nicotine availability (S^{D+}) together with a conditioned stimulus associated with nicotine self-administration (CS^+), induced a strong, long-lasting nicotine-seeking behavior

in abstinent rats after repeated extinction trials and with no further drug presentation (Di Clemente et al. 2011; Cervo et al. 2013).

Thereby, the robust, long-lasting nicotine seeking induced by S^{D+}/CS^+ has strong face- and construct-validity, and may be useful for comparing the activity of anti-relapse pharmacological and non-pharmacological measures in a more potent within-subject experimental design, using fewer experimental subjects.

Extinction-reinstatement paradigm: potential pitfalls

It has been argued that the reinstatement model does not mimic most situations that lead to drug abstinence in humans and thus may not be suitable to model relapse (Marlatt 1996; Katz and Higgins 2003). It is also important to notice that extinction in these model, relates not to the extinction of drug CSs, (i.e. like for cue exposure therapy in clinical setting) but to the instrumental behavior of lever pressing, (i.e. drug taking response without drug taking). This type of extinction of drug taking is not present in humans and may limit the face validity of the model (Everitt 2014).

Despite these limitations, the reinstatement model has good predictive validity because conditions that reliably reinstate drug-seeking in laboratory animals such as drug re-exposure, drug-associated cues and stress also provoke drug relapse and craving in human. Thus, the model can be used to study neuronal mechanisms underlying relapse in drug-seeking behavior despite the fact that the conditions that lead to drug abstinence in laboratory animals are not identical to those in human.

1.3 Glutamate and drug addiction

DAergic mechanisms are well known to be important in the field of drug addiction. The acute rewarding effects of addictive drugs are mediated by enhancing DA release in the striatum, mainly the Nacc, (Di Chiara and Imperato 1988), thus reinforcing reward learning (Berridge and Robinson 1998). Activation of the midbrain DA system drives also the incentive salience to stimuli in the environment (Robinson and Berridge 1993) and promotes performance of goal-directed behavior (Salamone 2007). Nevertheless, DA-independent mechanisms and in particular glutamatergic (GLU) mechanisms seem to be involved in the loss of behavioral control over drug-seeking and chronic relapse after protracted abstinence (Koob and Volkow 2010). In addition, much evidence highlights that DA-independent reinforcement occurs especially in Nacc (Koob and Weiss 1992; Nestler 2005). In this Chapter, I will summarize some of the findings showing long-lasting alterations at the level of the GLUergic system produced by contingent exposure to addictive drugs.

The first evidence that GLU transmission might be involved in the effect of addictive drugs was the finding that NMDARs mediated the primary rewarding properties of cocaine (Pierce et al. 1996) and that AMPARs were important for the behavior elicited by the stimuli previously associated with the drug action (Cervo and Samanin 1995). Following these observations, Cornish et al. (1999) demonstrated that after cocaine self-administration AMPAR stimulation in the Nacc consistently induced relapse to drug-seeking behavior without altering food-seeking. Furthermore, AMPAR antagonists in Nacc blocked the reinstatement of cocaine-seeking behavior (Cornish and Kalivas 2000).

Since the main GLU afferents in the Nacc are from the mPFC, these results were the first to highlight the importance of the mPFC-Nacc pathway in the reinstatement of cocaine-seeking (Park et al. 2002). Physiologically, corticostriatal projections are needed for changing behaviors in response to the context, thereby generating new adaptive actions (Everitt and Robbins 2016). Addictive drugs impair the ability of this system to inhibit drug-seeking in response to cues contingencies. This is demonstrated also by the finding that addicts have difficulty to suppress

drug-seeking behaviors even when provided with information that should prevent the behavior. As proposed by Kalivas (2009) this impairment could be generated either by a pathological strength of drug-seeking behavior or by an impairment in the capacity to control drug-seeking behavior.

1.3.1 Central glutamate and glutamate receptors

Long before the discovery of GLU as a neurotransmitter, it was recognized that certain amino acids, such as GLU and aspartate were found at high concentrations in the brain with a powerful stimulatory effects on neuronal activity (Curtis and Watkins 1960). However, it was extremely difficult to isolate the role these amino acids play in the neural metabolism from those as neurotransmitters. Thus, it was only in the late 1970s that it became widely recognized that GLU is the principal excitatory transmitter within the vertebrate nervous system.

In neurons L-GLU is synthesized in the synaptic boutons. It can derive from glucose through the Krebs cycle with the transamination of α -oxoglutarate. GLU can also be synthesized from glutamine derived from glial cells, which is transported in the presynaptic neurons and locally converted into GLU (Figure 1.3.1). In the synaptic boutons GLU is stored in vesicles that release GLU by a Ca^{2+} dependent process (Cotman et al. 1981). There are three types of vesicular GLU transporter (vGluT1, 2, 3). vGluTs pack GLU into synaptic vesicles using a proton gradient that is created by hydrolyzing ATP. vGluT1 and 2 are widely distributed and are mainly expressed in the terminals of GLU synapses. vGluT3, instead, is mainly expressed in neurons that release other neurotransmitters such as γ -aminobutyric acid (GABA), serotonin (5-HT) and catecholamines. It is also distributed in both axons and somatodendritic spines (Iversen et al. 2009). The GLU concentration within the vesicle is 100 mmol/L and release of a single vesicle produces an excitatory postsynaptic potential (EPSP) that derives primarily from AMPAR activation.

The synaptic release of GLU is controlled by a wide range of presynaptic receptors including

group II and group III metabotropic GLU receptors (mGluRs), cholinergic, adenosine, kappa opioid, GABA_B, cholecystokinin and neuropeptide Y receptors (Meldrum 2000). The activity of synaptic GLU is controlled by a high-affinity GLU transporter on the presynaptic neurons and glial cells. In the glial cells, GLU is converted by glutamine-synthetase into glutamine, which is then transported via low-affinity process into the neighboring nerve terminals. In astrocytes, glutamine can also be oxidized in the Krebs cycle into α -ketoglutarate, which can be actively transported into the neuron to replace the α -ketoglutarate lost during the neuronal GLU synthesis (Iversen et al. 2009).

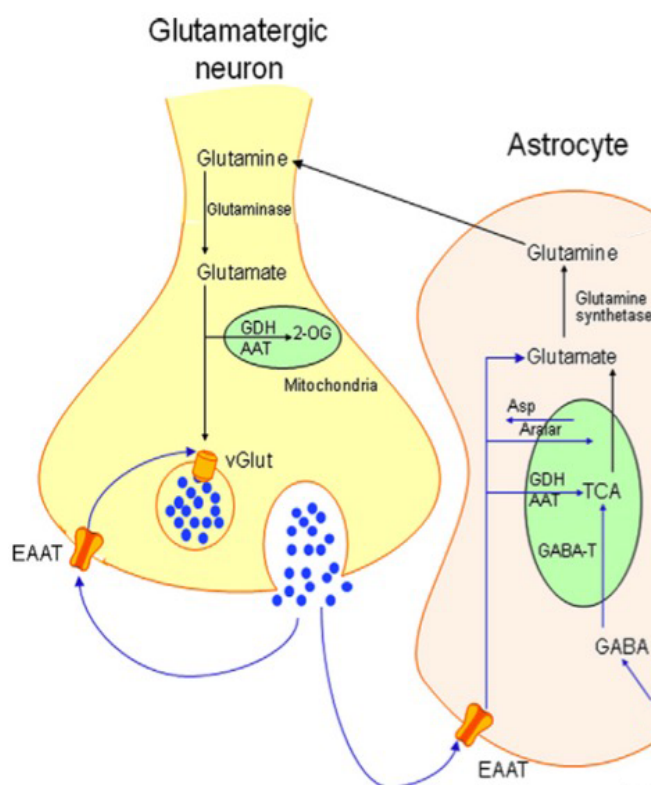


Figure 1.3.1. Synthesis, packaging, release, transport, and metabolism of glutamate (GLU). Glutaminase converts glutamine to glutamate. The neurotransmitter is subsequently transported by vesicular transporters into vesicles for release. Upon release, glutamate is taken up by high affinity membrane transporters into neurons and surrounding glia where it can be recycled or metabolized via several enzymes.

Abbreviations: 2-OG, 2-oxo-glutarate; AAT, aspartate amino-transferase; Aralar, aspartate-glutamate carrier; Asp, aspartate; EAAT, excitatory amino acid transporter; GDH, glutamate dehydrogenase; TCA, tricarboxylic acid cycle; vGluT, vesicular GLU transporter adapted by Rowley et al. (2012).

Glutamate ionotropic receptors Three families of ionotropic receptors (iGluRs) were defined by their pharmacology and molecular biology:

NMDARs are ligand-gated ion channels permeable to Ca^{2+} and Na^{+} and gated by Mg^{2+} in a voltage-dependent manner. NMDARs have two subunits: GluN1, that has several possible splice variants and GluN2 in which A, B, C or D subunits have different kinetics properties and

reactivity towards antagonists (Meldrum 2000). NMDARs appear to have a pivotal role in long-term depression (LTD) and long-term potentiation (LTP). Over activation or prolonged stimulation NMDARs can cause excitotoxicity. NMDARs have many different sites through which is possible to pharmacologically modulate their activity. Moreover, they have high degree of interaction with many other membrane and cytoplasmic proteins (Iverson et al. 2009).

AMPARs mediate fast excitatory synaptic transmission. AMPARs have different subunits termed GluA1-4, as well as auxiliary subunits (transmembrane AMPAR regulatory proteins [TARPs]) that modulate AMPAR trafficking and channel function (Straub and Tomita 2012). AMPARs are highly mobile proteins with fast trafficking in the synapses. Changes in AMPAR number and their subunit composition mediate LTP and LTD (Henley and Wilkinson 2013). All AMPARs are permeable to Na^+ and K^+ while Ca^{2+} permeability is brought about by an editing process of GluA2 RNA, whereby the conversion of a neutral glutamine codon (Q) to a positively charged arginine (R) renders the channel impermeable to Ca^{2+} . For this reason, GluA2-containing AMPA receptors are primarily Ca^{2+} impermeable (CI-AMPARs) (Tanaka et al. 2000). In contrast, GluA2-lacking AMPARs Ca^{2+} are permeable AMPARs (CP-AMPARs). In the Nacc of drug-naive rodents, CP-AMPARs account for only 5-10% of evoked excitatory postsynaptic currents (EPSCs) amplitude and the predominant CI-AMPARs are comprised mainly of GluA1A2-containing receptors (Pierce and Wolf 2013).

Kainate receptors (KARs) are tetramers assembled from the combination of five different subunits (GluK1-5). In contrast to AMPARs and NMDARs, KARs seem to have more diverse functions regulating the activity of neural circuits. KARs can be expressed postsynaptically where they mediate EPSCs of small amplitude and slow decay. Postsynaptic KARs are present on GABAergic interneurons in the Hipp, cortex and amygdala (Amy) (Contractor et al. 2011).

Glutamate metabotropic receptors mGluRs are linked to G proteins and second messenger systems and are divided into three groups: Group I mGluRs, include mGluR1 and 5, are predominately found postsynaptically where they couple to Gq-proteins to activate

phospholipase C and Ca^{2+} signal transduction. In addition, mGluR1/5 interact with intracellular Homer proteins that play a crucial role in mGluR trafficking in and out of the synapses. They also connect mGluRs to iGluRs in particular facilitating NMDARs (Markou 2007). In accord with this positive coupling between Group I mGluRs and NMDARs, evidence suggests that Group I mGluRs play an important role in NMDA-dependent synaptic plasticity (Olive 2010).

Group II mGluRs include mGluR2 and 3, and are coupled to Gi/o proteins to negatively regulate adenylyl cyclase activity. mGluR2/3 are found on glial cells and presynaptic terminal, where they inhibit GLU release decreasing neurons excitability (Markou 2007). Activator of G protein signaling 3 (Ags3) inhibits mGluR2/3 function by binding to the inactive form of the Gi coupled receptor (Kalivas et al. 2003).

Group III mGluRs include mGluR4, 6, 7 and 8, and are also negatively linked to cAMP activity but with a different agonist preference from that of mGluR2/3. Type III mGluR are found in the active zone of presynaptic terminals where they inhibit neurotransmitter release (Iversen et al. 2009).

Glutamate transporters Five Na^+ -dependent GLU transporters have been cloned. Two are expressed mostly in glia [glial GLU and aspartate transporter (GLAST) and glial GLU transporter (GLT-1)] and three in neurons [excitatory amino acid transporter EAAT 1, 4, 8] (Seal and Amara 1999). All the five GLU transporters catalyze the exchange of 1H^+ , 3Na^+ , and 1K^+ with GLU molecule (Danbolt 2001). GLU transporters are also differentially expressed in the brain, i.e. GLT-1 is predominant in the rat Hipp, whereas GLAST is predominant in the cerebellum (Lehre and Danbolt 1998). GLT-1 location close to the synaptic cleft enables it to buffer released GLU (Pendyam et al. 2009). Moreover, GLT-1 is responsible for the removal of almost all the GLU as shown by the finding that GLT-1 knockout mice have an almost complete loss (about 95%) of GLU uptake activity (Danbolt et al. 2016).

1.3.2 Glutamatergic circuits involved in drug addiction

A common feature for all mammals is that they can learn the relationship between their actions and outcomes, and modulate their actions according to their expectation for the desired outcome. A critical role for this aspect of animal behavior is played by the basal ganglia and their connections with the cortex. In particular, the striatum receives massive projections from almost all cortical areas, and from thalamic nuclei (Yin and Knowlton 2006). The corticostriatal circuitry mediates the processing of sensory information into adaptive behaviors (Everitt and Robbins 2016). This circuitry can be divided in two sub-circuits: 1) limbic sub-circuit, which comprises limbic brain regions such as mPFC, the Amy, the Nacc and the VTA; 2) motor sub-circuit, which contains the motor cortex, the dorsal striatum and the substantia nigra (Kalivas 2009). Within these circuits the Nacc plays a key role in action selection, integrating cognitive and affective information to augment the efficiency of appetitive or aversively motivated behaviors (Floresco 2015). Indeed, the Nacc is situated strategically to receive limbic information from Amy, mPFC, and Hipp that could be converted into action response through its connections with the extrapyramidal motor system (Koob and Volkow 2010).

When a novel stimulus capable of motivating an adaptive behavioral response, such as food, is encountered, the limbic circuit is engaged. In this way, an animal can implement a previously learnt behavior, thus forming new behavioral strategies. If for several times the action results in the desired outcome, then a gradual shift towards more organized activity of the motor system takes place (Kalivas 2009). In contrast, if the contingency between action and outcome changes, the activity of the motor system become less organized allowing the limbic circuit to update the change (Yin and Knowlton 2006).

Repeated assumption of drugs of abuse impairs the ability of the limbic circuit to effectively process environmental contingencies. The previously learnt, well-established drug-seeking becomes the dominating behavior and is less susceptible to extinction (Everitt and Robbins 2016). Consistently with this theory, the greater the duration of the self-administration training the more is the involvement of the motor circuit as proved by neuroimaging studies in

primates showing that early cocaine self-administration activate all the striatal regions but after protracted training there is a more prominent activation of the dorsal striatum (Porrino et al. 2004). Moreover, after protracted training using the second-order schedule of reinforcement it is the inactivation of the dorsolateral striatum that prevents drug-seeking (Everitt and Robbins 2016). Another characteristic of an extended periods of self-administration training is that animals are less responsive to environmental stimuli that disrupt drug-seeking (Vanderschuren and Everitt 2004). Together these data are consistent with drug-induced alterations of the striatal mechanisms where addictive drugs, by altering Nacc activity may cause a greater recruitment of the limbic circuit in the early phases of exposure and of the motor circuit after repeated use (Kalivas 2009; Everitt 2014).

1.3.3 Nucleus accumbens connectivity

As the dorsal striatum, the Nacc receives DAergic input from the ventral mesencephalon and GLUergic input from cortical and thalamic regions, and sends output GABAergic neurons to the basal ganglia (Scofield et al. 2016) (Figure 1.3.2). The Nacc is composed of 90-95 % of GABAergic medium spiny neurons (MSN) which can be further divided into D1 and D2 expressing neurons (Gerfen and Surmeier 2011). D1-containing MSN express also dynorphin, substance P and M4 cholinergic receptors, while D2-containing MNS express enkephalin, neurotensin, and A2a receptors (Lobo et al. 2006). Traditionally, within the striatum, D1 MSN are associated to the direct pathway, thereby facilitating motor responses while D2 MSN are associated to the indirect pathway inhibiting motor responses (Kravitz et al. 2010). Nevertheless, it was recently shown that D1 and D2 MSN of the Nacc both send projections to the ventral pallidum and unlike the dorsal striatum, D1 and D2 afferents to the ventral pallidum do not distinguish between direct or indirect basal ganglia pathways (Kupchik et al. 2015). The 5-10% of Nacc' neurons are classified as GABAergic interneurons (Kawaguchi et al. 1995). There are also cholinergic interneurons that are the main source of ACh in the striatum and that are responsive to both rewarding and aversive stimuli (Scofield et al. 2016).

The Nacc is divided into two structurally and functionally different subregions: the core and the shell (Floresco 2015). The Nacc core is responsible for the evaluation of reward and initializing reward-related motor action. The Nacc shell is responsible for reward prediction and reward learning (Shiflett and Balleine 2011).

The Nacc core is essential for acquiring drug-taking and cue-elicited drug-seeking behavior. In particular, GLU afferents from the prelimbic cortex (PLC) are necessary for the reinstatement of drug-seeking (McFarland et al. 2003). The Nacc shell instead is connected with regions such as the hypothalamus and Amy, thus positioned to process motivationally relevant information with emotion, autonomic response and motor response (Heimer et al. 1997). In particular, the GLUergic projections from the infralimbic cortex (ILC) to the Nacc shell are necessary for extinction learning (LaLumiere et al. 2012).

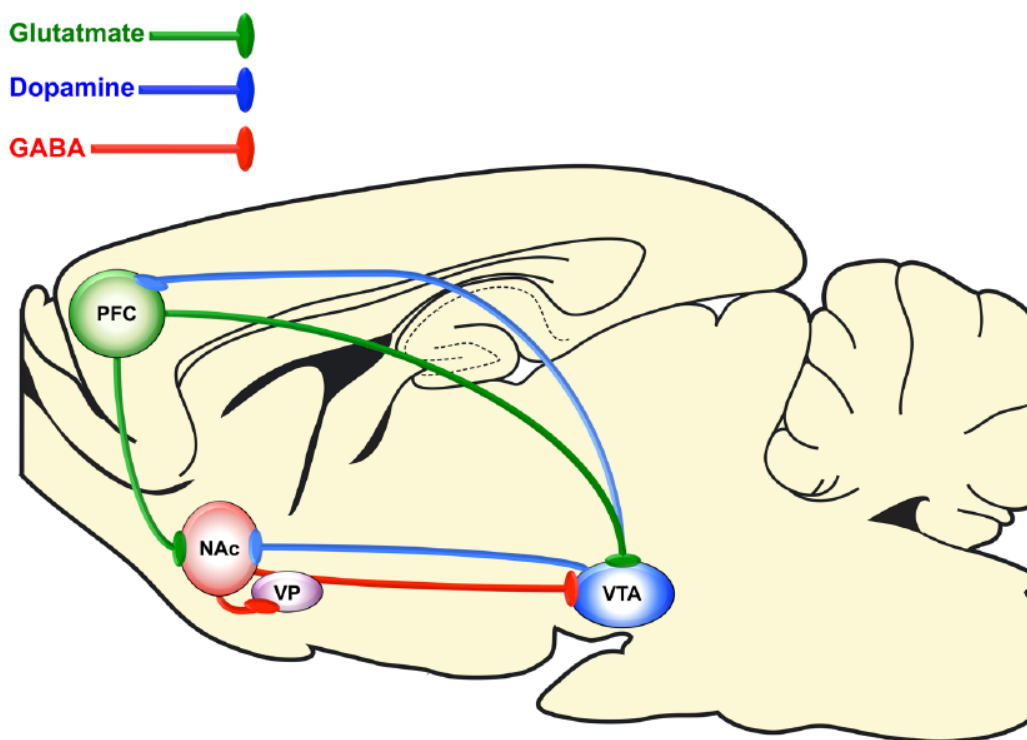


Figure 1.3.2. Nucleus accumbens (Nacc) connections. Schematic representations of the brain regions connected to the Nacc. The prefrontal cortex (PFC) is shown in green. The Nacc in red, the ventral pallidum (VP) in purple and the ventral tegmental area (VTA) in blue. Adapted from Scofield and Kalivas (2016).

1.3.4 Alteration in glutamate homeostasis in the nucleus accumbens mediate relapse

A large body of evidence reveals that a common aspect that link together nicotine, heroin and cocaine addiction are the alterations in GLU homeostasis in Nacc.

Regulation of extrasynaptic glutamate In vivo microdialysis studies have revealed that extracellular GLU levels in the Nacc are largely unaffected by blocking synaptic transmission with voltage-dependent Na⁺ or Ca²⁺ channel antagonists (Timmerman and Westerink 1997). In the NAcc core, ~60% of the basal extracellular GLU is derived from constitutive cystine–GLU exchange (system Xc) in 1/1 stoichiometry (Baker et al. 2002) and this non-synaptic GLU can modulate the activity of neurons (Rodriguez et al. 2013). It has been shown that cocaine and nicotine self-administration reduce membrane levels of the catalytic subunit, xCT of the exchanger, in the Nacc (Madayag et al. 2007; Knackstedt et al. 2009) and that animals treated with repeated contingent cocaine and nicotine have a 50% reduction of the basal levels of extracellular GLU in the Nacc core (Baker et al. 2003; Gipson 2013). These alterations in GLU levels differently affect GLU receptors: iGluRs are concentrated in the synaptic cleft and are activated primarily in a phasic event by presynaptic GLU release; mGluRs have a dissociation constant for GLU in the high-nanomolar range, thus lower than iGluRs and since the basal activity of the system Xc- maintains extrasynaptic GLU concentration in the low micromolar range mGluRs are tonically stimulated (Schoepp and True 1992; Moran et al. 2005).

It has been shown *in vitro* that activation of system Xc- in the Nacc by physiological levels of cystine stimulates mGluR2/3, decreasing synaptic GLU release probability (Moran et al. 2005). Thus, modulation of mGluR2/3 through system Xc- accounts not only for the reduction in basal extrasynaptic levels of GLU after chronic cocaine administration, but also for the increase in synaptic release of GLU into the Nacc core during cue-, stress- and drug-induced reinstatement of cocaine-, heroin- and alcohol-seeking (McFarland et al. 2003; Madayag et al. 2007; LaLumiere and Kalivas 2008; Meinhardt et al. 2013). The importance of this mechanism is highlighted by the finding that both the drug-seeking behavior and the concomitant rise in

synaptically released GLU in the Nacc core are abolished by inactivation of the PLC, indicating that prelimbic afferents to the Nacc core are the main source of synaptic GLU release (McFarland et al. 2003; LaLumiere and Kalivas 2008).

It was recently shown that GLT-1 too is down-regulated in the Nacc core after chronic exposure to cocaine, nicotine and ethanol (Kalivas 2009; Sari and Sreemantula 2012; Gipson 2013), making the down-regulation of GLT-1 a pivotal maladaptive response to drug exposure. Interestingly, expression levels of GLT-1 are reduced immediately following cocaine self-administration in both core and shell, whereas extended withdrawal causes further down-regulation only in the core (Fischer-Smith et al. 2012). Therefore, the overflow of synaptic GLU during the reinstatement of drug-seeking behavior is brought about not only by the enhanced release of synaptic GLU, but also by reduced elimination of GLU from the extracellular space. The drug-induced reduction of GLU uptake slows the removal of transmitter from the synaptic cleft, potentially amplifying the magnitude of GLU transmission thus allowing synaptic GLU to gain access to non-synaptic compartments (Kalivas 2009). This spill-over of GLU could potentially activate mGluR5 and GluNR2B-containing NMDARs (Kalivas et al. 2003) increasing intracellular Ca^{2+} and synaptic efficacy. This indicates that GLU homeostasis might involve co-regulation of glial GLU release through system Xc- and elimination of GLU by GLT-1. Therefore it has been suggested that the reduced GLU tone onto mGluR2/3 as well as the down regulation of the system Xc- and GLT-1 might account for the imbalance in GLU homeostasis in the Nacc and mediate drug-seeking behavior (Kalivas 2009).

Morphological changes in dendritic spines One of the most compelling examples of whereby experience at one point in life changes behavior and psychological function for a lifetime, is addiction. Drug addiction produces experience-dependent plasticity, causing functional impairments mainly at GLUergic synapses in the Nacc (Luscher and Malenka 2011). These adaptations are thought to impair the ability of the Nacc to process information, adaptively regulate reward-seeking behaviors, and thereby contribute to relapse to drug use in substance use disorders. For example, a cocaine challenge increases spine head diameter only in

rats previously trained to self-administer cocaine (Shen et al. 2009). Furthermore, discrete cue-induced reinstatement of cocaine-seeking (Gipson 2013) and context-induced cocaine relapse elicit rapid, transient increases in spine head diameter in the Nacc core (Stankeviciute et al. 2014). It is therefore clear that reinstating drug-seeking, regardless of whether initiated by cue, context, or a challenge of the drug increases spine head diameter. A recent study by Dumitriu and co-workers (2012) employing single cell microinjections and advanced 3D imaging gives some insight into cocaine-mediated regulation of dendritic spines in the core versus shell subregion of the mouse Nacc. Over a broad time course (4 hours, 24 hours, or 28 days) of withdrawal from chronic cocaine self-administration, they found that after 4 h spines are up-regulated in the shell but down-regulated in the core, while at a longer period of withdrawal (28 days) the only difference was spine elimination in the core. This may suggest that cocaine-induced spine plasticity is more enduring in the core than in the shell (Dumitriu et al. 2012).

Not only psychostimulants but also other classes of abused drugs induce changes in dendritic spine morphologies. Addiction to opiates alters dendritic arborisation and spine density (Diana et al. 2006) and chronic exposure to experimenter-administered morphine followed by one month of withdrawal decreased the complexity of dendritic branching and the number of dendritic spines on MSN in the Nacc shell (Shen et al. 2009). Moreover, extinction of morphine reward produced significant structural changes in dendritic branching in the Nacc core, but not in the Nacc shell (Leite-Morris et al. 2014). Similarly, chronic exposure to moderate levels of alcohol reduces dendritic spine density by decreasing the formation of mature spines (Romero et al. 2013).

In summary, different drugs of abuse produce alteration in spines morphology but while chronic administration of depressive drugs such as opiates and alcohol is accompanied by a decrease in the spine density and dendritic branching, administration of psychostimulants and nicotine increases spine density and dendritic branching in Nacc MSNs.

Alterations in synaptic proteins Some works have shown the requirement of AMPARs for GLU transmission into the Nacc in the development of many behavioral aspects related to drug addiction (Wolf 1998; Pierce et al. 1996). A growing body of evidence indicates that Nacc AMPARs contribute significantly to the reinstatement of cocaine-seeking (Park et al. 2002; McFarland et al. 2003) as well as heroin-seeking (LaLumiere and Kalivas 2008). In addition, AMPARs transmission in the Nacc is also required for drug-seeking under second-order schedules of reinforcement (Di Ciano and Everitt 2004) and for cue-induced seeking after prolonged abstinence (Conrad et al. 2008).

GluA1 trafficking is regulated by phosphorylation at the carboxy-terminal region of the GluA1 subunit that contains many phosphorylation sites including Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII). DA stimulation of D1-like receptors activates L-type Ca^{2+} channels so that the resulting activation of CaMKII facilitates the reinstatement of cocaine-seeking by promoting the synaptic incorporation of GluA1-containing AMPA receptors in the Nacc shell (Anderson et al. 2008). Indeed, CaMKII activity in the Nacc shell may be seen as an essential link between the DA and GLU systems contributing to the neuronal plasticity underlying cocaine craving and relapse. Interestingly the rapid increase in spine head diameter during the first 45 min after cocaine administration is consistent with an increase in GluA1 surface insertion while the marked reduction in spine diameter at 120 min after cocaine injection is consistent with internalization of AMPARs (Kalivas 2009).

The trafficking of the GluA2 subunit is altered also by exposure to addictive drugs. Phosphokinase C (PKC) phosphorylation of GluA2 at Ser880 influences the trafficking of GluA2 containing AMPARs increasing the rate of internalization (Anggono and Huganir 2012). Increased phosphorylation of GluA2 at PKC site was observed during cocaine-induced reinstatement (Famous et al. 2008).

Subsequent works confirmed the presence of CP-AMPARs in the Nacc core (Ferrario et al. 2011) and shell (Mameli et al. 2009) after extended access to cocaine self-administration. These results show that CP-AMPA accumulation in the Nacc is specifically linked to extended access

to cocaine self-administration, since it does not occur in rats treated with experimenter-given cocaine (McCutcheon et al. 2011) or following limited access to cocaine self-administration (Pierce and Wolf 2013). mGluR1 normally exerts a braking influence on CP-AMPA accumulation. A loss of mGluR1 tone may help enabling CP-AMPA accumulation during withdrawal (Loweth et al. 2014). The suggestion that mGluR1 stimulation might blunt cocaine-seeking behavior (McCutcheon et al. 2011) appears to be at odds with data indicating that group I mGluR antagonists attenuate the reinstatement of cocaine-seeking (Olive 2009). However, once CP-AMPA receptors are present in Nacc synapses, stimulation of mGluR1, as opposed to blockade of mGluR5, may be a more effective way to decrease excitatory transmission in the Nacc and reduce cocaine-seeking behavior (Pierce and Wolf 2013).

Group II mGluR2/3 are down-regulated after chronic experimenter-given cocaine administration and after nicotine self-administration (Xi et al. 2002). Decreased mGluR2/3 function could result from reduced protein expression, increased receptor phosphorylation and/or upregulated expression of activator of G protein 3 (AGS3) (Kalivas 2009). Similar up-regulation of AGS3 and inhibition of mGluR2/3 function is observed following chronic exposure to ethanol self-administration (Moussawi and Kalivas 2010). Decreasing expression of AGS3 with an antisense strategy in the Nacc core has been shown to inhibit the reinstatement of heroin-seeking (Yao et al. 2005). In a manner independent of AGS3, extended access to methamphetamine self-administration (6 h/day) followed by a period of abstinence inhibits mGluR2/3 signaling in the Nacc via down-regulation of mGluR2/3 surface expression in both the PFC and Nacc core (Schwendt et al. 2012). It has been suggested that these adaptations could be sensitive to environmental contexts as extinction training reversed the methamphetamine-induced alterations in mGluR2/3 expression observed in the Nacc core but not in the PFC (Schwendt et al. 2012).

mGluR1/5 and their intracellular binding protein Homer1b/c are down-regulated in the Nacc after withdrawal from chronic cocaine self-administration (Kalivas 2009). The possibility that the down-regulated Homer levels contribute to blunted mGluR1/5 signaling is supported

by the fact that restoration of Homer1b in the Nacc prevents acute cocaine injections inducing behavioral sensitization or the concomitant increase in extracellular GLU levels (Szumlinski et al. 2006). The potential importance of the down-regulation of mGluR1/5–Homer or mGluR2/3 signaling that occurs after chronic cocaine administration is indicated by the fact that inhibiting pharmacologically or deleting genetically mGluR5 as well as down-regulating Homer 1 levels attenuates cocaine, ethanol and nicotine-seeking and locomotor sensitization (Kalivas 2009). By contrast stimulation of mGluR2/3 signaling attenuates the reinstatement of nicotine-, cocaine- and heroin-seeking (Kalivas 2009). Notably, it has been proposed that drug-induced down-regulation of mGluR5–Homer signaling may be a compensatory effect, whereas the down-regulation of mGluR2/3 signaling may be a drug-induced effect that promotes drug-seeking (Kalivas 2009).

Metaplasticity in glutamate synaptic physiology The term metaplasticity is referred to changes in the ability to generate synaptic plasticity. Although addictive drugs have different pharmacological mechanisms, they ultimately remodel the brain's reward circuitry, which may explain behavioral changes associated with the addiction cycle. Changes in synaptic plasticity have been probed using two primary endpoints: induction of LTD or LTP and the relative strength of AMPA- and NMDA-induced synaptic currents. An increase in the AMPA/NMDA current ratio indicates that the synapses are in a relatively potentiated state, whereas a decrease indicates a de-potentiated state. It has been suggested that after several exposures to the drugs of abuse the initial plasticity in the VTA triggers changes in synaptic plasticity in the Nacc (Creed and Luscher 2013). The majority of Nacc neurons are MSNs which receive excitatory inputs from PFC, Hipp, BLA, VTA and thalamus (Sesack and Grace 2010). Indeed various changes induced by chronic drug treatment have been identified in the activity of MSNs in the Nacc. Acute cocaine exposure decreased AMPA/NMDA ratio in the Nacc whereas extended withdrawal from chronic cocaine produces an increase in the AMPA/NMDA ratio (Kourrich et al. 2007). Although the increase in AMPA/NMDA ratio would predict enhanced rather than reduced LTD, the induction of both LTP and LTD after chronic cocaine self-administration

were attenuated (Moussawi et al. 2011). A possible explanation for the bidirectional loss of synaptic plasticity is that system Xc- provides GLUergic tone onto mGluRs, and presynaptic mGluR2/3 and postsynaptic mGluR5 regulate the expression of LTP and LTD, respectively (Kalivas 2009). Thus, the tone on mGluRs provided by system Xc- facilitates the induction of both LTP and LTD, and the downregulation of system Xc- by chronic drug administration contributes to the bidirectional loss of synaptic plasticity.

1.3.5 Pharmacotherapies acting on glutamate

The approved medications for drug addiction have been used for helping people to stay abstinent, but the high relapse rate highlight the need of more efficacious treatment (WHO, 2012). Compounds that act on GLUergic system can be effective for different types of addictive drugs. In the next Section I will review clinical data concerning the efficacy of medications that acting on GLUergic transmission have shown promising results across different types of drugs.

Acamprosate is a pharmacological treatment approved to facilitate the maintenance of abstinence in alcohol dependent subjects (Plosker 2015). Although its mechanism of action is not completely understood, preclinical data suggest that it may interact with both GLUergic and GABAergic systems (Plosker 2015). In particular, acamprosate seems to prevent the hyper-GLUergic activity in the brains of alcohol-dependent patients by acting on NMDARs and mGluR5 (Spanagel and Heilig 2005). Few data are available on the use of acamprosate in other type of addictions. Recently a small double-blind placebo-controlled trial on cocaine-dependent patients, showed no effect of acamprosate on cocaine use, craving, or withdrawal symptoms compared to placebo (Kampman et al. 2011).

