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Vernon Garcia Rivas

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THÈSE PRÉSENTÉE

POUR OBTENIR LE GRADE DE

**DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX**

ÉCOLE DOCTORALE « SCIENCES DE LA VIE ET LA SANTÉ »

SPÉCIALITÉ NEUROSCIENCES

Par Vernon GARCIA-RIVAS

**CORRELATS PSYCHOBIOLOGIQUES DES VARIATIONS
INDIVIDUELLES DANS LE CONTROLE DE LA
RECHERCHE DE DROGUE PAR LA NICOTINE ET LES
INDICES ENVIRONNEMENTAUX ASSOCIES A LA
NICOTINE**

Sous la direction de : Véronique DEROCHE-GAMONET

Soutenue le 7 Décembre 2018

Membres du jury :

M. FAURE Philippe, Directeur de recherche, Université Pierre et Marie Curie
M. SOLINAS Marcello, Directeur de recherche, Université de Poitiers
M. NAASSILA Mickaël, Directeur de recherche, Université de Picardie Jules Verne
Mme CAILLE-GARNIER Stéphanie, Chargée de recherche, Université de Bordeaux

Président
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Par Vernon GARCIA-RIVAS

**PSYCHOBIOLOGICAL CORRELATES OF
INDIVIDUAL VARIATIONS IN THE CONTROL OF
NICOTINE SEEKING BY NICOTINE AND
NICOTINE-ASSOCIATED CUES**

Sous la direction de : Véronique DEROCHE-GAMONET

Soutenue le 7 Décembre 2018

Membres du jury :

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Résumé

Le tabagisme est la cause de longues maladies, responsables chaque année de 6 millions de décès. Le principal composant du tabac, la nicotine, est l'un des psychotropes les plus addictifs. L'abandon du tabac est difficile et les pharmacothérapies les plus efficaces, telles que la varénicline, ne viennent en aide qu'à une proportion limitée des 70% de fumeurs qui souhaitent stopper.

Des études cliniques et précliniques ont démontré que plusieurs mécanismes psychopharmacologiques différents contribuent au maintien de la prise de nicotine. Des données psychologiques, génétiques et neurobiologiques, issues d'études cliniques, indiquent désormais que le poids respectif de ces mécanismes psychopharmacologiques pourrait varier d'un fumeur à l'autre. Cette hétérogénéité pourrait contribuer à l'inégale efficacité de la varénicline, dont les cibles psychopharmacologiques sont encore mal connues, ainsi qu'à la faible validité prédictive des modèles précliniques, qui ne tiennent pas compte de cette possible hétérogénéité individuelle.

Dans ce travail de thèse, au moyen de l'auto-administration intraveineuse de nicotine chez le rat, nous avons exploré les variations individuelles dans la sensibilité aux effets renforçants primaires de la nicotine et aux effets de la nicotine sur la sensibilité aux effets renforçants de stimuli environnementaux associés. Nous avons mis en évidence trois sous-populations d'individus dont la recherche de nicotine est contrôlée par une contribution différente de ces deux types d'effets de la nicotine. Les phénotypes de ces sous-populations ont été validés par des marqueurs comportementaux préexistants à la consommation de nicotine (l'approche conditionnée pavlovienne), par des marqueurs du métabolisme de la nicotine et des marqueurs neurobiologiques des neurotransmissions cholinergique et dopaminergique dans des structures cérébrales clés. En parallèle, nous avons exploré les cibles psychopharmacologiques de la varénicline. En utilisant une nouvelle approche qui permet de manipuler, pendant l'autoadministration, les effets de la nicotine sur les effets renforçants d'un stimulus environnemental associé, nous avons montré que la varénicline antagonise à la fois ces effets de la nicotine et ses effets renforçants primaires. Néanmoins, dans le premier cas, la varénicline agit

d'autant plus que la sensibilité individuelle aux effets de la nicotine est élevée, alors que l'intensité de son effet ne dépend pas de l'amplitude des effets renforçants primaires de la nicotine.

Ce travail de thèse met en évidence et valide des variations individuelles dans les mécanismes qui régissent le comportement de recherche de nicotine dans un modèle préclinique. Il offre pour perspective d'explorer les mécanismes neurobiologiques responsables de ces variations individuelles et l'impact à long terme de ces variations sur le développement de la dépendance à la nicotine, ainsi que de tester si la varénicline est plus efficace chez l'une des sous-populations identifiées.

Mots clés: nicotine, auto-administration, différences individuelles

Abstract

Tobacco use leads to 6 million deaths every year due to severe long lasting diseases. The main component of tobacco, nicotine, is recognized as one of the most addictive drugs, making smoking cessation difficult, even when 70% of smokers wish to do so. Critically, even the most effective pharmacotherapies for smoking cessation, such as varenicline, have only limited efficacy.

Clinical and preclinical studies have demonstrated consistently that nicotine seeking is a complex behavior involving various psychopharmacological mechanisms. Critically, converging psychological, genetic and neurobiological data from clinical studies support that the mechanisms controlling nicotine seeking may vary from individual to individual. This heterogeneity could explain the unequal efficiency of treatments, notably of varenicline, whose psychopharmacological targets are still poorly understood, and the poor predictive validity of preclinical models, which do not consider possible individual variations in the mechanisms of nicotine seeking.

In this PhD work, using intravenous nicotine self-administration in the rat, we have explored individual variations in the control of nicotine seeking, by the primary reinforcing effects of nicotine, nicotine's impact on environmental cues, or both. We have evidenced three sub-populations of individuals whose nicotine seeking is controlled by distinct contributions of nicotine primary reinforcing effects and nicotine-cue interactions. Their phenotypes of nicotine seeking have been supported and validated by pre-existing behavioral markers of Pavlovian conditioned approach, as well as by markers of nicotine metabolism, and neurobiological markers of cholinergic and dopamine transmissions in key brain structures. In parallel, we have explored psychopharmacological targets of varenicline. Using a novel approach that allows manipulating the reinforcing-enhancing effects of nicotine on cues, during nicotine self-administration, we evidenced that varenicline antagonizes both these cue reinforcing-enhancing effects and the primary reinforcing effect of nicotine, but as a function of the individual response amplitude for the former, and not for the latter.

This PhD work evidences and validates preclinical individual variations in the mechanisms of nicotine seeking. It opens the perspective of exploring the neurobiological causal mechanisms for these individual variations, their long term impact on the development of nicotine dependence and whether varenicline efficacy benefits more to the subpopulation mostly driven by nicotine-induced enhancement of cue reinforcing effects.

Keywords: nicotine, self-administration, individual differences

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LIST OF ABBREVIATIONS

AL: Ambient light

BLA: Basolateral amygdala

CD: Change in dose

cNAcc : Nucleus accumbens core

CS: Conditioned stimulus

dACC: Dorsal anterior cingulate cortex

DCT: Delta of normalized Ct values (PCR)

IP: Intraperitoneal route of administration

IV: Intravenous route of administration

mHB: Medial habenula

mPFC: Medial prefrontal cortex

NAcc: Nucleus accumbens

nAChR: Nicotinic acetylcholinergic receptor

NPF: Non-pharmacological factor

PCA: Pavlovian conditioned approach

PCC: Posterior cingulate cortex

PCR: Polymerase chain reaction

PF: Pharmacological factor

SA: Self-administration

SEM: Standard error of the mean

shNAcc: Nucleus accumbens shell

SNP: Single nucleotide polymorphism

US: Unconditioned stimulus

VTA: Ventral tegmental area

Preface

Tobacco has one of the highest prevalence of use of all drugs of abuse (Peacock et al., 2018), and its associated chronic use carries a heavy burden of premature mortality worldwide (World Health Organization, 2018). Despite this, clinical and preclinical studies on the mechanisms that drive tobacco dependence appear not to capture the complexity of the neuropsychopharmacology of nicotine, its major psychoactive compound. This failure is ultimately translated into limited therapeutic options for smokers struggling to quit their harmful addiction.

The general aim of this doctoral dissertation is to bring into light the existence of individual differences in the mechanisms that drive nicotine seeking. The evidencing of these individual differences could help reframe the ongoing discussions about nicotine addiction vulnerability, explain some of the complexity observed in human and animal studies, as well as providing insights on why “one-size-fits-all” therapeutic approaches fail to meet the desired clinical efficacy.

This doctoral dissertation is divided into five chapters. The **first chapter** serves as an introduction, in which we first present psychological, genetic and neurobiological data, collected from clinical and preclinical studies, which strongly suggest that existence of individual differences in the mechanisms of nicotine seeking, in particular in the complex interactions between nicotine and surrounding environmental cues (“nicotine-cue interactions”). In the **second chapter**, we introduce the aims of our experimental work. Our **third chapter** oversees the identification and characterization of different clusters of rats, which differ in the strength in nicotine-cue interactions behind their nicotine seeking behavior. We validated this characterization using behavioral, metabolic, and neurobiological correlates. Our **fourth chapter** is devoted to a first step in refining the psychopharmacology of one of the most important pharmacotherapies against nicotine dependence, varenicline, where we show that it powerfully targets the reinforcing-enhancing effects of nicotine on surrounding cues, with only a limited effect on the primary reinforcing actions of nicotine. This raises important implications in its therapeutic use against tobacco dependence. We conclude with a **fifth chapter**, where we provide a general discussion of this doctoral dissertation, emphasizing the relevance of individual differences in the mechanisms of nicotine seeking, as well as providing future directions for further research.