D-cycloserine (DCS) is an NMDARs partial agonist that activate the glycine binding site and enhances Ca^{2+} influx through these receptors without causing neurotoxicity. DCS activating NMDARs may induce LTP, which is thought to play a key role in associative learning (Martina et al. 2004). DCS facilitate the extinction of fear responses in anxiety disorder patients during cue exposure therapy (CET) in numerous clinical studies (Olive 2010). On the basis of this

evidence, DCS has been used to facilitate extinction on neural reactivity to drug cues. Nevertheless, DCS activity on drug addiction is not proved yet. Preliminary findings suggest that DCS may be beneficial in augmenting the effects of Cue Exposure Therapy (CET) during attempts at cessation of cigarette smoking (Santa Ana et al. 2009), while in a double-blind, placebo-controlled study DCS had no effect on craving for smoking in cocaine dependent subjects (Yoon et al. 2013). Moreover a randomized, placebo-controlled study showed that DCS did not facilitate extinction of cocaine cue reactivity in cocaine-dependent individuals (Price et al. 2013). These contrasting results may be explained by the fact that if DCS is given before CET it may increase craving (induced by drug-related memories), and for this reason it may delay the acquisition of the inhibitory learning typically associated with extinction observed in the placebo group (Prisciandaro et al. 2013). In contrast, DCS has shown promising results for the treatment of alcohol dependence since in two placebo control studies DCS-treated subjects reported significant short-term reductions in drinking (Kiefer et al. 2015; MacKillop et al. 2015).

Gabapentin is approved by Food and Drug Administration (FDA) as an anticonvulsant for partial epilepsy. It has multiple mechanisms of action, including inhibition of presynaptic voltage-gated Na^+ and Ca^{2+} channels, thereby inhibiting the release of neurotransmitters including GLU (Rogawski and Loscher 2004). The inhibitory activity on GLU transmission was evaluated for the treatment of dependence to several types of drugs. Numerous studies have shown that gabapentin is efficacious in alleviating the somatic symptoms, relapse-associated symptoms and craving in alcohol dependence (Mason et al. 2014) and methamphetamine use and craving (Ling et al. 2012). Moreover, a double-blind, placebo-controlled clinical trial was conducted in 50 male and female outpatients revealed that relative to placebo, gabapentin significantly reduced cannabis use as measured both by urine toxicology and significantly decreased withdrawal symptoms as measured by the Marijuana Withdrawal Checklist (Mason et al. 2013). Gabapentin was also studied in heroin dependence where 60 outpatients under treatment with methadone reported a reduction in the cumulative dose of methadone during gabapentin co-administration (Moghadam and Alavinia 2013). Although some studies have also

shown that gabapentin decreases active cocaine use and craving (Olive et al. 2010) caution is needed since a Cochrane review reported no evidence for the clinical use of anticonvulsant medications in the treatment of cocaine dependence (Minozzi et al. 2015). In summary, high doses of gabapentin seem to have some clinical value especially for treating alcohol dependence.

Memantine is a NMDAR antagonist used in the treatment of Alzheimer's disease. Memantine also blocks the serotonin type 3 receptor (5-HT₃) and nAChRs. Memantine reduced withdrawal symptoms in alcoholics (Krupitsky et al. 2007) although a larger placebo-controlled study indicated it does not reduce on-going drinking behavior (Evans et al. 2007). Memantine has also been reported to decrease the subjective effects of cigarette smoking (Jackson et al. 2009). The effect of low-doses of memantine were studied in opioid dependence and a randomized, double-blind clinical trial showed that the memantine-treated group required slightly lower methadone than the placebo group (Lee et al. 2015) while also improving cognitive performance (Chang et al. 2015). In summary, even though memantine efficacy for treating addiction to other drugs of abuse remains unknown it seems promising for alcohol- or opiate-dependent patients.

Modafinil is an analeptic drug approved for the treatment of narcolepsy. More recently it was also used as a cognition-enhancing agent (Greely et al. 2008). Modafinil acts by stimulating adrenoceptors as well as suppressing GABA release. Moreover, it can also elevate extracellular levels of GLU in numerous brain regions including the striatum (Ferraro et al. 1998). Although it may seem counterintuitive, the resulting increase in GLU level can activate mGluR2/3 thereby reducing GLU transmission (Olive et al. 2010). For this reason, modafinil has been recently studied for the treatment of drug addiction. A randomized, double-blind, placebo-controlled, crossover study reported that modafinil can control impulsivity in alcohol-dependent patients thus, reducing one of the phenotype associated with drug addiction (Schmaal et al. 2013). Moreover, high doses of modafinil can be used to reduce cocaine use (Nuijten et al. 2015).

Topiramate is an anticonvulsant with several mechanisms of action, including inhibition of presynaptic voltage-gated Na⁺ and Ca²⁺ channels, as well antagonism of AMPA/kainate subtype of GLU receptors. In addition to the attenuation of alcohol withdrawal symptoms it also has

beneficial effects in cigarette smokers, where some small studies have reported it can promote abstinence from smoking or reducing overall smoking behavior (Kampman et al. 2013). Another double-blind, randomized, placebo-controlled study show that topiramate is more efficacious than placebo at increasing cocaine non-use in cocaine dependent subject (Johnson et al. 2013). Similarly, topiramate does not appear to promote abstinence in methamphetamine-addicts that were still assuming the drug during the treatment while it reduced the relapse rate in those treated during abstinence (Elkashef et al. 2012). Recently it has been proposed that topiramate together with cognitive-behavioral therapy is a promising treatment for cocaine addiction (Kim and Lawrence 2014). Nevertheless, one limitation of the use of topiramate is its great variety of adverse effects including paresthesia, anorexia, difficulties with memory or concentration, and taste disturbances (Markind 1998).

N-Acetylcysteine (N-AC) has a dedicate Section (Section 1.5), here I will summarize only the clinical results obtained on drugs of abuse other than nicotine. A recent systematic review about the clinical trials involving of N-AC has clearly reported that it can be effective in many psychiatric and neurological disorders including drug addiction (Deepmala et al. 2015). The more promising effect of N-AC seems to be on cocaine addiction where it reduces cocaine craving (LaRowe et al. 2006; Amen et al. 2011) and the days of abstinence (LaRowe et al. 2013). Contrasting effects have been reported for the use of N-AC in cannabis and methamphetamine dependence. In cannabis addicts N-AC reduce the use of marijuana (Gray et al. 2012) while no effects were present on craving (Roten et al. 2013). In methamphetamine addicts N-AC was effective only during treatment with no carryover effects (Mousavi et al. 2015).

Ketamine a non-competitive antagonist of NMDARs (Parson 1995), was recently shown to have promising effects 24 h post-infusion when given at low doses on motivation to quit cocaine and on cue-induced craving, (Dakwar et al. 2014).

Oxytocin acts as a neuro-hormone on peripheral targets to promote uterine contraction and lactation. Nevertheless, oxytocin is also centrally released in the brain by various mechanisms thus, interacting with several neurotransmitters including DA and GLU (Lee et al. 2016). In

particular, oxytocin, by inhibiting GLU release from PFC, seems to be a potential treatment for drug addiction (McGregor and Bowen 2012).

1.4 Nicotine

Among the approximately 4000 substances found in tobacco-containing products, nicotine is the principal psychoactive component that reinforces smoking behavior

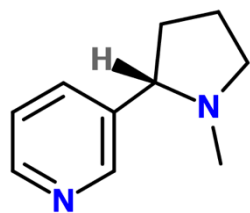


Figure 1.4.1
Nicotine molecule

(Henningfield and Goldberg 1983). The 1988 U.S. Surgeon General's report - *The Health Consequences of Smoking: Nicotine Addiction* - carries

three major conclusions: cigarettes and other forms of tobacco are

addictive, nicotine is the drug that causes this addiction, and the

pharmacological and behavioral processes that determine tobacco

addiction are similar to those that determine addiction to drugs such as heroin and cocaine. For

this reason, due to its addictive potential, nicotine has long been removed from pharmacopoeias

and from medical practice.

1.4.1 History

There are over sixty species of *Nicotiana*. Apart from a few which appear to be native to

Australia, most are indigenous to America. *Nicotiana*

tabacum (Figure 1.4.2), the plant now raised for

commercial tobacco production, is probably of

South American origin and *Nicotiana rustica*, the

other major species, which was carried around the

world, came from North America (Charlton 2004).

Tobacco plants were used well before the discovery

of new world as testified in a Mayan pottery vessel

(dating to the 11th century) that depicts a man

smoking a roll of tobacco plants (Figure 1.4.2).

Nevertheless, it is only after Colombo's expeditions

Figure 1.4.2. The first published illustration of *Nicotiana tabacum* by Pena and De L'Obel, 1570–1571.



to the new world that tobacco grew in popularity. Columbus found Native Americans growing and using tobacco, sometimes for its pleasurable effects but often for treatment of various



Figure 1.4.3. Madrid Codex (circa 900–1521 AD), one of the few survived Mayan’s historical records, has multiple images of people smoking.

illnesses. It was in 1500 that the notion of tobacco as a panacea became prevalent. In that year, a Portuguese explorer, Pedro Alvarez Cabral, in Brazil, reported the use of the herb tobacco for treating ulcerated abscesses, fistulas, sores, inveterate polyps and many others, and said it was called the holy herb because of its powerful virtue in desperate cases (Charlton 2004). Jean Nicot was a French ambassador to Portugal. While in Lisbon Nicot was presented with an herb by the keeper of

a prison he was visiting. It was described as a strange plant brought from Florida. Nicot learnt the medical properties of tobacco that would ultimately make him famous (indeed the main active ingredient, *nicotine*, is derived from his name). In 1560, Nicot sent tobacco to the queen of France Catherine de Medicis, including the medical application he discovered (from cancer to gout to headache). It is believed that Nicot by crushing the leaves into a powder cured the queen’s headache making this remedy famous in the French court and eventually Europe. Tobacco use became such widespread that early in the 17th century King James I wrote the first treatise to restrict its use: *“a custom loathsome to the eye, hateful to the nose, harmful to the brain, dangerous to the lungs, and in the black stinking fumes thereof, nearest resembling the horrible Stygian smoke of the Pit that is bottomless”*. Although the parliament refused to ban tobacco, the taxes imposed on it remained (from Iversen et al. 2009).

When tobacco trade grew the soil used for cultivation becomes exhausted, requiring 20 years to become revitalized. The need of new planting land induced U.S. farmer to move west where they encountered light sandy soil that produced a thin, lightly flavored and yellow tobacco leaf the famous *Bright leaf*. Legend has it that the flue curing process, the peculiarity of bright leaf, was initially discovered by accident in 1939 by a slave who added charcoal to increased heat.

The absence of smoke and the increased heat turned leaf a bright yellow and the *Bright leaf* or *Bright Yellow* tobacco was born (Iversen et al. 2009).

Until the discovery of flue curing of American Tobacco, most tobacco was consumed by smoking cigars or pipes or by chewing. The use of tobacco vastly increased after the discovery of the curing method and the mass manufacturing of cigarettes. When Spanish troop returned from the Crimean war (1853-1856), they brought with them the habit of smoking small sticks of tobacco wrapped in paper, what we know now as cigarette.

1.4.2 Pharmacokinetics

Absorption The most used form of nicotine is clearly cigarette, although also cigars, pipes, snuff, suns and chewing are sold. Also smokeless products contain nicotine along with several other toxic ingredients (Hatsukami 2008). Depending on the brand, each cigarette contains around 6-11 mg of nicotine resulting in blood concentration of 1-3 mg in the smoker (Benowitz and Hatsukami 1998). Smokers may feel the central stimulant effect of one puff within 7-10 second since the arterial flow delivers the dose directly from the lungs to the brain. Through cigarettes, nicotine, reach the central nervous system (CNS) with a higher speed than intra venous (i.v.) infusions, rapidly activating the mesocorticolimbic system that generates the rewarding properties described by smokers (Hukkanen et al. 2005). Cigars and pipes deliver nicotine more slowly by dissolution into the saliva and mostly absorbed into the buccal mucosa. These forms of tobacco have a venous blood level of nicotine that peaks 20-30 minutes after commencing to smoke and decline more slowly (Iversen et al. 2009). Snuff and chewing tobacco have much higher nicotine content than that used for cigarettes, while the absorption rate is similar to the one of cigars and pipes.

Acute intoxication is fast and adverse effects can be attributed to the stimulation of the adrenal medulla releasing adrenalin as well as direct stimulation of the sympathetic ganglia. However, repeated use leads to rapid tolerance due to receptor desensitization. Symptoms include nausea, vomiting, salivation, abdominal pain and generalized weakness. While breathing

difficulties arise later with a collapse followed by convulsions and death, which occurs within a few minutes from respiratory arrest (Iversen et al. 2009). The lethal dose of nicotine in the adult is 60 mg (Henningfield et al. 2009) .

Distribution The levels of nicotine that reach the CNS and other organs depend on the dose and type of assumption with a steady-state volume of distribution averaging 2.6 L/kg. Cigarette smoke brings nicotine to lung where it is absorbed. Through the pulmonary veins nicotine reaches the left atrium than the left ventricle and finally goes into the arterial circulation through which it can reach the CNS. Nicotine's half-life in the blood after an i.v. infusion or a cigarette is around 2 h (Benowitz et al. 2009).

Metabolism Nicotine is extensively metabolized by the liver. The most important metabolite is cotinine (in humans 70/80% of nicotine is converted to cotinine). This transformation involves two steps. First, the cytochrome CYP2A6 produces nicotine-1(5)-iminium ion, which is in equilibrium with 5-hydroxynicotine. Second, the cytoplasmic aldehyde oxidase produces cotinine (Shigenaga et al. 1988). Another primary metabolite is Nicotine N-oxide. About 4–7% of nicotine is converted in this form by flavin-containing monooxygenase 3. Nicotine N-oxide is not further metabolized, except by reduction back to nicotine in the intestines, which may lead to recycling of nicotine in the body. In addition, nicotine is metabolized by two non-oxidative pathways, methylation of the pyridine nitrogen giving nicotine isomethonium ion (also called N-methylnicotinium ion) and glucuronidation (Benowitz et al. 2009).

Some factors may alter nicotine metabolisms: hepatic blood flow increases about 30% and nicotine clearance increases about 40% after a meal (Gries et al. 1996); menthol and grapefruit juice are moderate inhibitor of CYP2A6, therefore may inhibit metabolism of nicotine to cotinine and nicotine (Gelal et al. 2005; Hukkanen et al. 2006); oral contraceptive by reducing cytochrome activity, induced increases in nicotine and cotinine clearance by 28 and 30%, respectively (Johnstone et al. 2006). Nicotine clearance is also negatively influenced by age since in the elderly (age>65) nicotine clearance is reduced (Molander et al. 2001), and neonates have

diminished nicotine metabolism, with a nicotine half-life three/four times longer than adults (Dempsey et al. 2000).

Excretion Nicotine is excreted in the kidney by glomerular filtration and tubular secretion, with variable re-absorption depending on urinary pH. Renal clearance account for the elimination of about 5% of total clearance, but if the urine pH is acid, nicotine is mostly ionized and tubular re-absorption is reduced while in alkaline urine, a larger fraction of nicotine is unionized, allowing net tubular re-absorption (Benowitz et al. 2009).

1.4.3 Nicotinic acetylcholine receptor

The molecular basis for the behavioral and physiological effects of nicotine is binding of the drug to nicotinic acetylcholine receptors (nAChRs) (Romano and Goldstein 1980), both in the brain and in the periphery at the level of neuromuscular junction and autonomic ganglia. nAChRs are ionotropic receptors with a pentameric structure composed of four different subunits (α , β , γ , δ), with each subunit having four transmembrane domains. The nAChR channel is made up of the second transmembrane segments from each subunit (Figure 1.4.4).

Acetylcholine (ACh) binding to the extracellular domain leads to opening of the channel to allow the influx of Na^+ and K^+ (Karlín 2002). Binding of ACh to the nAChR is communicated to the ion channel extremely rapidly within tens of microseconds (Sine and Engel 2006).. ACh is normally hydrolyzed rapidly by acetylcholinesterase (AChE). However, if AChE is inhibited, or exogenous drugs such as nicotine (that are not metabolized locally) are applied, the responsiveness of nAChRs diminishes over time, despite the sustained presence of the agonist. This phenomenon is referred to as receptor desensitization (Dani and Balfour 2011).

The receptor subtype $\alpha_3\beta_4$ and $\alpha_3\beta_2$ are most abundant in autonomic ganglia while the subtype $\alpha_4\beta_2$ is mostly found in the brain. α_7 and α_9 subunits often form homomeric receptors with some distinctive properties, including very high permeability to Ca^{2+} and a propensity to desensitize rapidly. α_4 and β_2 subunits are relatively abundant in cortex, thalamus and DA

neurons, whereas the α_7 subunit is highly expressed in hippocampus (Hipp). Moreover α_7 nAChRs are more commonly associated with glutamate (GLU) terminals and γ -Aminobutyric acid (GABA) neurons where they contribute to synaptic plasticity, including long-term potentiation (LTP) (Dajas-Bailador and Wonnacott 2004). The nAChRs and the mechanisms that mediate the reinforcing effect of nicotine are discussed in next Section.

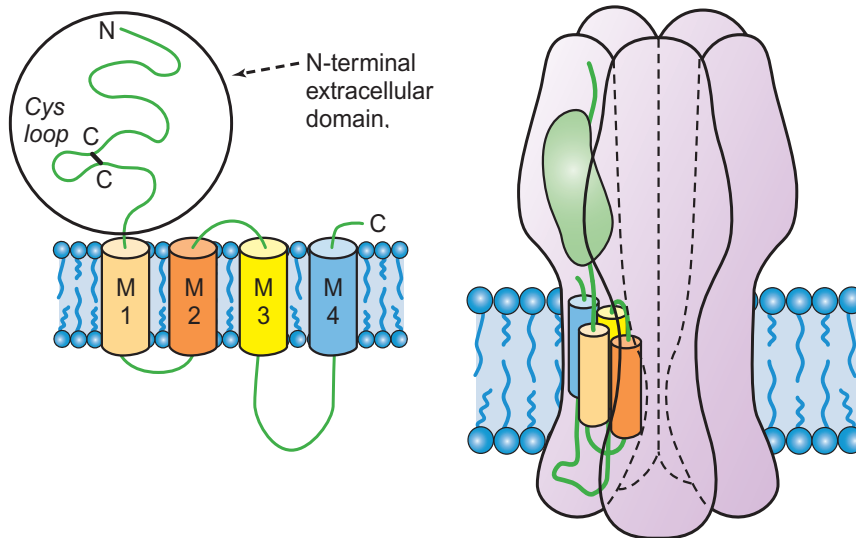


Figure 1.4.4. Structural features of the nicotinic acetylcholine receptor (nAChR).

Left: in the N terminus cys loop is common to all these subunits.

Right: schematic of an assembled nAChR, with five subunits arranged to create a central ion channel, lined by M2. (adapted from Fisher and Barik 2009)

1.4.4 Pathophysiology of nicotine addiction

The rapid rate of nicotine absorption and high amount of nicotine attained in the brain from smoking are two crucial factors that promote nicotine addiction.

Acute rewarding effects The systemic administration of nicotine promotes DA release by acting on nAChRs that are abundantly expressed in the ventral tegmental area (VTA) and Nacc (Figure 1.4.5). This increase of DA levels mediates the acute rewarding effect produce by nicotine. In the VTA, nAChRs are found on DAergic cell bodies ($\alpha_4\alpha_5\beta_2$, $\alpha_4\alpha_6\beta_2$, α_7), GABAergic cell bodies ($\alpha_4\beta_2\alpha_5$, α_7) and terminals ($\alpha_4\beta_2$) as well as GLUergic terminals (α_7) while in the Nacc are found on DAergic ($\alpha_4\beta_2$, $\alpha_4\alpha_5\beta_2$, $\alpha_4\beta_2\beta_3$, $\alpha_4\alpha_6\beta_2\beta_3$), GABAergic and GLUergic terminals ($\alpha_4\beta_2$) (Pierce and Kumaresan 2006). The systemic administration of nicotine increases extracellular DA levels in the Nacc (Picciotto et al. 1998) particularly in Nacc shell (Pontieri et al. 1996). DA release in the Nacc mainly results from direct stimulation of

nAChRs on DAergic terminals (Di Chiara 2000).

The main source of DA release in Nacc is through the stimulation of nAChRs in the

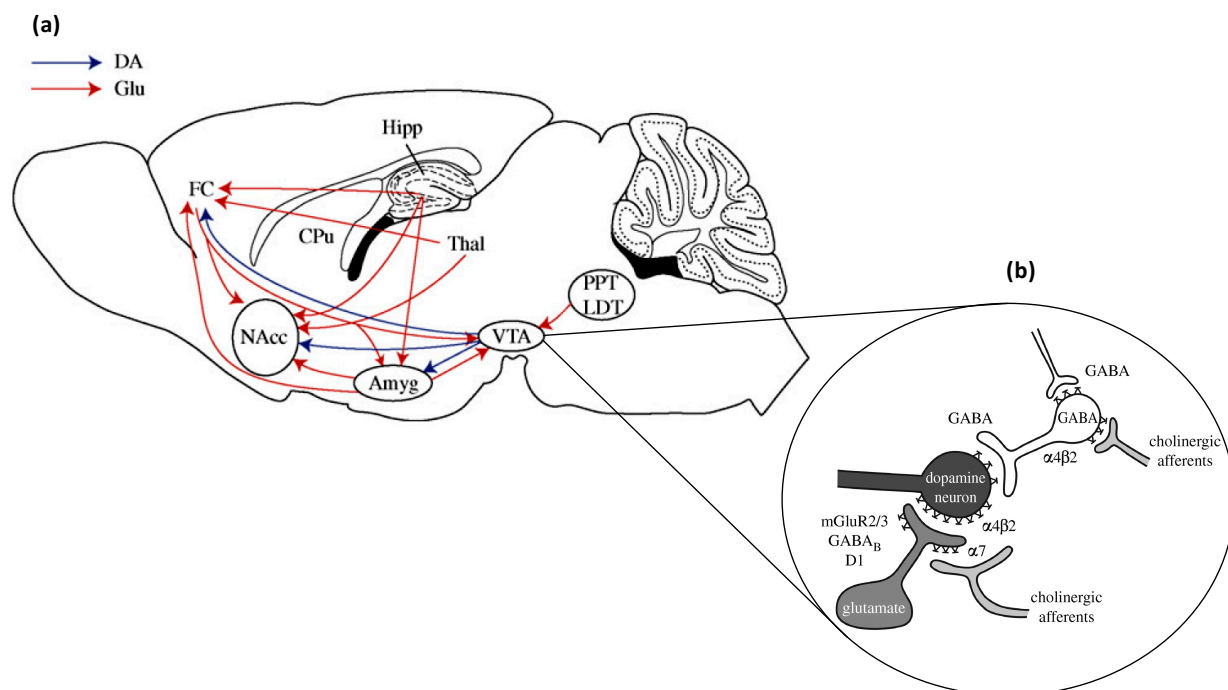


Figure 2.5. (a) The mesolimbic dopaminergic system (blue arrows) and glutamatergic connections (red arrows). (adapted from Gass and Olive 2006). Thalamus (Thal), Frontal cortex (FC), caudate putamen (CPu), Latero dorsal and pedunculopontine tegmentum (LDT and PPT) **(b)** Nicotine–acetylcholine–glutamate–GABA–dopamine interactions in the VTA. Schematic depicting neurotransmitter interactions in the VTA, which are hypothesized to be critically involved in mediating various effects of nicotine with relevance to nicotine dependence and withdrawal. Neuroadaptations have been shown to develop to several of these receptor and transmitter systems with the development of nicotine dependence. Adapted from Mansvelder and McGehee (2002).

VTA (Nisell et al. 1997). nAChRs in the VTA desensitize rapidly (Pidoplichko et al. 2004) while nicotine-induced DA release in the Nacc can persist for over one hour (Di Chiara and Imperato 1988) and this can be explained by the finding that nicotine also increases GLU and GABA transmission in the VTA but GABA activation desensitize rapidly (Mansvelder et al. 2002). Thus, following nicotine administration there appears to be a net shift in the balance of excitatory and inhibitory inputs to DAergic neurons in the VTA such that inhibitory GABAergic transmission is decreased and excitatory GLUergic transmission is increased. Since GLUergic terminals in the VTA mainly express nAChRs including α_7 subunits, it has been proposed that α_7 subunit-containing receptors in the VTA desensitize more slowly than other nAChRs resulting in prolonged GLU release and stimulation of DAergic neuronal activity in the VTA

(Mansvelder et al. 2002). However, α_7 subunit-containing receptors in the VTA are not solely responsible for nicotine-induced DA release in the striatal complex since this effect is attenuated also in mutant mice lacking nAChRs containing β_2 (Picciotto et al. 1998) or α_4 subunits (Marubio et al. 2003).

Nicotine increases also GLU release by acting on presynaptic nAChRs on GLUergic terminals in various brain areas, including the VTA, Nacc, PFC and Hipp.

Considerable evidence suggests that these actions partly mediate the reinforcing effects of acute nicotine. Specifically, in the VTA, the blockade of postsynaptic mGluR5 or NMDARs decreased intravenous nicotine self-administration in rats and mice without altering the responses for food reinforcement under similar schedules of reinforcement (Markou 2008). Finally, mGluR2/3 receptor agonist, injected systemically or directly into the posterior VTA or the Nacc shell, dose-dependently decreased nicotine self-administration at doses that had no effect on responding for food (Markou 2008).

Long-lasting modifications The neuroadaptations that occur in response to chronic nicotine exposure induce tolerance to nicotine effects. Thus, within hours upon cessation of nicotine exposure, a nicotine withdrawal syndrome emerges characterized by depressed mood, irritability, mild cognitive deficits and physiological symptoms (Shiffman et al. 2004). The avoidance of these withdrawal syndrome, as well as the positive subjective effects of nicotine, motivates nicotine use. In addition, as with other psychomotor stimulants learning processes also contribute to nicotine dependence. Environmental stimuli associated with either the positive subjective effects of nicotine or the induction of nicotine withdrawal motivate nicotine-seeking and eventually drug consumption (Paterson and Markou 2004; Kenny and Markou 2006)

GLU plays a critical role in the long-term effects of nicotine (and other drugs of abuse, see Section 1.3.4). With chronic exposure to nicotine and the development of nicotine dependence, adaptations in GLUergic system at the level of the mesocorticolimbic system occurs. A decrease in mGluR2/3 receptor function in the Nacc indicates impaired negative feedback control on

GLUergic terminals (Liechti et al. 2007), possibly to counteract the decreased GLU transmission that characterizes early nicotine withdrawal (Wang et al. 2007). Additionally, decreased expression levels of NMDAR (GluN2A, GluN2B) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) (GluA2) subunits were observed in the PFC (Kenny et al. 2009).

After nicotine self-administration, rats have also an altered GLU homeostasis (see also Section 1.3.3) in the Nacc produced by a decreased expression of the cystine/GLU exchanger (system Xc-) and the glial GLU transporter 1 (GLT-1) (Knackstedt et al. 2009). Moreover, following nicotine self-administration in the Nacc, rats have an increased head diameter of dendritic spines and increased levels of AMPAR (mainly GluA1 subunit) and NMDAR (GluN2A and GluN2B subunits) (Gipson et al. 2013). It has been hypothesized that nicotine-induced neuroadaptation at the level of GLU system in the Nacc may favor nicotine relapse, a finding supported by the observation that nicotine-related cues increased GLU release in the Nacc core in abstinent rats (Gipson et al. 2013).

1.4.5 Treatments for nicotine addiction in human

Currently the greatest quit rates are achieved when non-pharmacologic support is combined with pharmacotherapy. In particular nicotine replacement therapy (NRT), in combination with behavioral support was found to be the most effective strategy (Thurgood et al. 2016). However, many smokers do not accept referral to a group program (Rennard and Daughton 2014). There are four specific issues that the clinician should anticipate with each quit attempt in order to improve the relapse prevention rate: withdrawal symptoms, cravings, depression, and weight gain (Rennard and Daughton 2013).

Medications approved as aids to smoking cessation comprise nicotine replacement (NRT), bupropion, and varenicline and two others available off-label such as nortryptiline and clonidine, which have documented efficacy and are recommended as alternative therapies in current guidelines.

There are different formulations of NRTs, the transdermal systems are available over the counter while nasal spray and a nicotine inhaler are available with a prescription. Recently other preparations, like nicotine toothpicks and e-cigarettes, have been developed and marketed although their efficacy and safety in smoking cessation remain to be properly investigated.

Bupropion appears to act on a number of brain pathways also implicated in dependence. It is known to act as an inhibitor of DA and noradrenaline transporters (Ascher et al. 1995), and has been shown to also act as an antagonist of nAChRs (Slemmer et al. 2000). It is used as an antidepressant but is effective also as an aid for smoking cessation. Bupropion approximately doubles quit rates compared with placebo and when combined with NRT seems to be more effective than either agent alone (Jorenby et al. 1999). Side effects include insomnia, xerostomia, and headaches. Bupropion is associated with weight loss in some patients and may attenuate the usual weight gain seen after quitting smoking (Parsons et al. 2009).

Varenicline is a partial agonist at the $\alpha_4\beta_2$ nAChR (Coe et al. 2005). Thus, it partially activates the receptor thereby reducing withdrawal symptoms and when nicotine is taken it reduces nicotine reinforcement effects. Both reduction in withdrawal symptoms and reduction in the rewarding effects of smoking a cigarette have been reported in clinical trials where it improves the success in quitting from up to 4-fold compared to control (Cahill et al. 2012). The most common adverse reactions associated with varenicline are nausea, insomnia, visual disturbances, syncope, and skin reactions. Varenicline has the same boxed warning as bupropion, indicating that patients and their caregivers should be alerted to the possibility of neuropsychiatric symptoms, and patients should be monitored for changes in behavior, hostility, agitation, depressed mood, suicidal ideation, and suicide attempts (Rennard and Daughton 2014).

The following two off-label medications have documented efficacy for smoking cessation.

Nortriptyline is a tricyclic antidepressant. Its efficacy in aiding smoking cessation is supported by both individual studies and meta-analyses (Stead and Lancaster 2012). Major adverse effects of nortriptyline include drowsiness and dry mouth. As with other tricyclics, central nervous system and cardiovascular effects, including arrhythmias, may occur.

Clonidine is an α_2 -adrenergic agonist used to treat hypertension. Several clinical trials have shown trends toward efficacy as an aid to smoking cessation, which is supported by a meta-analysis (Gourlay et al. 2004). The most common important adverse effects are drowsiness, fatigue, xerostomia, and postural hypotension.

New technologies to help in engagement and treatment

There has been an explosion in technological interventions to help people quit tobacco use, which includes smartphone apps, websites and social media. These and other technology options are advancing faster than the evidence base supporting these interventions, and there is a need to consider how clinicians might evaluate the best option for their patients (Das et al. 2016). For example, the positive effects of text messaging-based interventions offering a mixture of motivational messages and quitting advice were reported in a Cochrane review of six studies and a subsequent analysis of 13 studies which found a significant 36 % improvement in quit rates with text messaging methods and outcomes similar to phone call in quit-line services (Spohr et al. 2015).

1.5 N-acetylcysteine

N-acetylcysteine (N-AC) is a derivative of the non-essential amino acid cysteine with an acetyl group attached to its nitrogen atom. N-AC is a well-tolerated and safe medication that has been used for many decades all across the world as an over-the-counter nutritional supplement with antioxidant properties (Berk et al. 2013). Although N-AC is mostly known as a mucolytic agent in view of its central action it has been recently studied in many diseases/disorders and in particular in psychiatric and neurological disorders (Deepmala et al. 2015).

1.5.1 Chemistry

N-AC is a thiol therefore it can be oxidized by many reactive species including radicals. Moreover, N-AC can also react as a nucleophile since the presence of the N-acetyl and carboxylate groups [instead of the respective $-\text{NH}_3^+$ and $-\text{CONH}^-$ moieties in glutathione (GSH) and peptides] both stabilize the high electron density and the concomitant high alkalinity and strong reducing power of the thiolate site: in fact it reacts rapidly with $\cdot\text{OH}$, $\cdot\text{NO}_2$, $\text{CO}_3^{\cdot-}$, thiyl radicals and nitroxyl (HNO) (Noszal et al. 2000). In the plasma, N-AC can be found also as cysteine and as the cysteine dimer, cystine.

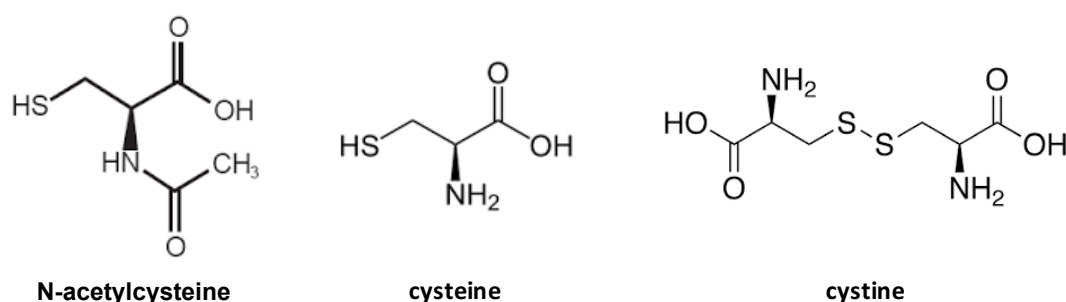


Figure 1.5.1. Chemical structures of N-acetylcysteine and its derivate

Redox exchange reactions between N-AC, cysteine and cysteine proteins in the plasma rapidly produce disulphides with proteins and also N-AC–cysteine and N-AC–N-AC dimers (Samuni et al. 2013). N-AC is a strong reducing agent since N-AC thiol–disulfide pair has a redox potential higher than those of GSH/glutathione disulfide (GSSG) and cysteine/cysteine. For

this reason, N-AC can easily reduce disulfide bonds in proteins, thus reducing also the disulfide bonds in cross-linked mucous proteins thus explaining N-AC's, mucolytic activity (Samuni et al. 2013).

1.5.2 Pharmacokinetic

Absorption, bioavailability N-AC reaches the peak plasma concentration (C_{max}) within 120 min (Olsson et al. 1988). N-AC is rapidly oxidized before it reaches the general circulation and the bioavailability is 4% for the reduced form and 9.1% for the total drug (Olsson et al., 1988). The relatively low bioavailability of N-AC is associated with its N-deacetylation in the intestinal mucosa and first pass metabolism in the liver. The fact that N-AC rapidly forms disulphides bonds in the plasma prolongs the presence of the drug in plasma for up to 6 h (MacNee et al. 1991). A single oral dose of N-AC increases N-AC levels in plasma without accumulating (Borgstrom and Kagedal 1990) although its oxidized metabolites can accumulate (Cotgreave et al. 1987).

Distribution The volume of distribution of total N-AC ranges from 0.33 to 0.47 L/kg highlighting its binding to plasma proteins (Olsson et al., 1988). After 2 h N-AC distributed also in the kidney, liver, adrenal gland, lung, spleen, blood, muscle, brain and urine in decreasing concentration (Sheffner et al. 1966).

Metabolism and excretion In plasma and tissue N-AC free form and metabolites bound to the proteins by disulphide linkages, and a fraction incorporated into protein peptide chains (Figure 1.5.2) (De Caro et al. 1989). From 1 h after administration, 50% of total N-AC in the plasma is present in a covalent protein-protein bound (Olsson et al., 1988). The major urinary excretory product is inorganic sulfate together with taurine (Sheffner et al. 1966).

Renal clearance of N-AC is around 0.2 L/h/kg and approximately 70% of plasma clearance was non-renal (Olssona et al., 1988). The total N-AC concentration with intravenous administration has a total clearance of 0.11 L/h/kg and a $t_{1/2}$ of 5.6 h (Olsson et al., 1988).