Chapter 1

INTRODUCTION

*“Tobacco, divine, rare superexcellent tobacco,
which goes far beyond all panaceas,
potable gold and philosopher's stones,
a sovereign remedy to all diseases...”*

Robert Burton – “Anatomy of Melancholy” (1621)

*“...Quoi que puisse dire Aristote, et toute la philosophie,
il n'est rien d'égal au tabac, c'est la passion des honnêtes gens;
et qui vit sans tabac, n'est pas digne de vivre.
non seulement il réjouit, et purge les cerveaux humains,
mais encore il instruit les âmes à la vertu,
et l'on apprend avec lui à devenir honnête homme...”*

Molière – “Dom Juan ou le Festin de Pierre” (1665)

*“...For I hate, yet love, thee so,
That, whichever thing I shew,
The plain truth will seem to be
A constrained hyperbole,
And the passion to proceed
More from a mistress than a weed [...]
For I must (nor let it grieve thee,
Friendliest of plants, that I must) leave thee.
For thy sake, TOBACCO, I
Would do any thing but die,
And but seek to extend my days
Long enough to sing thy praise...”*

Charles Lamb – “A Farewell to Tobacco” (1805)

General Introduction

The Health and Economical Burden of Tobacco Use

Tobacco is a drug of choice for an estimated of 1.1 billion people worldwide (World Health Organization, 2018), ranking as the drug of abuse with the highest prevalence of use, only behind alcohol (Peacock et al., 2018). Tobacco use is particularly high in the European Union, where 24% of individuals above 15 years of age are current smokers, of which 80% are daily users (Eurostat, 2016). The health burden of these numbers is not insignificant, as habitual smoking, as well as indirect exposure to tobacco smoke, are direct causes for premature mortality, being responsible for 6 million deaths worldwide every year (World Health Organization, 2018). Indeed, in Europe tobacco is responsible for as much as 26% of all deaths among adults aged 30-69 years (Goodchild et al., 2018). Lung cancer, normally associated with poor prognosis and high mortality, is the prime cause of smoking-attributable mortality, with 90% of all diagnosis linked to active smoking (Centers for Disease Control and Prevention (CDC), 2018). Moreover, smoking has been also linked to pharynx, esophagus, cervix, kidney and colorectal cancers, as well as being a strong risk factor for chronic obstructive respiratory disorders, cardiovascular diseases and diabetes (Centers for Disease Control and Prevention (CDC), 2018). These smoking-attributable diseases represent around 6% of global health expenditure, and in Europe, an estimated 2.5% of the European Union's gross domestic product (Goodchild et al., 2018). Despite social and political measures developed in the last fifteen years to curtail smoking, it continues to be the leading cause of preventable deaths worldwide (World Health Organization, 2018).

The Problem of Tobacco Dependence

Initiation of smoking and experimentation with tobacco normally occurs in adolescence, with more than 90% of adult smokers having had initiated smoking before their twentieth year of age (Glynn et al., 1993; Institute of Medicine (US), 1994), in a developmental period characterized by heightened risk taking and novelty seeking (Bava and Tapert, 2010). A combination of social, environmental, economic, familiar and biological factors need to be in place to foster smoking initiation (Hawkins et

al., 1992; Brown and Rinelli, 2010; Lovato et al., 2011; Audrain-McGovern et al., 2012; García-Rodríguez et al., 2014; Leventhal, 2016). While a discussion of these factors is well beyond the scope of this doctoral dissertation, it is important to note that not all individuals that have ever experimented with tobacco during their adolescence would continue to do so in the future, or transit into tobacco dependence into adulthood (Hiroi and Agatsuma, 2005; Audrain-McGovern et al., 2012)). Individuals who pass the first stage of tobacco experimentation normally progress into irregular, non-daily smoking, with varying degrees of intermittency between one cigarette and the following (DiFranza, 2015), representing roughly 25% of all smokers (Jamal et al., 2015). From this group, a large proportion will later escalate smoking towards habitual daily use (Coggins et al., 2009), with a minority escalating to more than 25 cigarettes per day (Wilson et al., 1992)

What drives the need to reach for the next cigarette? Addiction has been globally defined as a compulsive drive to take a drug despite serious adverse consequences (Volkow and Li, 2004), and for some smokers, tobacco is no exception (Anthony et al., 1994; Nutt et al., 2007; van Amsterdam et al., 2010). Between 30 and 50% of all smokers meet diagnostic criteria for tobacco dependence (Breslau et al., 1993; Anthony et al., 1994; Breslau et al., 1994; National Survey on Drug Use and Health, 2013), the proportion depending on sample size and diagnostic tool used (Smith and Fiore, 1999; Hiroi and Scott, 2009). Despite 70% of all smokers wanting to quit, only 50% of all smokers attempt to quit in a year, but only 6% of them will succeed without medical intervention (Benowitz, 2010; National Center for Health Statistics, 2012; Rigotti, 2012). Non-daily smokers are not spared from the addictive profile of tobacco, and also report high rates of cessation failure (Tindle and Shiffman, 2011). In fact, occasional non-daily smokers can experience strong cravings that cannot be explained by pharmacokinetics alone (Fernando et al., 2006), but which nonetheless strongly predict smoking cessation failure (Killen and Fortmann, 1997; Potvin et al., 2015). Exacerbating this problem, approved pharmacotherapies against tobacco dependence show efficacy only in a limited proportion of individuals wanting to quit (Schuit et al., 2017). For instance, varenicline, despite being the most efficacious of the available therapies against tobacco dependence (Cahill et al., 2013; Hartmann-Boyce et al., 2014), can only sustain long-term abstinence from tobacco in about 20% of users beyond 6

months post-quitting (Oncken et al., 2006; Niaura et al., 2008; Jordan and Xi, 2018). Biomedical research efforts in the fight against tobacco have thus aimed their attention in understanding the biological mechanisms of tobacco dependence, which could improve the use, and development, of therapies and help improve cessation rates.

Nicotine drives Tobacco Dependence

As their name suggests, tobacco products incorporate extracts from the tobacco plant, *Nicotiana tabacum*, native to the Americas and introduced to Europe at the dawn of the 16th century (Russo et al., 2011). Among the thousands of compounds contained in combusted tobacco (Stedman, 1968; Löfroth, 1989), one of them, an alkaloid named nicotine, has been long identified as the main responsible for the addictive potential of tobacco (Benowitz, 1992). Nicotine is an agonist at the nicotinic acetylcholinergic receptors (nAChRs), so named after the discovery that nicotine selectively activates them (Changeux et al., 1970; Klett et al., 1973). As a family of receptors, nAChRs are all ionotropic (Dajas-Bailador and Wonnacott, 2004), although recent evidence suggests that they may couple with G-proteins in certain cases (Kabbani et al., 2013). Different subtypes of nAChRs are assembled as pentamers through different combinations of their constituent α and β subunits, which determine the receptor gating properties, i.e. the speed of opening and desensitization (Sargent, 1993; Dani, 2015). The most common nAChR assembly in the brain is the $\alpha 4\beta 2$ configuration, allowing for high affinity for nicotine, fast activation, but also rapid desensitization (Lippiello et al., 1987; Dani et al., 2000; Gotti et al., 2006)

nAChRs, as part of the brain cholinergic system, play key roles in attention, memory, arousal, mood, and reward (Pich et al., 1997; Levin and Simon, 1998; Phillips et al., 2000; Dani and Bertrand, 2007; Wallace and Bertrand, 2013; Kutlu and Gould, 2016; Gandelman et al., 2018). Of particular interest for tobacco dependence, $\alpha 4\beta 2$ -containing nAChRs are highly expressed in the ventral tegmental area (VTA), where they play a role in modulating the burst firing of dopaminergic neurons (Picciotto et al., 1998; Klink et al., 2001; Pidoplichko et al., 2004). The monomeric $\alpha 7$ nAChRs are also important in the VTA, as their pre-synaptic location facilitate glutamate release into VTA neurons (Jones and

Wonnacott, 2004; Gao et al., 2010), and as they desensitize less rapidly than $\alpha 4\beta 2$ nAChRs (Wooltorton et al., 2003), allow for a sustained modulation of dopamine neurotransmission (Schilström et al., 2003; Pidoplichko et al., 2004). The dopaminergic projections from the VTA to the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) constitute the most central pathway involved in reinforcement, and not surprisingly, in drug addiction (Pich et al., 1997; Koob et al., 2004; Pidoplichko et al., 2004; Everitt and Robbins, 2005; Salamone et al., 2007), explaining its central role in the development of tobacco dependence (Picciotto et al., 1998).

Despite this, nicotine has surprisingly only limited primary reinforcing properties, compared to other drugs of abuse (Henningfield and Goldberg, 1983). Previous studies have shown that nicotine, by itself, is poorly self-administered (Caggiula et al., 2001, 2002). The primary reinforcing effects of nicotine are not able, by themselves, to explain the pervasiveness of tobacco use (Chaudhri et al., 2006; Caggiula et al., 2009). Thus, in the last fifteen years, research has shifted attention to the powerful effects that nicotine exerts on surrounding stimuli instead (Caggiula et al., 2002; Donny et al., 2003; Chaudhri et al., 2006; Palmatier et al., 2007; Caggiula et al., 2009). It is widely known that nicotine can transform surrounding environmental cues into conditioned reinforcers (Caggiula et al., 2001; Cohen et al., 2005; Rose, 2006; Donny et al., 2007), which can later trigger craving and facilitate relapse (Conklin and Tiffany, 2002; Ferguson and Shiffman, 2009; Rupperecht et al., 2015). Newer evidence has also identified that nicotine can enhance the reinforcement value of stimuli that are already reinforcers by themselves (Palmatier et al., 2006, 2007; Caggiula et al., 2009; Perkins and Karelitz, 2013; Perkins et al., 2017; Constantin and Clarke, 2018). Not surprisingly, this potentiation appears to rely on the dopaminergic system (Palmatier et al., 2014). Although the precise neurobiology of these actions are yet to be explored, it is possible that they could involve structures related to reward-cue interactions, such as NAcc, hippocampus, mPFC and the basolateral amygdala (BLA), structures that express both nAChRs and dopamine receptors (Gasbarri et al., 1997; Palermo-Neto, 1997; Paterson and Nordberg, 2000; Kröner et al., 2005; Zhu et al., 2005; Gotti et al., 2006; Raybuck and Gould, 2010; Kutlu and Gould, 2016).

Altogether, this evidence supports that smokers would seek nicotine for two main reasons: (1) for the psychopharmacology of nicotine in and of itself, whether through primary reinforcement or withdrawal alleviation, and (2) for the psychopharmacology of nicotine in modulating environmental stimuli (“nicotine-cue interactions”), whether through Pavlovian conditioning, or through acute enhancement of the incentive value of non-nicotine primary reinforcers.