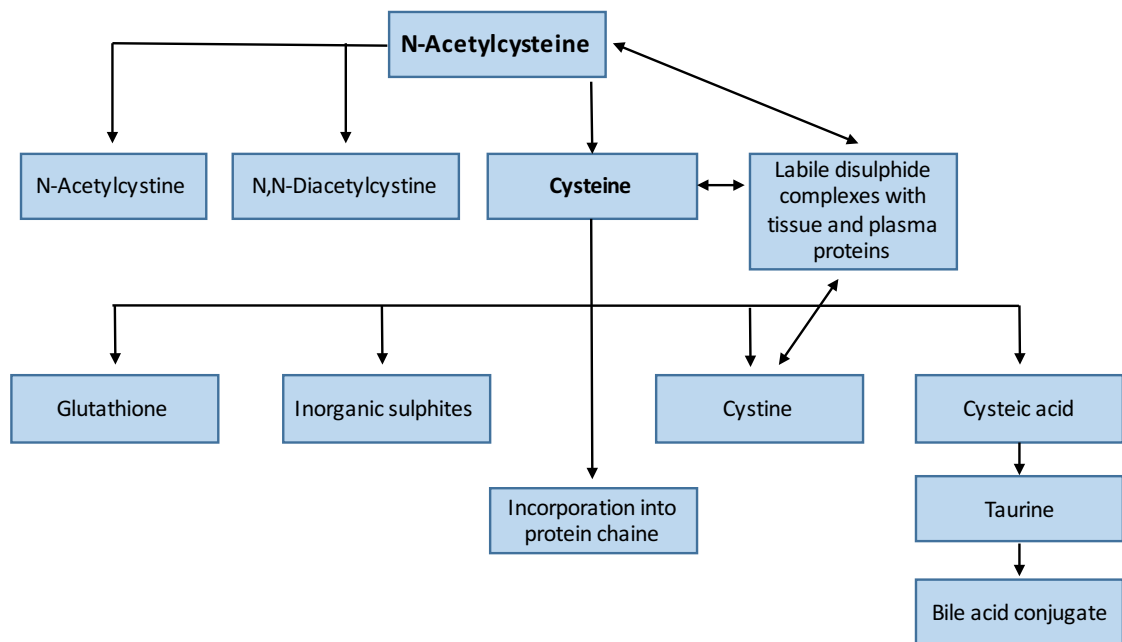


Figure 1.5.2. N-AC metabolic pathway. Adapted from DeCaro et al. (1989).

Side effects and drug interactions Generally N-AC has mild side effects. The most common are vomiting and diarrhea. A variety of symptoms are noted with one or more doses of N-AC but none more than 5% of the time (increased blood pressure, chest pain, hypotension, rectal bleeding, respiratory distress, headache, lethargy, fever and skin allergy) (Miller and Rumack 1983). The lethal dose 50 (DL50) after oral administration is > 10000 mg/kg (rat and mouse). After intravenous administration the DL50 is 4600 mg/kg (mouse) and 2800 mg/kg (rat) (Johnston et al. 1983). N-AC may inactivate antibiotics (tetracycline, eritromicine, amphotericin B) if given in the same preparation.

1.5.3 Biological activities

The fact that N-AC interact with several important pathways makes it difficult to understand the mechanisms underlying the therapeutic applications of N-AC. Physiological functions and therapeutic effects of N-AC are mainly associated with its antioxidant activity through maintaining the levels of intracellular GSH. Nevertheless, N-AC has been shown to interact with various metabolic pathways although these are only partially understood.

The main cellular activity of GSH is the detoxification of electrophilic xenobiotics to which

GSH can be conjugated by the action of glutathion-s-transferases. GSH is synthesized by two ATP-depended reactions, γ -glutamylcysteine synthetase (that combine GLU and cysteine) and glutathione synthetase (that add glycine). Cysteine concentration is the limiting step in GSH synthesis. This is particularly important in the CNS where contrary to other cells type cysteine is transported through astroglial cells membrane in a sodium independent manner (Figure 1.5.3) in exchange for GLU by cystine–GLU exchanger (system Xc-) (Cho and Bannai 1990).

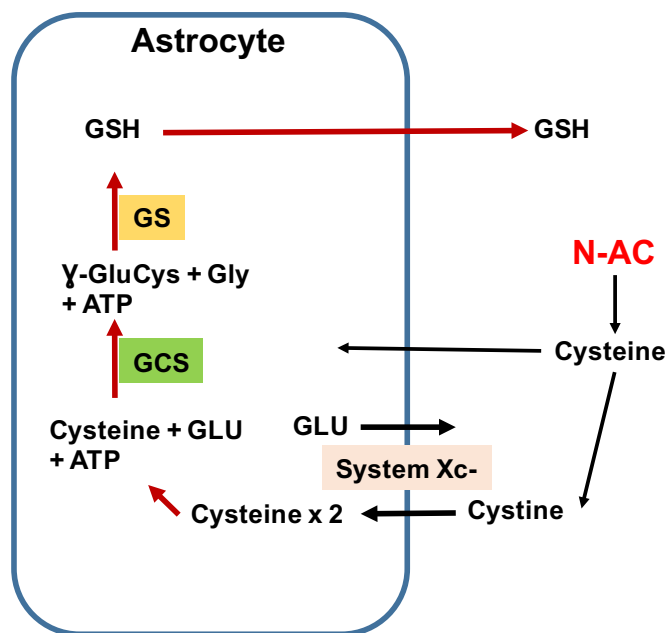


Figure 1.5.3. Schematic representation of the activity of N-acetylcysteine (N-AC) on astrocyte.

Glutamate (GLU), Glutathione (GSH), γ -glutamylcysteine synthetase (GCS), GSH synthetase (GS).

N-AC is a source of cysteine since it can be de-acetylated to cysteine, which is rapidly oxidized to cystine (Bannai et al. 1989) thus transported by system Xc- into the cells, where it is reduced back to cysteine and used for GSH synthesis. Although cysteine can be absorbed quite easily from the systemic circulation, it has a toxic effect that limits its therapeutic applications. N-AC can be a source of cystine bypassing the toxic effect of cysteine and raising the level of GSH by 41% in astroglial cells (Kranich et al. 1998). However, higher concentration of N-AC are required because transport and enzymatic deacetylation may limit the ability of low concentration of N-AC to increase GSH (Dringen et al. 2000). Recently it has been reported that N-AC may also elevates cysteine level in astroglial cells independently from system Xc- by the activity of specific cysteine transporters (Arakawa and Ito 2007; Kupchik et al. 2012). Finally

N-AC elevates also indirectly GSH levels by decreasing the activity of GSH peroxidase, which catalyzes the reaction between GSH with other species (Chen et al. 2007).

Other biological activities of N-AC In-vivo and in-vitro studies have shown that N-AC is effective in modulating apoptosis (Parasassi et al. 2005) by activating Ras-extracellular signal regulated kinase (ERK), inducing immediate early genes such as c-fos and c-jun, and inhibiting DNA synthesis and proliferation (Yan et al. 1995). N-AC may also affect the signal transduction pathway by inhibiting the activity of the specific kinase of NF- κ B needed for the dissociation of the inhibitor of NF- κ B thus, preventing the nuclear translocation of NF- κ B (Fukami et al. 2004). It has been shown that N-AC has an immuno-modulatory activity by inhibiting the production of polyclonal immunoglobulins (Ig) from B cells (Samuni et al. 2013). Clinically, N-AC can increase the activity of natural killer and T-cell, and delay the reduction in CD4+ levels in Immune deficiency virus (HIV) patients (Arranz et al. 2008). Animal studies have shown that long term treatment with N-AC can improve both heart- and brain-mitochondrial activities in rats (Cocco et al. 2005), and protect against age-related decline, significantly increasing the specific activities of complex I, IV and V in hepatic mitochondria of mice (Miquel et al. 1995).

1.5.4 N-AC brain permeability

There was uncertainty as to whether N-AC could effectively cross the blood-brain barrier (BBB). N-AC has a – COOH group (pKa = 3.31) and a – SH group (pKa = 9.87), and at pH 7.4 is negatively charged and its neutral, membrane permeating form, constitutes as little as 0.001% of the total N-AC (Samuni et al. 2013). The neutral form of N-AC that would allow membrane penetration becomes predominant only at pH below 3.3. Thus, N-AC can leave the blood vessels only after N-deacetylation or by a carrier-mediated active transport. Similar to N-AC, GSH is in its ionic form at pH 7.4 does not cross the cell membrane and BBB, but its precursor cysteine (N-AC deacetylate form) is a neutral species at pH 1.9-8.2 so it can cross the cell membrane and BBB. Cysteine is also transported across membranes by alanine–serine–cysteine sodium-dependent transport (Krzyzanowska et al. 2014).

Although some studies report that ^{14}C -N-AC resulted in its uptake into most tissues tested, excepted the brain (McLellan et al. 1995; Arfsten et al. 2007), Farr et al. found that N-AC crosses the BBB in mice at a rate of about $2.41 \mu\text{L}/\text{g}\cdot\text{min}$ and about 0.4% of an i.v. injected dose is taken up by brain. N-AC influx rate in the brain is less than that of other essential amino acids but it is much higher than that of many other centrally active compounds. In particular, i.v. N-AC reaches the brain in a concentration about 4, 5 and 20, times greater than the values for acetaminophen, interleukin-1 alpha and morphine, respectively (Banks et al. 1991; Preston and Hynie 1991; Banks and Kastin 1994).

More recently, radiolabeled N-AC has been shown to cross the BBB in humans (Katz et al. 2015). In this study, the patients received either 7, 35 or 70 mg/kg N-AC orally twice a day for two days. The higher N-AC dose produced CSF concentrations of N-AC comparable or higher than that of CSF cysteine concentrations, suggesting that the levels achieved were biologically significant. Their results showed that the majority of CSF N-AC was in its reduced form, and that reduced and total N-AC concentrations increased in parallel with oral N-AC administration. Furthermore, magnetic resonance spectroscopy studies indicate also increased brain GSH levels following intravenous administration of N-AC (Holmay et al. 2013).

1.5.5 N-AC and central glutamate

As already described in Section 1.5.1, N-AC can boost the levels of GSH in glial cells providing the cystine needed for the activation of the exchanger system Xc-. Thus, while increasing the levels of intracellular cysteine system Xc- concomitantly increases extrasynaptic GLU level. This N-AC activity is especially important in the brain, where the system Xc- is mainly located on astrocytes. Once N-AC is administered it is easily deacetylated to cysteine. A large fraction of cysteine is then oxidized to its dimer cystine and it is this dimer that can be transported into glial cells by system Xc- to be used for GSH synthesis (McBean 2002). In this way N-AC while increasing the intracellular level of cyst(e)ine is also increasing extracellular GLU level in the extrasynaptic space (Figure 1.5.1).

In particular, this source of GLU can restore the homeostasis in extrasynaptic GLU levels (Baker et al 2003) and increases GLUergic tone on presynaptic mGluR2/3 thereby inhibiting excitatory transmission (Moran et al. 2005) (Figure 1.5.4).

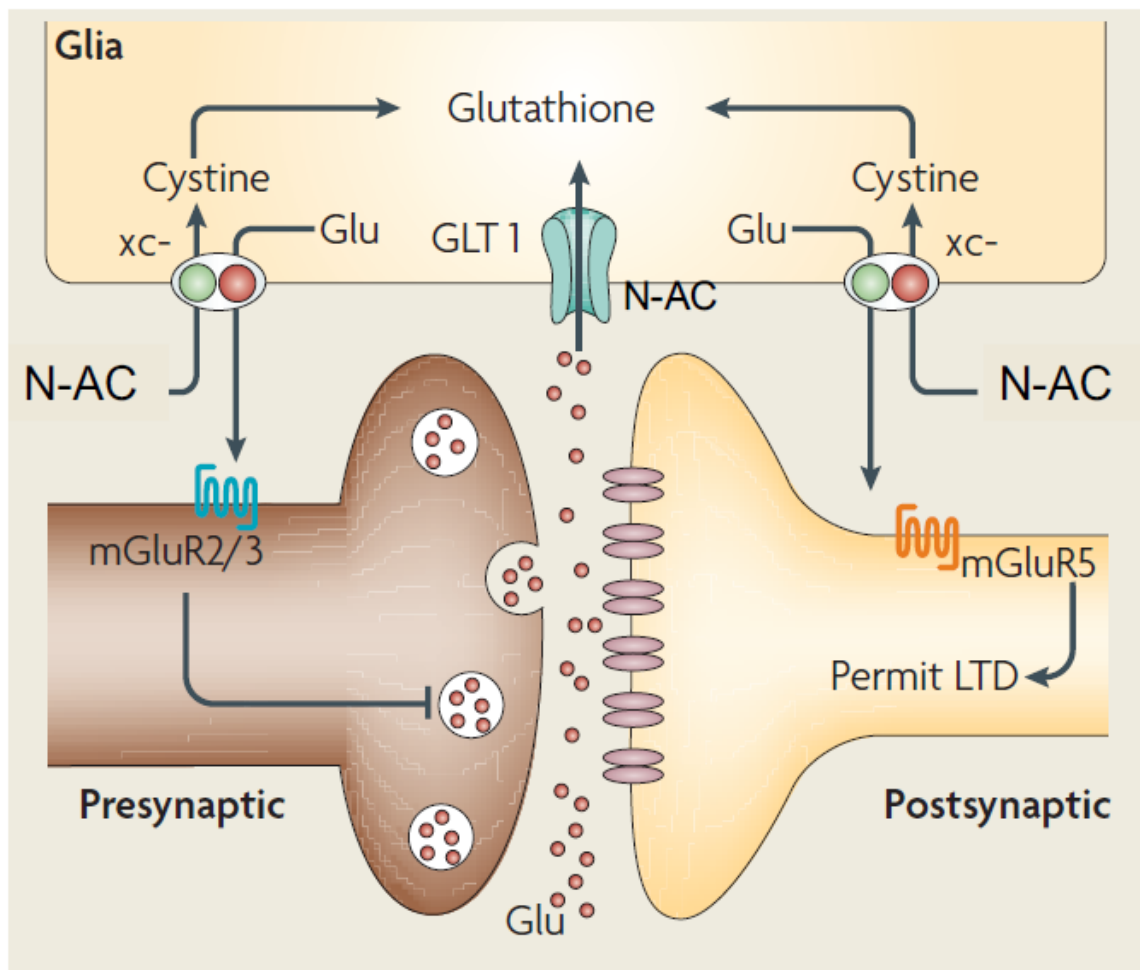


Figure 1.5.4. Schematic drawing summarizing N-AC activity on GLU homeostasis. N-AC, deacetylated after administration dimerizes to cystine, which is transported into astrocyte by system Xc in exchange with intracellular GLU downregulated cystine–glutamate exchange in the Nacc after cocaine administration results in reduced extracellular GLU. N-AC by activating system Xc- help to maintain tone on perisynaptic mGluR2/3. Moreover, NAC restores the bidirectional loss of long-term potentiation (LTP) and long-term depression (LTD) at PF–Nacc synapses that is produced by chronic cocaine administration, an action that is inhibited by blocking mGluR2/3 (for LTP) or mGluR5 (for LTD). Recently chronic N-AC was shown to interact also with glutamate transporter 1 (GLT1) increasing its expression and helping to reduce over-increase activity of extrasynaptic GLU. Adapted from Kalivas (2009).

1.5.6 N-AC and drug addiction

Starting from the observation that chronic cocaine reduced basal concentration of extracellular GLU in Nacc but potentiates GLU release during cocaine-primed reinstatement (Baker et al. 2003; McFarland et al. 2003), Baker and colleagues demonstrated that the modulation of extracellular GLU level by N-AC could prevent priming-induced relapse to cocaine-seeking in rats (Baker et al 2003). They found that similar to cysteine, administration of N-AC, systemically or directly into the Nacc, elevated extracellular GLU in rats treated with repeated cocaine injections and withdrawn for 3 weeks and that reverse dialysis of the system Xc- antagonist (*S*)-4-carboxyphenylglycine (CPG) into the Nacc blocked the elevation in extracellular GLU. Moreover, they found that N-AC pre-treatment (60 mg/kg, s.c., 4h before) also blocked the reinstatement of cocaine-primed but not food-primed reinstatement of lever pressing without altering basal or cocaine-induced locomotor activity. In agreement with this finding, Baker et al. (2003) demonstrated that in rats with an history of cocaine self-administration acute N-AC increased basal levels of GLU and a cocaine priming injection caused no further increase in GLU release that instead is present in vehicle treated rats (Figure 1.5.5).

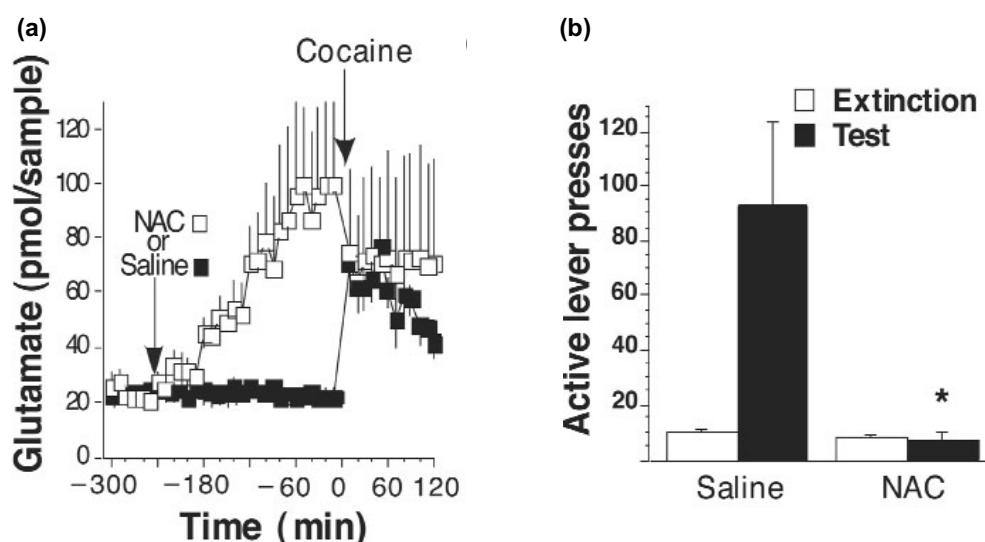


Figure 1.5.5. Acute effect of *N*-acetylcysteine (NAC) on cocaine experienced rats (Baker et al. 2003). Cocaine-primed reinstatement of lever pressing and the increase in extracellular glutamate were blocked by pre-treatment with NAC. (a) NAC (60 mg/kg, s.c.) elevated extra-cellular glutamate (evaluated with microdialysis) and (b) prevented cocaine-primed reinstatement. Adapted from Baker et al. (2003).

These results indicate that the reduction in non-vesicular GLU release from system Xc- after withdrawal from cocaine increased relapse susceptibility, and that ameliorating this reduction might be a viable therapeutic strategy in treating cocaine addiction.

Other experiments were carried out to better characterize the mechanism by which N-AC may exert its anti-relapse activity. It has been shown the effect of acute N-AC inhibition of cocaine-induced reinstatement by activation of the system Xc- was prevented by blocking mGluR2/3 (Moran et al. 2005). Furthermore, withdrawal from cocaine attenuated both LTD and LTP in the Nacc and N-AC (100 mg/kg i.p. 2.5 h pre-treatment) restored the capacity to induce both LTP and LTD by stimulation of mGluR2/3 and mGluR5 respectively (Moussawi et al. 2009). N-AC not only decreased the tendency to relapse but also helped to break drug cue-elicited cocaine-seeking habits. This was demonstrated by the observation that enduring relapse protection by daily N-AC (100 mg/kg i.p.) was achieved pre-treating rats 2 h before 12 extinction sessions after self-administration. Moreover, this correlates with a restored extracellular GLU level in cocaine trained rats that was maintained even 2-3 weeks after the last N-AC injection. Even the long-lasting N-AC anti-relapse activity involved mGluR2/3 since the selective antagonist LY341495 injected directly in the Nacc-core, blocked its activity (Moussawi et al. 2009).

It was also shown that N-AC long-lasting anti-relapse activity may be also influenced by the contingences in which it is administered. Herof, Reichel and colleagues observed that N-AC (100 mg/kg i.p.) exerts more profound effects especially when it modifies the neuroadaptations resulting from explicit extinction training (Reichel et al. 2011).

Other evidence indicates that N-AC can be effective not only in cocaine addiction but also in preventing relapse from heroin- (Zhou et al. 2007) and nicotine-seeking (Ramirez-Nino et al. 2013). Nevertheless, the majority of preclinical studies have been conducted on cocaine and more evidences are needed to confirm that N-AC is effective in preventing cue-induced nicotine-seeking as well as its underling mechanism of action.

N-AC effects on GLU homeostasis are summarized in Figure 1.5.4 All this convincing

preclinical evidence formed the basis for a series of clinical trials to evaluate N-AC activity in many substance use disorders (Table 1.5.1). Most of this studies are based on small clinical trials, but the results seem to be sufficiently promising to suggest the need for larger better designed studies.

Controlled studies	Participants # Group (M, F); age (SD)	Treatment	Study design	Effect of N-AC
Cannabis				
Gray et al. (2012)	N-AC: 58 (39M, 19F); 18.9 (1.5) PB: 58 (45M, 13F); 18.8 (1.5)	2.4 g/d NAC or PB for 8 wk	DBPC parallel	OR in favor of NAC
Rolen et al. (2013)	N-AC: 45 (gender NRY); 15–21 PB: 44 (gender NRY); 15–21			No significant differences
Cocaine				
LaRowe et al. (2006)	13 (6M, 7F); 37.1 (7.6)	1.2 g/d NAC or PB, every 12 h dosing for 4 doses	DBPC crossover	Reduced CSSA, craving and self-reported use
LaRowe et al. (2007)	15 (7M, 8F); 37.4 (7.1)			Reduced viewing time, desire and interest. No change in physiologic response and craving
Amen et al. (2011)	6 (4M, 2F); 41.8 (7.4)	2.4 g/d NAC or Baclofen for 16 days	Single blind cross over	NAC reduced cravings- No effect on rush, high
LaRowe et al. (2013)	2.4 g NAC: 33 (25M, 8F); 43 (9) 1.2 g NAC: 40 (30M, 10F); 44 (10) PB: 38 (28M, 10F); 43 (9)	2.4 g/d or 1.2 g/d NAC or PB for 8 wk	DBPC parallel	No significant effect; improved BSCS. CSSA days to relapse only in initially abstinent individuals
Methamphetamine				
Grant et al. (2010)	N-AC: 14 (8M, 6F); 37.2 (8.2) PB: 17 (14M, 3F); 36.1 (6.6)	Up to 2.4 g/d N-AC+200mg Naltrexone or PB for 8 wk 1.2 g/d N-AC or placebo for 4 wk	DBPC parallel	No significant differences
Mousavi et al. (2015)	32 (26M, 6F); 29.2 (4.9)		DBPC crossover	Significant improvement on N-AC during treatment but no carryover effects
Nicotine				
Bernardo et al. (2009)	Nicotine, alcohol and caffeine in BPAD N-AC: 38 (15M, 23F); 44.6 (11.2) PB: 37 (15M, 22F); 46.6 (13.8)	1–2 g N-AC or PB for 24 wk	DBPC parallel	No change in alcohol and tobacco use; significant decrease in caffeine use in N-AC group at 2wk
Krackstedt et al. (2009)	N-AC: 14 (9M, 5F); 51.3 (10.1) PB: 15 (10M, 5F); 48.6 (10.5)	2.4 g/d NAC or PB for 4 wk	DBPC parallel	No significant differences
Schmaal et al. (2011)	N-AC: 10 (42% M); 21.4 (2.07) PB: 12 (40% M); 20.25 (1.14)	3.6 g/d NAC or PB for 3 days and 1.8 g the 4th day	DBPC parallel	Reduced MNWS, VAS reward, no effect on craving
Grant et al. (2014)	28 (23M, 5F); 47.6 (10.9) N-AC: 13 PB: 15	1.2–3.0 g/d NAC or PB for 12 wk	DBPC parallel	Improvement at 6 wk but not at 12 and 24 wk
McClure et al. (2014)	32M, 36F; 18.8 (1.4) N-AC: 34 PB: 34	2.4 g/d N-AC or PB for 8 wk	DBPC parallel	No significant differences
Prado et al. (2015)	Treatment resistant tobacco use disorder N-AC: 17 (7M, 10F); 51.9 (7.02) PB: 14 (2M, 12F); 50.8 (11.8)	3 g/d NAC or PB for 12 wk	DBPC parallel	Significant difference in all outcomes

Table 1.5.1. Clinical trials evaluating N-acetylcysteine (N-AC) use on drug addiction. Legend: BSCS, Brief Substance Craving Scale; CSSA, Cocaine Selective Severity Assessment; DBPC, Double Blind Placebo Control Trial; MNWS, Minnesota Nicotine Withdrawal Scale; OR, odds ratio; PB, placebo; VAS, Visual Analog Scale. (adapted from Decmala et al. 2015)

Chapter Two – Aims of the thesis

The fundamental goal of the work described in this Thesis was to evaluate the anti-relapse activity of a compound that acting on the GLUergic system may prevent or reduce cue-induced nicotine-seeking behavior. The preceding pages have set out the general background to this work. This has included material from psychopathology, pharmacology, neurochemistry, behavioral pharmacology. With such a diverse background it is clear that in pursuing this thesis's aim experimental methods have concentrated on behavioral, neurochemical and biochemical techniques.

Nicotine associated cues can generate craving and relapse in human as well as drug-seeking behavior and relapse in animal models of addiction. In spite of the fact that nicotine non-selectively activates nicotinic ACh receptors several evidences reviewed in the introduction chapter demonstrated that GLU homeostasis could play a relevant role in cue-induced nicotine-seeking behavior (Gipson et al. 2013). As reviewed in the introduction chapter an effective strategy to control GLU dysfunctions, which has been shown to mediate drug-seeking behavior and relapse could be the use of compounds that acting on proteins crucial for GLU homeostasis may counteract these dysfunctions (Kalivas 2009). Among these drugs N-AC seems to be a promising candidate for the treatment of nicotine addiction both preclinically and clinically.

The work has four main parts:

- 1) To evaluate a new extinction-reinstatement procedure based on discriminative learning paradigm. The nicotine-seeking behavior was also tested after reintroduction of a single component of nicotine-associated cues. In addition, in order to have a control procedure for nicotine reinstatement in subsequent experiments the same nicotine-cues were paired to a palatable reinforcer (saccharine) and the duration of seeking behavior induced by saccharine and nicotine associated cues examined (Chapter 4).
- 2) To exam the behavioral and neurochemical effects of acute N-AC. (a) Determine the effects of acute N-AC on extracellular levels of GLU in the nucleus accumbens by microdialysis technique (Chapter 5); (b) examine the acute effects of N-AC on seeking

- behavior induced by reintroduction of nicotine-associated cues (Chapter 6); (c) study the involvement of mGLU2/3 receptors in mediating N-AC's acute effect (Chapter 6); (d) verify the selectivity of N-AC effects towards nicotine but not saccharine-seeking behavior (Chapter 6); (e) examine the effects of N-AC on spontaneous locomotor activity of rats with an history of nicotine self-administration (Chapter 6).
- 3) To determine the optimal condition at which chronic administration of N-AC could elicit an enduring reduction in cue-induced reinstatement. This was done by studying whether relapse prevention can be achieved by treating rats with N-AC during either a period of extinction of the instrumental response (LP-EXT) or during the exposure to the same cues and context associated with nicotine self-administration (CET). In an additional experiment rats were tested for nicotine-seeking behavior after chronic treatment with N-AC during a period of abstinence (absence of any nicotine associated stimuli) (Chapter 7).
 - 4) To evaluate whether proteins involved in GLU homeostasis were associated with the long-lasting anti-relapse effects of chronic N-AC on nicotine-seeking. First, this was done to confirm the behavioral results showing that chronic N-AC plus CET produced a long-lasting anti-relapse activity. Second, western blotting technique was employed to evaluate changes in proteins involved in GLU homeostasis such as Xct, GLT-1, mGLU2, GluN1, GluN2B and GluN2A in rats treated with vehicle or chronic N-AC plus CET and sacrificed at different time points after the end of combined treatment (Chapter 8).

In conclusion it was hoped that the planned behavioral, neurochemical and biochemical studies could provide new information on the physiological mechanisms involved in ability of N-AC to control nicotine reinstatement and relapse. The data presented in this thesis could provide a better understanding of the mechanism of action of N-AC, and therefore could help in the design of future clinical trials aiming at assessing the value of N-AC in the treatment of nicotine addiction and relapse.

Chapter Three – General materials and methods

3.1 Animals

Naïve male Wistar rats (Harlan Laboratories, San Pietro al Natisone, Udine, Italy) weighing 250-275 g at the beginning of the experiments were used. They were housed individually at constant room temperature ($21\pm 1^{\circ}\text{C}$) and relative humidity (60%) under an inverted light/dark schedule (light on 7:30 PM-7:30 AM) with food and water *ad libitum*. All experimental work was done during the dark phase. Rats were allowed to adapt to the vivarium conditions for at least two weeks and were handled daily during this period. After this, all rats received a maintenance diet of 20-25 g/rat of laboratory chow/day (Global Diet 2018S, Harlan Laboratories) in the early evening and over weekends for the duration of the experiments. This dietary regime was selected since in our previous nicotine reinstatement studies rats remained healthy and gained weight at 1-3 g/day (Di Clemente et al. 2011; Cervo et al. 2013).

3.2 Animal care

Procedures involving animals were conducted at the IRCCS - Istituto di Ricerche Farmacologiche “Mario Negri” which adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments (Quality Management System Certificate - UNI EN ISO 9001:2008 - Reg. No. 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). The Statement of Compliance (Assurance) with the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals has been recently reviewed (9/9/2014) and will expire on September 30, 2019 (Animal Welfare Assurance #A5023-01).

3.3 Drugs

(-)-Nicotine hydrogen bi-tartrate salt (Sigma-Aldrich, Milan, Italy) expressed as free-base was dissolved in sterile 0.9% NaCl and the solution was adjusted to pH 7.1-7.3 with NaOH. Saccharin solution was prepared by dissolving 50 mg saccharin (Sigma-Aldrich) in 1 L of sterile water. Stock solutions were prepared in a laminar airflow cabinet, filtered through a 20- μ m syringe filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and stored in aliquots at 4°C. Stock solutions were freshly remixed every four days.

N-acetylcysteine (N-AC; Sigma-Aldrich) was dissolved in 1 mL/kg of sterile saline with the pH solution adjusted to 7.1-7.3 with NaOH and given intraperitoneally (i.p.) 2.5 h before tests. LY341495 disodium salt 2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid disodium salt; (Tocris Bioscience, Bristol, UK) expressed as free acid was dissolved in 1 mL/kg of sterile saline with the pH adjusted to 7.1–7.3 with HCl and given i.p. 2 and 0.5 h before tests. Solutions were freshly prepared immediately before use.

3.4 Chronic jugular catheter for nicotine self-administration and surgery

Catheters were made in-house using guide cannulae (C313G 5UP, Plastic One Inc., Roanoke, VA, USA), silicon tubing (0.30 x 0.60 and 0.64 x 1.19 mm i.d. x o.d., Degania Silicone LTD., Israel), dental cement (Paladur, Heraeus Kulzer GmbH, Wehrheim/Ts., Germany) and silicon rubber (Elastosil E43, Wacker-Chemie GmbH, München, Germany) according to Cervo et al. (2003).

The right external jugular vein was catheterized in rats pre-treated with carprofen 5 mg/kg subcutaneous (s.c.) as analgesic (Rimadyl[®], Pfizer Italia s.r.l. Roma-Latina, Italy) under isoflurane anesthesia (induction 5.0% isoflurane in N₂O/O₂ (70/30%); maintenance 1.5-2.0% isoflurane in the same mixture), with standard aseptic surgical techniques as previously reported (Cervo et al., 2003, 2007). Starting the day before surgery and during the five-day recovery period, rats received one (s.c.) daily injection of 7.5 mg/kg of Baytril[®] (Enrofloxacin, Alcyon Italia S.p.A., Marene (CN), Italy). Catheters were kept patent by daily (i.v.) infusions of

0.1 mL heparinised sterile 0.9% saline (30 units/mL, Opocrin S.p.A., Corlo di Formigine, Italy) given during the surgery recovery period and before and after each self-administration session. If rats behaved differently from the normal baseline during the self-administration training, catheter patency was checked by injecting 0.05 mL i.v. of a solution containing 2.5 mg/mL of Zoletil® (Virbac, Carros Cedex, France). Animals with patent catheters display clear signs of sedation within 3-second with full recovery in 30-45 second. In the present experiments none of the rats failed this test.

3.5 Self-administration training

3.5.1 Apparatus and conditioning

Rats were trained in 16 identical operant chambers (ENV-007, MED Associates Inc., St Albans, VT, USA). Rats designated for nicotine self-administration were surgically prepared with jugular catheters and given a week of recovery before self-administration training commenced.

Independent groups of rats were food-deprived overnight and trained to associate a white noise (20 dB above background) that lasted throughout the session as a discriminative stimulus (S^{D+}) with the availability of nicotine (0.03 mg/kg/65 μ L/2-second/infusion) or saccharin (100 μ L of a 50 mg/L solution in water). Sessions started with extension of active and inactive levers and reinforced response was followed by light cue (6-second) on the active lever signaling 20-second Time Out (CS^+).

3.5.2 Nicotine self-administration training

Rats were trained according to the experimental procedure described previously (Di Clemente et al. 2012; Cervo et al. 2013) with a substantial modification: they did not receive the initial lever-pressing training for food reinforcement but were immediately trained for nicotine self-administration. Rats were trained to press the active lever to self-inject nicotine under continuous reinforcement [fixed ratio 1 (FR1), meaning each lever press resulted in a nicotine

infusion]. The first two sessions, lasting 2 h or until 20 infusions were earned, started with extension of the active lever and concurrent presentation of S^{D+} for nicotine availability. After each infusion, during the presentation of CS^+ the lever remained inactive for 20s TO to prevent accidental overdosing. On days 3 and 4 of training, the FR was raised to 2 and the session duration reduced to 1-h. From Day 5, sessions started with extension of the active and inactive levers, and the infusion limitation was removed. Responses on the inactive lever were recorded but had no programmed consequences. Thus, from day 10 rats were placed on a “discrimination learning” regimen.

3.5.3 Discrimination learning training

After 10 days of self-administration training in which a FR2 was imposed, rats were placed on a “discrimination learning” regimen comprising a second non-reward daily session. These second daily session started with extension of active and inactive levers and together with the illumination of the house light that remained on throughout the session and served as discriminative stimulus (S^D) for no reward (65 μ L/2-s/infusion of sterile saline as a control for nicotine or 100 μ L of H_2O as a control for saccharin). Reinforced response was followed by an intermittent tone (7 KHz, 70 dB) signaling 20-s Time Out (CS^-). The “discrimination learning” phase comprised two daily 1-h sessions, separated by 1-h rest in the home cage, when either nicotine or saccharin and no-reward were available as the only solution, in a random sequence. This training was conducted daily five-days/week until individual reinforced responding was stable ($\pm 15\%$ over 3 consecutive sessions).

3.5.4 Saccharin self-administration training

Rats were food-deprived overnight and then trained to press the active lever to self-administer saccharin under FR1. The first two sessions, lasting 1h, started with extension of the active lever and concurrent presentation S^{D+} for saccharin availability. After each delivery, during the presentation of CS^+ , the lever remained inactive for 6s TO. From day 3 to day 7 of

training the TO was raised to 10s and sessions started with extension of the active and inactive levers. On days 8 and 9 the TO was raised to 15s.

Thus, from day 10 rats were placed on a “discrimination learning” regimen comprising two daily 1-h sessions, when either saccharin or H₂O was available as the only reinforcers under a FR2TO20s, in a random sequence, separated by 1-h rest in the home cage. Stimuli used during H₂O sessions were the same used in for the saline sessions of the nicotine experiments.

This training was conducted daily until saccharin and H₂O responding stabilized (\pm 20% over 3 consecutive sessions).

3.5.5 Extinction of lever presses

After rats acquired a stable self-administration performance on discrimination learning regimen they were then placed under the extinction condition until the end of the experiments where no S^Ds were presented and the instrumental lever response produced neither the reinforcer nor the CSs (Torregrossa et al. 2010; Cervo et al. 2013). One daily 1-h extinction session was conducted until there were 3 consecutive sessions in which the number of responses/session was less than 20% of the number at reinforced training criteria or \pm 15% over 3 consecutive extinction sessions.

3.5.6 Reinstatement

Reinstatement tests began one day after individual animals met the extinction criterion. Tests lasted 1 h during which rats were exposed to non-contingent S^{D+} or S^{D-} under conditions identical to those during discrimination learning phase, except that reinforcers or no-reward were not available. Two responses on the previously active lever were followed by activation of the pump motor followed by a 20-s CS⁺ or CS⁻ presentation. Each reinstatement test was separated by at least 3 extinction sessions in which the lever presses returned to the criterion.

3.6 Microdialysis procedures

Concentric dialysis probes were made with Cuprophan membrane (216- μm outer diameter, 3,000 Da cutoff, Sorin Biomedica, Italy) and assembled as described in Invernizzi (2013). The length of the dialysis membrane exposed to the brain tissue was 2 mm. The guide cannulae were built cutting a segment from a syringe needle. The internal diameter of the guide cannula (22 G) was chosen to permit the precise insertion of the probes (Figure 3.1). Rats were anesthetized with isoflurane (as described in Section 3.2), positioned on a stereotaxic frame (Kopf Instruments, Tujunga, CA, U.S.), and then 3 small screws were fixed on the skull and a guide cannula was implanted aiming at the center of the Nacc core. The stereotaxic coordinates referred to the tip of the guide cannulae were: AP +1.5; L +2.5 mm (with the stereotaxic arm fixed at a -8° angle to avoid damage to ventricles) and DV -3 mm (final DV after probe insertion is -8.5, Figure 3.1) from bregma (Paxinos and Watson 1986). The guide cannulae were fixed to the skull with dental cement and a stylet inserted inside to avoid clogging. Rats were allowed to recover for at least 5 days after surgery. The night before testing, the dialysis probes were inserted in the guide cannulae and slowly lowered into the Nacc. Probe and guide cannula were finally secured with dental cement. The probes were perfused with artificial cerebrospinal fluid (aCSF, composition in mM: NaCl 140, CaCl₂ 1.26, KCl 3, MgCl₂ 1, Na₂HPO₄ 1.2, glucose 7.2; pH 7.4 with 0.6 M NaH₂PO₄) overnight at 0.2 $\mu\text{L}/\text{min}$ with a CMA/100 pump (CMA Microdialysis, Kista, Sweden). In the morning the flow rate was increased to 1 $\mu\text{L}/\text{min}$ and after at least 2.5 h washout 3 consecutive samples were collected as basal levels. Rats were then treated with N-AC or vehicle by i.p. injection or the normal aCSF was changed with the one containing 60 mM K⁺. Samples of dialysate were collected every 20 min. The resulting 20 μL samples were stored at -20°C and subsequently analyzed for GLU quantification whereas for DA quantification aliquots were stored at 4°C with the addition of an antioxidant solution and immediately processed.

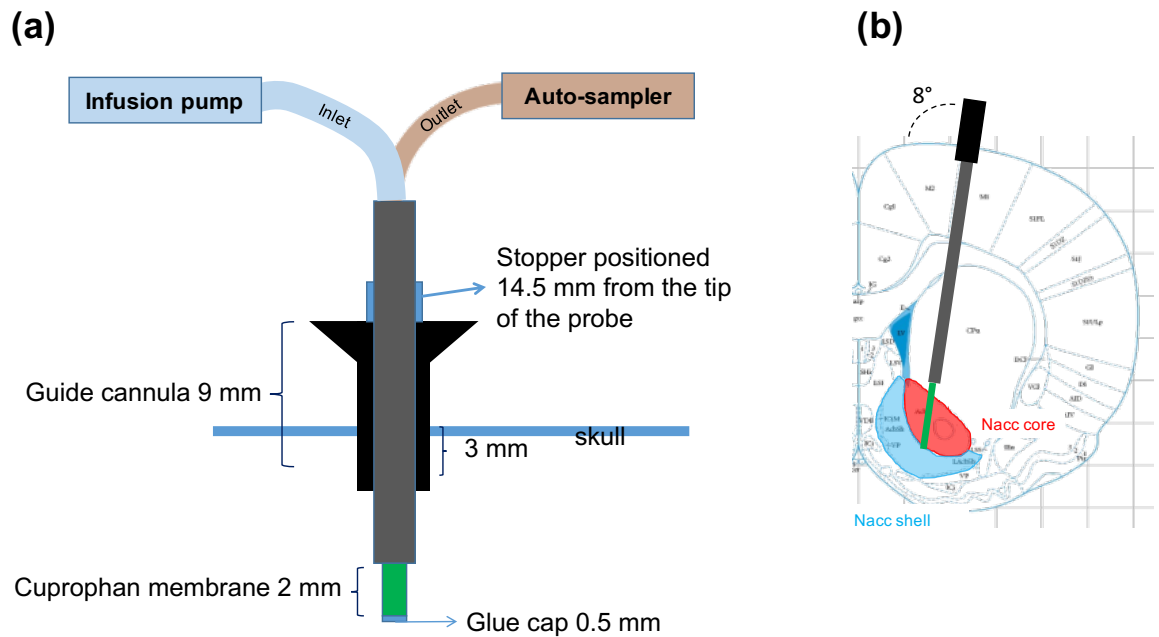


Figure 3.1. (a) schematic representation of guide cannula and probe. The guide cannula is lowered 3 mm from the surface of the skull. The probe is inserted into the guide cannula the night before microdialysis and lowered till the stopper that block the probe in its final position. The infusion pump connects to the inlet bring aCFS directly to the probe, while the outlet brings the dialysate to the auto sampler. (b) shows the final position of the membrane (green) mostly into the nucleus accumbens (Nacc) core. The guide cannula is fixed with an angle of 8° to avoid damage to the ventricles.

3.6.1 Glutamate and dopamine quantification

The concentrations of GLU in dialysate samples were determined by HPLC with fluorometric detection after precolumn derivatization with o-phthaldialdehyde (OPA)/ β -mercaptoethanol reagent according to Ceglia et al. (2004). The derivatizing reagent was prepared by dissolving 27 mg OPA (Sigma-Aldrich, Milan, Italy) in 1 mL methanol followed by 5 μ L of β -mercaptoethanol and 9 mL of 0.1 M sodium tetraborate buffer (pH 9.3 adjusted with NaOH 5 M). This stock solution was stored for about 5 days at 4 C° in a sealed vial darkened with aluminum film. Before using it was diluted 1:4 with 0.1 M sodium tetraborate. Twenty-five μ L of the diluted reagent was added to 5 μ L sample and the reaction was allowed to proceed for 2 min at room temperature before injection onto the chromatographic system. GLU was separated by a reverse phase column (HR-80, 80 x 4.6 mm, 3 μ m packing; Thermo Scientific, USA) protected with a guard column (New Guard RP-18, 7 μ m, 15 x 3.2 mm; Perkin Elmer, Norwalk,

CT, USA). The mobile phase consisted of 0.1 M Na₂HPO₄, 28% CH₃OH, adjusted to pH 7.2 with 85% H₃PO₄, pumped at 1 mL/min with a LC-20ADvp pump (Shimadzu, Milan, Italy). GLU was detected by a scanning fluorescence detector (FP-2020 Plus, Jasco, Tokyo, Japan) at 335 nm (excitation) and 450 nm (emission). The assay was calibrated daily with GLU standard solutions (10, 3, 1, 0.3 pmol/5 µL GLU) made up in aCSF.

DA quantification was also performed by HPLC with electrochemical detection without prior purification for the determination of monoamines as detailed in Invernizzi (2013).

3.7 Brain micro-dissection procedure

Rats were killed by decapitation 7 days or 51 days after the end of the chronic N-AC treatment. Whole brains were frozen on dry ice and stored at -80°C for later micro-dissection. Coronal sections (220 µm) were cut through the brain in a cryostat at -15°C, mounted onto glass slides and rapidly cooled with dry ice. Using a dissecting microscope, the Nacc core and shell were micro-dissected from Bregma +2.76 to Bregma +0.84 mm according to the rat brain atlas (Paxinos and Watson 2007) using a needle with a sharp cutting tip of 1 mm diameter (Harris Uni-Core, Ted Pella inc.). Separate, sterile needles were used to punch each area, to eliminate the possibility of contamination by tissue carry-over. At the completion of micro-dissection, brain regions of interest were rapidly frozen in dry ice and stored at -80°C until protein extraction for molecular analysis.

3.8 Protein extraction and western blot analysis

Micro-dissected brain regions were homogenized by sonication using a cold buffer containing 0.32 M sucrose, 1 mM Hepes, 0.1 mM EGTA, 0.1 mM PMSF, pH=7.4, in the presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. Total proteins were measured in the total homogenate using the Bio-Rad Protein Assay (Bio-Rad Laboratories). Ten µg of proteins for each sample were run on a sodium dodecyl sulfate-8% polyacrylamide gel under reducing conditions and then electrophoretically transferred onto

nitrocellulose membranes (GE Healthcare, Milan, Italy). Blots were blocked 1 hour at room temperature with 10% non-fat dry milk in tris buffered saline + 0.1% Tween-20 buffer and then incubated with antibodies against the proteins of interest. The conditions of the primary antibodies were the following: anti-GLT-1 (1:5000, Abcam, Cambridge, UK) Anti-XCt (1:1000, Abcam) anti-GluN2B (1:1000, Santa-Cruz Biotechnology, Santa Cruz, CA, USA) anti-GluN2A (1:1000, Invitrogen, Carlsbad, CA, USA) anti-GluN1 (1:1000, Invitrogen) anti-mGluR2 (1:500, Abcam) and anti- β -actin (1:10000, Sigma-Aldrich, Milan, Italy). Results were standardized using β -actin as the control protein, which was detected by evaluating the band density at 43 kDa. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories, Milan, Italy) and analyzed using the Image Lab software from Bio-Rad.

3.9 Staistical analysis

In the self-administration training period, the number of lever presses during the last 3 sessions of nicotine self-administration, saccharin-maintained behavior or no-reward and the last 3 sessions of extinction, before and between the different reinstatement sessions were analyzed separately by one-way analysis of variance (ANOVA) with repeated measures. Since there were no differences between sessions, the last 3 days for each condition were pooled for further statistical analysis.

In Chapters 4, 5 and 6 the effects of reintroduction of nicotine- or saccharin-associated cues to revive seeking behavior were analyzed by one-way ANOVA with repeated measures with test sessions as the main factor. The effects of N-AC on seeking behavior induced by reintroduction of saccharin-associated cues were analyzed by mixed-factorial ANOVA, with treatment as between-subject factor and sessions as within-subject factor. The interactions between LY341495 and N-AC were analyzed by two-way ANOVA with repeated measures (with LY341495 and N-AC as main factors). When appropriate, *post-hoc* comparisons were made with Newman-Keuls test. The cumulative number of responses for conditioned reinstatement

was analyzed by mixed-factorial ANOVA (with treatment as between-subject factor and time as within-subject factor), followed by simple effects analysis. Changes were considered significant when $P < 0.05$.

Data obtained in experiments presented in chapter 7 and 8 experiments were analysed with a mixed-factorial ANOVA with treatment as between-subject factor and sessions as within-subject factor was performed to analyze the experiments. When appropriate, post-hoc comparisons were made with the Newman-Keuls test.

Extracellular levels of GLU and DA were analyzed by ANOVA for repeated measures. Values were not corrected for in vitro recovery of the probes. In the experiment evaluating N-AC effect extracellular levels of GLU were expressed as percentages of basal values, a mixed ANOVA was performed with groups (Naive-Veh; Naive-N-AC; Nic-Veh; Nic-N-AC) as the between-subject factor and time as the within-subject factor. Post-hoc comparisons were performed with Tukey–Kramer’s test.

Protein levels were analyzed by two-way ANOVA with treatment and self-administration history as main factors. When appropriate, post-hoc comparisons were performed with Tukey’s multiple comparisons test.

Chapter Four – Extinction-reinstatement procedure: model based on discriminative learning paradigm

4.1 Introduction

This procedure was based on classical conditioning (Pavlovian conditioning) that is operationally defined by the ability of environmental stimuli (supposed to be neutral), after repeated association with an appetitive or aversive stimulus (unconditioned stimulus, US), to provoke one or several of the US-induced responses in the absence of the US (Conditioned response, CR). Stimuli that acquire the ability to elicit a response upon pairing with an US are defined conditioned stimulus (CS).

In the context of drug response, nicotine-associated stimulus can also act as a secondary reinforcer, i.e. contingent CS^+ can reinstate an extinguished self-administration behavior (Caggiula et al. 2001; Cohen et al. 2005). Also discriminative stimuli (S^D) may signal the availability of a reinforce and thereby set occasion to engage in behavior that brings the organism into contact with the reinforcing substance (McFarland and Ettenberg 1997). Recently, Cervo et al. (Di Clemente et al. 2011; Cervo et al. 2013) demonstrated that reintroduction of a S^{D+} predictive of nicotine availability, with a CS^+ associated with nicotine self-administration, induced strong, long-lasting nicotine-seeking behavior in abstinent rats after repeated extinction trials. This enduring behavioral effect resembles the persistence of conditioned cue reactivity and cue-induced craving in humans, a factor implicated in high rates of relapse (O'Brien et al. 1998). The robust, long-lasting nicotine-seeking induced by S^{D+}/CS^+ has strong face- and construct-validity, and may be useful for comparing the activity of anti-relapse pharmacological and non-pharmacological treatments in a more potent within-subject experimental design, using fewer experimental subjects.

A commonly used procedure is food self-administration training before the beginning of nicotine self-administration. Nevertheless, this training may generate an undesired

relationship between context and food that can possibly impact later evaluation of cue-induced nicotine-seeking behavior. In the present experiments I used the procedure previously used in my lab with a substantial modification: rats did not receive the initial lever-pressing training for food but were immediately trained for nicotine self-administration.

Moreover, I set up a procedure of saccharin self-administration in which sessions were paired with the same S^{D+}/CS^+ used for nicotine experiments. This was done in order to have a procedure in which S^{D+}/CS^+ were paired to a palatable reinforcer and that could be used as a control for nicotine effect in subsequent experiments.

4.2 Specific materials and methods

After training rats as described in section 3.5, I examined whether nicotine-associated cues reinstated seeking behavior and the resistance to extinction of response reinstatement under repeated nicotine-associated stimuli in 8 rats trained to self-administer nicotine without any prior operant instrumental training for food reinforcement. Rats were initially tested for reinstatement with saline-associated stimuli, then repeatedly tested after reintroduction of nicotine-associated cues.

4.2.1 Seeking behavior induced by reintroduction of the single components of nicotine-associated cues

In this experiment I tested how the single components of the complex cues presentation (S^{D+}/CS^+) associated to nicotine self-administration impacted the reinstatement level. To this end an independent group of rats was used to assess the seeking behavior induced by reintroduction of the single components either S^{D+} or CS^+ of nicotine-associated cues respectively.

4.2.2 Seeking behavior induced by reintroduction of saccharin-associated cues

To examine whether and how saccharin-associated cues reinstated seeking behavior and the resistance to extinction of response reinstatement under repeated saccharin-associated stimuli in 10 rats were first trained to self-administer saccharin. Afterwards rats were initially tested for reinstatement with non-reward-associated stimuli, and then tested once again after reintroduction of saccharin-associated cues.

4.3 Results

4.3.1 Seeking behavior induced by reintroduction of nicotine-associated cues

Figure 4.1a shows the responses on the active and inactive levers during the self-administration training, extinction, and reinstatement phases. Rats acquired nicotine-reinforced responding, with 21.5 ± 0.4 sessions needed to reach criterion. Note that this and all following quantitative data are expressed as mean \pm SEM, unless stated otherwise. In the last 3 days of self-administration, nicotine intake remained stable (20.5 ± 2.3 infusions corresponding to 51.7 ± 6.4 active lever presses). Responding was significantly lower during the last 3 saline sessions (4.4 ± 0.5 infusions corresponding to 9.8 ± 0.9 active lever presses, $P < 0.05$).

During extinction sessions, the number of presses on the active lever decreased, and all rats met the extinction criterion in an average of 10.0 ± 0.5 sessions. Reintroduction of nicotine-associated stimuli led to immediate recovery of responding on the active lever [$F(11,77) = 15.4$, $P < 0.05$], which was significantly higher than after introduction of no-reward-associated stimuli and the 3 preceding extinction sessions (both $P < 0.05$, Newman-Keuls test). The individual recovery of active lever presses in the first and eighth test sessions ranged from 53.2% to 190.7% and from 41.4% to 149.2% of the presses during nicotine self-administration. Active lever presses were the same during presentation of no-reward-associated stimuli as in the 3 preceding extinction sessions. The mean cumulative active lever responding to nicotine-associated cues was sustained throughout the reinstatement sessions and was clearly different from the saline-

associated cues [$F_{\text{Sessions}}(3,28)=6.0, P<0.05; F_{\text{Time}}(5,140)=47.6, P<0.05; F_{\text{Sessions} \times \text{Time}}(15,140)=3.6, P<0.05$] (Figure 3.1b).

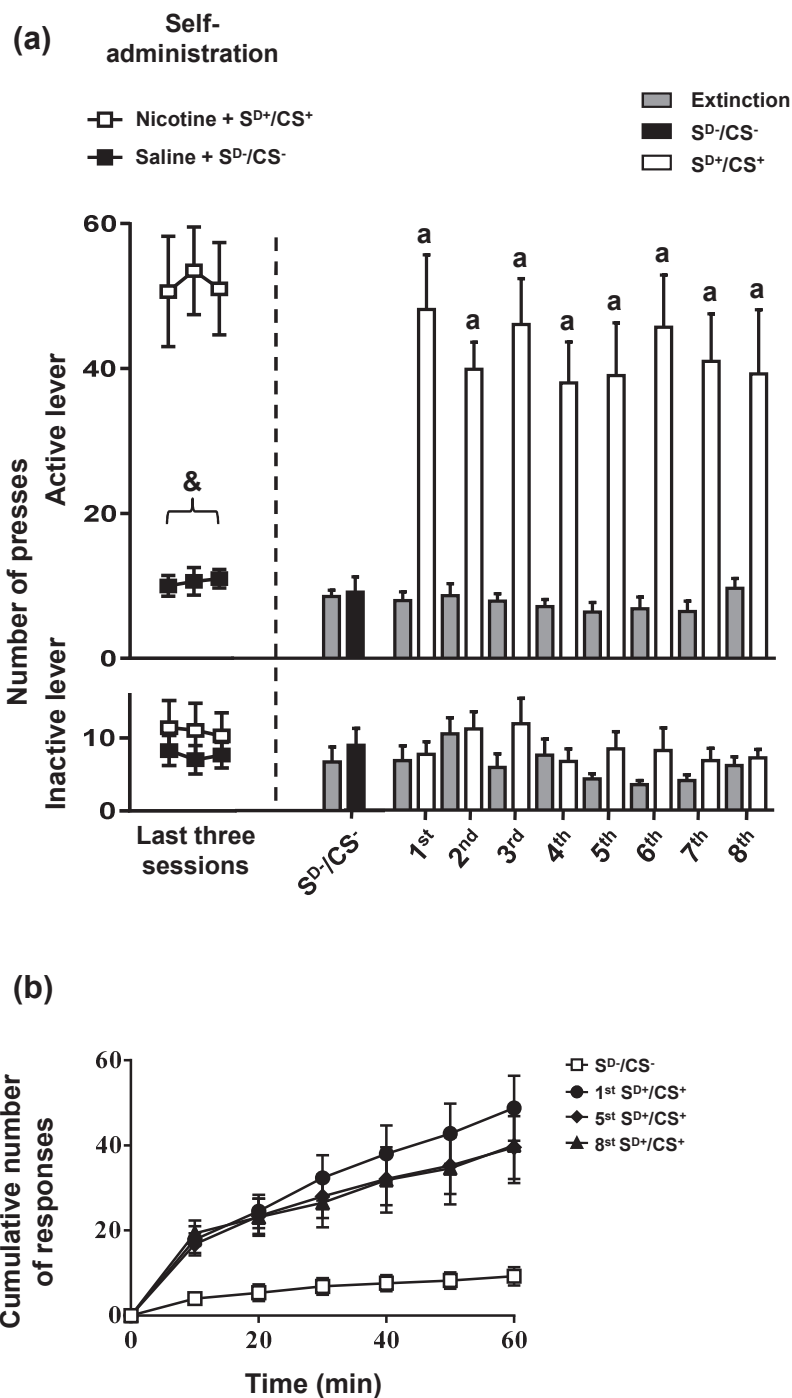


Figure 4.1. Seeking behavior induced by reintroduction of saline- and repeated nicotine-associated cues. **(a)** Number of active and inactive lever responses (8 rats; mean \pm S.E.M) during self-administration training (mean of last 3 sessions), extinction (last 3 sessions), and during reinstatement sessions under saline- (S^D-/CS^-) and repeated presentation of nicotine-associated stimuli (S^D+/CS^+) (see Methods for further details). **(b)** Cumulative number of responses (in 10-min intervals) throughout the 60-min reinstatement sessions (for clarity, only responses recorded during the first, fifth and eighth reintroductions of nicotine-associated cues are presented in comparison with responses induced by vehicle-associated cues). Solid symbols in panel **(b)** indicate $P<0.05$ vs. S^D-/CS^- , $\&P<0.05$, vs. nicotine + S^D+/CS^+ , $^aP<0.05$, vs. no-reward-associated stimuli and respective extinction sessions, Newman-Keuls test.

4.3.2 Effects on seeking behavior induced by reintroduction of the single components of nicotine-associated cues

A within-subject design was adopted to verify how much S^{D+} and CS^+ contributed to the nicotine-seeking behavior after S^{D+}/CS^+ reintroduction. Eight rats were trained as above and once the extinction criteria were met they were tested with either S^{D+} , CS^+ or S^{D+}/CS^+ (Figure 4.2a). To control for order effect stimuli were tested in a random sequence across reinstatement test sessions.

In this experiment rats acquired stable nicotine self-administration (an average of 20.2 ± 0.6 sessions needed to reach criterion). They maintained stable nicotine self-administration during the last 3 sessions of the conditioning phase (19.7 ± 2.4 infusions, corresponding to 46.3 ± 6.2 active lever presses). Responding was significantly lower during the last 3 saline self-administration sessions (3.8 ± 0.5 infusions corresponding to 10.2 ± 1.3 active lever presses, $P < 0.05$ versus nicotine, Newman-Keuls test). During lever extinction sessions the number of presses on the active lever gradually fell to 7.3 ± 0.6 and all rats met the criterion after an average of 9.1 ± 0.3 sessions. Inactive lever presses were minimal during these phases (6.2 ± 1.4).

Reintroduction of S^{D+}/CS^+ increased responding on the active lever [$F(4,49) = 47.62$, $P < 0.01$].

Rats made significantly more responses than after S^{D-}/CS^- or the preceding extinction sessions (both $P < 0.05$, Newman-Keuls test) and the S^{D+} or CS^+ presented alone ($P < 0.05$ Newman-Keuls test)(Figure 4.2a). Figure 4.2b shows the effect of S^{D+} , CS^+ and S^{D+}/CS^+ on the

cumulative responses rate during the 1h session (Figure 4.2b). [$F_{\text{cues}}(3,28) = 23.63$, $P < 0.01$;

$F_{\text{Time}}(5,140) = 86.5$, $P < 0.01$; $F_{\text{cues} \times \text{Time}}(15,140) = 15.01$, $P < 0.01$].

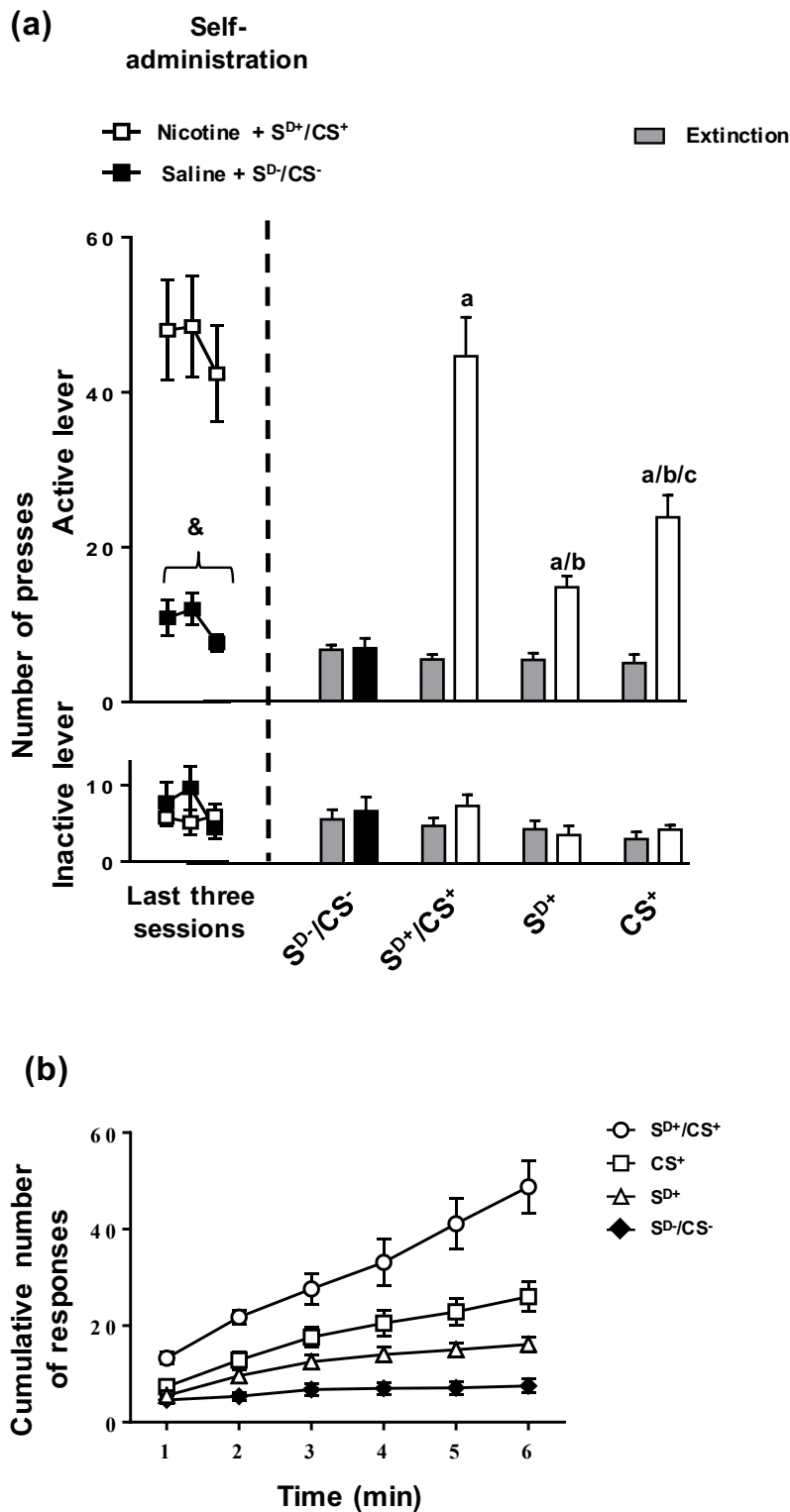


Figure 4.2. Effects on seeking behavior induced by reintroduction of the single components of the nicotine-associated cues (SD^+/CS^+ , SD^+ , CS^+). **(a)** Histograms represent the mean \pm SEM number of presses on the active and inactive levers (8 rats). For comparison, the figure also shows the average numbers of lever presses during self-administration training (mean of last 3 days), extinction (last 3 sessions) and with stimuli associated with no-reward during reinstatement (SD^-/CS^-). Reinstatement data were analyzed by one-way ANOVA for repeated measurement followed by Newman-Keuls *post-hoc* comparison. **(b)** Cumulative number of active lever presses (in 10-min intervals) throughout the 60-min reinstatement sessions. Solid in panel (b) symbols indicate $P < 0.05$ vs. SD^+/CS^+ , SD^+ , CS^+ . $\&P < 0.05$ vs. nicotine + SD^+/CS^+ , $^aP < 0.05$ vs. SD^-/CS^- and respective extinction, $^bP < 0.05$ vs. SD^+/CS^+ , $^cP < 0.05$ vs. SD^+ , Newman-Keuls test.

4.3.3 Seeking behavior induced by reintroduction of saccharin-associated cues

Figure 4.3a shows the responses on the active and inactive levers during the self-administration training, extinction, and reinstatement phases. Rats developed stable saccharin self-administration, and the number of lever presses for H₂O gradually decreased (27.4 ± 0.2 sessions needed to reach criterion). During the last 3 sessions of stable saccharin self-administration, there were significantly more active lever presses (17.7 ± 2.9 reinforcers corresponding to 44.0 ± 7.2 active lever presses) than during the H₂O sessions (4.3 ± 1 reinforcers corresponding to 11.4 ± 2.9 active lever presses, $P < 0.05$, versus saccharin, Newman-Keuls test). During extinction sessions, the number of presses on the active lever gradually decreased, and rats met the extinction criterion after 7.6 ± 0.2 sessions. As shown in Figure 4.3a reintroduction of saccharin-associated stimuli selectively increased the number of active lever presses during the first, second and the third reintroduction sessions [$F(8,72) = 23.7$, $P < 0.05$; $P < 0.05$ compared to reintroduction of H₂O-associated cues and preceding extinction sessions, Newman-Keuls test]. The individual recovery of active lever presses in the first test session ranged from 54.3 to 111.1% of the presses during saccharin self-administration. During the fourth and the fifth presentation of saccharin-associated cues the number of presses on the active lever was not significantly greater than those after H₂O-associated cues or during extinction. Indeed, the statistical analysis of data showed that the mean cumulative active lever responding to saccharin-associated cues was sustained and clearly different from the H₂O-associated cues throughout the first 3 reinstatement sessions, but not during the fourth and fifth reinstatement sessions [$F_{\text{Sessions}}(5,45) = 8.2$, $P < 0.05$; $F_{\text{Time}}(5,270) = 68.8$, $P < 0.05$; $F_{\text{Sessions} \times \text{Time}}(25,270) = 6.6$, $P < 0.05$] (Figure 3b).

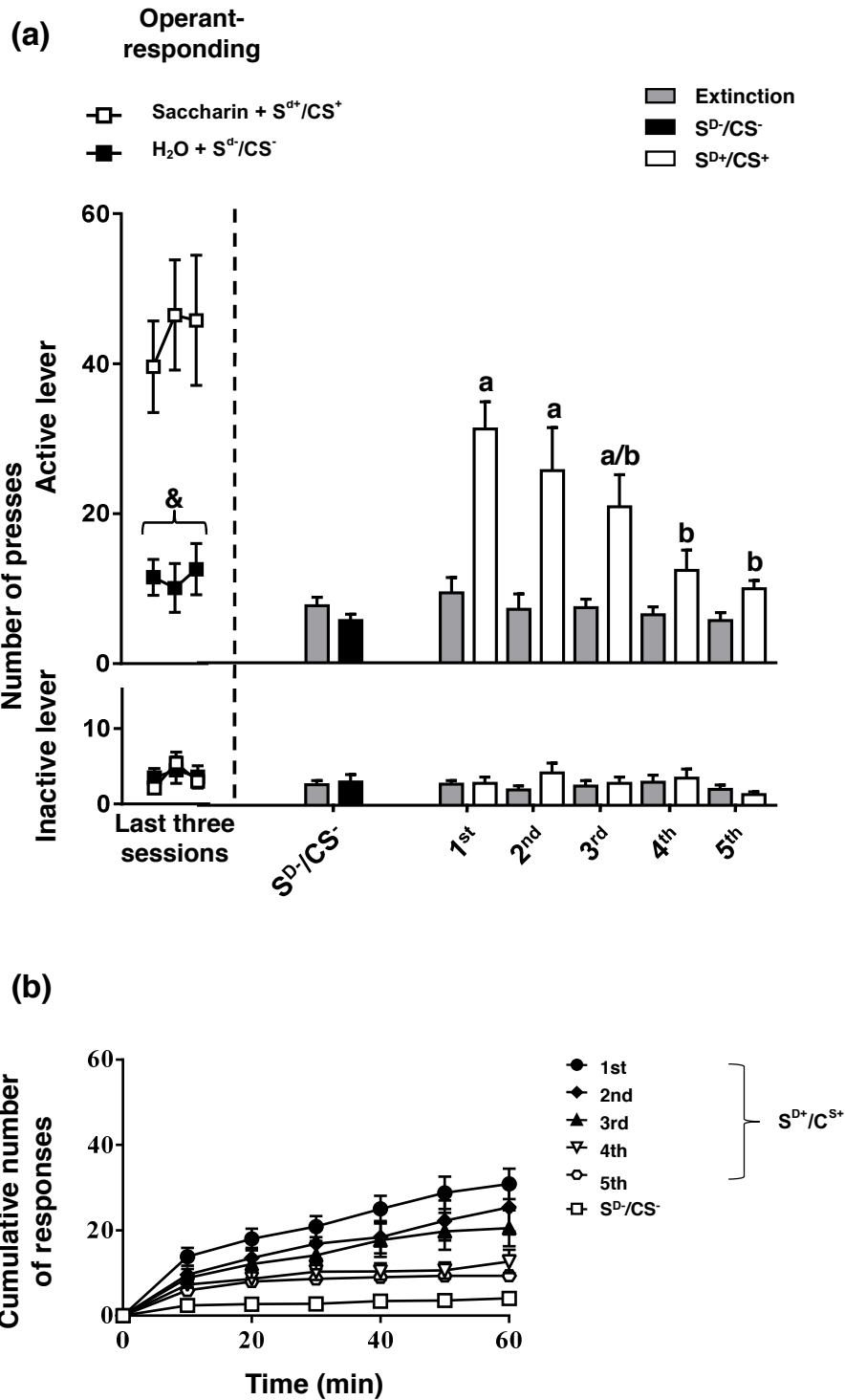


Figure 4.3. Seeking behavior induced by reintroduction of H₂O- (S^{D-}/CS⁻) and repeated saccharin- (S^{D+}/CS⁺) associated cues. **(a)** Histograms show the mean \pm SEM presses on the active and inactive levers (10 rats). For the sake of comparison, the figure also shows the average number of lever presses during self-administration training (last 3 sessions) and extinction (mean of last 3 days before and between the different reinstatement sessions). Data were analyzed by one-way ANOVA with repeated measures (with sessions as the main factor). **(b)** Cumulative number of active lever presses (in 10-min intervals) throughout the 60-min reinstatement sessions. Solid symbols in panel **(b)** indicate $P < 0.05$ vs. S^{D-}/CS⁻. &P < 0.05, different saccharin + S^{D+}/CS⁺, ^aP < 0.05 vs. S^{D-}/CS⁻ and respective extinction and ^bP < 0.05 vs. first S^{D+}/CS⁺, Newman-Keuls test.