Individual Differences in the Mechanisms of Nicotine Seeking

Despite that these distinct motivations for nicotine seeking are well-known (see (Rupprecht et al., 2015)), most preclinical studies focusing in understanding the neurobiological mechanisms of nicotine seeking have only explored them from what it is inferred from the mean observations in their study populations. Over the same time, preclinical addiction research in cocaine has demonstrated the importance of individual variability in factors governing initial drug intake, transition to habitual use, and progression to compulsive drug use (Piazza et al., 1998, 2000; Deroche-Gamonet et al., 2004; Kasanetz et al., 2010; Bardo et al., 2013; Lenoir et al., 2013; Piazza and Deroche-Gamonet, 2013; Pelloux et al., 2015), an approach also recently developed with alcohol (Augier et al., 2018). Despite their possible relevance, individual variations in the previously mentioned mechanisms contributing to nicotine seeking have been scarcely explored in human or animal research on nicotine addiction (Hiroi and Scott, 2009).

In our first publication (Garcia-Rivas and Deroche-Gamonet, 2018) presented below, we reviewed extensive evidence from clinical and preclinical studies, suggesting that individual differences in nicotine seeking behavior do exist, and that they have different neurobiological underpinnings. In our second publication (Garcia-Rivas et al., 2017), presented immediately after, we propose preclinical experimental strategies that would allow to capture, in a rodent model, these individual differences in the mechanisms that drive nicotine seeking. The aim of these strategies would not only be useful to explore the precise neurobiology of these different mechanisms, but also provide a tool to better tailor current pharmacotherapies, while also helping developing individualized approaches against tobacco dependence.

Not all smokers appear to seek nicotine for the same reasons: implications for preclinical research in nicotine dependence

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ABSTRACT

Tobacco use leads to 6 million deaths every year due to severe long-lasting diseases. The main component of tobacco, nicotine, is recognized as one of the most addictive drugs, making smoking cessation difficult, even when 70 percent of smokers wish to do so.

Clinical and preclinical studies have demonstrated consistently that nicotine seeking is a complex behavior involving various psychopharmacological mechanisms. Evidence supports that the population of smokers is heterogeneous, particularly as regards the breadth of motives that determine the urge to smoke.

Here, we review converging psychological, genetic and neurobiological data from clinical and preclinical studies supporting that the mechanisms controlling nicotine seeking may vary from individual to individual. It appears timely that basic neuroscience integrates this heterogeneity to refine our understanding of the neurobiology of nicotine seeking, as tremendous progress has been made in modeling the various psychopharmacological mechanisms driving nicotine seeking in rodents.

For a better understanding of the mechanisms that drive nicotine seeking, we emphasize the need for individual-based research strategies in which nicotine seeking, and eventually treatment efficacy, are determined while taking into account individual variations in the mechanisms of nicotine seeking.

Keywords animal model, cue, individual differences, nicotine, seeking, self-administration.

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Tobacco is recognized as one of the most addictive products, with more than 70 percent of smokers wishing to quit (National Center for Health Statistics 2012) and less than 10 percent succeeding without medical support (Rigotti 2012). Although tobacco dependence is not associated with obvious behavioral disruptions, alleviating it is a major public health concern and a main societal challenge, as it produces severe long-lasting health-related problems (WHO 2015). Available therapies for smoking cessation have limited efficacy (Schuit *et al.* 2017), warranting the need for developing better therapeutic strategies, which depend on understanding the mechanisms that underlie tobacco dependence. Compromising this pursuit, tobacco dependence in humans has been difficult to define (Hiroi & Scott 2009; Piper 2015; Potvin *et al.* 2015) as attested by the various available diagnostic tools assessing different dimensions of smoking behavior

(Shiffman, Waters, & Hickcox 2004; Etter 2005; Hiroi & Scott 2009; Baker *et al.* 2012). Despite that diagnostic tools in addiction should help clinicians in tailoring treatment for drug cessation (West & Miller 2011), accumulating evidence suggests that those developed for tobacco dependence are often incongruent between each other, and none of them accurately and consistently predicts cessation or treatment outcome against tobacco dependence (Heatherton *et al.* 1989; Breslau & Johnson 2000; Patten *et al.* 2001; Etter, Le Houezec, & Perneger 2003; Etter 2005; Baker *et al.* 2007; Donny & Dierker 2007; Hiroi & Scott 2009; Courvoisier & Etter 2010). This apparent inefficacy may be due to their inability to directly assess the heterogeneity of the population of smokers, which has been observed and reviewed repeatedly (Kassel *et al.* 1994; Hiroi & Scott 2009; Conway *et al.* 2010; Courvoisier & Etter 2010; Baker *et al.* 2012; Loukola

et al. 2014; Hall *et al.* 2015; Potvin *et al.* 2015), particularly as regards the breadth of motives and mechanisms that determine the urge to smoke (Donny *et al.* 2008; Hiroi & Scott 2009; Conway *et al.* 2010).

It is acknowledged that a major motive for smoking is seeking for nicotine, which is recognized as the main psychoactive compound of tobacco responsible for dependence (Benowitz 1992). The paradoxical contrast between the strong addictive profile of tobacco and the relatively weak primary reinforcing effect of nicotine (Caggiula *et al.* 2001; Rose 2006) has been explained by both clinical and preclinical studies consistently demonstrating that complex interactions between environmental cues and nicotine also play a critical role in promoting and maintaining nicotine seeking (Shiffman *et al.* 2012; Bani, Andorn, & Heidbreder 2014; McClernon *et al.* 2015; Stoker & Markou 2015; Shiffman, Dunbar, & Ferguson 2015). As comprehensively described by Rupperecht *et al.* (2015), data support that smokers would seek nicotine (1) for its primary reinforcing effects; (2) for its ability to relieve withdrawal symptoms; (3) in response to external or internal cues, which have acquired the ability to promote nicotine seeking due to their Pavlovian association with the primary reinforcing effects of nicotine, or the alleviation of withdrawal; and (4) for its ability to enhance the reinforcing value of natural reinforcers or the incentive value of environmental stimuli that have acquired reinforcing properties through conditioning to primary reinforcers.

Most studies in tobacco addiction have explored these mechanisms of nicotine seeking from what is inferred from the mean observations in their study populations. Over the last 15 years, preclinical addiction research, in particular in regards to cocaine, has shown an interest for individual variability in factors governing initial drug intake, transition to habitual use and the progression to compulsive drug use (Piazza *et al.* 1998, 2000; Deroche-Gamonet, Belin, & Piazza 2004; Kasanetz *et al.* 2010, 2013; Bardo, Neisewander, & Kelly 2013; Lenoir *et al.* 2013; Piazza & Deroche-Gamonet 2013; Pelloux, Murray, & Everitt 2015). Despite their possible relevance, individual variations in the previously mentioned mechanisms contributing to nicotine seeking have been scarcely explored in human or animal research on nicotine dependence, while they may contribute to explain the heterogeneity of smoking behavior (Baker *et al.* 2012), the inconsistent relationship between craving and smoking cessation outcome (Wray, Gass, & Tiffany 2013), the limited therapeutic predictive validity of the existing preclinical models (Lerman *et al.* 2007; O'Dell & Khroyan 2009; Le Foll *et al.* 2014; Schuit *et al.* 2017), the limited reliability of diagnostic clinical tools (Hiroi & Scott 2009) and the inconsistent success of approved therapies for tobacco cessation (Schuit *et al.* 2017). Based on this premise, we

recently emphasized the need for an individual-based preclinical research on the mechanisms of nicotine seeking (Garcia-Rivas, Cannella, & Deroche-Gamonet 2016).

Here, we present a review of behavioral and neurobiological data, both clinical and preclinical, that support the role of individual variations in the mechanisms underlying nicotine seeking. Experimental disentangling of the psychopharmacological mechanisms of nicotine seeking is complex, as some of these mechanisms are intimately linked and are difficult to be distinguished one from the other. Nevertheless, considering the decisive steps that have been taken in preclinical modeling of nicotine seeking and in molecular characterization of nicotine targets over the last 30 years, we propose that conditions are met for starting to explore individual variations in the mechanisms of nicotine seeking and their consequence in research strategies on therapeutic targets. Table 1 summarizes the different nicotine-related domains, in which individual variations from the acknowledged mean observation have been evidenced in tobacco smokers, as well as related relevant preclinical observations.

PSYCHOPHARMACOLOGICAL MECHANISMS OF NICOTINE SEEKING

Through the activation of nicotinic cholinergic receptors (nAChRs) in the dopaminergic neurons in the ventral tegmental area (VTA), nicotine directly increases dopamine release in the nucleus accumbens (NAcc) (Kenny & Markou 2006), which is thought to be central for the rewarding actions of nicotine that underlie positive reinforcement of tobacco use (Corrigall, Coen, & Adamson 1994; Ikemoto, Qin, & Liu 2006; Peng *et al.* 2017). However, there is plenty of evidence that the rewarding effects of nicotine are relatively poor, in comparison with other psychostimulants (Risner & Goldberg 1983; Caggiula *et al.* 2001). Furthermore, nicotine can produce very unpleasant aversive effects such as nausea and vomiting at high doses in regular smokers, but also particularly during the first cigarette ever smoked (Sartor *et al.* 2010; Agrawal *et al.* 2014). Why would then individuals sustain volitional administration of nicotine chronically? Factors other than the balance of nicotine reward and aversion seem to play a role. Since nAChRs are largely distributed in the central nervous system (Changeux 2010; Brunzell, Stafford, & Dixon 2015), nicotine can enhance cognitive function (Levin 1992; Warburton 1992; Sutton *et al.* 2016), regulate mood and affect (Kassel *et al.* 2007) and regulate appetite and body weight (Bowen, Eury, & Grunberg 1986; Grunberg, Popp, & Winders 1988; Huang, Xu, & van den 2011), all of which have been self-reported as primary sources of reinforcement by nicotine and motivations for smoking (Pulvers *et al.* 2014;

Table 1 Evidence of individual differences affecting nicotine seeking. Most studies in tobacco addiction have explored the mechanisms of nicotine seeking from what is inferred from the mean observations in their study populations. Summarized in this table is selected evidence of individual differences in domains that impact nicotine seeking in humans, as well as preclinical observations that can shed light on the mechanisms of this inter-individual variability. Highlighted in bold are the rare preclinical studies that have directly explored individual differences in nicotine seeking.