4.4 Discussion

The main finding of these experiments is that even without the commonly used initial instrumental training for non-drug reinforcement (usually food) the extinction-reinstatement procedure employed here induced strong and long-lasting nicotine-seeking that could be used in evaluating pharmacological and non-pharmacological anti-relapse therapies in a potent within-subject experimental design.

In good agreement with clinical observations that smoking-related cues enhance the desire for smoking (Droungas et al. 1995; Lazev et al. 1999) the reintroduction of stimuli predictive of and associated with nicotine infusion during self-administration sessions induced, once the rats had met a stable extinction criterion, a reliable and strong drug-seeking behavior in abstinent rats (Di Clemente et al. 2012; Cervo et al. 2013). Here, to reduce possible confounding interpretations for responding at the time of reinstatement, I show, as earlier reported by Garcia et al. (2014), Shram et al. (2008) and Peartree et al. (2012) that even rats with no prior operant training for food reinforcement acquire stable nicotine self-administration.

Once the rats met stable extinction, repeated non-contingent reintroduction of S^{D+}/CS^+ induced reliable strong and long-lasting drug-seeking behavior. This effect cannot be attributed to non-specific arousal or spontaneous recovery, since responding on the inactive lever remained negligible and responding with S^{D-}/CS^- remained at the extinction level. It may seem surprising that the limited period of nicotine self-administration (20-25 1-h sessions) induced such strong and lasting association with cues predictive of and associated with drug availability. However, as previously seen with alcohol (Ciccocioppo et al. 2001), heroin (Gracy et al. 2000) and cocaine (Weiss et al. 2001), the persistence of behavioral responses to drug-associated cues obtained with this procedure is quite reliable and may be attributable to the complex stimuli associated with the drug during the self-administration training. During self-administration sessions rats were not only exposed non-contingently to the nicotine S^{D+} , signaling its availability, but infusions were also paired with a response cue marking the 20-s time-out period acting as CS^+ . The finding that both S^{D+} and CS^+ reintroduction induced nicotine-seeking, even

if of lower intensity than with the S^{D+}/CS^+ reintroduction (Figure 4.2), suggests that during reinstatement sessions both S^{D+} and CS^+ contribute to the nicotine-seeking. Thus, non-contingent reintroduction of S^{D+} by signaling drug availability may set the conditions for behavior that brings the organism into contact with the reinforcing substance, and the contingent CS^+ may subsequently have maintained drug-seeking behavior (see Weiss et al. 2001; Cervo et al. 2013; Di Clemente et al. 2012). It is of interest that a condition often associated with drug craving in humans is cognitive awareness of drug availability (Mirin et al. 1976). It has been argued, therefore, that the manner in which drug-associated cues attain their incentive properties is likely to involve the predictive nature of these stimuli rather than only the classically conditioned stimulus-response associations (McFarland and Ettenberg 1997).

Although the impact of the early food-training on later nicotine-seeking seems irrelevant and both S^{D+} and CS^+ contribute to the nicotine-seeking, the precise nature of the behavioral control in response to these stimuli requires further investigation. This seems especially relevant in light of the persistence of high responding during reinstatement (i.e. Figure 4.1, eight reinstatement tests). It cannot be excluded that our training procedure may have generated a sort of “habitual-like” responding difficult to extinguish. However, these results unequivocally show that this procedure elicits strong, sustained nicotine-seeking behavior and could be useful to compare the value of pharmacological and non-pharmacological anti-relapse therapies in a within-subject experimental design.

In the experiment with saccharin, the chosen concentration of saccharin generated a rate of responding during reinstatement testing that closely resembles that observed during the nicotine-reinstatement. This was done in order to have a reward for which rats have a similar motivation than that for nicotine. However, in contrast to nicotine, saccharin is not addictive and the ability of saccharin-related cues to induce active lever presses was rapidly extinguished by rats (Figure 4.3). This is an important information to consider when designing the saccharin experiments since the present procedure cannot be used for multiple testing in a within-subject experimental design. For this reason, in the experiments evaluating the efficacy of N-AC to

reduce saccharin-seeking behavior independent groups of rats were used and rats were tested only once with saccharin related cues.

Chapter Five – Evaluation of acute N-AC effect on extracellular GLU levels in the nucleus accumbens

5.1 Introduction

It has been shown in rats that repeated cocaine self-administration induces a decrease in the extracellular GLU levels measured by microdialysis (Baker et al. 2003; Madayag et al. 2007). Moreover, previous studies have shown that N-AC may increase extracellular GLU levels mainly in the Nacc core but only in rats that have an altered GLU homeostasis caused by chronic cocaine self-administration (Baker et al. 2003). To date nobody has evaluated if in rats with a history of nicotine self-administration N-AC is able to increase extracellular GLU levels in the Nacc. This is an important aspect to clarify since it has been suggested that acute N-AC acts by activating the system Xc⁻ (see General Introduction Section 1.3) thus, increasing the extracellular GLU levels.

As discussed in General Introduction, extracellular GLU levels in the Nacc are primarily maintained by the activity of the system Xc⁻ on glial cells (Baker et al. 2002). Indeed, the extrasynaptic source of GLU is the main source of total levels of extracellular GLU. Although N-AC effect seems to reflect the increase of the extrasynaptic GLU levels, the precise mechanism of action of N-AC is not completely understood. For these reasons I have decided to use the in-vivo microdialysis technique to quantify extracellular GLU levels in rats with an history of nicotine self-administration. The dose of 100 mg/kg N-AC i.p. was chosen since it proved to be effective in previous studies on cocaine (Zhou and Kalivas 2008; Moussawi et al. 2009).

Microdialysis has long been used in neuroscience as a method that permits a precise quantification of neurotransmitters in interstitial tissue fluid in behaving animals. Thus, in order to answer the experimental question, I used an in vivo microdialysis procedure to collect samples of brain extracellular fluid and then measured the accumulation of GLU (Invernizzi

2013) by high-performance liquid chromatography (HPLC) coupled to fluorometry (Ceglia et al. 2004).

I validated my experimental procedure with a preliminary experiment in which I evaluated the effects of a depolarizing agent [high (60 mM) K⁺ containing aCSF] to raise neuronally released DA and GLU in the extracellular fluid (Invernizzi 2013). Since this preliminary experiment confirmed the responsiveness of GLU and DA neurons to the physiological challenge I proceeded with the evaluation of extracellular GLU levels after an acute challenge of N-AC (100 mg/kg i.p.) in naïve and nicotine self-administered rats.

5.2 Specific materials and methods

Rats in each experimental group used for testing the acute effect of N-AC were implanted with a catheter in the right jugular vein. After 1 week of recovery, rats designated for nicotine self-administration began the training (see General Methods section 3.5). The day after reaching the self-administration criteria, rats implanted with microdialysis probes were given N-AC and samples of dialysate collected during 4 hours for subsequent determination of GLU levels (see General Methods Section 3.7) (Figure 5.3a).

Two animals were excluded because the implant detached during microdialysis test. Two rats [one in (Nic - N-AC) and one in (Nic – Veh) group] were excluded because of an incorrect cannula and probe placement. Two rats in the Nic - N-AC group, 3 rats in the Nic – Veh group, and 1 rat in the Naive-N-AC group were excluded because their basal levels of GLU were under the limit of detection. One rat in the Nic - N-AC group with very high GLU levels was excluded as outlier after a Grubb test had been performed.

5.2.1 Histology

At the end of the experiments, rats were killed by decapitation. The brain was removed and frozen on dry ice. Correct probe and cannula placement was checked by visual inspection of the tracks on 30- μ m coronal sections (Figure 5.1). In its final position the membrane was

mostly in the core sub-region of the Nacc. Only rats with correct cannula and probe placements were included in the results (24 out of 26).

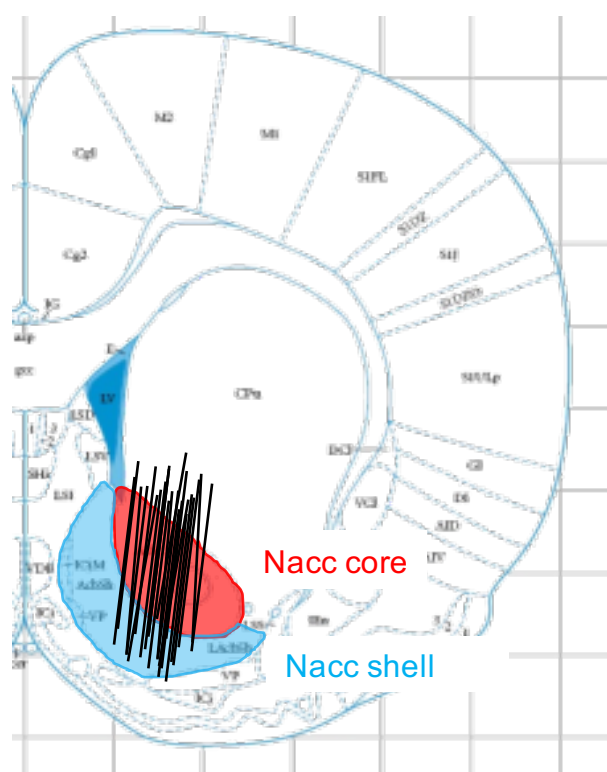


Figure 5.1. Locations of microdialysis probe membranes implanted in the Nacc (black bars) from all rats used to evaluate N-AC effect (n=24). Coronal brain section is taken from Paxinos and Watson (1997)

5.3 Results

5.3.1 Effects of elevated KCl in the aCSF on extracellular dopamine and glutamate in the nucleus accumbens

The increased release of neuro-transmitters produced by increasing the concentration of K^+ in the aCSF is commonly used as index of neuronal release. Indeed, Figure 5.2, show that raising K^+ in the aCSF was able to increase the release of both DA and GLU.

The data show a marked increase of DA [F(3,18)= 34.45, $P < 0.001$, ANOVA] and GLU [F(3,18)= 12.17, $P < 0.001$, ANOVA] in response to increased concentration of K^+ in the perfusion medium. Basal DA level increased from 13.61 ± 0.8 fmol/10 μ L to 175.7 ± 9.70 fmol/10 μ L, corresponding to about 13 times the basal level. Basal GLU levels increased from 2.1 ± 0.15 pmol/5 μ L to 5.98 ± 0.57 pmol/5 μ L, resulting in a 3-fold increase of the basal values.

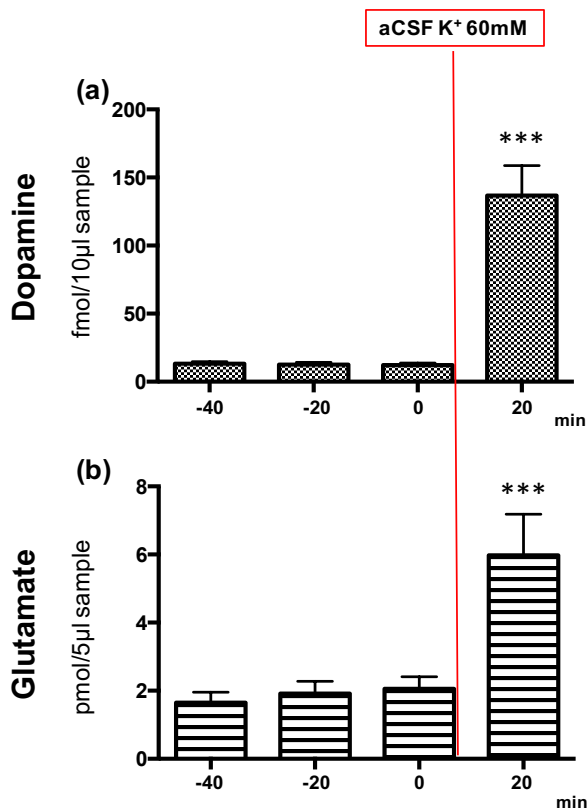


Figure 5.2. Effect of aCSF K⁺ 60 mM on extracellular dopamine (a) and glutamate (b) levels in the nucleus accumbens (n=7). Fractions collected every 20 min. ***P<0.001vs 0 min sample. Dunnett's multiple comparisons test.

5.3.2 Effect of N-AC 100 mg/kg on extracellular glutamate in the nucleus accumbens of rats after nicotine self-administration

Microdialysis began 24h after the last nicotine self-administration session (Figure 5.3.a). Basal extracellular concentrations of GLU in the Nacc were 1.45 ± 0.14 pmol/5 µl (n=24). These values were not corrected for in vitro recovery of the probes. Figure 5.3a shows a schematic representation of experimental conditions. Figures 5.3.b shows the responses on the active and inactive levers during the self-administration training [groups (Nic – N-AC) and (Nic – Veh)]. Rats acquired nicotine-reinforced responding [23.4 ± 0.4 (Nic – N-AC); 24 ± 0.5 (Nic – Veh) sessions needed to reach criterion]. During the last 3 days of self-administration, nicotine intake remained stable [20.8 ± 1 (Nic – N-AC); 17.3 ± 2.2 (Nic – Veh) infusions corresponding to 56.2 ± 2.6 (Nic – N-AC); 44.6 ± 5 (Nic – Veh) active lever presses] and responding was significantly lower during the last 3 saline sessions [5.1 ± 0.4 (Nic – N-AC), 3.3 ± 0.6 (Nic – Veh) infusions corresponding to 11.3 ± 0.9 (Nic – Veh), 8.7 ± 1.5 (Nic – Veh) active lever presses, $P < 0.05$]. Figure 5.3.c shows the effects of N-AC on basal extracellular GLU in the Nacc of

nicotine experienced and naïve rats. Extracellular GLU reached 185% of basal values 2.5 h after the i.p. injection of N-AC only in nicotine experienced rats. Mixed ANOVA showed a significant effect of time [F(12,249)=2.9, P<0.05] an interaction between time and groups [F(36,240)= 2.1, P<0.05] but not groups [F(3,20)= 3, P>0.05]. Post-hoc analysis showed that in the (Nic – N-AC) group GLU levels are significantly increased between 160 and 220 min after N-AC injection (P<0.05, Tukey’s test).

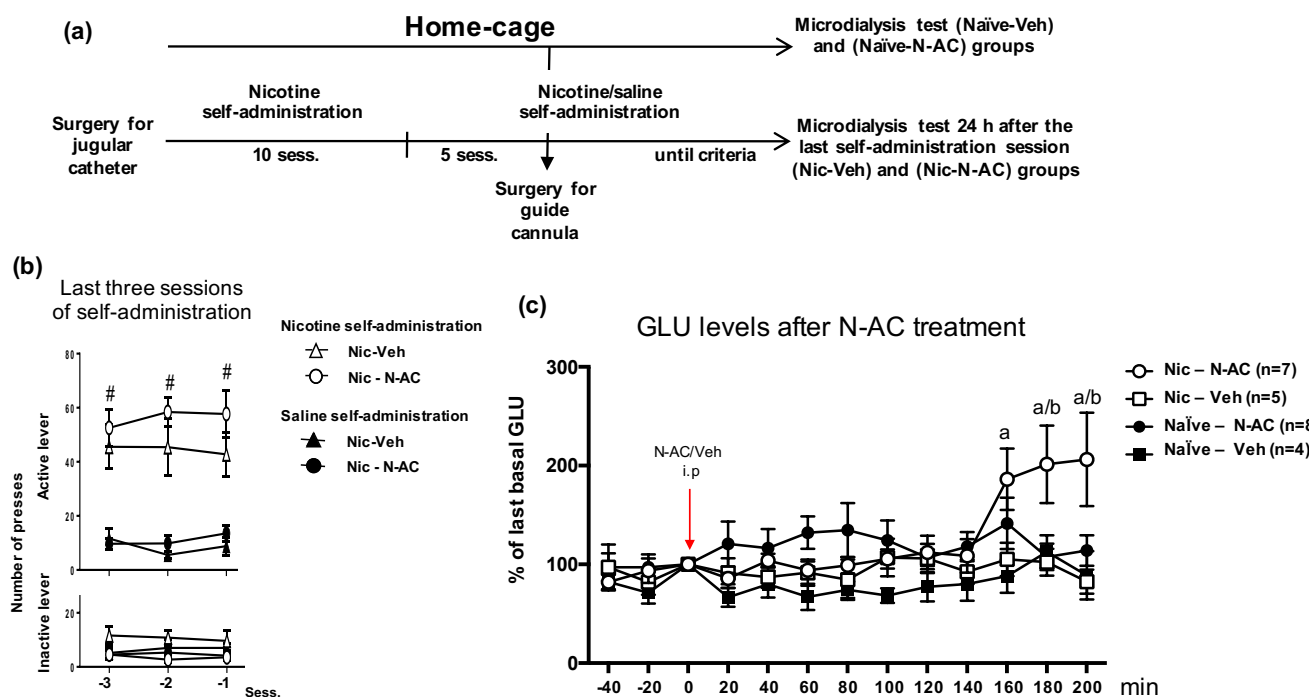


Figure 5.3. Timeline of the experiment (a). The last 3 sessions of nicotine and saline self-administration as well as active and inactive levers (b). Effect of N-AC (100 mg/kg, i.p.) on GLU levels in the Nacc. Injections were done immediately after the last basal (indicated by the arrow). For each rat data are expressed as % of the last basal and samples were collected every 20 min (c).

#P<0.05 vs. respective saline session; ^aP<0.05 vs. (Nic-Veh) and (Naïve-Veh); ^bP<0.05 vs. (Naïve-N-AC). Tukey’s multiple comparisons test

5.4 Discussion

The results of these experiments support the reliability of this procedure and demonstrate that N-AC (100 mg/kg) increased extracellular levels of GLU only in rats with an history of nicotine self-administration. With the stereotaxic coordinates I have used in these experiments the membranes of the probes resulted mostly in the core subregion of the Nacc, although limited part of the membrane was also sampling from dorsal striatum and/or shell

subregion of the Nacc (Figure 5.1).

In the present experiments microdialysis tests were performed between 14 and 18 h after probe insertion. This time window allows the recovery of the acute damage produced by probe insertion (van der Zeyden et al. 2008) and precedes glial response (Moussawi et al. 2011). To validate the current experimental procedure, I provided evidence that neurons responded as expected to high K^+ aCSF (Figure 5.2) with increased evoked release of GLU and DA as previously shown by (Paulsen and Fonnum 1989; Matos et al. 1990) thus, showing that the neuronal activity around the probe was not compromised by its insertion.

GLU levels in Nacc were increased 2.5 h after N-AC i.p. injections only in rats with an history of nicotine self-administration. This finding is in agreement with previous work on cocaine (Baker et al. 2003; Madayag et al. 2007). Indeed, the effect of the N-AC on extracellular GLU seems to be related to the fact that after deacetylation to cysteine and the oxidation of cysteine to cystine, it enhances the activity of the system Xc-, thus increasing extracellular GLU levels (Kalivas 2009). The reason of the delay (about 2.5 h) between N-AC injection and the increase of GLU levels is still not clear. It is possible that indirect mechanisms of N-AC or its metabolites, may be involved in this delayed effect. In fact, it has been recently shown that cysteine (a metabolite of N-AC) can be internalized directly via cysteine transporters leading to an increase in system Xc- activity. However, the intracellular signaling mechanism by which this occurs is unknown (Kupchick et al. 2011).

In summary, this experiment confirmed that similarly to what happened after cocaine self-administration, N-AC can increase extracellular GLU levels in the Nacc, only in nicotine-experienced rats. The increase in GLU levels occurred 2.5 h after N-AC injection, and I used this information to choose the time of N-AC pre-treatment for subsequent behavioral experiments..

Chapter Six – mGluR2/3 mediates short-term control of nicotine-seeking by acute systemic N-acetylcysteine

6.1 Introduction

Although pharmacotherapy and psychosocial support can help smokers quit (Fiore and Baker 2009), the high relapse rates indicate a pressing need for more efficacious therapies (Harmey et al. 2012).

Nicotine is the principal psychoactive component of tobacco smoke that reinforces and maintains smoking behavior (see Chapter 2). However, environmental stimuli associated with nicotine consumption play an important motivational role in maintenance and relapse (Niaura et al. 1989; Droungas et al. 1995; O'Brien et al. 1998; Lazev et al. 1999)(see also Chapter 8). The cues that have become associated with nicotine, especially by acting as conditioned reinforcers, induce craving and relapse in humans as well as drug-seeking and relapse behavior in animal models of addiction (Caggiula et al. 2002; Cervo et al. 2013). Thus, treatments that reduce vulnerability to nicotine conditioned cues in rodents may help prevent relapse to smoking.

As previously described (General Introduction Section 1.3.4), different classes of drugs of abuse alter GLU release in the Nacc after stimulation of the PFC. Synaptic plasticity changes within this circuitry may lead to a hypoglutamatergic state resulting in the preservation of drug-seeking behavior and drug-associated memories (Kalivas and Volkow 2011; van Huijstee and Mansvelder 2014). Thus, a systemically administered single dose of N-AC, a cysteine prodrug which activates the cystine/GLU exchange, system Xc⁻ (Baker et al. 2003) and indirectly restores tonic activation on presynaptic group II mGluR2/3, selectively prevents relapse to drug-seeking behavior in rats after chronic self-administration of cocaine (Moran et al. 2005; Moussawi et al. 2011; Kupchik et al. 2012). Similarly to cocaine, nicotine self-administration also reduces the expression of the catalytic subunit of system Xc⁻ (Knackstedt et al. 2009) as well as

the function of mGluR2/3 (Liechti et al. 2007). Moreover, acute N-AC attenuates cue-induced reinstatement of nicotine-seeking behavior (Ramirez-Nino et al. 2013).

By using the model described in Chapter 3, I tested whether the effect of N-AC on nicotine-associated cues reflect selective activity towards drug-seeking behavior or simply a more general ‘anhedonia’ by examining its effects on saccharin reinstatement. Then, I used the selective mGluR2/3 antagonist LY341495 (Kingston et al. 1998) to investigate whether the anti-relapse activity of N-AC on nicotine-seeking behavior required activation of mGLURs.

6.2 Specific materials and methods

All rats were trained as described in Chapter 3 (Section 3.5). Two rats were excluded, one because of lack of catheter patency (effects of N-AC on seeking behavior induced by reintroduction of nicotine-associated cues) and the other because the self-administration criterion was not reached (from the group of rats used to test the effects of N-AC on seeking behavior induced by reintroduction of saccharin-associated cues, saccharin-vehicle group)

6.2.1 Effects of N-AC on seeking behavior induced by reintroduction of nicotine-associated cues

In view of the lasting ability of nicotine-associated cues to revive drug-seeking behavior generated by this procedure, I investigated the effects of N-AC on nicotine-seeking behavior in a within-subject design. Eight rats were tested 4 times with S^{D+}/CS^+ , 2.5 h after i.p. vehicle or N-AC (30, 60 or 100 mg/kg). They were also tested once under S^D/CS^- , after vehicle. To control for order effects, different N-AC doses and vehicle were given in a random sequence across reinstatement sessions. Doses, administration route and pre-treatment time for N-AC were chosen on the basis of previous studies in rats (Zhou and Kalivas 2008; Reichel et al. 2011). Effects of N-AC on seeking behavior induced by reintroduction of the single components of nicotine-associated cues (S^{D+} , CS^+ and S^{D+}/CS^+) were also assessed in an independent group of 10 rats.

6.2.2 Interaction between LY341495 and N-AC on seeking behavior induced by re-introduction of nicotine-associated cues

Eight rats were trained as above and once the extinction criteria were met they were used to examine the interaction between the mGluR2/3 antagonist LY341495 (1 mg/kg i.p. at 2 and 0.5 h before test) or its vehicle, and N-AC 100 mg/kg i.p. or vehicle (2.5 h before test). All rats were tested 4 times with S^{D+}/CS^+ under the 4 treatment combinations. Rats were also tested once under S^{D-}/CS^- , after vehicles. To control for order effects N-AC, LY341495 and vehicle combinations were given in a random sequence across S^D s/ CS s reinstatement sessions. Doses and administration route for LY341495 were chosen on the basis of previous studies in rats (Moran et al. 2005; Baker et al. 2008; Kupchik et al. 2012).

6.2.3 Effects of N-AC on seeking behavior induced by reintroduction of saccharin-associated cues

Because of the diminishing ability of saccharin-associated cues to induce reinstatement (see Chapter 4, Section 4.2.3), a between-subjects experimental design was used to assess whether N-AC influenced seeking behavior for a high palatable reward. Naïve rats were trained as described in (Chapter 3, Section 3.5.4). Once they met the extinction criterion they were randomly allocated to 3 groups of 8 rats each to be tested after vehicle, 60 or 100 mg/kg N-AC. Each rat underwent two test sessions, one with S^{D+}/CS^+ and the other with S^{D-}/CS^- . The order of these two sessions was counterbalanced in each group. Test sessions were separated by at least 3 extinction sessions at criterion.

6.2.4 Effects of N-AC on spontaneous locomotor activity

The effect of N-AC on spontaneous locomotor activity was evaluated in separate groups of nicotine-experienced rats. Rats were trained to self-administered nicotine as stated above and as soon as they met the extinction criterion they were randomly allocated to 2 groups (n=7 for each group) to receive 2.5 h before the test 100 mg/kg i.p. N-AC or vehicle. Spontaneous

locomotor activity was recorded using gray non-transparent PVC cages (72x25x32 cm, LxWxH) with metal grid floor, equipped with 26 horizontal photocell beams along the long axis, 3 cm above the floor and 2.8 cm apart (TSE Systems, Bad Homburg, Germany). Locomotor activity was recorded for 1 h by a computer with dedicated software converting light beam interruptions into distance travelled (cm) in 10-min blocks of activity. Before and after tests, the cages were wiped with 70% ethanol and dried.

6.3 Results

6.3.1 Effects of N-AC on seeking behavior induced by reintroduction of nicotine-associated cues

Figure 6.1a shows the responses on the active and inactive levers during the self-administration training, extinction and reinstatement phases. Rats acquired nicotine-reinforced responding (with 20.1 ± 0.1 sessions needed to reach criterion). Nicotine self-administration remained stable during the last 3 sessions of the conditioning phase (19.2 ± 3.9 infusions, corresponding to 45.8 ± 7.1 active lever presses). Responding was significantly lower during the last 3 saline self-administration sessions [3.5 ± 2.0 infusions corresponding to 9.6 ± 1.4 active lever presses, $P < 0.05$ versus respective nicotine, $F(5,30) = 28$, Newman-Keuls test].

During extinction sessions, the number of presses on the active lever gradually decreased and rats met the criterion after an average of 12.6 ± 0.9 sessions. Inactive lever presses were minimal during these phases. Reintroduction of S^{D^-}/CS^- did not modify the number of active or inactive lever presses. In vehicle-treated rats, reintroduction of S^{D^+}/CS^+ increased responding on the active lever [$P < 0.05$ versus S^{D^-}/CS^- and extinction, Newman-Keuls test; $F(7,42) = 28.4$, $P < 0.05$] but not on the inactive ones. Individual recovery of active lever presses ranged from 89.7% to 129.5% of the lever presses during nicotine self-administration. N-AC reduced the number of active lever presses induced by S^{D^+}/CS^+ after 100 mg/kg N-AC, but not 30 and 60 mg/kg. The number of active lever presses after 100 mg/kg N-AC was significantly different from

vehicle+S^{D+}/CS⁺ ($P<0.05$, Newman-Keuls test) and similar to that during vehicle+S^{D-}/CS⁻ or the preceding extinction.

S^{D+}/CS⁺ reintroduction produced strong and sustained responding throughout the 1-h reinstatement session (Figure 6.1b). N-AC modified the cumulative response profile as reflected by a main effect for N-AC doses [$F(3,24)=4.4$, $P<0.05$], for time [$F(5,120)=87.4$, $P<0.01$] and a treatment x time (10-min intervals) interaction [$F(15,120)=3.0$, $P<0.01$]. Only 100 mg/kg N-AC reduced responding (simple effects, $P<0.05$).

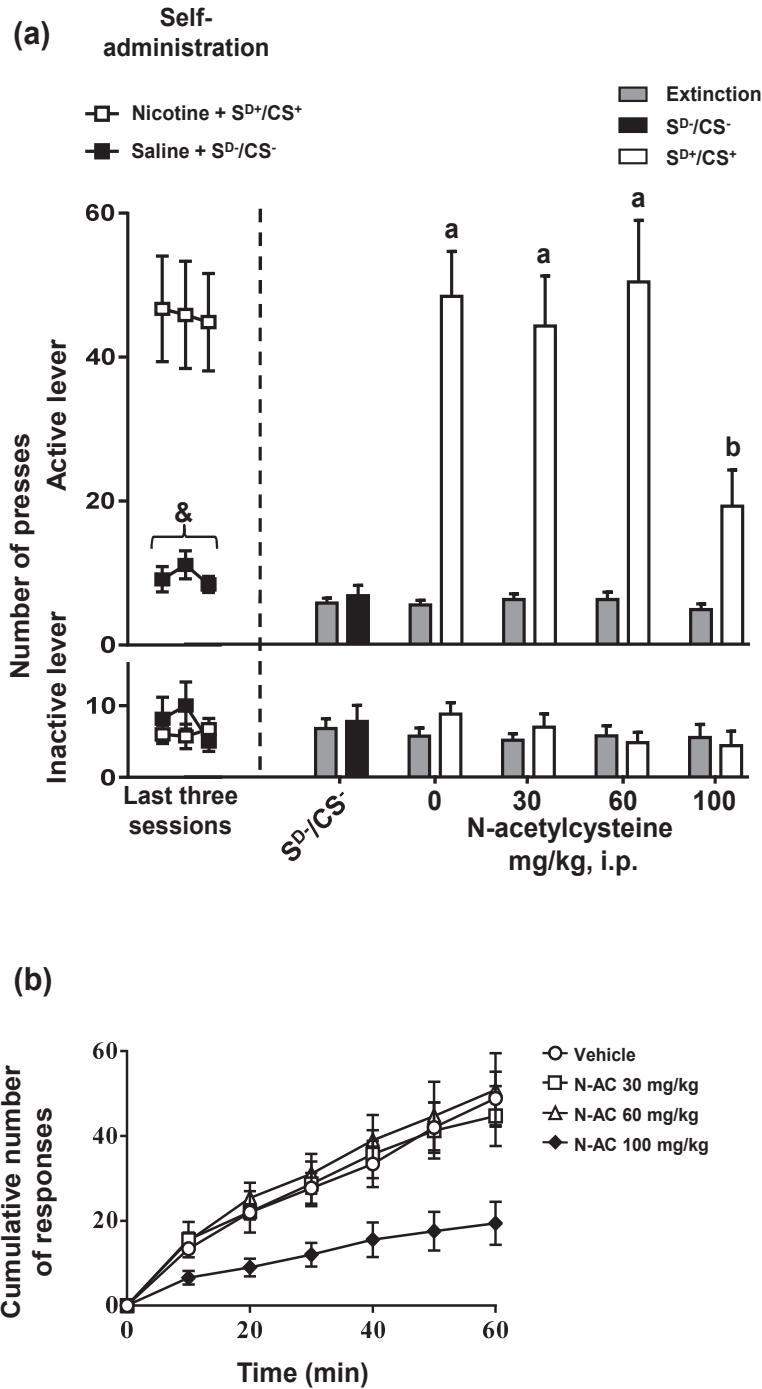


Figure 6.1. Effect of N-acetylcysteine (N-AC) on seeking behavior induced by reintroduction of nicotine-associated stimuli (S^{D+}/CS^+). **(a)** Histograms represent the mean \pm SEM presses on the active and inactive levers in a within-subject design (7 rats; see Methods for details). Also shown is the average number of lever presses during self-administration training (last 3 sessions), extinction (mean of last 3 sessions), and with stimuli associated with no-reward during reinstatement (S^{D-}/CS^-). Data were analyzed by one-way ANOVA with repeated measures. **(b)** Cumulative number of active lever presses (in 10-min intervals) throughout the 60-min reinstatement sessions. Solid symbols in panel (b) indicate $P < 0.05$ vs. vehicle. $^{\&}P < 0.05$, vs. nicotine + S^{D+}/CS^+ , $^aP < 0.05$ vs. respective S^{D-}/CS^- and respective extinction sessions, $^bP < 0.05$ vs. vehicle + S^{D+}/CS^+ , Newman-Keuls test.

6.3.2 Effects of N-AC on seeking behavior induced by reintroduction of the single components of nicotine-associated cues

A within subject design was adopted to verify how much S^{D+} and CS^+ by themselves contributed to the nicotine-seeking behavior after S^{D+}/CS^+ reintroduction and to evaluate the effects of N-AC on the single components of the complex stimuli. Ten rats were trained as above and once the extinction criteria were met they were tested with S^{D+} , CS^+ or S^{D+}/CS^+ after injection of vehicle or N-AC 100 mg/kg. Rats were also tested once under S^{D-}/CS^- , after vehicle. To control for order effect, the stimuli, N-AC and vehicle were given in a random sequence across reinstatement test sessions.

The effects of 100 mg/kg N-AC or vehicle on reintroduction on S^{D+} , CS^+ and S^{D+}/CS^+ are reported in Figure 6.2a. In this experiment rats acquired stable nicotine self-administration with an average of 22.0 ± 0.3 sessions needed to reach criterion. They maintained stable nicotine self-administration during the last 3 session of the conditioning phase (15.3 ± 1.4 infusions, corresponding to 40.2 ± 4.0 active lever presses). Responding was significantly lower during the last 3 saline self-administration sessions (3.0 ± 0.3 infusions corresponding to 7.7 ± 0.9 active lever presses, $P < 0.05$ versus respective nicotine, Newman-Keuls test). During lever extinction sessions the number of presses on the active lever gradually decreased to an average of 7.2 ± 0.6 and all rats met the criterion after an average of 9.4 ± 0.1 sessions. Inactive lever presses were minimal during these phases (average 4.5 ± 0.8).

In vehicle-treated rats, reintroduction of S^{D+}/CS^+ increased responding on the active lever [$F(2,18)=59.2$, $P < 0.05$], significantly more than after S^{D-}/CS^- or the preceding extinction sessions (both, $P < 0.05$, Newman-Keuls test). Reintroduction of S^{D+}/CS^+ induced more presses [$F(2,18)=13.9$, $P < 0.01$] than S^{D+} or CS^+ presented alone ($P < 0.05$ Newman-Keuls test). Independently from the cue presented, N-AC 100 mg/kg reduced the number of active lever presses after their reintroduction ($P < 0.05$ vs. vehicle, Newman-Keuls test), as shown by a main effect for treatment [$F(1,9)=75.2$, $P < 0.01$] and the treatment by cues interaction [$F(2,18)=3.8$, $P < 0.05$].