Domain	Well-known mean observation	Clinical evidence for individual differences	Preclinical observations
NICOTINE REWARD	Primary reinforcing effects, although much less than other psychostimulants (Risner & Goldberg 1983; Caggiula <i>et al.</i> 2001)	<ul style="list-style-type: none"> - Carriers of CHRNA4 rs6122429 allele: ↑ experience of euphoria after cigarette (Hutchison <i>et al.</i> 2007) - Carriers of CHRNA4 rs2236196 allele: ↑ experience of euphoria after cigarette (Hutchison <i>et al.</i> 2007), ↑ probability of being a smoker (Li <i>et al.</i> 2005; Breitling <i>et al.</i> 2009; Esterlis <i>et al.</i> 2016), ↑ cessation outcome when using nicotine nasal spray (Hutchison <i>et al.</i> 2007) 	<ul style="list-style-type: none"> - Hypersensitive alpha4-containing nAChRs ↑ the rewarding properties of acute nicotine (Tapper <i>et al.</i> 2004) - KO of alpha4 subunit in the ventral midbrain ↓ nicotine self-administration (Pons <i>et al.</i> 2008) and nicotine place preference (Peng <i>et al.</i> 2017) - Naturally-occurring CHRNA4 A529T SNP in mice: ↓ sensitivity to nicotine reward (Butt <i>et al.</i> 2005; Wilking <i>et al.</i> 2010) - CHRNA5 rs16969968 allele introduced in mice ↓ sensitivity to rewarding effects of low doses of nicotine, compared to WT (Frahm <i>et al.</i> 2011; Morel <i>et al.</i> 2014)
NICOTINE AVERSION	Aversive effects that can limit volitional administration at high doses (Norton & Barske 1977)	<ul style="list-style-type: none"> - Increased aversive side effects after first cigarette linked to ↓ risk for nicotine dependence (Sartor <i>et al.</i> 2010; Hoff <i>et al.</i> 2011; Svyryd <i>et al.</i> 2016) - Carriers of CHRNA5 rs16969968 risk allele: ↓ aversive symptoms after acute nicotine administration (Jensen <i>et al.</i> 2015) 	<ul style="list-style-type: none"> - KO of alpha5 subunit ↑ nicotine self-administration in mice, but rescued after reintroduction of alpha5 in medial habenula, key structure mediating aversion to nicotine (Fowler <i>et al.</i> 2011) - Overexpression of beta4 subunit in the medial habenula ↓ nicotine self-administration in rodents, which is rescued with introduction of CHRNA5 rs16969968 allele (Frahm <i>et al.</i> 2011) - CHRNA5 rs16969968 allele introduced in mice ↓ sensitivity to aversive effects of high doses of nicotine (Morel <i>et al.</i> 2014)
NICOTINE-ASSOCIATED CUES	Nicotine establishes paired environmental cues as conditioned reinforcers (Caggiula <i>et al.</i> 2001; Cohen <i>et al.</i> 2005; Feltenstein <i>et al.</i> 2012)	<ul style="list-style-type: none"> - Carriers of CHRNA5 rs16969968 risk allele: ↓ cue-reactivity to smoking cues (Janes <i>et al.</i> 2012) 	<ul style="list-style-type: none"> - In rats previously selected according to their high incentive salience to food cues ('sign trackers'), a nicotine-associated cue was a stronger conditioned reinforcer than in 'goal trackers' (Yager & Robinson 2015).

NICOTINE-INDUCED ↑ IN ATTENTION TO ENVIRONMENTAL CUES	Nicotine ↑ the attention to environmental cues (Witte <i>et al.</i> 1997)	<ul style="list-style-type: none"> - Carriers of CHRNA4 rs1044396 allele: ↑ risk for nicotine dependence (Feng <i>et al.</i> 2004; Kamens <i>et al.</i> 2013), ↓ basal visuospatial attention performance, but which ↑ after nicotine exposure (Ahrens <i>et al.</i> 2015; Behler <i>et al.</i> 2015) - Nicotine ↑ attentional performance to visual cues, but only in individuals that have poor attentional performance baseline (Hammersley <i>et al.</i> 2016) - Individuals with low hedonic capacity have ↑ risk for tobacco use and dependence, possibly due to nicotine incentive-enhancing effects (Perkins 2009; Audrain-McGovern <i>et al.</i> 2012; Leventhal 2016; Perkins <i>et al.</i> 2017) 	- Speed of nicotine metabolism predicted nicotine reinforcement threshold and degree of compensation when unit dose of nicotine was reduced in rats (Greibenstein <i>et al.</i> 2015).
NICOTINE-INDUCED ↑ OF INCENTIVE SALIENCE FOR REINFORCING ENVIRONMENTAL CUES	Nicotine ↑ the incentive value of environmental non-drug reinforcers (Caggiula <i>et al.</i> 2009; Grimm <i>et al.</i> 2012; Palmatier <i>et al.</i> 2013.b; Perkins & Karelitz 2013a.b)	<ul style="list-style-type: none"> <i>Slow nicotine metabolizers:</i> - ↓ cigarettes smoked (Styn <i>et al.</i> 2013; Wassenaar <i>et al.</i> 2011), - ↑ cessation success with NRTs (Kaufmann <i>et al.</i> 2015; Mamoun <i>et al.</i> 2015) <i>Fast nicotine metabolizers:</i> - ↑ cigarettes smoked, attempting to titrate falling nicotine plasma levels (Olsson <i>et al.</i> 2016) - ↑ risk for nicotine dependence in adulthood (Wassenaar <i>et al.</i> 2011; Sofuoglu <i>et al.</i> 2012) - ↑ cue-reactivity to smoking cues, compared to slow metabolizers (Tang <i>et al.</i> 2012; Falcone <i>et al.</i> 2016) 	
NICOTINE METABOLISM	Nicotine is metabolized to cotinine and has a half-life of 2 hours (Hukkanen, Jacob, & Benowitz 2005).		
PSYCHOLOGICAL TRAITS MODULATING NICOTINE SEEKING	Novelty/sensation seeking, anxiety and impulsivity/poor decision making can be associated with increased risk for tobacco dependence (Falco & Bevins 2015) <i>Controversial data:</i> Inconsistent association of impulsive choice with severity of tobacco dependence (Johnson <i>et al.</i> 2007; Sweitzer <i>et al.</i> 2008; Carim-Todd <i>et al.</i> 2016; Rasmussen <i>et al.</i> 2016).	<ul style="list-style-type: none"> - High impulsivity is associated with high smoking relapse only in subjects with high cue-induced craving (Bourque <i>et al.</i> 2013; Erblich & Michalowski 2015) - Carriers of CHRNA3 rs578776 allele: amount of nicotine consumed correlates with functional coupling in circuitry associated with risky decision making (Hong <i>et al.</i> 2010, Wei <i>et al.</i> 2016) 	- High impulsive rats show ↑ cue-induced relapse and ↓ control of nicotine seeking during self-administration under increasing workload (Diergaarde <i>et al.</i> 2012) and during abstinence (Diergaarde <i>et al.</i> 2008).

Hall *et al.* 2015). In addition, avoidance and/or relief of pharmacological withdrawal from nicotine can be a major reinforcing effect of nicotine driving smoking behavior (Hughes 2007; Allen *et al.* 2008; Scott & Hiroi 2011).

Abundant evidence also suggests that the role of surrounding cues accompanying nicotine intake is pivotal to nicotine seeking, much more than in other drugs of abuse (Caggiula *et al.* 2001; Donny *et al.* 2003; Rupprecht *et al.* 2015). Clinical evidence shows that the sight, smell or touch of cigarettes, observing others smoking, visiting the habitual places for smoking or consuming alcoholic beverages might act as powerful motivators for nicotine seeking (Niaura *et al.* 1992; Conklin & Tiffany 2001; Van Gucht *et al.* 2010; Shiffman *et al.* 2015). Behavioral rituals in anticipation to smoking, such as rolling a cigarette, can by themselves trigger craving (Baker *et al.* 2006; Perkins *et al.* 2008), and expectation of withdrawal, as well as expectation of tobacco availability, can trigger nicotine craving (Wertz & Sayette 2001; Dar *et al.* 2010; Scott & Hiroi 2011). On this regard, in some smokers, the use of non-nicotine-containing electronic cigarettes can decrease craving (Van Heel *et al.* 2017), most probably through the sensorimotor cues accompanying smoking. The powerful role of nicotine-associated cues in modulating nicotine seeking has also been demonstrated in rodents self-administering nicotine: nicotine alone is poorly self-administered, but when its delivery is paired with a discrete cue light, self-administration is enhanced synergistically (Caggiula *et al.* 2001; Donny *et al.* 2003). Alone, the cue can maintain self-administration over an extended number of sessions; the behavior resisting to extinction much longer than for the other drugs of abuse (Cohen *et al.* 2005). Additionally, in rats trained to extinguish seeking for both nicotine and associated cue, only presentation of both nicotine and cue induces a strong reinstatement, with a weaker effect of nicotine and cue when presented alone (Feltenstein, Ghee, & See 2012).

The first mechanism evoked for this control of nicotine seeking by cues is the establishment of paired environmental cues as strong conditioned reinforcers driving nicotine seeking through Pavlovian conditioning. More recently, it was evidenced that nicotine can further increase the incentive value of these classically conditioned cues through a non-associative mechanism (Palmatier *et al.* 2007), which also applies to, and has been particularly characterized for, non-nicotine conditioned cues. Indeed, nicotine can increase the incentive value of sensory cues that have gained secondary reinforcing properties after association with a primary reinforcer, such as sucrose (Chaudhri *et al.* 2006; Liu *et al.* 2007; Palmatier *et al.* 2007, 2013; Caggiula *et al.* 2009; Grimm *et al.* 2012). Nicotine also directly enhances the reinforcing effect of non-drug or drug reinforcers (Grimm *et al.* 2012;

Palmatier, O'Brien, & Hall 2012). Supporting a non-associative mechanism, it is also noteworthy that these effects of nicotine can occur in nicotine-naïve individuals, suggesting that it is part of the acute psychopharmacology of nicotine, does not involve a learning process and is not due to nicotine dependence (Rupprecht *et al.* 2015; Perkins, Karelitz, & Boldry 2017).

The term initially used to define these effects of nicotine was 'reinforcer-enhancing'. Now, mechanisms have been refined (Palmatier *et al.* 2013,b) supporting that nicotine increases the incentive salience of cues and not just their reinforcing effects. Both these 'incentive-enhancing' (Palmatier *et al.* 2013) and 'reinforcer-enhancing' (Donny *et al.* 2003) effects of nicotine occur regardless of schedule of nicotine delivery, that is, whether it is contingent or not to reinforcer or conditioned cue presentation (Chaudhri *et al.* 2006; Liu *et al.* 2007; Palmatier *et al.* 2007, 2012). This contrasts with the classical Pavlovian conditioning mechanism, where an otherwise non-salient cue becomes salient, acquiring drug-like reinforcing properties, only when drug and cue are paired contingently. Altogether, this evidence suggests that the influence of nicotine depends on the intrinsic nature of the environmental cue and the timing and coincidence of cue and nicotine presentation.

Then, in addition to attributing nicotine-associated cues with specific and stronger incentive salience (Cohen *et al.* 2005; Yager & Robinson 2015), this non-associative mechanism confers other natural and non-natural reinforcers (Grimm *et al.* 2012; Palmatier *et al.* 2012) and their associated cues with increased reinforcing and incentive properties under nicotine effect (Palmatier *et al.* 2013), which could be an additional motive for nicotine seeking. Although the attention to this non-associative mechanism in clinical studies has been widely overlooked (Perkins *et al.* 2017), there is evidence supporting that nicotine increases the reinforcement of non-nicotine sensory stimuli that are primary reinforcers by themselves, such as auditory (Perkins & Karelitz 2013a,b) and visual rewards (Perkins & Karelitz 2014), but not of monetary rewards (Perkins *et al.* 2017). In addition, acute nicotine withdrawal may involve sensory anhedonia that can be reversed with nicotine (Dawkins *et al.* 2006; Dawkins, Acaster, & Powell 2007; Cook *et al.* 2015, 2017). Thus, it is possible that tobacco cessation attempts prove difficult also due to nicotine making daily activities, natural reinforcers and environments much more pleasurable (Leventhal *et al.* 2009; Perkins 2009; Perkins *et al.* 2017).

Taken together, the motivations and mechanisms for nicotine seeking are diverse and the interplay between them is complex. As they involve distinct neurobiological mechanisms (Antolin-Fontes *et al.* 2015; Stoker & Markou 2015), they offer multiple sources of individual

variations, eventually leading to a different contribution of these diverse mechanisms from one smoker to the other.

INDIVIDUAL DIFFERENCES IN THE PSYCHOPHARMACOLOGICAL MECHANISMS OF NICOTINE SEEKING MAY CONTRIBUTE TO THE HETEROGENEITY OF SMOKER POPULATION

Evidence of differences in the mechanisms of nicotine seeking among smokers comes notably from the work of S. Shiffman and colleagues (for review Coggins *et al.* 2009). Using cue-reactivity and ecological momentary assessments, which involves the self-report of current behaviors and experiences in real time during habitual daily life, they have notably shown that factors driving seeking are different in daily and non-daily intermittent smokers, with environmental cues associated with smoking playing a stronger role in determining nicotine seeking among non-daily intermittent smokers (Shiffman, Stone, & Hufford 2008; Shiffman *et al.* 2014). Although they solicit global impressions of smokers' behaviour, studies using questionnaires have also proposed a predominant role of smoking-associated environmental cues in non-daily smokers (Pulvers *et al.* 2014; Scheuermann *et al.* 2015). The acute psychopharmacological effects of nicotine in enhancing the incentive salience of environmental cues could be a possible explanation for the importance of nicotine-associated cues in this population (Perkins & Karelitz 2013a,b). Some of these non-daily smokers progress into daily smoking, and it is proposed that in these individuals nicotine seeking is determined more by withdrawal alleviation and/or avoidance rather than environmental cues, thus requiring smoking at regular intervals to titrate nicotine intake (Shiffman *et al.* 2012, 2015; Bani *et al.* 2014; Piasecki *et al.* 2014; Roberts *et al.* 2015; Ferguson *et al.* 2016; Shiffman & Terhorst 2017). However, this shift is incomplete, as some degree of responsiveness to environmental cues is maintained even among heavy daily smokers (Baker *et al.* 2012; Shiffman *et al.* 2015). Only those who progress to very high daily tobacco consumption appear to be less sensitive to environmental stimuli (Ferguson *et al.* 2016). Altogether, this data suggests a wide range of smokers, where those non-daily irregular smokers who transit to become daily smokers become more susceptible to pharmacologically-induced nicotine craving and less reliant on environmental stimuli signaling smoking opportunities.

However, non-daily and daily smoker populations are more heterogeneous than this proposed view, as evidence accumulates that individual vulnerabilities appear to modulate, independently from each other, smoking

behavior, smoking frequency, craving, withdrawal, relapse timing and cessation success (McCarthy *et al.* 2006; DiFranza *et al.* 2007; Tindle & Shiffman 2011; Rigotti 2012; Piper 2015; Potvin *et al.* 2015). For instance, it has been shown that about 72 percent of the non-daily smokers, traditionally neglected in the discussions about nicotine dependence, fail to maintain abstinence for more than 90 days after a quit attempt (Tindle & Shiffman 2011). Despite suggestions that all non-daily smokers are 'social smokers' (Philpot *et al.* 1999), recent evidence suggests that only a small subset of them smoke exclusively in social settings and in the presence of other smokers, while the other non-daily smokers appear less dependent on these factors (Shiffman *et al.* 2014, 2015). In addition, despite the prediction that nicotine withdrawal may play a major role in heavy daily smokers, not all of them respond positively to nicotine replacement therapies (NRTs) (Rigotti 2012). Other daily smokers report reductions in craving scores upon switching to denicotinized cigarettes, suggesting that in these individuals, factors other than nicotine pharmacology contribute to smoking (Gross, Lee, & Stitzer 1997; Pickworth *et al.* 1999; Dallery *et al.* 2003; Bickel & Kirshenbaum 2004; Donny & Jones 2009). Among those daily smokers who eventually reach abstinence, some have responded to approved pharmacological therapies (Tang, Law, & Wald 1994; Cepeda-Benito, Reynoso, & Erath 2004; King *et al.* 2012), others required joint pharmacological and psychological support (Willemsen *et al.* 1998), while others achieve abstinence without any therapy (Rigotti 2012). Although limited, there is evidence suggesting that individuals experiencing low hedonic effect of natural reinforcers are more likely to become tobacco smokers later in life (Audrain-McGovern *et al.* 2012; Stone, Audrain-McGovern, & Leventhal 2017). The exact mechanisms behind this association are still to be explored, but it remains a possibility that these individuals may seek nicotine for its 'reinforcer-enhancing' effect on environmental cues, contributing to differences in prevalence, dependence score, progression into heavier use and cessation success among smokers (Audrain-McGovern *et al.* 2012; Leventhal 2016; Perkins *et al.* 2017).

Heterogeneity among daily and non-daily smokers is further evidenced in the dynamic shifts observed between these subpopulations: some non-daily smokers continue occasional and infrequent smoking, while others progress to become daily smokers, and a proportion of the latter revert to non-daily smoking habits (Shiffman *et al.* 2015). The exact mechanisms of such dynamic shifts are poorly understood (Coggins *et al.* 2009), as the timing between the transitions is highly variable, but recent epidemiological evidence suggests that individual neurobiological vulnerabilities may be responsible. Some individuals can reduce their smoking behavior following newer

tobacco policies and increasing negative societal pressures (National Center for Health Statistics 2012), while others with specific psychobiological traits or psychiatric comorbidities are more likely to become and remain heavy daily smokers, despite such deterrents (Thorgeirsson & Stefansson 2008; Piasecki *et al.* 2014; Steinberg, Williams, & Li 2015; Parikh, Kutlu, & Gould 2016; Talati, Keyes, & Hasin 2016; Shiffman & Terhorst 2017). Taken together, this behavioral data supports the notion that smokers with the same smoking pattern of consumption may differ in the mechanisms that drive their smoking behavior, their vulnerability for dependence and their response to therapy. Although increased smoking, through neurobiological adaptations following chronic high nicotine use (Hiroi & Agatsuma 2005), may be a source of such variation, biologically predetermined factors may also account for differences in nicotine seeking, smoking and cessation outcome, which occur independently from, or even are causal for, smoking severity. Unfortunately, the vast majority of clinical and preclinical studies in nicotine addiction do not consider individual variability, thus limiting the exploration of the sources of individual differences in human tobacco smoking. Despite this, there is new neurobiological evidence, described below, that sheds light on the possible mechanisms of this individual variability in the mechanisms of nicotine seeking.

NEUROPSYCHOBIOLOGICAL BASIS FOR THE INDIVIDUAL DIFFERENCES IN THE MECHANISMS OF NICOTINE SEEKING

A wide range of specific gene mutations, biological factors and personality traits appear to contribute to, or protect against, smoking initiation, maintenance and cessation (Bierut 2007; Thorgeirsson & Stefansson 2008; Doran *et al.* 2009; Gold & Lerman 2012; Fowler & Kenny 2014; Loukola *et al.* 2014; Erblich & Michalowski 2015; Lee *et al.* 2015; Olfson *et al.* 2016). It has been shown that combinations of risk and protective factors cumulatively affect smoking behavior (Greenbaum & Lerer 2009; Haller *et al.* 2014; Yang *et al.* 2015). Although only a limited body of research has explored the mechanisms of these vulnerabilities, this research suggests that they contribute differently to the different mechanisms of nicotine seeking.

Individual differences in nAChR subunit function can modulate the risk for dependence and the mechanisms of nicotine seeking

Alpha5-containing nAChRs

The pentameric nAChRs are the primary sites of action of nicotine and are composed by a combination of alpha and

beta subunit assemblies (Changeux 2012). Genome-wide associated studies have linked vulnerability to nicotine dependence to single nucleotide polymorphisms (SNPs) in the CHRNA5/CHRNA3/CHRNA4 gene cluster, which encodes the alpha5, alpha3 and beta4 subunits. In particular, a SNP in the rs16969968 allele of the CHRNA5 gene has been associated with smoking that is heavy, out of control, and manifests in strong craving (Saccone *et al.* 2007; Thorgeirsson *et al.* 2008; Chen *et al.* 2012; Gabrielsen *et al.* 2013). Converging evidence suggests that rs16969968 allele reduces susceptibility to the rewarding and aversive aspects of nicotine, but also to cue-induced craving, paradoxically leading to a 'heavy smoker' phenotype, as explained below.