The effect of N-AC on reintroduction the S^{D+} , CS^+ and S^{D+}/CS^+ is also seen in the cumulative responses rate during a 1h session (Figure 6.2b). Independently from the type of cue, N-AC reduced response rates throughout the session [$F_{\text{Treatment}}(5,48)=6.8, P<0.01; F_{\text{Time}}(5,240)=91.5, P<0.01; F_{\text{Treatment} \times \text{Time}}(25,240)=4.3, P<0.05$].

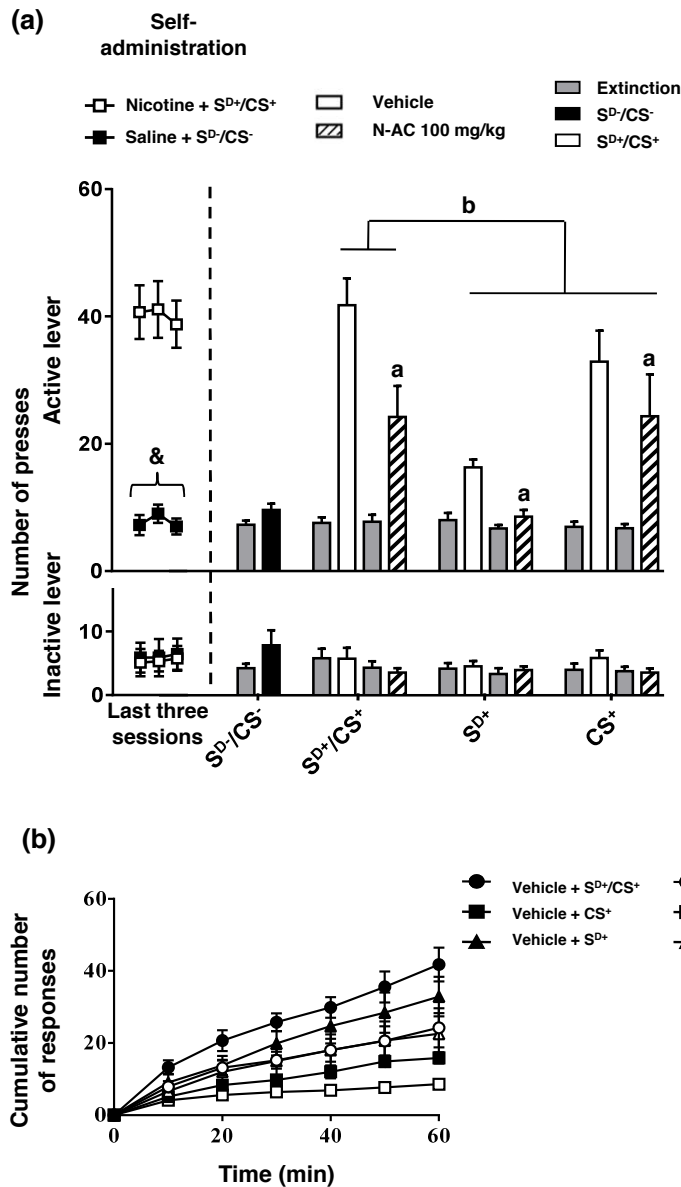


Figure 6.2. Effects of N-AC on seeking behavior induced by reintroduction of the single components of the nicotine-associated cues (S^{D+}/CS^+ , S^{D+} , CS^+). **(a)** Histograms represent the mean \pm SEM number of presses on the active and inactive levers (10 rats). For comparison, the figure also shows the average numbers of lever presses during self-administration training (last 3 days), extinction (mean of last 3 sessions) and with stimuli associated with no-reward during reinstatement (S^{D-}/CS^-). N-AC 100 mg/kg i.p. was injected 2.5 h before tests. Reinstatement data were analyzed by two-way ANOVA (with treatment and test session as between-subject factors). **(b)** Cumulative number of active lever presses (in 10-min intervals) throughout the 60-min reinstatement sessions. Solid symbols in panel (b) indicate $P<0.05$ vs. N-AC. $\&P<0.05$, vs. nicotine + S^{D+}/CS^+ , $^aP<0.05$ vs. respective vehicle, $^bP<0.05$ vs. respective S^{D+}/CS^+ .

6.3.3 Effects of N-AC on reintroduction of nicotine-associated cues and interaction with LY341495

Figure 6.3a reports the effect of co-administration of 1 mg/kg LY341495 on the reduction of nicotine-seeking behavior induced by N-AC 100 mg/kg. Rats acquired nicotine-reinforced responding (24.6 ± 0.6 sessions needed to reach criterion). They maintained stable nicotine self-administration during the last 3 sessions of the conditioning phase (14.1 ± 2.8 infusions, corresponding to 35.5 ± 6.7 active lever presses). Responding was significantly lower during the last 3 saline self-administration sessions (2.1 ± 0.3 infusions corresponding to 4.5 ± 0.9 active lever presses, $P < 0.05$ versus respective nicotine, Newman-Keuls test). During extinction sessions the number of presses on the active lever gradually decreased and all rats met the criterion after an average of 10.3 ± 0.6 sessions. Inactive lever presses were minimal during these phases.

In vehicle-treated rats, reintroduction of S^{D^+}/CS^+ increased responding on the active lever [$F(2,14)=70.1$, $P < 0.05$], which were significantly higher than S^{D^-}/CS^- and the 3 preceding extinction sessions (both $P < 0.05$ Newman-Keuls test) with individual recovery ranging from 54.3 to 111.4% of the presses during nicotine self-administration. N-AC 100 mg/kg reduced the ability of nicotine-associated stimuli to revive active lever presses [$F(1,7)=11.7$, $P < 0.05$]. LY341495 on its own had no effect [$F(1,7)=4.5$, $P > 0.05$] but it completely abolishes N-AC activity [N-AC x LY341495 interaction: $F(1,7)=61.1$, $P < 0.05$]. N-AC and LY341495, injected alone or in combination, had no effects on the number of inactive lever presses.

The ability of LY341495 to reverse N-AC activity is also shown in the cumulative response rate during a 1h session (Figure 6.3b). N-AC but not N-AC + LY341495 reduced the response rate throughout the session [$F_{\text{Treatment}}(3,28)=5.3$, $P < 0.01$; $F_{\text{Time}}(5,140)=75.0$, $P < 0.01$; $F_{\text{Treatment} \times \text{Time}}(15,140)=2.1$, $P < 0.05$].

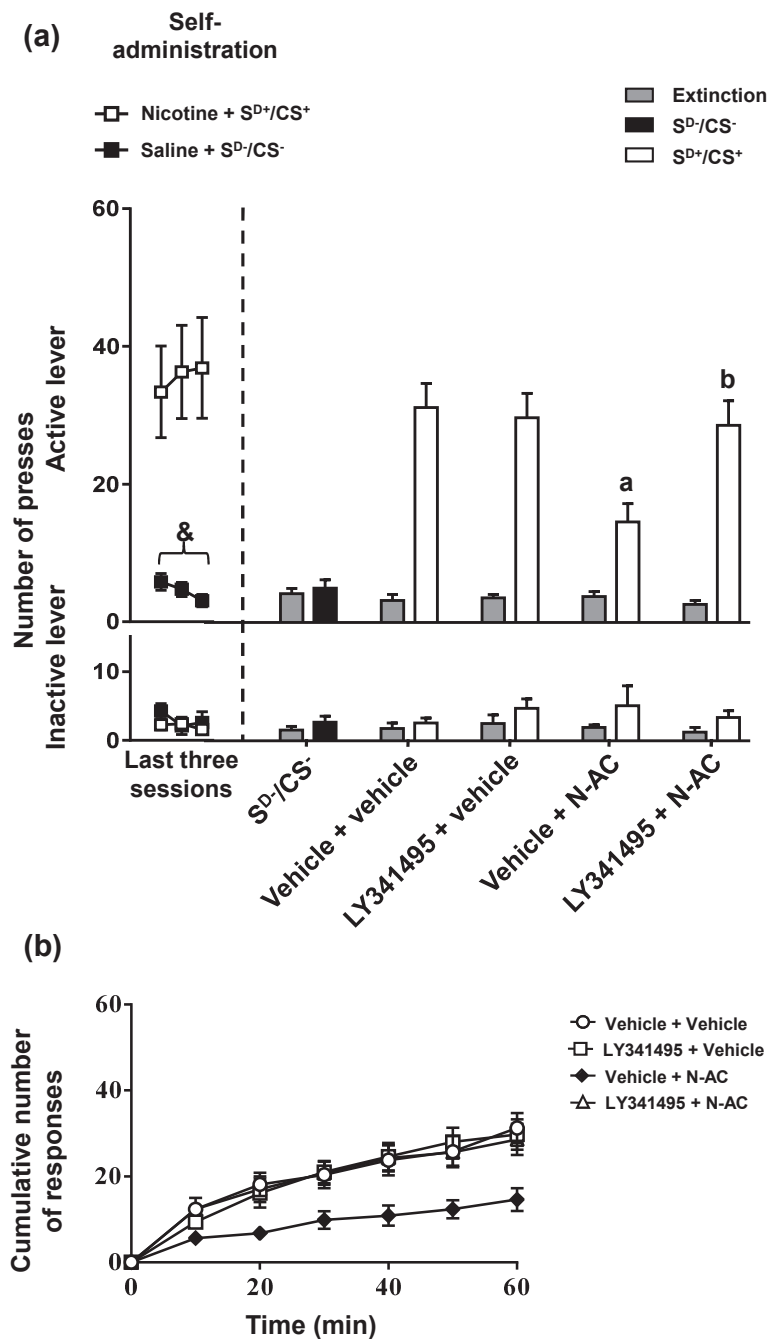


Figure 6.3 Effect of LY341495 on the reduction in nicotine-seeking behavior induced by N-acetylcysteine (N-AC). **(a)** Histograms show the mean \pm SEM presses on the active and inactive levers (8 rats) after re-introduction of nicotine-associated cue (S^{D+}/CS^+). For comparison, the figure also shows the average numbers of lever presses during self-administration training (last 3 days), extinction (mean of last 3 sessions) and with stimuli associated with no-reward during reinstatement (S^{D-}/CS^-). N-AC 100 mg/kg i.p. was injected 2.5 h before tests; LY341495, 1 mg/kg i.p. was given 2 and 0.5 h before tests. Reinstatement data were analyzed by two-way ANOVA (with treatment and test session as between-subject factor). **(b)** Cumulative number of active lever presses (in 10-min intervals) throughout the 60-min reinstatement sessions. Solid symbols in panel (b) indicate $P < 0.05$ vs. vehicle + vehicle. $\&P < 0.05$, vs. nicotine + S^{D+}/CS^+ , $^aP < 0.05$ vs. respective vehicle + vehicle, $^bP < 0.05$ vs. N-AC + vehicle, Newman-Keuls test.

6.3.4 Effects of N-AC on reintroduction of saccharin-associated cues

Figure 6.4a reports the effects of N-AC on seeking behavior triggered by saccharin-associated cues. Rats developed stable saccharin-reinforced responding and lever presses during no-reward sessions gradually decreased (27.3 ± 0.2 days required to meet the training criterion). During the last 3 training sessions all rats in the 3 groups earned similar amounts of saccharin (56.8 ± 9.4 , 54.4 ± 11.9 and 58.0 ± 13.3 active lever presses, $P > 0.05$, Newman-Keuls test). Responding was significantly lower during the last 3 no-reward sessions (18.0 ± 4.2 , 14.5 ± 3.7 and 16.9 ± 3.7 , $P > 0.05$, Newman-Keuls test). During extinction, responding on the active lever gradually decreased and rats met the extinction criterion after an average of 8.1 ± 0.3 sessions. Responding on the inactive lever was always not significant.

No significant differences were found between the 3 groups of animals during the last 3 sessions of saccharin-maintained responding. No differences were observed in reward and extinction on either the active or inactive lever. Mixed factorial ANOVA indicated a significant effect of S^{D+}/CS^+ on the active [$F_{\text{treatment}}(2,80)=0.1$, $P > 0.05$; $F_{\text{test sessions}}(4,80)=50.3$, $P < 0.05$ and $F_{\text{treatment} \times \text{test sessions}}(4,80)=0.1$, $P > 0.05$], but not the inactive lever. There were no significant effects of treatment and no interaction between N-AC and S^{D+}/CS^+ reintroduction on active or inactive lever presses. The revived active lever presses were similar to those observed during the sessions of saccharin-maintained behavior and significantly higher than those during no reward sessions ($P < 0.05$, Newman-Keuls test). Individual recovery of active lever presses ranged from 38.2 to 111.1%, 36.7 to 108.9% and 32.6 to 108.6% for rats treated with vehicle, 60 and 100 mg/kg N-AC compared to those observed during the sessions of operant responding for saccharin, respectively.

N-AC lack of activity on saccharin-seeking behavior was also evident from the cumulative number of active lever responses (Figure 6.4b). Mixed-factorial ANOVA found a significant effect of time [$F(5,100)=66.7$, $P < 0.05$] but no effect for treatment [$F(2,20)=0.2$, $P > 0.05$] and no treatment x time (10-min intervals) interaction [$F(10, 100)=0.5$, $P > 0.05$].

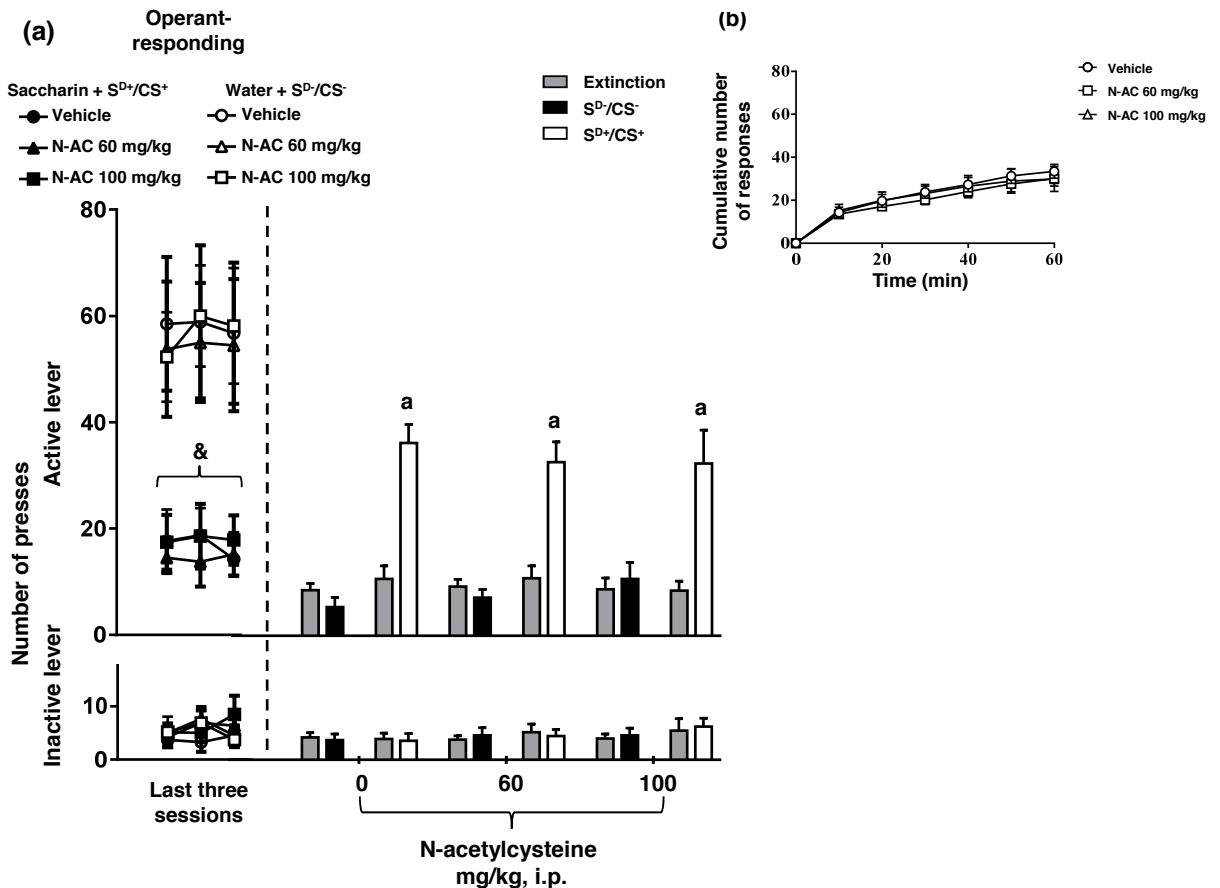


Figure 6.4. Effects of N-acetylcysteine (N-AC) on seeking behavior induced by reintroduction of saccharin-associated cues (S^{D+}/CS⁺). **(a)** Histograms show the mean ± SEM presses on the active and inactive levers during reinstatement sessions in a between-subjects experimental design (Vehicle = 7, N-AC 60 mg/kg = 8, N-AC 100 mg/kg = 8). Also shown is the average number of lever presses during self-administration training (last 3 sessions), extinction (last 3 days before and between the different reinstatement sessions) and with stimuli associated with no-reward during reinstatement (S^{D-}/CS⁻). Data were analyzed by mixed-factorial ANOVA (with treatment as between-subject factor and sessions as within-subject factor). **(b)** Cumulative number of active lever presses (in 10-min intervals) throughout the 60-min reinstatement sessions. ^a*P* < 0.05 vs. S^{D-}/CS⁻, and respective extinction, Newman-Keuls test.

6.3.5 Effects of N-AC on spontaneous locomotor activity

N-AC effect on the distance travelled during the 60-min observation period was analyzed by Student *t*-test. Changes were considered significant when *P* < 0.05.

As reported in Figure 6.5a, 100 mg/kg i.p. N-AC did not modify spontaneous locomotor activity of rats with a history of nicotine self-administration. In fact, the distance travelled by the animals treated with N-AC was not statistically different from that of vehicle-treated rats (*t* = 1.8, *df* = 14, *P* > 0.05, Student *t*-test). The lack of N-AC effect was also evident in the

cumulative distance travelled by rats in 10-min blocks (Figure 6.5b). Mixed-factorial ANOVA found a significant effect of time [$F(5,70)=82.9, P<0.01$], but not of treatment [$F(1,14)=0.1, P>0.05$] and no interaction of treatment x time [$F(5,70)=0.1, P>0.05$].

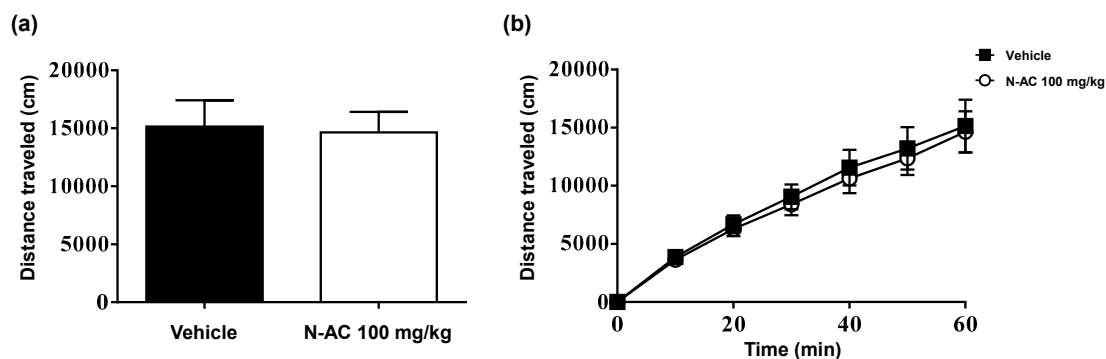


Figure 6.5. (a) Effects of N-AC (100 mg/kg, i.p. 2.5 h pre-treatment) on the distance traveled (cm) in the activity cages during the 60-min observation (7 rats). Histograms show the mean \pm SEM of seven rats. Data were analyzed by Student's *t*-test. (b) Cumulative distance traveled in the activity cages (10-min blocks of activity) of seven rats. Data were analyzed by mixed-factorial ANOVA (with treatment as between-subject factor and time as within-subject factor) followed by simple effects analysis.

6.3.6 Short but not long-lasting N-AC effect on nicotine-seeking behavior

Seven rats, treated firstly with 100 mg/kg N-AC and then during the following reinstatement session treated with vehicle, were selected (pooled from Figure 6.1, 6.2, 6.3). Data were analyzed by one-way ANOVA for repeated measure with test sessions as the main factor. Figure 6.6 clearly shows that one single N-AC injection while acutely decreasing the number of active lever presses had no effect on the following reinstatement test [$F(3,18)=102.9, P<0.01$, Newman-Keuls test]. This was also in agreement with N-AC pharmacokinetics, since it has a terminal $t_{1/2}$ of 6.25 h.

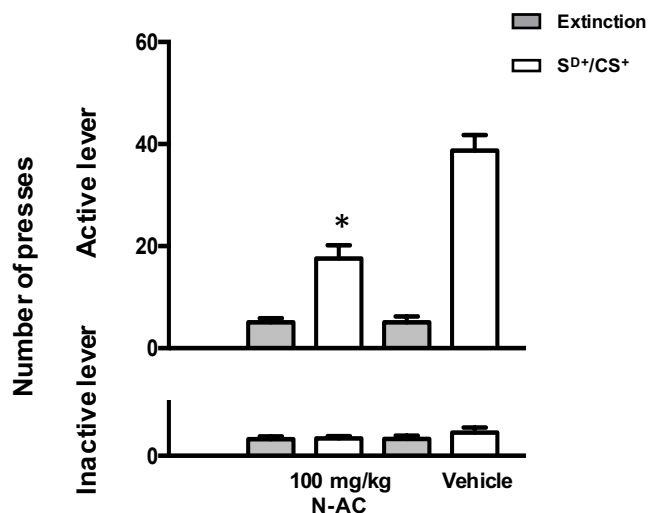


Figure 6.6. The figure shows active and inactive lever presses during two consecutive presentation of SP⁺/CS⁺ and extinction (mean of last 3 sessions). The acute effect of 100 mg/kg N-acetylcysteine on the first presentation of SP⁺/CS⁺ was not maintained when the same rats (n=7) were tested the following time with SP⁺/CS⁺. Data were taken from acute experiments. * $p<0.01$ vs. following SP⁺/CS, Newman-Keuls test.

6.4 Discussion

Here I show that systemic dose of N-AC (100 mg/kg) reduced nicotine-seeking behavior after reintroduction of drug-associated stimuli, without influencing either the responding elicited by stimuli conditioned to saccharin or spontaneous locomotor activity of rats with a similar nicotine self-administration history. In addition, an important finding presented in this Chapter is that, blocking mGluR2/3 with the selective antagonist LY341495 (1 mg/kg) completely prevented the effect of N-AC on nicotine-seeking behavior.

A single dose of 100 mg/kg N-AC significantly attenuated the nicotine-seeking behavior observed on reintroduction of S^{D+} , CS^+ and S^{D+}/CS^+ . This effect seems behaviorally specific since N-AC did not influence inactive lever presses. Moreover, N-AC at the dose effective in reducing nicotine-seeking did not alter the spontaneous locomotor activity of rats with the same nicotine self-administration experience. In addition, this dose did not affect the conditioned reinstatement of seeking behavior triggered by S^{D+}/CS^+ associated with a highly palatable reinforcer (saccharin).

The fact that N-AC reduced nicotine-seeking behavior agrees with the results of Ramirez-Niño and et al. (2013), although we found that 100 but not 60 mg/kg of N-AC reduced the reinstatement level. Methodological differences between our protocol and that used in previous reports on N-AC activity could have influenced our results. A common procedure used by Ramirez-Niño et al (2013) and other groups (Corrigall and Coen 1989; Vazquez-Sanroman et al. 2016) is to initially food-train animals and to run one daily session of nicotine self-administration during which rats are exposed only to the CS. However, we used a more complex S^{D+}/CS^+ cue presentation and trained the rats to discriminate between nicotine and saline self-administration. This may have led to a stronger link between drug and cues that might have required a higher dose of N-AC to be disrupted.

On the other hand, the lack of N-AC activity on saccharin-seeking seems to contrast with the ability of the compound to reduce food-seeking behavior as described by Ramirez-Niño et al. (2013). However, in contrast to the large difference in the rate of responding that is commonly

reported when assessing nutritive or highly palatable reinforcer-seeking behavior (Hopkins et al. 2012; Ramirez-Nino et al. 2013), we used a concentration of saccharin, a non-caloric sweeteners, that generated a rate of responses during reinstatement testing that closely resembles those observed during evaluation of conditioned nicotine-seeking behavior. Although our results suggest that the acute effect of N-AC appears to be selective on nicotine-versus saccharin-seeking, this does not exclude the possibility that N-AC can reduce the strong seeking behavior induced by a primary reinforcer, such as the food in hungry rats (Ramirez-Nino et al. 2013). Future studies are required to better understand whether food-seeking behavior could be attenuated by N-AC since similar doses of the compound did not influenced priming-induced food-seeking (Baker et al. 2003).

The neuronal mechanism by which N-AC reduced nicotine-seeking behavior by re-introduction of drug-associated cues is not completely known. Different classes of drugs of abuse alter GLU release in the Nacc after stimulation of the PFC and changes in synaptic plasticity within this circuit may contribute to the preservation of drug-seeking behavior and drug-associated memories (Gass and Olive 2008; Kalivas 2009). Baker et al. (2002) showed that in the Nacc, ~60% of basal extracellular GLU is derived from constitutive cystine-GLU exchange (system Xc⁻). System Xc⁻, which exchanges cysteine for intracellular Glu, is the rate-limiting step in GSH synthesis and the main source of extracellular GLU (McBean 2002). Down-regulation of system Xc⁻ may account for the changes in basal GLU levels after chronic cocaine (Baker et al. 2003; Madayag et al. 2007). Basal levels of GLU dropped after cocaine self-administration and GLU release was enhanced during priming-induced heroin- and cocaine-seeking behavior (LaLumiere and Kalivas 2008). Environmental contexts and cues are also important factors affecting the ability of cocaine and nicotine to raise extracellular levels of GLU in the Nacc (Hotsenpiller et al. 2001; Gipson et al. 2013). An impaired GLU homeostasis in the Nacc was also found after nicotine self-administration as demonstrated by the findings that a) the catalytic subunit of system Xc⁻ was decreased in rats withdrawn from chronic 12 h/day nicotine self-administration (Knackstedt et al. 2009) and b) GLT-1 expression was decreased

after both chronic 12 h/day and 2 h/day nicotine self-administration (Knackstedt et al. 2009; Gipson et al. 2013). Moreover, cue-induced reinstatement of nicotine-seeking produced an increase in extracellular GLU in the Nacc (Gipson et al. 2013). All these findings stress the point that, similarly to other drugs of abuse, the expression of nicotine-seeking behavior may depend on altered GLU transmission in the Nacc. Many recent studies have also shown that extracellular GLU levels in the Nacc-core regulate reinstatement of cocaine and heroin seeking, through stimulation of group II mGluRs (mGluR2/3s) (Baptista et al. 2004) (Moran et al. 2005; Bossert et al. 2006; Peters and Kalivas 2006; Xi et al. 2010; Moussawi et al. 2011).

My hypothesis is that like cocaine, N-AC interacts with system Xc- and the glial GLU transporter GLT-1 by restoring homeostasis of the basal GLU level in the Nacc-core (Kalivas 2009), increasing tonic activation of mGluR2/3. This suggestion is supported by data presented here showing that co-administration of the antagonist LY341495 prevented N-AC effect on reinstatement. To the best of my knowledge, these data are the first to demonstrate that nicotine requires mGluR2/3 for an acute N-AC effect. This may reflect the fact that the hypoglutamatergic state generated by nicotine self-administration prevents tonic activation of mGluR2/3. LY341495 alone has no effect since the activity of these receptors was already down-regulated. When N-AC enhances extrasynaptic GLU levels in the presence of LY341495, mGluR2/3 control on pre-synaptic GLU release could no longer be restored. Our results show that N-AC effect required the activation of mGluR2/3, likely due to N-AC activation of system Xc- resulting in an increase of extrasynaptic GLU levels (Chapter 5). This is in agreement with acute N-AC effect on cocaine-seeking behavior (Moran et al. 2005). Of note, Reissner et al. (2015) have recently demonstrated that repeated N-AC prevents cue-induced cocaine-seeking behavior, mainly restoring the activity of GLT-1 thereby reducing the activation of mGluR5. Thus, it may possible that chronic N-AC treatment involves pathways that are different from the ones activated acutely. Further study will be needed to clarify whether for nicotine too, a chronic treatment with N-AC involves GLT-1, system Xc- or both.

Our findings support the evidence that alterations in mGluR2/3 presynaptic control may be a common feature of the chronic use of different classes of drugs of abuse as well as a key protein for acute N-AC anti-relapse activity.

So far only small clinical trials have evaluated the use of N-AC as a smoking cessation treatment (for a review, see Deepmala et al. 2015). Recently a 12-week double blind randomized controlled trial evaluating the effect of N-AC (3 g/day) in smokers found that the treatment significantly reduced the daily number of cigarettes smoked, the exhaled carbon monoxide and that more patients were able to quit smoking compared to placebo (Prado et al. 2015). Moreover Froeliger et al. (2015) reported that smokers treated with N-AC (2.4 g/day, for 3.5 days) not only maintained abstinence and reported less craving but also have a recovery of the dysregulation in corticostriatal connectivity compared to the placebo group (Froeliger et al. 2015). Finally, its high safety profile opens up the possibility to use N-AC in combination with other therapeutic strategies to boost smoking cessation (McClure et al. 2014). Since no data are available on the use of N-AC in humans for preventing cue-induced nicotine craving, our results support the use of N-AC as a therapeutic aid for acute cue-controlled nicotine-seeking during the withdrawal period. Further work is needed to clarify in what conditions chronic N-AC could be useful as a lasting anti-relapse medication.

Considering the short-lasting anti-relapse activity of N-AC 100 mg/kg (Figure 6.6), in the next Chapter I will report the results obtained by evaluating whether repeated N-AC injections may produce a long-lasting effect on cue-induced nicotine-seeking.

Chapter Seven – Chronic N-AC treatment induces long-lasting prevention of cue-induced nicotine-seeking behavior

7.1 Introduction

With the progressive engagement of different pavlovian and instrumental learning systems in the brain, neutral environmental cues and contexts become strongly associated with the reinforcing properties of nicotine (Henningfield and Goldberg 1983).

A non-pharmacological strategy aiming to reduce the impact of drug-related cues is the extinction therapy. Extinction is not forgetting but is an active learning process involving neural substrates that subserve other forms of learning and memory (Bouton 2004). Thus, it is likely that also other forms of learning, such as extinction learning may induce enduring synaptic changes through mediation of GLUergic mechanisms (Conklin and Tiffany 2002; Peters and De Vries 2012).

Thus far cue extinction therapies have shown limited effectiveness in preventing relapse (Conklin and Tiffany 2002) and their clinical benefits are still debated (Hajek et al. 2013). In agreement with clinical observations, preclinical evidence of the efficacy of extinction training appears to be very limited (Figure 8.1). It has been proposed that a more ethological cue exposure therapy (CET) targeting drug-associated cues in the same context where the associative over-learning and consolidation took place (i.e. becoming “over-learned”) could be a better strategy to improve CET efficacy. Nevertheless, extinction efficacy might be also limited by dysfunctions of GLU transmission in memory systems induced by chronic drug abuse needed for extinction learning and consolidation (Conklin and Tiffany 2002).

Despite the fact that acute N-AC reduces nicotine-seeking behavior by activating mGluR2/3 and restoring GLU homeostasis (see Chapter 6), the effect of acute N-AC treatment was short-lasting (Figure 6.6) and a drug regimen that induces long-lasting repair of nicotine-induced GLU-mediated neuroplasticity might have greater therapeutic value.

It has been shown that repeated N-AC persistently restored non-synaptic GLU tone thus, normalizing the alterations in the cortico-accumbens synaptic transmission and glial cell activity produced by chronic cocaine self-administration (Moussawi et al. 2010). Moreover, repeated N-AC markedly reduced relapse especially when given during the extinction of the instrumental response (LP EXT) (Reichel et al. 2011). For this reason, I speculated that restoring nicotine-induced dysfunction in the GLUergic system by repeated N-AC administration might increase the extinction of the over-learned relationship between nicotine, conditioned-cues and instrumental response. Nevertheless, in preclinical settings extinction of drug-related memories has been only studied using the extinction/reinstatement model focusing on the extinction of the instrumental response (LP EXT) used to self-administered the drug, the LP EXT (Carter and Tiffany 1999; See 2002). However, it is important to bear in mind that LP EXT does not have an equivalent in clinical setting (Everitt 2014), thus limiting the predictive validity of the model. To date few preclinical studies have specifically evaluated the impact of drug conditioned cues extinction (Torregrossa and Taylor 2013).

With the aim of targeting nicotine-associated cues, firstly I evaluated the effects of chronic N-AC effects when given during LP EXT (Exp. 1). Then, I evaluated the effects of chronic N-AC effects when given during a model of experimental CET (Exp. 2) obtained by refining the experimental model previously used in my lab (Chapter 8) by adding nicotine-associated cues during the phase of LP EXT. Finally, the effect of the pure pharmacological treatment was also assessed in rats receiving N-AC during abstinence (Exp. 3).

7.2 Specific materials and methods

All rats were trained for nicotine self-administration and discriminative learning training as described previously. Five rats were excluded, 2 because of lack of catheter patency (1 in Exp. 2 and 1 in Exp. 3), 2 because the self-administration training criterion was not reached (1 in Exp. 1 and 1 in Exp. 2), and 1 rat (Exp. 2: 100 mg/kg N-AC) was excluded from all the analysis because treatment was never effective and it was identified as an outlier (Groub's test).

After the 1st reinstatement, test rats were randomly assign to one of the 3 groups (Veh, 60 mg/kg, 100 mg/kg N-AC).

7.2.1 First reinstatement test

All the rats used in Exp. 1, 2 and 3 underwent nicotine self-administration training and discriminative learning training (Figure 7.2.1). Thus, rats learned to associate S^{D^+}/CS^+ to nicotine self-administration and S^{D^-}/CS^- to saline self-administration.

At the end of the training the lever press extinction phase was omitted and the day after the end of the self-administration training rats were immediately tested a first time either with nicotine-associated cues (S^{D^+}/CS^+) (half of the rats in each experiment) or saline-associated cues (S^{D^-}/CS^-) (half of the rats in each experiment). The day after the test, the order was switched (i.e. 1st day S^{D^+}/CS^+ , 2nd day S^{D^-}/CS^- and vice versa). The rats were then randomly allocated to the 3 different experimental groups (Figure 7.2.1).