In terms of reducing the rewarding actions of nicotine, it has been demonstrated that introduction of the human rs16969968 allele in mice causes a rightward shift of the dose-response curve for nicotine self-administration (Frahm *et al.* 2011; Morel *et al.* 2014), suggesting a decreased sensitivity to the rewarding effects of low nicotine doses. This is also evidenced by higher nicotine levels needed to engage the dopaminergic neurons in the VTA to NAcc pathway, involved in nicotine reward, compared to wildtype mice (Morel *et al.* 2014). In exploring the molecular basis for this poor sensitivity, studies have identified that the rs16969968 risk allele decreases receptor function of alpha5-containing nAChRs (Bierut *et al.* 2008; George *et al.* 2012). In the particular case of (alpha4beta2)2-alpha5 nAChRs, which are also expressed in the VTA and are deemed central for nicotine reinforcement, the rs16969968 allele promotes loss of permeability for Ca²⁺ ions and a faster desensitization of this receptor (Kuryatov, Berrettini, & Lindstrom 2011; Sciacaluga *et al.* 2015). Since nAChR desensitization can also contribute to modulating nicotine reward (Rice & Cragg 2004; Zhang & Sulzer 2004), carriers of the rs16969968 risk allele may increase nicotine intake to titrate for the desired activation of the mesolimbic dopaminergic pathway.

In terms of reducing the aversive effects of nicotine, the alpha5-containing nAChRs in the medial habenula (mHb) appear to play a pivotal role (Fowler *et al.* 2011; Tuesta, Fowler, & Kenny 2011; Fowler & Kenny 2012; Fowler, Tuesta, & Kenny 2013; Tuesta *et al.* 2017). By reducing the function of alpha5-containing nAChRs in the mHb, the rs16969968 risk allele decreases sensitivity to nicotine aversion. Confirming this, and in a manner similar to the behavior observed in mice lacking the alpha5 subunit (Fowler *et al.* 2011), mice with forced expression of the rs16969968 risk allele in the mHb self-administer nicotine at quantities that are aversive to wildtype mice (Morel *et al.* 2014). This body of evidence suggest that this risk allele prevents the habenular nAChRs from inducing the inhibitory signal intended to limit intake of

nicotine (Bierut *et al.* 2008), thus promoting the intake of high nicotine doses. Supporting this notion, at least one clinical study with rs16969968 risk allele human carriers has reported lower aversive effects of intravenous nicotine following an overnight abstinence, compared to non-carriers (Jensen *et al.* 2015).

As regards the effect on nicotine-related cues, the rs16969968 allele has been associated with circuit deficiencies that could impair the formation of proper nicotine-cue associations that drive cue-induced nicotine craving. Firstly, the rs16969968 allele has been associated with lower resting state functional connectivity between the dorsal anterior cingulate cortex (dACC) and the ventral striatum (Hong *et al.* 2010), regions whose coupling has been recently linked to tobacco craving (Janes *et al.* 2014). Secondly, this risk allele has been associated to impairments in the n-back test, which assesses working memory (Winterer *et al.* 2010). Finally, and most importantly, carriers of this allele have reduced cue-responsiveness to smoking cues in the posterior cingulate cortex (PCC), caudate nucleus and hippocampus (Janes *et al.* 2012), brain regions implicated in learning, conditioning and habit formation (Heimer 2003). This evidence suggests that these individuals, despite their heavy smoking, may be less sensitive to smoking-associated cues.

Taken together, this evidence suggests that the heavy, daily smoking of the rs16969968 phenotype is surprisingly due to a lower sensitivity to the pharmacological effects of nicotine, whether reinforcing or aversive, as well as low sensitivity to environmental cues signaling smoking opportunities (Johnson *et al.* 2010). It seems that this risk allele may strengthen nicotine seeking as a means to alleviate or avoid nicotine withdrawal after heavy use (Gabrielsen *et al.* 2013), but other risk or protective factors may modulate the extent at which this phenotype is ultimately expressed. In fact, not all carriers of the rs16969968 allele respond to NRT (Leung *et al.* 2015; Tyndale *et al.* 2015), but some do in specific environmental situations (Chen & Bierut 2013; Chen *et al.* 2014), possibly due to the interplay between different genetic variants influencing nicotine seeking (Barrie *et al.* 2017).

Alpha4-beta2 containing nAChRs

Variations of Alpha4-beta2 in the rewarding properties of nicotine. The alpha4-beta2-containing nAChRs constitute the most abundant nAChR type in the brain (Whiting & Lindstrom 1986) and play a central role in the rewarding actions of nicotine (Tapper *et al.* 2004). It follows that genetic variability in the CHRNA4 gene, encoding for the alpha4 subunit, can modulate the subjective rewarding actions of nicotine, impacting the

motivations to smoke and the potential therapeutic options for these individuals (Hutchison *et al.* 2007). Preclinical studies have shown that hypersensitive alpha4-containing nAChRs enhance the rewarding properties of acute nicotine administration (Tapper *et al.* 2004). Conversely, deletion of the alpha4 subunit in the ventral midbrain decreases nicotine self-administration (Pons *et al.* 2008) and nicotine place preference (Peng *et al.* 2017). In humans, the CHRNA4 risk allele rs2236196 has been associated with a higher probability for being a smoker (Li *et al.* 2005; Hutchison *et al.* 2007; Breitling *et al.* 2009; Esterlis *et al.* 2016), with greater self-reported euphoria after nicotine consumption and a better response to rapid release NRTs compared to non-carriers (Hutchison *et al.* 2007). New evidence suggests that the rs2236196 risk allele increases the relative upregulation of cerebellar and cortical beta2-containing nAChRs after nicotine exposure, compared to non-carriers (Esterlis *et al.* 2016). Thus, carriers of this risk allele appear more sensitive to the rewarding aspects of nicotine, and this could be due to a higher upregulation of beta2-containing nAChRs in key brain areas.

Variations of Alpha4-beta2 in the effects of nicotine on selective attention. The alpha4beta2-containing nAChRs also play a central role in cognition and in selective attention to environmental stimuli (Witte, Davidson, & Marrocco 1997; Phillips *et al.* 2000; Wallace & Bertrand 2013), thus providing a potential source of individual differences in the modulatory effects of nicotine on sensory cues. The high incidence of tobacco smoking among populations with trait attention deficits has led to the hypothesis that some individuals may smoke to counteract for these deficits (Poirier *et al.* 2002; Gardner, Dishion, & Posner 2006). Indeed, recent evidence suggests that individuals with particular SNPs may benefit more of the effect of nicotine on attentional performance. In fact, the rs1044396 SNP of the CHRNA4 gene, involved in nicotine dependence (Feng *et al.* 2004; Kamens *et al.* 2013), is also involved in visuospatial and auditory attention deficits (Parasuraman *et al.* 2005; Greenwood *et al.* 2009). In particular, individuals that are homozygotes for the rs1044396 SNP C risk allele have an attention deficit to surrounding cues, but which may be alleviated upon nicotine administration. When asked to fix their attention on a particular visual cue, these individuals are more likely to disengage their attentional focus to the targeted cue, and respond quicker to interfering cues appearing outside the target zone (Espeseth *et al.* 2010). Notably, nicotine enhances the selective attention to cues and improve distractor suppression (Thiel, Zilles, & Fink 2005; Hahn *et al.* 2009; Ahrens

et al. 2015; Behler, Breckel, & Thiel 2015), but only when the baseline performance is low, as it is the case with these individuals (Ahrens *et al.* 2015; Behler *et al.* 2015; Hammersley *et al.* 2016). Thus, it could be argued that in the rs1044396 SNP C/C homozygotes, the primary reinforcing properties of nicotine come from its ability to compensate for their cue attention deficits, compared to non-carriers (Espeseth *et al.* 2010).

The precise mechanisms behind the effects of the rs1044396 SNP on both attentional performance and vulnerability to nicotine dependence are poorly understood, but newer evidence shows that gene-gene interactions may solve part of the puzzle. On one side, the effect of the rs1044396 SNP C/C homozygote on the scaling of attentional focus manifests itself only if the individuals are also T/T homozygote for the CHRM2 rs8191992 allele, an SNP of the muscarinic M2 receptor (Greenwood *et al.* 2009). Furthermore, nicotine-enhancing effects on attentional performance were strong in the rs1044396 SNP C carriers, but only if they were also carriers of the dopaminergic DRD2 T allele, an SNP for the dopaminergic D2 receptor (Ahrens *et al.* 2015; Breckel *et al.* 2015). This new evidence highlights the complex interplay between genetic factors in brain networks, which could shape individual differences in the psychopharmacology of nicotine (Ahrens *et al.* 2015) and by extension, in the vulnerability for nicotine dependence.

Variations in nAChR subunits can protect against nicotine dependence

Genetic variability in nAChR subunits can also protect individuals against nicotine dependence. Genome-wide associated studies have identified rare missense SNPs in the alpha4 (Xie *et al.* 2011), beta2 (Hoft *et al.* 2011; Svyryd *et al.* 2016) and beta4 (Haller *et al.* 2012) subunits, which appear to decrease risk of nicotine dependence in humans, and are thus underrepresented in smoker populations (McClure-Begley *et al.* 2014). Protection against nicotine dependence through these rare SNPs in the CHRB2 gene appears mediated by increased sensitivity to the subjective aversive effects of nicotine, including increased nausea, heart palpitations and sweating compared to non-carriers (Hoft *et al.* 2011; Svyryd *et al.* 2016), which can deter further experimentation with nicotine. Since alpha5beta4-containing nAChRs are central in nicotine aversion, the protection given by beta4 variants may also be due to increased sensitivity to the aversive effects of nicotine (Haller *et al.* 2014; McClure-Begley *et al.* 2014). Supporting this view, there is evidence that forced overexpression of the beta4 subunit in the mHb limits nicotine self-administration in rodents (Frahm *et al.* 2011). Finally, genetic variability at the level of the CHRNA4 gene can also protect against

nicotine dependence through decreased sensitivity to nicotine reward (Butt *et al.* 2005; Wilking *et al.* 2010) or to decreased benefit of the attention-enhancing effects of nicotine (Feng *et al.* 2004; Greenwood, Parasuraman, & Espeseth 2012).

Individual differences in CYP2A6 function can modulate the mechanisms of nicotine seeking

Genetic variability in the systemic clearance of plasma nicotine levels, determined by the metabolic activity of the hepatic enzyme CYP2A6, has been linked to differences in nicotine seeking, vulnerability to nicotine dependence and cessation outcome (Lerman *et al.* 2015; Mamoun *et al.* 2015). Slow CYP2A6 metabolizers have approximately 50 percent reduction in nicotine metabolism compared to fast metabolizers (Benowitz *et al.* 2006). Even though adolescent slow nicotine metabolizers progress faster into habitual cigarette consumption (Audrain-McGovern *et al.* 2007; Olsson *et al.* 2016, O'Loughlin *et al.* 2004), once they reach adulthood they have reduced risk for nicotine dependence (Rubinstein *et al.* 2008; Wassenaar *et al.* 2011; Sofuoglu *et al.* 2012; Olsson *et al.* 2016), are more likely to smoke less cigarettes per day than fast metabolizers (Wassenaar *et al.* 2011; Styn *et al.* 2013), but also experience less withdrawal symptoms (Mamoun *et al.* 2015), and report higher cessation success with NRTs (Kaufmann *et al.* 2015; Mamoun *et al.* 2015).

The association between decreased nicotine dependence risk and low smoking profile among low metabolizers can be explained by the peculiar nicotine pharmacokinetics and the effects of prolonged nicotine exposure on neural activity in these individuals: despite the sustained presence of nicotine in their system, the neurobiological changes produced by nicotine over time can be easily overcome by NRTs, thus easing their cessation success dramatically. Recent evidence suggest that slow and fast metabolizers do not differ in their risk for smoking initiation (Olsson *et al.* 2016), and, among non-smokers, individuals with slow and fast CYP2A6 metabolism did not differ in the activity of striatal-cingulate neural circuits computing for reward and impulsivity (Li *et al.* 2017). However, once they become smokers, slow metabolizers experience less functional connectivity between the VS and the dACC, which is translated into deficits in reward processing and inhibitory control during abstinence, but which are alleviated after exposure to nicotine through a patch (Li *et al.* 2017). Remarkably, for smokers who are fast metabolizers, absence or presence of nicotine did not alter their neural response in VS and dACC in the same experimental conditions. These data suggest that the prolonged exposure to nicotine in slow metabolizers changes the neuroplasticity of these

neural networks, over time reducing their sensitivity to reward processing and response inhibition in the absence of nicotine. However, their slow metabolism of nicotine ensures that only a small number of cigarettes are needed to achieve this nicotine-induced alleviation of these changes in network connectivity. In fact, there is evidence that slow metabolizers have reduced thalamic nAChRs during early abstinence (Dubroff *et al.* 2015), which are normally upregulated during chronic nicotine exposure in normal and fast metabolizers. Activation of low nAChR densities in the thalamus following slow transdermal delivery of nicotine may reduce background craving associated with abstinence (Allenby *et al.* 2016). Since NRTs appear to mimic the same pharmacokinetic profile of their smoking patterns, slow metabolizers are thus more likely to benefit from this cessation strategy than fast metabolizers.

The increased heaviness in the smoking behavior of fast nicotine metabolizers can be explained by an attempt to titrate nicotine intake given its rapid clearance (Olfson *et al.* 2016). Furthermore, increased sensitivity to both the rewarding and withdrawal-alleviating actions of nicotine appears to play a role in the nicotine seeking in these individuals (Benowitz, Hukkanen, & Jacob 2009; Sofuoglu *et al.* 2012). Fast metabolizers report higher subjective craving scores (Patterson *et al.* 2008), dose-dependent effect of nicotine in subjective withdrawal alleviation (Faulkner *et al.* 2017) and greater rewarding effects of intravenous nicotine following an overnight abstinence (Sofuoglu *et al.* 2012). Although preclinical evidence for these observations is still scarce, a study from Grebenstein *et al.* (2015) showed in rats that nicotine clearance predicts nicotine reinforcement threshold and degree of compensation when decreasing nicotine dose, i.e. the fastest the clearance, the lowest the reinforcement threshold and the highest the degree of compensation. This raises important implications for cessation strategies in fast metabolizers, as switching to low dose nicotine cigarettes in an attempt to curtail nicotine dependence could lead to the opposite: an increase desire to titrate nicotine dose, and thus, increase smoking heaviness (Grebenstein *et al.* 2015).

Furthermore, reaching faster for the next cigarette also increases the opportunity for stronger temporal relationship between nicotine intake and its effects on surrounding stimuli, including both Pavlovian conditioning and the reinforcer-enhancing effect of nicotine (Donny *et al.* 2008; Caggiula *et al.* 2009). This could explain the reported higher smoking cue-reactivity in several brain areas of fast metabolizers, including amygdala, hippocampus, caudate and cingulate cortex (Tang *et al.* 2012; Falcone *et al.* 2016) in comparison with slow metabolizers. Interestingly, a recent study done by Faulkner *et al.* (2017) suggests that nicotine dose, more than the ritualistic and sensory

cues accompanying smoking, alleviates craving and withdrawal better in fast metabolizers, compared to slow metabolizers. However, this study was only conducted in young smokers; thus, it is possible that a more prolonged history of tobacco smoking is needed to observe higher cue reactivity among fast metabolizers. Altogether, this evidence suggests that individuals with fast nicotine metabolism relapse more frequently, probably due to a combination of increased sensitivity to nicotine, nicotine withdrawal and higher reactivity to environmental cues signaling nicotine availability.

Individual differences in psychological traits can modulate the mechanisms of nicotine seeking

Three main personality traits, anxiety, novelty/sensation-seeking and poor decision making/impulsivity have been associated with increased risk or severity of nicotine dependence (Falco & Bevins 2015). We take here the example of impulsive choice, because data are controversial, and controversy could result from individual variations. Higher delay discounting scores, a measure of impulsive choice, have been observed among highly dependent daily smokers compared to daily smokers with lower Fagerström Test for Nicotine Dependence scores (Sweitzer *et al.* 2008) or to non-daily smokers (Heyman & Gibb 2006). However, other studies have failed to see this differential relationship between delay discounting and smoking behavior (Johnson, Bickel, & Baker 2007; Carim-Todd, Mitchell, & Oken 2016; Rass, Ahn, & O'Donnell 2016).

Rather than a risk for dependence, evidence suggests that impulsive choice could be a risk factor for increased difficulty to control nicotine seeking, once craving is triggered. Supporting this, not all high impulsive smokers relapse more than low impulsive smokers, but only those with high cue-induced craving (Bourque *et al.* 2013; Erbllich & Michalowski 2015). This impulsivity-driven enhancement of smoking relapse seems also to involve increasing cue-induced neural reactivity, suggesting that impulsive individuals react more to nicotine-associated cues and are less likely to resist the accompanying urges. Studies show that the PCC, involved in trait impulsivity and control of craving to psychoactive substances (Brody *et al.* 2007; Potvin *et al.* 2015), is negatively coupled with the insula, dACC and dorsolateral prefrontal cortex, involved in nicotine cue reactivity (Bourque *et al.* 2013). In fact, hypofunction of the PCC is correlated with higher impulsivity, and at the same time with increased function of the insula, dACC and dorsolateral prefrontal cortex after smoking cue presentation (Bourque *et al.* 2013). Supporting a decreased inhibitory control by impulsivity, rats that score higher in impulsive choice, measured via delay discounting, not only show increased cue-induced relapse, but also fail to inhibit nicotine seeking during

abstinence (Diergaarde *et al.* 2008) and show a less elastic demand for nicotine (Diergaarde *et al.* 2012), i.e. they maintain nicotine intake while price (workload) increases. Highly impulsive individuals are thus more likely to react to cues signaling nicotine availability, and are less likely to control their urges, resulting in higher relapse rates.

Impulsivity might be pre-existing, but data suggest that it could also be induced or amplified by nicotine itself. Individuals carrying a the risk allele of the alpha3 nAChR subunit, rs578776, have higher resting functional coupling between the dACC-thalamus (Hong *et al.* 2010), a coupling recently implicated with risky decision making (Wei *et al.* 2016). Interestingly, the observed increased dACC-thalamus resting state functional connectivity was correlated to the amount of cigarettes smoked before the test session (Hong *et al.* 2010), suggesting that in these individuals, nicotine may act as positive feedback for riskier behavior, predisposing them for more deleterious consequences of craving episodes.

CONCLUSIONS

In this review, we have sought to provide converging epidemiological, clinical and preclinical evidence that individuals may differ in the neurobiological mechanisms behind nicotine seeking, and this may explain the variety of smoking behaviors, and the individual profiles for craving, withdrawal, relapse timing, responsiveness to therapy and cessation success. Combinations of risk and protective factors do not necessarily have the same impact in the different mechanisms of nicotine seeking, but they shape the overall vulnerability for nicotine seeking, and eventually, transitioning into nicotine dependence. To complicate the picture, it is interesting to note that expression (hence contribution to tobacco seeking) of individual differences, notably individual differences in reward-enhancing effects of nicotine, might be influenced by socioeconomic or health conditions associated with abundant or poor opportunities for reward (Perkins 2009; Leventhal 2016). However, most studies in tobacco addiction have neglected individual variations in the mechanisms of nicotine seeking, obscuring the existence of subpopulations with specific characteristics that could better define their treatment.

Preclinical animal models of nicotine seeking have been proved useful, as they have helped in understanding the mechanisms underlying nicotine seeking. These models, in line with clinical studies, have consistently demonstrated that pharmacological and non-pharmacological factors interact in a complex manner to exert control on nicotine seeking. They have poorly considered, however, that these interactions may vary among nicotine users, and thus individual differences in nicotine-seeking and nicotine-taking in these preclinical

models are scarcely described in the literature. Over the last 15 years, tremendous progress has been made in modeling nicotine seeking in rodents using intravenous self-administration (Cohen & George 2013). Procedures are available (Palmatier *et al.* 2013; Robinson *et al.* 2014) that appear useful for exploring individual differences in the mechanisms by which nicotine attributes incentive salience to rewards, reward-related cues and contexts (Yager & Robinson 2015).

In a recent review on the individual differences in the behavioral effects of nicotine, Falco and Bevins (Falco & Bevins 2015) refer only to four self-administration studies, which investigated the relationships between nicotine self-administration and psychobehavioral traits associated with tobacco dependence (impulsivity, sensation-seeking and anxiety). Nevertheless, these studies (Suto, Austin, & Vezina 2001; Guillem *et al.* 2005; Diergaarde *et al.* 2008, 2012) do not explore whether differences in nicotine self-administration between high and low scoring animals involve differential control of nicotine seeking by nicotine and/or nicotine-associated cues, for example, thus highlighting the need to further explore these questions in available animal models.