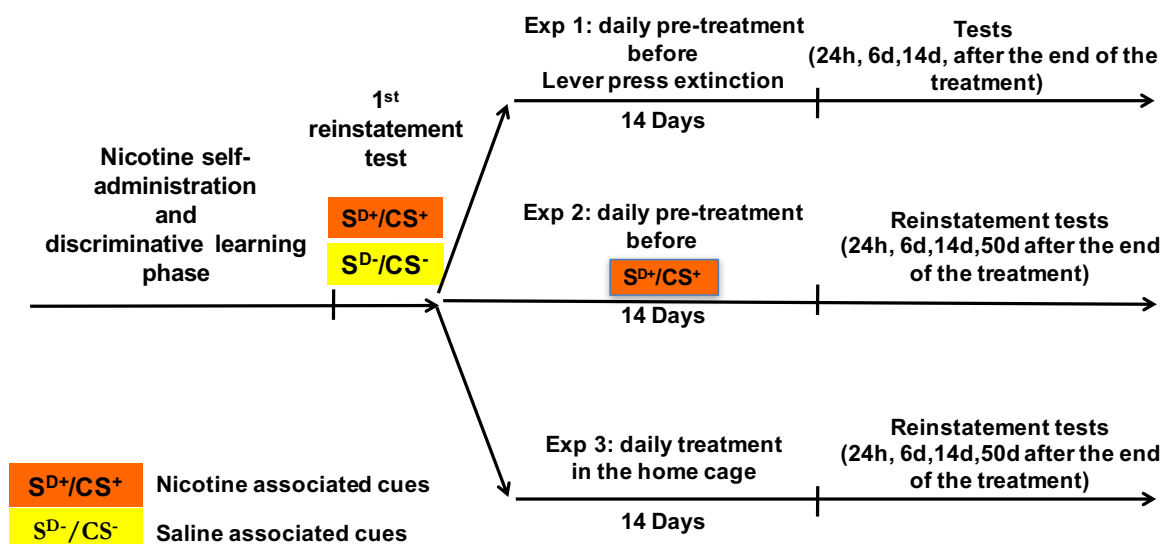
7.2.2 Chronic treatments and reinstatement tests after the end of the treatments

A total of 72 rats were used divided in three independent experimental groups. Exp. 1 24 rats were used: vehicle (n=8), N-AC 60 mg/kg (n=8) and N-AC 100 mg/kg (n=8). Exp. 2 24 rats were used: vehicle (n=8), N-AC 60 mg/kg (n=8) and N-AC 100 mg/kg (n=8). Exp. 3 24 rats were used: vehicle (n=8), N-AC 60 mg/kg (n=7) and N-AC 100 mg/kg (n=9). In Exp. 1 and 2 every day for 14 days, rats received the appropriate treatment i.p. 2.5 h before either 1h of lever press extinction training (LP EXT) or cue extinction training (CET), respectively.

In Exp. 3 the rats were appropriately treated i.p. once a day while in the home-cage for 14 days. At the end of the treatment, in order to evaluate any effect on nicotine cue-induced reinstatement rats were tested twice with S^{D^+}/CS^+ (1 and 6 days after treatment). On days 14 and 50, half of the rats were tested with S^{D^+}/CS^+ and the other half with S^{D^-}/CS^- . The day after the test, the order was switched (i.e. day 14 S^{D^+}/CS^+ , day 15 S^{D^-}/CS^- and vice versa): thus, rats

were tested also on days 15 and 51. For the sake of simplicity, I pooled the data obtained from the same cues and indicated them as day 14 and 50 after the end of the treatment.

Figure 7.2.1 Timeline of the experiments



7.3 Results

7.3.1 Training and first reinstatement tests before the beginning of the N-AC treatment

As shown in Table 7.1, rats in the 3 experiments developed stable nicotine self-administration and lever presses in the saline sessions gradually decreased (data not shown). During the last 3 sessions of self-administration training, all experimental groups earned similar amount of nicotine as demonstrated by the similar number of infusions. Responding on the inactive lever was always non significant.

In the 1st reinstatement test in all groups S^{D+}/CS^+ , but not S^{D-}/CS^- renewed active lever presses ($P < 0.05$ vs. S^{D-}/CS^- , Newman Keuls test) to a similar extent in the different groups ($P > 0.05$ vs. S^{D+}/CS^+ , Newman Keuls test). The revived active lever presses were similar to those during the sessions of nicotine self-administration and significantly higher than during the saline self-administration sessions. Individual recovery of active lever presses during the first reinstatement was: Exp. 1 (veh) $105.9 \pm 11.7\%$, (N-AC 60 mg/kg) $108.5 \pm 15.8\%$ and (N-AC 100 mg/kg)

116.2±12.0 % of the presses during the sessions of operant responding for nicotine; Exp. 2
(veh) 121.6±21.7%, (N-AC 60 mg/kg) 87.4±9.9% and (N-AC 100 mg/kg) 98.1±10.5%; Exp.
3 (veh) 96.1±9.2%, (N-AC 60 mg/kg) 91.6±6.8% and (N-AC 100 mg/kg) 121.3±7.0%.

Table 7.1. Self-administration training and 1st reinstatement test

	Self-Administration Training (mean of last 3 days)						1 st Reinstatement Test			
	Sessions to criteria	Number of Nic Infusions	Act. Lever Presses Nic	Lever Presses Sal	Inact. Lever Presses Nic	Lever Presses Sal	Act. Lever Presses S ^{D+} /CS ⁺	S ^{D-} /CS ⁻	Inact. Lever Presses S ^{D+} /CS ⁺	S ^{D-} /CS ⁻
Experiment 1 Treatment + EXT										
Vehicle	12.6±0.2	16.4±2.1	37.7±5.5	6.0±0.6	4.4±0.8	3.9±1.1	37.8±4.8*	6.1±1.5	3.3±0.6	4.9±1.5
N-AC 60 mg/kg	12.3±0.2	15.5±1.8	36.4±4.5	6.3±0.6	5.3±1.9	6.2±1.9	38.4±6.6*	6.1±1.2	6.0±1.7	4.1±1.0
N-AC 100 mg/kg	12.4±0.2	13.8±2.2	34.0±5.7	7.1±1.1	5.7±1.5	5.8±2.0	36.8±3.3*	6.1±1.2	5.9±1.5	4.9±2.8
Experiment 2 Treatment + CFT										
Vehicle	12.0±0.3	13.9±2.5	34.7±5.5	5.0±1.1	3.3±1.9	5.5±1.7	37.6±5.0*	7.8±0.6	6.4±1.5	8.1±3.0
N-AC 60 mg/kg	12.0±0.3	13.2±2.5	34.7±6.3	8.1±2.1	1.6±0.4	3.8±1.3	29.3±5.7*	5.5±1.2	3.8±0.9	5.2±3.0
N-AC 100 mg/kg	12.0±0.2	13.5±2.2	33.3±6.2	5.9±1.3	1.2±0.3	2.5±0.6	31.4±5.5*	5.4±1.4	3.4±1.3	2.4±1.2
Experiment 3 Treatment + ABS										
Vehicle	11.5±0.2	13.8±1.8	35.6±4.5	6.1±0.6	5.5±1.2	3.6±1.6	33.3±5.5*	5.9±1.3	5.5±1.2	3.4±1.7
N-AC 60 mg/kg	11.3±0.2	15.2±1.5	37.8±3.2	5.8±1.0	11.1±2.0	5.5±2.9	35.2±4.5*	8.5±2.2	6.6±3.1	3.8±1.8
N-AC 100 mg/kg	11.7±0.2	13.7±2.2	35.6±4.1	6.3±1.1	6.5±3.1	5.1±1.1	43.0±5.8*	7.7±1.0	11.1±2.0	4.8±1.1

Table 7.1. Data are expressed as means±S.E.M. After that rats met the discrimination criteria (±15% over 3 consecutive sessions and the mean of the last 3 days of saline self-administration is less the 20% of nicotine sessions), 24h after the self-administration training half of the rats were exposed to stimuli predictive of (S^{D+}) and associated to nicotine availability (CS⁺) and half to stimuli predictive of (S^{D-}) and associated to saline availability (CS⁻). Day after rats tested with S^{D+}/CS⁺ were tested with S^{D-}/CS⁻ and vice versa. * p<0.05 vs. respective S^{D-}/CS⁻, Newman-Keuls test.

7.3.2 Evaluation of chronic N-AC treatment during LP EXT (Exp. 1)

Figure 7.2b shows the responses on the active lever during the self-administration (mean of the last 3 sessions), the 1st reinstatement test, the 14 LP EXT sessions of rats daily pre-treated with N-AC or vehicle, and reinstatement tests after the end of treatment.

A mixed-factorial ANOVA found a significant effect of sessions [$F(23,460)=88.20, P<0.01$] and interaction treatment x sessions [$F(46,460)=2.04, P<0.05$] with no effect of the treatment [$F(2,20)=2.34, P>0.05$]. Responses during the last 3 sessions of nicotine self-administration were similar between groups ($P>0.05$, Newman Keuls) and significantly higher than the means of last 3 saline self-administration sessions ($P<0.05$ vs. nicotine self-administration, Newman–Keuls test). In the 1st reinstatement, test S^{D^+}/CS^+ revived active presses in all groups ($P<0.05$ vs. S^{D^-}/CS^- , Newman–Keuls test).

During LP EXT sessions, the number of active lever presses between days was similar in all groups of rats. They were always similar to those emitted during S^{D^-}/CS^- and lower than in presence of S^{D^+}/CS^+ ($P<0.05$, Newman–Keuls test) at the 1st reinstatement test. Thus, no effect of N-AC was detectable during the treatment.

After the end of the treatment, S^{D^+}/CS^+ , but not S^{D^-}/CS^- , revived active presses in all groups of rats ($P<0.05$ vs. respective S^{D^-}/CS^- before and after treatment; Newman–Keuls test). In vehicle and 60 mg/kg N-AC treated rats, the active lever presses at 6, 14 and 50 days after the end of the treatment were significantly higher than during the 1st reinstatement test ($P<0.05$, Newman–Keuls test), indicating a potential “drug seeking incubation”.

A partial reduction in the number of active lever presses after 100 mg/kg N-AC was observed at 24 h, 6 and 50 days, but not at 14 days, after the end of the treatment ($P<0.05$ vs. respective vehicle group, Newman–Keuls test).

Inactive lever responses remained negligible throughout the experiment.

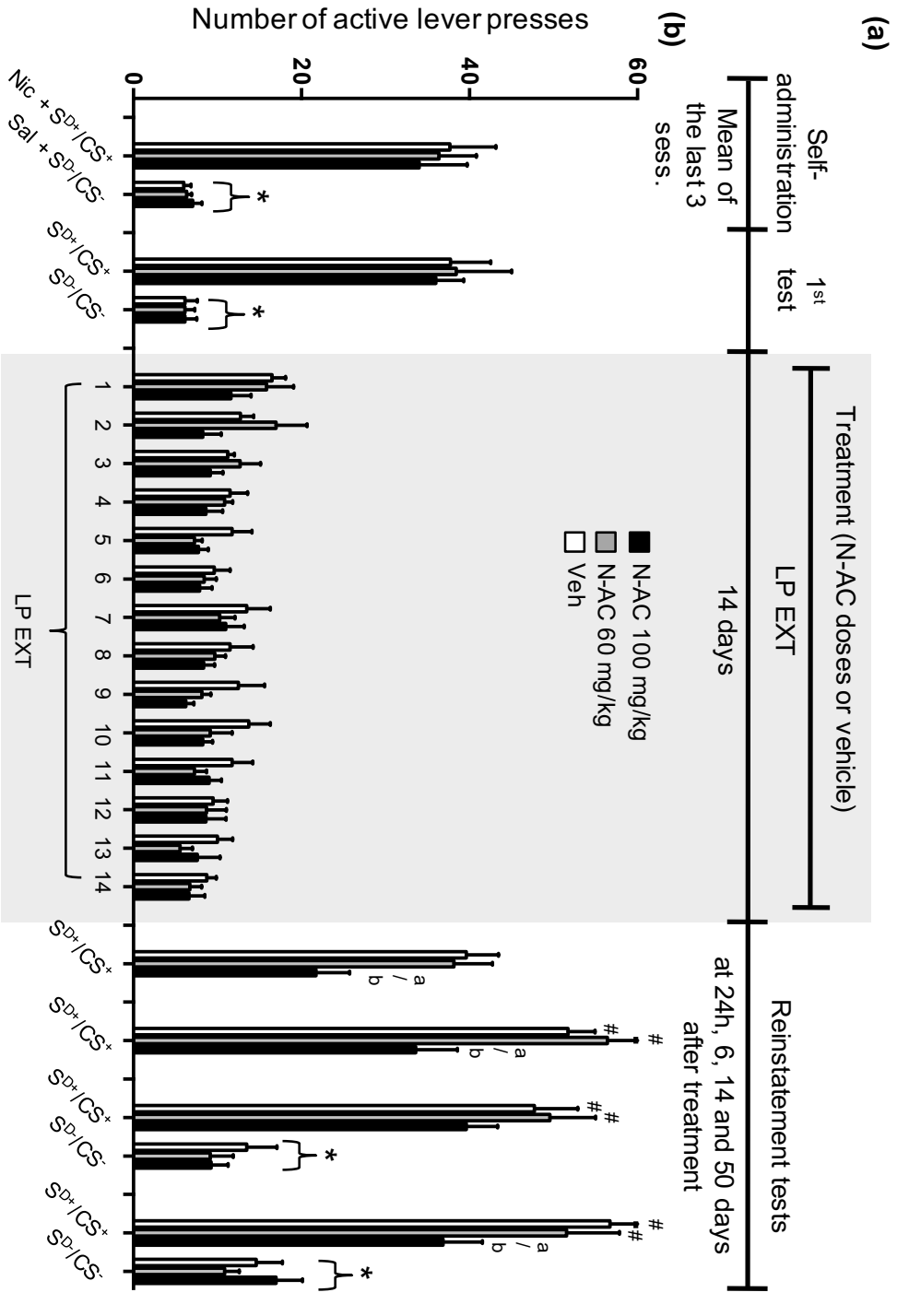


Figure 7.2. N-AAC + LP EXT: Effects of chronic treatment with 60 ($n=7$), 100 ($n=8$) SD^D/C^S mg/kg (i.p.) N-AAC or vehicle ($n=8$) during lever press extinction (LP EXT) on repeated reintroduction of stimuli predictive of (SD^+) and associated to nicotine availability (C^{S+}). **(a)** time course for experiment 1. After a 1st reinstatement test rats were treated daily during LP EXT. At the end of the treatment rats were tested with SD^+/C^{S+} and SD^+/C^{S+} at different time points. **(b)** Mean \pm SEM number of presses on the active lever in a within between-subject design. Also shown is the number of lever presses during self-administration training (mean SEM of last 3 sessions) and in response to stimuli predictive of and associated with saline availability (SD^D/C^{S-}). * $P < 0.05$ vs. respective SD^+/C^{S+} , # $P < 0.05$ vs. vehicle, ^a $P < 0.05$ vs. vehicle, ^b $P < 0.05$ vs 60 mg/kg N-AAC-treated group Newman-Keuls *post hoc* comparison.

7.3.3 Evaluation of chronic N-AC treatment during CET (Exp. 2)

Figure 7.3b shows the responses on the active lever during self-administration (mean of the last 3 sessions), the 1st reinstatement test, the 14 CET sessions of rats daily pre-treated with N-AC or vehicle, and reinstatement tests after the end of treatment.

A mixed-factorial ANOVA of active lever presses showed a significant effect of session [$F(23,414)=19.42$, $P<0.01$], treatment [$F(2,18)=1.47$, $P<0.001$] and interaction treatment x sessions [$F(46,414)=2.74$, $P<0.01$].

Responses during the last 3 sessions of nicotine self-administration were similar between groups ($P>0.05$, Newman-Keuls test) and significantly higher than the last 3 saline self-administration sessions ($P<0.05$ vs. nicotine self-administration, Newman-Keuls test).

In the 1st reinstatement test S^{D^+}/CS^+ revived active presses in all groups ($P<0.05$ vs. S^{D^-}/CS^- , Newman-Keuls test).

During treatment sessions S^{D^+}/CS^+ always revived the number of active lever presses in vehicle and 60 mg/kg N-AC ($P<0.05$ vs. S^{D^-}/CS^- at the 1st reinstatement, Newman-Keuls test).

In all test days with the exception of test days 7-10 and 12, 100 mg/kg N-AC significantly reduced the number of active lever presses ($P<0.05$ vs. vehicle group, Newman-Keuls test).

During treatment the level of responses in 100 mg/kg N-AC treated rats was similar to that of the S^{D^-}/CS^- in the 1st reinstatement test ($P>0.05$, Newman-Keuls test). The effect of 100 mg/kg N-AC showed no tolerance since was present also in the last 2 days of treatment ($P<0.05$ vs. vehicle group, Newman-Keuls test).

After the end of treatment, at all testing times reintroduction of S^{D^+}/CS^+ , but not S^{D^-}/CS^- , revived active presses in vehicle and 60 mg/kg N-AC treated rats ($P<0.05$ vs. S^{D^-}/CS^- before and after treatment, Newman-Keuls test). N-AC 100 mg/kg completely blocked the renewed active lever presses induced by reintroduction of nicotine associate cues ($P<0.05$ vs. respective vehicle group, $P>0.05$ vs. S^{D^-}/CS^- before and after treatment, Newman-Keuls test). Inactive lever responses remained negligible throughout the experiment.

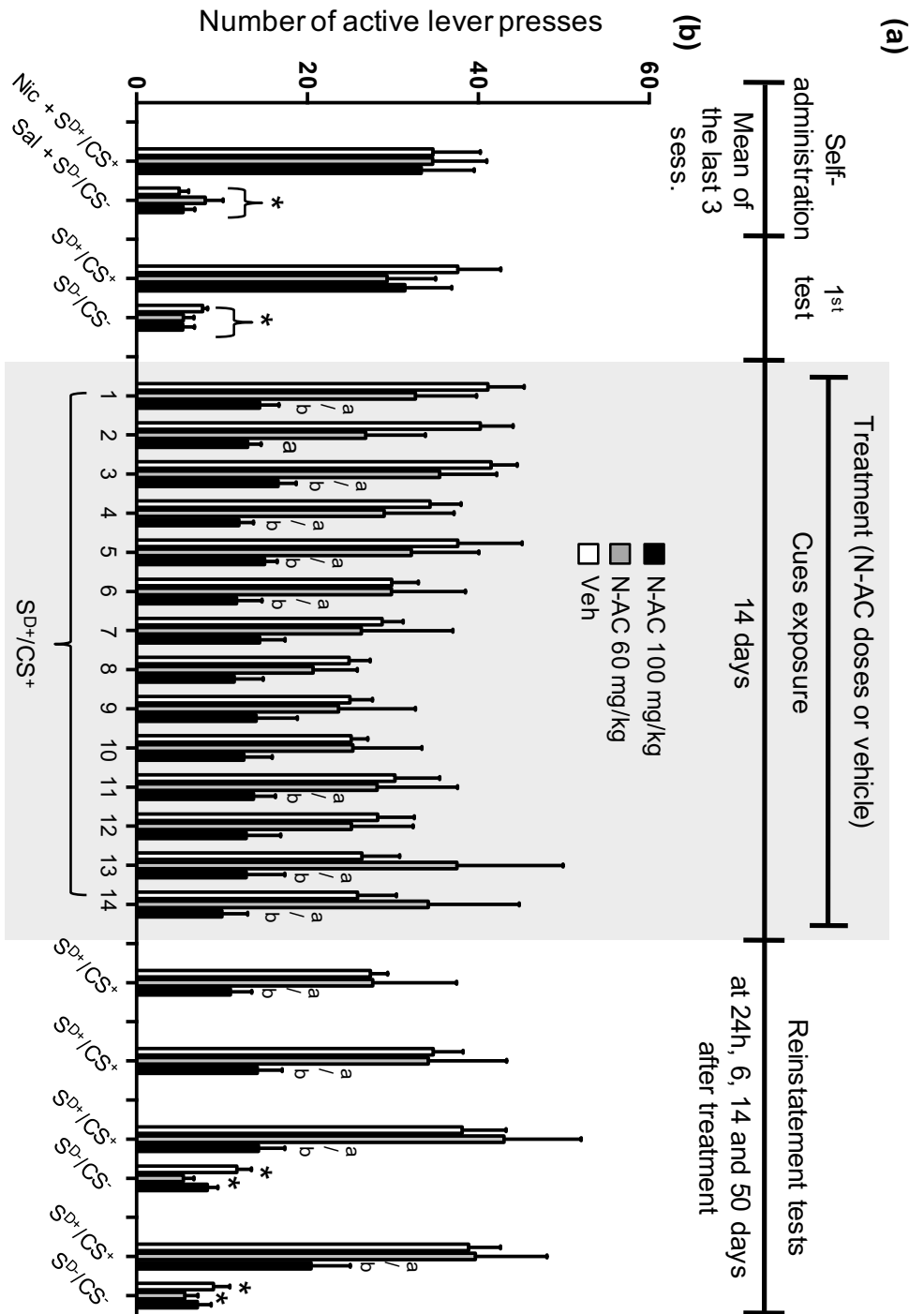


Figure 7.3. N-AC + CET: Effects of chronic treatment with 60 ($n=6$), 100 ($n=7$) SP⁻/CS⁺ mg/kg (i.p.) N-AC or vehicle ($n=8$) during experimental cue exposure therapy (CET) on repeated reintroduction of stimuli predictive of (SD⁺) and associated to nicotine availability (CS⁺). **(a)** time course for experiment 1. After a 1st reinstatement test rats were treated daily during cue exposure. At the end of the treatment rats were tested with SD⁺/CS⁺ and SP⁺/CS⁺ at different time points. **(b)** Mean \pm SEM number of presses on the active lever in a within between-subject design. Also shown is the number of lever presses during self-administration training (mean SEM of last three sessions) and in response to stimuli predictive of and associated with saline availability (SP⁻/CS⁺).
 * $P < 0.05$ vs. respective SP⁺/CS⁺, ^a $P < 0.05$ vs. vehicle, ^b $P < 0.05$ vs 60 mg/kg N-AC -treated group Newman-Keuls *post hoc* comparison.

7.3.4 Evaluation of chronic N-AC treatment during abstinence (Exp. 3)

Figure 7.4b shows the responses on the active lever during the self-administration (mean of the last 3 sessions) and reinstatement tests before and after the end of treatment.

A mixed-factorial ANOVA of active lever presses found a significant effect of session [$F(9,171)=40.94, P<0.001$] but not treatment [$F(2,19)=0.08, P>0.05$] and interaction treatment x sessions [$F(18,171)=0.53, P>0.05$].

The number of responses after the reintroduction of S^{D-}/CS^{-} and S^{D+}/CS^{+} were different as revealed by the main effect of the sessions but N-AC treatment never altered the numbers of responses during tests. Inactive lever responses remained negligible throughout the experiment.

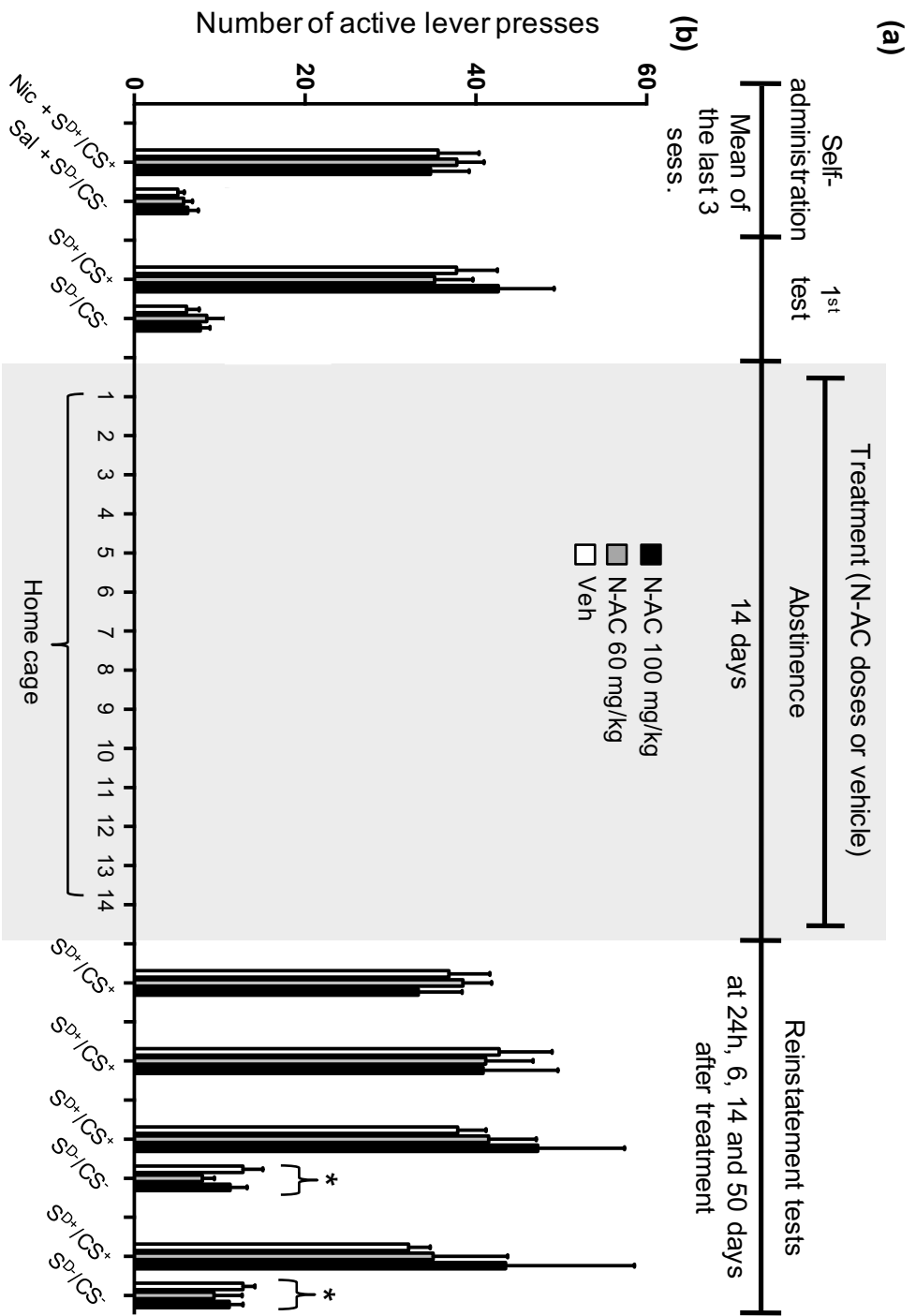


Figure 7.4. N-AAC + ABS: Effects of chronic treatment with 60 ($n=6$), 100 ($n=8$) SP⁻/CS⁻ mg/kg (i.p.) N-AAC or vehicle ($n=8$) during abstinence on repeated reintroduction of stimuli predictive of (SD⁺) and associated to nicotine availability (CS⁺). **(a)** time course for experiment 1. After a 1st reinstatement test rats were treated daily during in the home cage. At the end of the treatment rats were tested with SP⁺/CS⁺ and SP⁻/CS⁻ at different time points. **(b)** Mean \pm SEM number of presses on the active lever in a within between-subject design. Also shown is the number of lever presses during self-administration training (mean SEM of last 3 sessions) and in response to stimuli predictive of and associated with saline availability (SP⁻/CS⁻).

7.4 Discussion

These experiments have 4 main findings: i) 14 days of LP EXT, CET or ABS in the absence of N-AC treatment did not alter the seeking-behavior induced by nicotine-associated cues tested at 24h, 6, 14 and 50 days; ii) 14 days of N-AC (100 mg/kg) during CET completely blocked cue-induced nicotine-seeking behavior during treatment and the effects lasted for at least 50 days after the end of N-AC treatment; iii) 14 days of N-AC (100 mg/kg) during LP EXT attenuated cue-induced nicotine-seeking behavior during N-AC treatment and this attenuation lasted for 50 days after withdrawal of N-AC ; and iv) 14 days of N-AC during ABS did not alter cue-induced nicotine-seeking behavior after the end of the treatment.

The present study demonstrates that repeated non-contingent reintroduction of S^{D+} predictive of nicotine availability, together with the contingent presentation of the conditioned reinforcer, CS^+ , induces reliable and long lasting drug-seeking behavior even after 14 days of LP EXT, CET or ABS. These effects cannot be attributed to non-specific activation since responding on the inactive lever and with S^D/CS^- were not significant. It may be surprising that the limited period of nicotine self-administration (19–22 sessions of 1-hour) induced such a strong-lasting association with cues predictive of and associated with drug availability. However, as discussed in Chapter 4, the persistence of behavioral responses to drug-associated cues may be attributable to the complex stimuli associated with the drug during the self-administration training. This enduring behavioral effect resembles the persistence of conditioned cue reactivity and cue-induced craving in humans, and has been implicated as a key factor in lasting relapse risk and high rates of recidivism (O'Brien *et al.* 1998). Thus, the robust, lasting nicotine-seeking induced by S^{D+}/CS^+ in the present procedure may permit an assessment of the effects of treatments to promote extinction of the learned relationship between drug-associated cues and the conditioned responses (O'Brien *et al.* 1990; Havermans & Jansen 2003).

Two other points arise from the analysis of results. Rats that were subjected to the LP EXT did not show the typical extinction behavior (Figure 7.2b). In fact, the number of lever presses did not gradually decrease during LP EXT, but from the first day they were significantly

reduced in respect to those during the self-administration period. This probably reflects the absolute control of nicotine-associated cues (S^{D+}/CS^+) on their operant behavior. Rats would emit operant responding only in the presence of salient stimuli, i.e. those associated to nicotine-self administration. Even though the experimental CET was conducted in “a more ethological way” (i.e. with the same stimuli presented in the same context) it was not sufficient to reduce the salience of nicotine-associated cue during reinstatement tests.

As far as the different results of chronic N-AC are concerned, I have no clear explanation at the present. However, the above considerations may help in understanding the effects induced by chronic N-AC treatment. It could be that the restoring GLU homeostasis should be contingent with the devaluation of the salience of nicotine-conditioned cue. In particular, all the salient stimuli should be devaluated as in the Exp. 2 (N-AC + CET) to get a complete block of later reinstatement of nicotine-seeking behavior, while a partial devaluation of nicotine-associated cue, as in Exp. 1 (N-AC + LP EXT), would later produce just an attenuation of drug-seeking behavior. This hypothesis is supported by the lack of effect of chronic N-AC treatment given during abstinence on later nicotine conditioned cue reintroduction. In fact, giving chronic N-AC during abstinence results in restoring GLU homeostasis in the absence of any nicotine conditioned stimuli.

These results are partially in agreement with those obtained restoring GLU homeostasis after cocaine and heroin-seeking behavior giving chronic N-AC + LP EXT (Riechel et al. 2011; Zhou and Kalivas 2008), since in their extinction/reinstatement procedure the instrumental response and the context were unequivocally associated to drug-self-administration.

Ample evidence indicates that extinction memory involves a new learning (i.e. a new CS-US association) (Bouton 1993). Like other forms of learning, extinction of drug-conditioned cue memories is mainly mediated by the activity of GLURs in several brain areas including the Nacc (Myers et al. 2011). The importance of GLU transmission is further supported by the findings that GLUergic plasticity in the Nacc shell is a consequence of extinction training (Sutton et al.

2003) and that among the various GLU afferents to the Nacc those that originate from the ILC mediate extinction of CS associated to addictive drugs (Millan et al. 2011).

On the basis of this evidence, several drugs that regulate GLU transmission have been recently tested as tools to improve the extinction of drug-associated memories. D-cycloserine (DCS) (Section 1.3.4), a partial NMDARs agonist acting at the glycine-binding site, has been used to facilitate extinction memory formation (Davis et al. 2006), and reduced cue-induced cocaine-seeking when given both systemically or directly into the Nacc after LP EXT (Torregrossa et al. 2010).

Repeated treatment with N-AC or with the beta-lactam antibiotic ceftriaxone given during LP EXT by increasing the expression of GLT-1 and Xct in the Nacc, has been shown to reduce cue-induced cocaine-seeking behavior (Knackstedt et al. 2010; Moussawi et al. 2011). Multiple overlapping effects on GLU transmission in the Nacc are induced by extinction training and repeated N-AC. Indeed, it is possible that these two forms of neuroadaptation are needed for an enduring relapse protection. For this reason, in order to provide a molecular correlate to the persistent anti-relapse prevention achieved after N-AC + CET treatment, I examined the expression of proteins associated with GLU transmission in the Nacc.

Chapter Eight – Evaluation of protein expression in the Nacc after chronic N-AC treatment

8.1 Introduction

In the previous chapter I have provided evidence that 100 mg/kg N-AC given i.p. during 14 days of experimental CET is very effective in reducing cue-induced nicotine-seeking even 50 days after the end of the treatment (Chapter 7).

It has been shown that in rats self-administering cocaine the expression of Xct (the catalytic subunit of the system Xc-) and GLT-1 in the Nacc is decreased, and that chronic 100 mg/kg N-AC restored the expression of these proteins back to their normal level (Knackstedt et al. 2010). Reissner et al. (2015) also reported that, at least for cocaine, the main anti-relapse activity of chronic N-AC relays on the increased expression of GLT-1 in the Nacc. Thus, N-AC might promote the tonic activation of mGluR2/3 autoreceptors thereby decreasing presynaptic GLU release during cue-induced cocaine reinstatement (Kalivas 2009).

Moreover, nicotine self-administration alters protein expression in the Nacc. In particular, a reduction of proteins that govern GLU transmission, like Xct and GLT-1, has been implicated in cue-induced nicotine-seeking behavior (Knackstedt et al. 2009; Gipson et al. 2013). As for cocaine, nicotine self-administration decreased the function of mGluR2/3 (Liechti et al. 2007). All these data indicate that cocaine and nicotine self-administration alter the expression of similar proteins within the Nacc. Indeed, N-AC might be a good candidate to revert nicotine-induced alteration of GLU transmission. In an attempt to provide a molecular mechanism underlying the observed behavioral results, I replicated the results obtained with N-AC + CET (Chapter 7, Section 7.3.2). To this end, separate groups of rats were killed at different time points to verify whether the level of expression of the proteins thought to mediate N-AC effect, i.e. Xct, GLT-1 and mGluR2, were restored by the treatment.

8.2 Specific materials and methods

Rats were trained as described in the previous Chapter for nicotine self-administration. One rat (N-AC treated, killed at 51 d days) was excluded from all the analysis because treatment was never effective and it was identified as an outlier (Groub's test).

8.2.1 Treatment with N-AC + CET and reinstatement tests of rats killed at 7 and 51 days after the end of the treatment

After treatment (N-AC + CET), 14 rats (Veh=7; N-AC=7) were killed 24 h after the reinstatement tests at 6 days. As a control, on the same day, naïve rats treated for 14 days with 100 mg/kg N-AC (n=6) or vehicle (n=6) were also killed (corresponding to 7 days after the last dose)(Figure 8.1a). A separate group of 18 (Veh=9; N-AC=9) rats were treated with N-AC + CET and killed 24 h after the reinstatement tests at 50 days from the end of the treatment. As a control, on the same day, naïve rats treated for 14 days with 100 mg/kg N-AC (n=6) or vehicle (n=6) were also killed (corresponding to 50 days after the last dose)(Figure 8.2a).

8.3 Results

8.3.1 Training and 1st reinstatement tests before the beginning of the chronic treatment

As shown in table 8.1, rats in the three experiments developed stable nicotine self-administration and lever presses in saline sessions gradually decreased (data not shown). During the last 3 sessions of self-administration training, all experimental groups earned similar amount of nicotine as demonstrated by the similar number of infusions. Responding on the inactive lever was always not significant.

In the 1st reinstatement test in all groups S^{D^+}/CS^+ , but not S^{D^-}/CS^- renewed active lever presses ($P < 0.05$ vs. S^{D^-}/CS^- , Newman Keuls test) to a similar extent in the different groups ($P > 0.05$ vs. S^{D^+}/CS^+ , Newman Keuls test). The revived active lever presses were similar to those during the sessions of nicotine self-administration and significantly higher than during the saline self-administration sessions. Individual recovery of active lever presses during the 1st reinstatement

tests was: 7 days (Veh) 105.7 ± 12.5 % and (N-AC-100mg/kg) 103.3 ± 6.3 %; 51 days (Veh) 103.0 ± 6.4 % and (N-AC 100 mg/kg) 102.2 ± 6.5 % of the presses during the sessions of operant responding for nicotine.

Table 8.1. Self-administration training and 1st reinstatement test

	Self-Administration Training (mean of last 3 days)						1 st Reinstatement Test			
	Sessions to criteria	Number of Nic. Infusions	Act. Lever Presses		Inact. Lever Presses		Act. Lever Presses		Inact. Lever Presses	
			Nic	Sal	Nic	Sal	S ^{D+} /CS ⁺	S ^{D-} /CS ⁻	S ^{D+} /CS ⁺	S ^{D-} /CS ⁻
<u>Rats killed at 7 d</u>										
Vehicle	20.3±0.2	14.6±1.4	37.8±4.4	5.1±0.8	3.3±1.5	4.7±0.8	42.4±10.2*	6.2±1.2	8.4±3.2	4.6±1.0
N-AC 100 mg/kg	20.6±0.2	13.0±2.2	32.4±5.2	5.3±1.3	2.2±0.5	2.3±0.5	32.9±5.0*	7.0±1.7	3.0±0.8	3.0±0.9
<u>Rats killed at 51 d</u>										
Vehicle	20.3±0.2	13.7±2.1	35.1±5.0	6.0±0.9	1.1±0.5	1.9±0.6	35.6±5.0*	7.0±1.1	1.6±0.4	2.0±0.7
N-AC 100 mg/kg	20.9±0.3	12.9±1.7	31.2±3.6	5.1±0.7	2.4±0.5	2.4±0.3	30.9±2.8*	5.0±1.1	4.4±2.0	2.9±1.0

Table 12.1. Data are expressed as mean±SEM. After that rats met the self-administration criteria ($\pm 15\%$ over 3 consecutive sessions and the mean of the last 3 days of saline self-administration is less the 20% of nicotine sessions). 24h after the self-administration training half of the rats were exposed to stimuli predictive of (S^{D+}) and associated to nicotine availability (CS⁺) and half to stimuli predictive of (S^{D-}) and associated to saline availability (CS⁻). Day the day after rats tested with S^{D+}/CS⁺ were tested with S^{D-}/CS⁻ and vice versa.

* $P < 0.01$ vs. respective S^{D-}/CS⁻, Newman-Kuels test.

8.3.2 Behavioral results and proteins analysis of rats killed 7 days after the end of the treatment

Figure 8.1b shows the responses on the active lever during self-administration (mean of the last 3 sessions), the 1st reinstatement test, the 14 CET sessions of rats pre-treated daily with 100 mg/kg i.p. N-AC or vehicle and reinstatement tests after the end of treatment.

A mixed-factorial ANOVA of active lever presses found a significant effect of session [$F(19,228)=14.12$, $P < 0.01$], treatment [$F(1,12)=9.07$, $P < 0.01$] and interaction treatment x sessions [$F(19,228)=4.35$, $P < 0.001$]. The means of the last 3 sessions of nicotine self-administration were similar between groups ($P > 0.05$, Newman-Keuls test) and significantly higher than the last 3 saline self-administration sessions ($P < 0.05$ vs. nicotine self-administration, Newman-Keuls test). In the 1st reinstatement test S^{D+}/CS⁺ revived active presses in all groups ($P < 0.05$ vs. S^{D-}/CS⁻, Newman-Keuls test).

During treatment sessions S^{D+}/CS^+ always revived the number of active lever presses in vehicle group ($P < 0.05$ vs. S^{D-}/CS^- at the 1st reinstatement, Newman-Keuls test). In all test days with the exception of test days 2 and 4-8, 100 mg/kg N-AC significantly reduced the number of active lever presses ($P < 0.05$ vs. vehicle group, Newman-Keuls test). During treatment the responses in 100 mg/kg N-AC treated rats were similar to that of the S^{D-}/CS^- in the 1st reinstatement test ($P > 0.05$, Newman-Keuls test). The effect of 100 mg/kg N-AC showed no tolerance since it was present in the last 2 days of treatment ($P < 0.05$ vs. vehicle group, Newman-Keuls test).

After the end of treatment, at all testing times reintroduction of S^{D+}/CS^+ , but not S^{D-}/CS^- , revived active presses in vehicle treated rats ($P < 0.05$ vs. S^{D-}/CS^- before and after treatment, Newman-Keuls test). N-AC 100 mg/kg completely blocked the renewed active lever presses induced by reintroduction of nicotine associate cues ($P < 0.05$ vs. respective vehicle group, $P > 0.05$ vs. S^{D-}/CS^- before treatment, Newman-Keuls test). Inactive lever responses remained negligible throughout the experiment.

Table 8.2 shows that when the effect of N-AC treatment on Xct expression in the Nacc core was compared between naïve and nicotine self-administered rats there was a significant main effect of N-AC [$F(1,22)=7.93$, $P < 0.05$] and the main effect of the nicotine self-condition [$F(1,22)=9.18$, $P < 0.01$] but no nicotine self-condition x treatment interaction [$F(1,22)=0.29$, $P > 0.05$].

An effect of N-AC treatment was present on GLT-1 expression in the Nacc shell [F self-condition (1,22)=2.95, $P > 0.05$; F treatment(1,22)=17.13, $P < 0.05$; F interaction (1,22)=5.01, $P < 0.05$]. Compared to Naïve/Veh group, the expression of GLT-1 in nicotine self-administered rats treated with vehicle + CET was reduced ($P < 0.05$ vs. Naïve/Veh group, Tukey's test) and N-AC (Nic/N-AC group) restored protein expression to that of naïve rats ($P > 0.05$ vs. naïve/Veh group, Tukey's test). Moreover, the expression of GluN2B was affected by N-AC treatment in the Nacc shell [F self-condition (1,22)=2.76, $P > 0.05$; F treatment(1,22)=4.11, $P > 0.05$; F interaction(1,22)=5.19, $P < 0.05$]. Compared to naïve rats, the expression of GluN2B in nicotine self-administered rats treated with vehicle + CET was increased ($P < 0.05$ vs.

Naïve/Veh group, Tukey's test) and N-AC (Nic/N-AC group) restored protein expression to the level of naïve rats ($P > 0.05$ vs. Naïve/Veh group, Tukey's test) (Table 8.2). N-AC treatment did not alter proteins expression in naïve animals (Table 8.2).

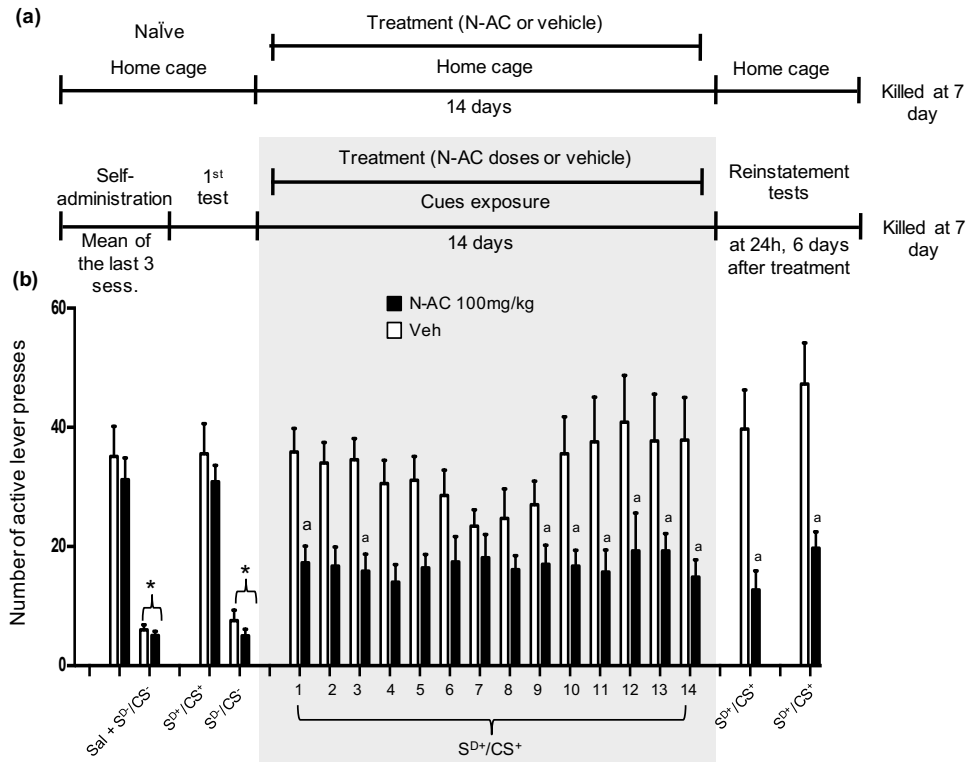


Table 8.2. Proteins analyzed from rats killed at 7 days after the end of the treatment

Exp. Group	Brain Area	Proteins levels						
		Killing time	GLT-1	xCT	mGluR2	GluN1	GluN2B	GluN2A
Naïve/Veh	Nacc-shell		100±5	100±7	100±39	100±10	100±4	100±10
	Nacc-core		100±8	100±8	100±25	100±10	100±2	100±8
Naïve/N-AC	Nacc-shell		111±9	106±17	135±45	109±16	105±8	109±11
	Nacc-core		103±13	118±12	106±28	109±15	90±12	84±14
nic/Veh	Nacc-shell		77±2 [#]	99±16	86±19	136±17	136±11 [#]	111±11
	Nacc-core		94±5	72±6	90±35	111±17	78±2	90±7
nic/N-AC	Nacc-shell		114±4 ^{***}	91±12	69±5	121±10	103±8 [*]	120±9
	Nacc-core		99±7	98±5	54±6	108±16	72±7	93±12

Figure 8.1. Effects of chronic treatment with i.p. 100 mg/kg N-AC ($n=7$) or vehicle ($n=7$) during experimental cue exposure therapy (CET) on repeated reintroduction of stimuli predictive of (SD^+) and associated to nicotine availability (CS^+). (a) time course of the experiment. (b) Mean \pm SEM number of presses on the active lever in a within between-subject design. Also shown is the number of lever presses during self-administration training (mean \pm SEM of last 3 sessions) and in response to stimuli predictive of and associated with saline availability (SD^-/CS^-). **Table 8.2** western-blot analysis of rats killed 24h after the reinstatement test at 6 days [Naïve/Veh ($n=6$), Naïve/N-AC ($n=6$), Nic/Veh ($n=7$), Nic/N-AC ($n=7$)]. Behavioral data were analyzed by mixed-factorial ANOVA followed by Newman-Keuls test. Proteins data were analyzed by two-way ANOVA followed by Tukey's test.

** $P < 0.05$ vs. respective SD^+/CS^+ , ^a $P < 0.05$ vs. Veh. In Table 8.2 * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. Nic-Veh; # $P < 0.05$ vs. Naïve-Veh

8.3.3 Behavioral results and proteins analysis in rats killed 51 days after the end of the treatment

Figure 8.2b shows the responses on the active lever during self-administration (mean of the last 3 sessions), the 1st reinstatement test, the 14 CET sessions of rats daily pre-treated with N-AC or vehicle, and reinstatement tests after the end of treatment.

A mixed-factorial ANOVA of active lever presses showed a significant effect of session [$F(23,368)=20.50$, $P<0.001$], treatment [$F(1,16)=13.56$, $P<0.01$] and interaction treatment x sessions [$F(23,368)=4.90$, $P<0.001$]. The means of the last 3 sessions of nicotine self-administration were similar between groups ($P>0.05$, Newman-Keuls test) and significantly higher than the last 3 saline self-administration sessions ($P<0.05$ vs. nicotine self-administration, Newman-Keuls test). In the 1st reinstatement test S^{D^+}/CS^+ revived active presses in all groups ($P<0.05$ vs. S^{D^-}/CS^- , Newman-Keuls test). During treatment sessions S^{D^+}/CS^+ always revived the number of active lever presses in vehicle ($P<0.05$ vs. S^{D^-}/CS^- at the 1st reinstatement, Newman-Keuls test). In all test days with the exception of test days 7-13, 100 mg/kg N-AC significantly reduced the number of active lever presses ($P<0.05$ vs. vehicle group, Newman-Keuls test). During treatment the response in 100 mg/kg N-AC treated rats was similar to that of the S^{D^-}/CS^- in the 1st reinstatement test ($P>0.05$, Newman-Keuls test). The effect of 100 mg/kg N-AC showed no tolerance since it was present in the last day of the treatment ($P<0.05$ vs. vehicle group, Newman-Keuls test).

After the end of treatment, at all testing times reintroduction of S^{D^+}/CS^+ , but not S^{D^-}/CS^- , revived active presses in vehicle treated rats ($P<0.05$ vs. S^{D^-}/CS^- before and after treatment, Newman-Keuls test). N-AC 100 mg/kg completely blocked the renewed active lever presses induced by reintroduction of nicotine associate cues ($P<0.05$ vs. respective vehicle group, $P>0.05$ vs. S^{D^-}/CS^- before and after treatment, Newman-Keuls test). Inactive lever responses remained negligible throughout the experiment.

Table 8.3 shows protein expression of rats killed 51 days after N-AC + CET.

An effect of N-AC was present on the expression of GLT-1 [F self-condition(1,26)=2.53, $P>0.05$; F treatment(1,26)=1.07, $P>0.05$; F interaction(1,26)=6.07, $P<0.05$] and mGluR2 [F self-condition(1,26)=2.90, $P>0.05$; F treatment(1,26)=3.39, $P>0.05$; F interaction(1,26)=4.3, $P<0.05$] in the Nacc shell. Compared to the Nic/Veh group the expression of GLT-1 and mGluR2 in nicotine self-administered rats treated with CET + N-AC were increased ($P<0.05$, Tukey's test). Moreover, the expression of mGluR2 was increased in the Nacc core [F self-condition(1,26)=0.37, $P<0.05$; F treatment(1,26)=7.25, $P<0.05$; F interaction(1,26)=4.28, $P<0.05$] of Nic/N-AC groups ($P<0.05$ vs. Nic/Veh, Tukey's test). N-AC treatment had no effects on proteins expression in naïve animals ($P>0.05$, Tukey's test) (Table 8.3).

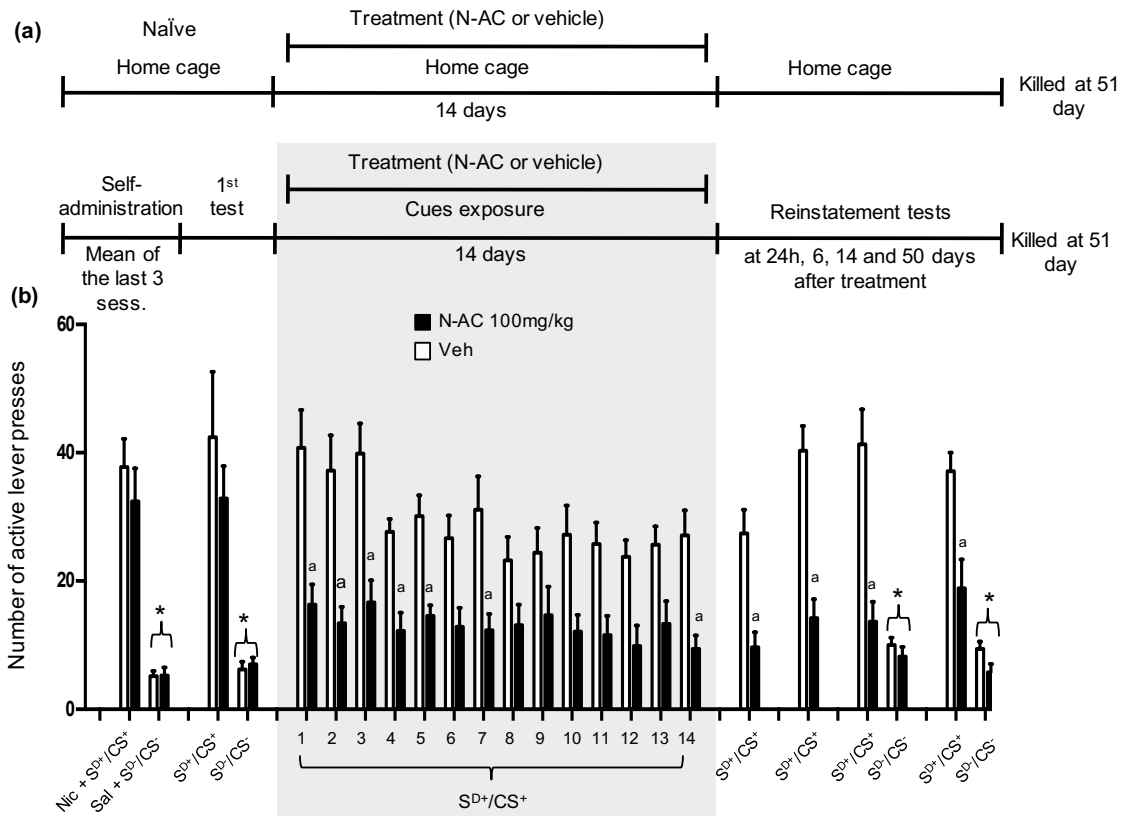


Table 8.3 Proteins analyzed from rats killed at 51 days after the end of the treatment

Exp. Group	Brain Area	Proteins levels						
		Killing time	GLT-1	xCT	mGluR2	GluN1	GluN2B	GluN2A
Naïve/Veh	Nacc-shell		100±8	100±3	100±5	100±12	100±6	100±9
	Nacc-core		100±7	100±3	100±5	100±5	100±10	100±8
Naïve/N-AC	Nacc-shell		95±7	93±3	93±9	96±13	105±18	87±7
	Nacc-core		105±9	100±3	110±9	106±11	96±13	126±20
nic/Veh	Nacc-shell		93±3	100±6	96±11	90±8	97±9	86±7
	Nacc-core		105±7	85±5	76±6	108±8	101±9	93±6
nic/N-AC	Nacc-shell		115±5 [#]	110±5	165±28 [*]	84±11	112±5	111±7
	Nacc-core		105±6	92±10	154±25 ^{**}	103±13	107±8	87±6

Figure 8.2. Effects of chronic treatment with i.p. 100 mg/kg N-AC ($n=9$) or vehicle ($n=9$) during experimental cue exposure therapy (CET) on repeated reintroduction of stimuli predictive of (SD^+) and associated to nicotine availability (CS^+). **(a)** time course of the experiment. **(b)** Mean±SEM number of presses on the active lever in a within between-subject design. Also shown is the number of lever presses during self-administration training (mean±SEM of last 3 sessions) and in response to stimuli predictive of and associated with saline availability (SD^-/CS^-). **Table 8.3** Western-blot analysis of rats killed 24h after the reinstatement test at 50 days [Naïve/Veh ($n=6$), Naïve/N-AC ($n=6$), Nic/Veh ($n=7$), Nic/N-AC ($n=7$).

Behavioral data were analyzed by mixed-factorial ANOVA followed by Newman-Keuls test. Proteins Data were analyzed by two-way ANOVA followed by Tukey's test.

* $P<0.05$ vs. respective SD^+/CS^+ , ^a $P<0.05$ vs. Veh. In Table 8.3 * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ vs. Nic-Veh; # $P<0.05$ vs. Naïve-Veh

8.4 Discussion

In these experiments I replicated previous behavioral findings (see Chapter 7) showing that N-AC + CET reduced cue-induced reinstatement during the 14 treatment-days and that after the end of the treatment the anti-craving activity was maintained for up to 50 days. This was important since the degree of concordance between the results performed in the same laboratory by the same experimenter at different time points, is a fundamental prerequisite for acceptance of any experimental evidence.

My result showed that in the Nacc, 7 days after N-AC + CET treatment the expression of Xct, GLT-1 and GluN2B was restored by N-AC, while at later time point (50 day) the over-expression of mGluR2 in both Nacc subregions may reflect an increased control on presynaptic GLU release in N-AC treated rats.

Previous studies have reported that in the Nacc during the withdrawal from nicotine self-administration there is a decreased expression of Xct (Knackstedt et al. 2009) and GLT-1 (Knackstedt et al. 2009; Gipson et al. 2013), and an elevated expression of GluN2B subunit of NMDARs (Gipson et al. 2013). Moreover, Xct and GLT-1 are also found to be decreased during withdrawal from cocaine (Knackstedt and Kalivas 2007; Madayag et al. 2007) as well as ethanol (Alhaddad et al. 2014) self-administration. Indeed, alterations of proteins crucial for GLU homeostasis are really consistent finding across different types of drugs of abuse and could account for the long-lasting behavioral responses produced by drug-related cues (Scofield et al. 2016).

In general, my results are in agreement with these findings since 7 days after the end of the CET, and more than 21 days after the last nicotine self-administration session, the expression of Xct and GLT-1 in the Nacc (Table 8.2) was reduced when compared to naïve rats. Furthermore, the expression of GluN2B was increased in nicotine self-administered rats (Table 8.2).

The fact that proteins expression was different in the two subregions of the Nacc is not surprising. The Nacc is a very complex area that mediates the reinforcing effect of abused drugs

and integrates cognitive and affective information processed by frontal regions to augment the strength of appetitive motivated behaviors (Floresco 2015). The Nacc core and Nacc shell mediate different aspects related to drug addiction. The ILC has a major projection to the Nacc shell, and these connections are mostly implicated in maintaining the extinction of cocaine- and morphine-seeking (Peters et al. 2008). The PLC instead sends projections to the Nacc core that are activated during cue-induced drug-seeking behavior (McFarland and Kalivas 2001). Although the Nacc core is the region that has been mostly implicated in mediating drug-seeking behavior (Kalivas 2009), I found that proteins in the Nacc shell were also changed. It is important to consider that the Nacc shell seems to mediate the devaluation of the reward and extinction of drug-related cues (West and Carelli 2016). Indeed, a restored GLU homeostasis in the Nacc shell may account for the fact that rats treated with N-AC + CET have extinguished/attenuated the susceptibility of nicotine-associated cues to induce seeking behavior.

However, it is important to consider that in the present experiment, rats were not only treated with N-AC, but were also daily exposed to CET. Indeed, the results of the present experiments should be viewed as a combination of a pharmacological (N-AC) and behavioral (CET) treatment. The evaluation of CET on the expression of proteins mediating GLU transmission in the Nacc was outside the scope of my thesis and further studies are needed to examine this point. Nevertheless, experimental evidence indicates that GLU adaptations at the Nacc level could be sensitive to environmental contexts, as demonstrated by the fact that extinction training reverses the methamphetamine-induced alterations in mGluR2/3 expression observed in the Nacc but not in the PFC (Schwendt et al. 2012). Furthermore, extinction of morphine and heroin reward produces structural changes in dendritic branching in the Nacc core (Leite-Morris et al. 2014; Chen et al. 2016).

At 51 days after the end of the N-AC + CET treatment, i.e. more than two months after the last nicotine self-administration session, a drastically different protein profile was detected. Nicotine-related changes were no more present, probably reflecting the fact that in the Nacc

these proteins returned back to control levels. In contrast, in N-AC treated rats there was a clear increase of mGluR2 expression (Table 8.3), possibly underlining an increased control of GLU release, and an over-expression of GLT-1. The reason for evaluating mGluR2 and not mGluR3 was based on previous studies showing that systemic administration of selective mGluR2 antagonist decreases nicotine cue- and nicotine priming-induced seeking (Justinova et al. 2015) as well as cue-induced methamphetamine-seeking behavior (Caprioli et al. 2015).

It might seem singular that protein expression had changed between 7 and 51 days. This evidence suggests that behavioral and proteins results data are not directly correlated. To the best of my knowledge, nobody has investigated protein levels in the Nacc after such a long period following nicotine self-administration and CET + N-AC treatment. It is possible that at 7 days from the end of the treatment nicotine-induced modifications of protein expression were still present and the treatment was reverting these changes. In contrast, at 51 days nicotine-induced changes were no longer present. A complex interaction between the neuroplasticity induced by nicotine self-administration and the learning and memory system might account for these results (Fuchs et al. 2006). Nevertheless, it could be speculated that the over-expression of mGluR2 might counteract the increased GLU release in the Nacc caused by the activation of PFC afferents during cue-induced reinstatement and the increased expression of GLT-1 might further prevent the activation of post-synaptic receptor in the Nacc, thus blunting GLU transmission.

Overall, these results provide evidence for the ability of N-AC to regulate the expression of proteins that mediate GLU transmission in the Nacc, and further support the use of chronic N-AC as a medication.

Chapter Nine – General discussion

In this Thesis, I first refined the extinction/reinstatement procedure previously used in my laboratory (Di Clemente et al. 2012; Cervo et al. 2013) by removing the instrumental training for a food-reinforcer before the beginning of nicotine self-administration (Moro et al. 2016). Using this procedure, I then provided evidences that N-AC reduces the level of cue-induced nicotine-seeking behavior. In particular, two were the main findings: 1) N-AC has anti-relapse activity when it is administered acutely before the reintroduction of nicotine-associated cues, and its effects depends on the activation of mGLU2/3 receptors, 2) Chronic N-AC treatment produces an enduring anti-relapse activity especially when the drug is injected daily during reintroduction of nicotine-associated cues.

I used rats trained to self-administered nicotine and to associate nicotine to a complex cue presentation (S^{D+}/CS^+) consisting of a discriminative stimulus (S^{D+}) predictive of nicotine availability and a conditioned stimulus (CS^+) associated with nicotine infusions. As shown in Chapter 4, both these cues were needed in order to have the maximum level of reinstatement, while S^{D+} and CS^+ alone reinstated the active lever pressing at a lower extent. Moreover, rats had to discriminate between nicotine self-administration and saline self-administration sessions. Saline sessions were paired with a different set of stimuli (S^D and CS^-). Therefore, after training rats learned that if a specific set of stimuli was present nicotine would be available. It was interesting to observe that using this training, even after long period of abstinence, rats reinstated when exposed to stimuli associated to nicotine similarly to what happens in abstinent smokers (O'Brien et al. 1990). Compared to others experimental procedures that use only the drug self-administration session, the experimental procedure I used has a stronger face validity since rats learn that, like for humans, different cues are associated to different outcomes. Moreover, this procedure has proved helpful in evaluating anti-relapse treatment (Di Clemente et al. 2011; Cervo et al 2013). For the chronic experiments in Chapter 7 and 8, the experimental procedure was further refined. In particular, the lever press extinction phases before and

between the reinstatement sessions were removed. This was possible since the double self-administration training sessions allowed us to verify whether nicotine-related cues (S^{D+}/CS^+) induced a seeking behavior by comparing the reinstatement level produced by the reintroduction of saline-related cues (S^{D-}/CS^-). Indeed, in the 1st reinstatement session the number of active lever presses after reintroduction of nicotine-associated cues was always higher than that of saline-associated cues. The finding that nicotine self-administration changed the expression of proteins known to be altered by different paradigms of chronic nicotine self-administration further supports the validity of revised behavioral procedures that I have developed. In fact, I observed a reduction of the expression of system Xc- and GLT-1, the same proteins that were found to be reduced after 6 h daily nicotine self-administration sessions (Knackstedt et al. 2009). Indeed, even the short access to nicotine (1-h sessions) used for present experiments produced changes in these proteins.

I provided evidences that as for cocaine, acute N-AC increases extracellular GLU levels in the Nacc only in rats with an history of nicotine self-administration. Moreover, I showed the acute and short-lasting reduction of cue-induced nicotine-seeking behavior produced by N-AC required the activation of mGluR2/3. Although N-AC could reduce the level of cue-induced nicotine-seeking behavior, it was also important to assess the selectivity of this activity by verifying that N-AC did not alter the hedonic state of the rats, thus excluding potential side effects of the treatment. For this purpose, I used saccharin self-administration and cue-induced saccharin-seeking behavior. Saccharin was chosen because it is a caloric substance whose concentration can be regulated in order to generate a number of active lever presses during the self-administration sessions that closely resembles the one of nicotine self-administration. Other groups have used different procedures to control the selectivity toward drugs of abuse. Generally, when evaluating anti-relapse medication to exclude an unspecific effect, food (Ramirez-Nino et al. 2013) and sucrose-self-administration (Hopkins et al. 2012) are the most used procedures. However, these procedures generate a strong desire in rats as indicated by the very high levels of active lever presses during both self-administration and reinstatement. The

great salience of food and sucrose seems to produce in rodents even more craving than that of addictive drugs. Thus these models should be used carefully when evaluating anti-craving medications. For this reason, I decided to use saccharin self-administration with a concentration of saccharin that generated a reinstatement level similar to that of nicotine.

To date repeated N-AC has been evaluated only in clinical setting, so my results highlight the possibility to use N-AC acutely to reduce nicotine craving after a period of abstinence.

In the chronic experiments, I evaluated whether repeated N-AC treatment could be a strategy for long lasting relapse prevention. I evaluated three different contingences of N-AC treatment and cue exposure. The results showed that, chronic N-AC may have a long-lasting anti-craving protection but only when given repeatedly before or together with nicotine related cue presentation. Interestingly, I show that it is the contingency of repeated N-AC treatment and cue exposure therapy (CET) that ensure the more consistent and lasting effect. In fact, chronic N-AC treatment alone given during abstinence was not effective to reduce the number of active lever presses in later reinstatement tests. This is an important finding because it demonstrates for the first time that only increasing the level of GLU with repeated N-AC treatment in the absence of nicotine related cues was not enough to produce extinction of drug related memories. These findings were further supported by data showing that CET alone or N-AC treatment alone had no effect on protein expression (Chapter 8). A possible explanation could be that N-AC by restoring altered GLU system homeostasis in the presence of nicotine associated cues may restore the neuroplasticity required for extinction learning.

It was also interesting to notice that vehicle treated rats exposed to 14 daily nicotine related cues presentation (CET) did not extinguish drug related memories (Chapter 7). It is possible to speculate that our nicotine self-administration training has generated a habitual like behavior difficult to extinguish. In keeping with this finding is a report that rats trained with a second order schedule of reinforcement have a shift from an action outcome response early in the training to a more habitual behavior later in the training that became difficult to devalue with an extinction procedure (Murray et al. 2012). The shift from a goal direct behavior to a

habitual one is also accompanied by a parallel shift in neural activity from dorsomedial (DSM) to dorsolateral striatal (DLS) regions (Corbitt et al. 2012; Murray et al. 2012). Thus it could not be excluded that our procedure generating a habitual response has also produced neuroadaptation in dorsolateral striatum. This suggestion is supported by a recent finding that N-AC restores the expression of GLT-1 and zif268 in the DLS in a model of cocaine addiction (Ducret et al. 2016). This raises the possibility that N-AC by interfering with mechanisms in the DLS may also restore the ability to properly devalue the habitual response produced by nicotine-related cues. This possibility seems to be supported at least in part by the lack of correlation in the Nacc between behavior and protein expression. Further studies are needed to examine the involvement of DLS and its role in the persistence of this habitual response to nicotine cues and in the ability of N-AC to extinguish it.

The importance of the contingency between N-AC treatment and extinction training has been reported before for other abused drugs. Reichel et al. (2011) showed that in cocaine self-administered rats, N-AC exerted a more profound anti-relapse activity when given during lever press extinction (LP EXT) sessions than during forced abstinence. Moreover, the fact that chronic N-AC given during LP EXT might have an enduring relapse protection is in agreement with previous studies on heroin (Zhou and Kalivas 2008) and cocaine (Moussawi et al. 2011).

The results of my Thesis highlight a number of similarities between N-ACs effects across different types of drug-induced reinstatement. In particular, as for cocaine (Baker et al. 2003), for nicotine too N-AC increased extracellular GLU levels by activating system Xc- in the Nacc but only in drug self-administered rats. Moreover, as shown by Kupchik and colleagues (2012) and Moussawi and colleagues N-AC activity requires the function of mGluR2/3 for its acute reduction of cue-induced nicotine-seeking behavior. Furthermore, the ability of chronic N-AC treatment to restore the expression of Xct and GLT-1 in self-administered rats has already been reported for cocaine (Knackstedt et al. 2010).

Growing evidence indicates that drugs that interact with the GLU system may be a valid approach as anti-craving medications. The cephalosporin ceftriaxone which enhances GLT-1

and Xc- expression in the Nacc (Fischer et al. 2013) has been found. Moreover, ceftriaxone was found to be effective in reducing ethanol consumption (Sari et al. 2013) and cue-induced reinstatement in cocaine- and heroin-seeking individuals (Sondheimer and Knackstedt 2011; Shen et al. 2014). The cognitive-enhancer modafinil, among the variety of its target, activates system Xc- thereby activating mGluR2/3 and preventing cocaine-primed reinstatement (Mahler et al. 2014) and cue- and context-induced methamphetamine-seeking behavior (Reichel and See 2010). The design of new drugs able to restore the balance in GLU homeostasis in the Nacc as well as further characterization of the already available pharmacological tools can be valid strategies for treating not only nicotine addiction but drug addiction in general.

These promising preclinical data and the high safety profile of N-AC have allowed a series of clinical trials to examine its activity in drug addiction (Deepmala et al. 2015). Human data concerning N-AC activity in nicotine addiction have mostly evaluate the efficacy of repeated administration of N-AC (Knackstedt et al. 2009; Schmaal et al. 2011; Grant et al. 2014; Prado et al. 2015). Even though high doses of N-AC are needed both in humans and in rats in order to observe any effect in the brain, N-AC safety profile has allowed chronic N-AC administration for long period of time.

My results may be of great value for the design of future clinical trials since they highlight the possibility that N-AC treatment may show greater efficacy if abstinent subject are concomitantly treated with cue extinction therapy (CET). This possibility is further supported by recent finding showing that repeated N-AC administered to smokers treated during smoking-focused group behavioral therapy were more prone to quit smoking compared to the placebo group (Prado et al. 2015). Interestingly, it has been also shown recently that a greater cue specificity for a behavioral therapy such as CET can be achieved with virtual reality approach (Giovancarli et al. 2016). It would be of great interest to evaluate if increasing CET specificity in combination with chronic N-AC treatment may provide a long lasting anti-craving prevention.

In conclusion, the results of my Thesis support the validity of N-AC treatment in nicotine addiction. In particular, the finding that N-AC can be more effective when

administered during the experimental CET provides interesting indication for clinical trials. In fact, when evaluating N-AC activity in smoking cessation it should be also considered that a combined N-AC and CET therapy could drastically improve the final outcome.

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