Advances in rodent models of genetic risk markers for nicotine dependence (Morel *et al.* 2014), as well as in *in vivo* tracing and manipulation of neuronal circuit activity (Cruz *et al.* 2013; Jennings & Stuber 2014) have been made recently. They could be incorporated to study the neurobiological mechanisms underlying individual differences in the mechanisms driving nicotine seeking. It is becoming evident that an individual-based strategy of the neurobiology of nicotine self-administration could increase the predictive validity of preclinical models of nicotine dependence and help develop individual-based therapeutic strategies for tobacco dependence, as we have recently suggested (Garcia-Rivas *et al.* 2016).

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Perspective

Individual Variations in the Mechanisms of Nicotine Seeking: A Key for Research on Nicotine Dependence

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Tobacco users are estimated to be at least 1.1 billion worldwide. Although tobacco dependence is not associated with obvious behavioral disruptions, alleviating it is a major public health concern and a main societal challenge, as it produces severe long-lasting health-related problems, leading to 6 million deaths every year. Available therapies for smoking cessation have limited efficacy, warranting the need for developing better therapeutic strategies, which depend on understanding the mechanisms that underlie tobacco dependence, in which nicotine is considered to have a central role.

The relevance of preclinical animal models to nicotine dependence is questioned because their predictive validity is considered poor (Hong *et al*, 2010). In spite of this, preclinical animal models of nicotine seeking have been proved useful, as they have helped in understanding the mechanisms underlying nicotine seeking. These models, in line with clinical studies, have consistently demonstrated that both pharmacological factors (PF) and non-pharmacological factors (NPF) promote nicotine seeking and have helped identify underlying neurobiological mechanisms (Stoker and Markou, 2015).

Increasing psychological, genetic, and neurobiological data support the notion that the contribution of PF and NPF to nicotine seeking differ from smoker to smoker, thus possibly contributing to the observed heterogeneity in the population of smokers (Hiroi and Scott, 2009). It could also contribute to the incongruence between the multiple diagnosis tools, the inconsistent relationship between craving and smoking cessation outcome, and the limited predictive validity of animal models. This might also eventually reduce our ability to identify relevant therapeutic targets for smoking cessation (Potvin *et al*, 2015).

For instance, a single-nucleotide polymorphism of the *CHRNA5* gene has been associated with heavy smoking, which appears to be caused by a lowered sensitivity to the aversive effects of nicotine (Fowler and Kenny, 2014). In

smokers who carry this *CHRNA5* risk allele, compared with noncarriers, nicotine seeking through PF is increased, namely through withdrawal avoidance, whereas susceptibility to NPF, in particular cue-induced craving (Janes *et al*, 2012), is decreased. This supports the notion that the two types of factors (PF/NPF) can vary independently. Fast nicotine metabolism is another risk factor for heavy smoking and low cessation rates (Mamoun *et al*, 2015). Fast nicotine metabolizers are more sensitive to withdrawal than their slow metabolizer counterparts, but differently from the *CHRNA5* case, they are also more responsive to drug cues (Falcone *et al*, 2016); a difference possibly due to their specific nicotine pharmacodynamics that might favor cue conditioning. Altogether, specific combinations of risk and protective factors, like these and many others, will determine the individual mechanisms underlying nicotine seeking.

We propose that the predictive validity of animal models might increase by taking into account these individual variations. This implies determining, within the same individuals, the influence of both PF and NPF in the control of nicotine seeking, and evaluating how individual psychobiological factors and length of nicotine exposure (early vs late nicotine use) affect this balance (Figure 1).

The exploration of individual differences in nicotine self-administration is at a very early stage. A recent exhaustive review by Falco and Bevins (2015) on individual differences in the behavioral effects of nicotine refers to only four self-administration studies that investigated the relationships between impulsivity-related psychobehavioral traits and nicotine self-administration. However, even this very limited body of research has produced inconsistent results and has yet to identify underlying mechanisms of nicotine seeking.

Now important conditions are met for investigating individual variations in nicotine seeking. Over the last 15 years, tremendous progress has been made in modeling nicotine taking in rodents using intravenous self-administration. Newer developments include the exploration of alternative routes of administration (Cohen and George, 2013) or even self-administration (www.simply-lab.com) (ie, vapor inhalation), as well as the influence of social factors on nicotine taking (Wang *et al*, 2014). Thanks to these models,

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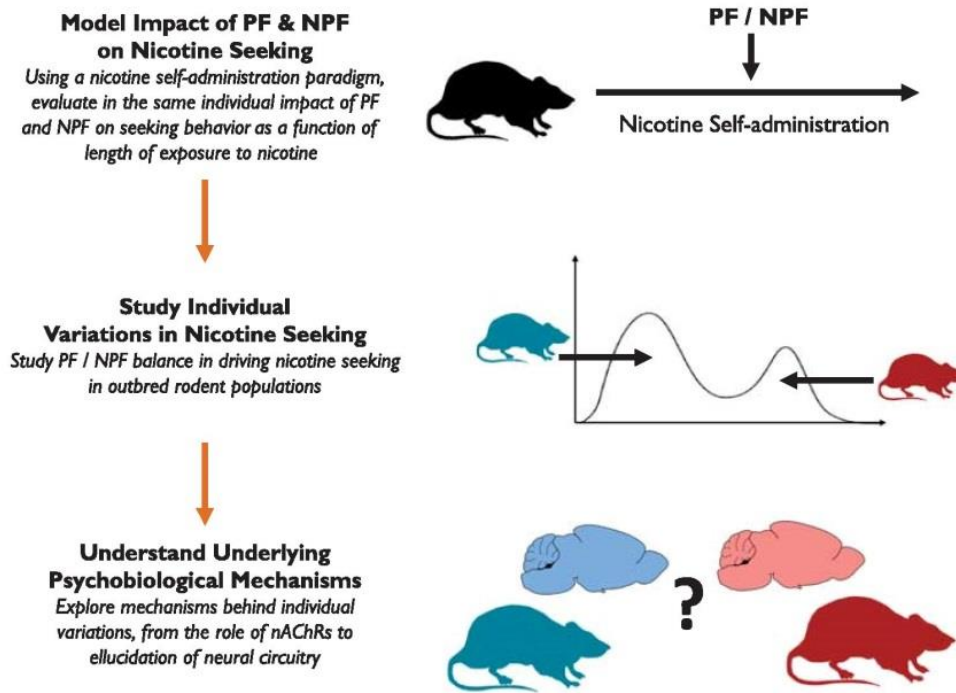


Figure 1 Individual-based approach for the mechanisms of nicotine seeking. We propose a three-level strategy for an individual-based animal model of nicotine seeking: (1) modeling, in the same individuals, and as a function of duration of nicotine self-administration, the impact of pharmacological factors (PF) and non-pharmacological factors (NPF) on nicotine seeking; (2) exploring individual variations in the balance between PF and NPF in the control of nicotine seeking, based on such a model, and (3) exploring the underlying psychobiological mechanisms of these individual differences, from a circuitry down to a molecular level, using complementary tools and approaches, such as outbred rodents characterized for personality traits associated with risk for nicotine dependence (impulsivity, novelty seeking and so on), genetically-manipulated rodents with altered expression of specific nAChR subtypes, or reproducing human allelic variations in nAChR subtypes associated with increased risk for nicotine dependence, and tools such as opto- and chemogenetics, viral tracing techniques, and brain calcium imaging in freely behaving rodents that allow for precise investigations of neural circuit function, including those relevant to nicotine seeking.

key information has been collected on the control of nicotine seeking by PF and notably NPF, thanks to the seminal work of Anthony Caggiula and Eric Donny (Pittsburgh University, USA) on the complex control of nicotine seeking by conditioned factors. Also significant, investigating the mechanisms by which nicotine alters contextual learning, Thomas J Gould and colleagues (Temple University, Philadelphia, USA) have contributed to the current view on how nicotine withdrawal might promote seeking through cognitive impairments and negative affective state.

Over the same time period, new mouse genetic models have been developed to reproduce human allelic variations in nAChR subtypes associated with increased risk for nicotine dependence (Morel *et al*, 2014). Furthermore, newer experimental tools, such as opto- and chemogenetics, viral tracing techniques, and brain calcium imaging in freely behaving rodents allow for precise investigations of neural circuit function, including those relevant to nicotine seeking. Personality traits, like reactivity to novelty, anxiety, and impulsivity, associated with increased risk for nicotine dependence in humans, are now also well characterized in rodents, providing the opportunity to study their role in nicotine seeking.

More recently, two promising studies support the notion that consistent individual differences in the reinforcing and incentive effects of nicotine can be observed. In an outbred strain of rats, Grebenstein *et al* (2015) reported individual

variations in nicotine self-administration. Superficially, the nicotine reinforcement threshold and degree of compensation when decreasing nicotine dose were predicted by nicotine clearance, suggesting that nicotine seeking might be controlled by PF in some individuals, and less so in others, through biochemical differences affecting pharmacokinetics. Echoing the fast/slow metabolizer phenotypes in humans, the observations of Grebenstein *et al* (2015) offer interesting perspectives for studying the PF vs NPF balance in controlling nicotine seeking by identifying individual differences in nicotine metabolism.

In search for the mechanisms of individual vulnerability to cocaine or opioid addiction, sophisticated procedures have been developed to investigate psychobehavioral factors such as attribution of incentive salience to drug cues that might influence drug seeking. In the so-called sign trackers rats (STs), food- or drug- (cocaine, opioid) associated discrete cues are both more attractive (elicit approach) and more wanted (are conditioned reinforcers) than in goal trackers rats (GTs), in which presentation of reward-associated cues elicits approach to the location of reward delivery. For nicotine, Yager and Robinson (2015) showed that if STs rats want more a nicotine-associated cue, they approach a nicotine cue similarly to GTs, demonstrating nicotine-specific mechanisms of salience attribution. This model not only opens perspectives for the study of the mechanisms of salience attribution in general, but offers the opportunity to

study the control of nicotine seeking by NPF and PF as a function of individual differences in salience attribution to nicotine cues.

CONCLUSION

Tobacco use is the net balance between factors promoting smoking maintenance ('seeking mechanisms') and factors promoting smoking cessation ('motivation to stop'). Even if capturing motivation to stop is a challenge in animal models, they allow exploring mechanisms promoting nicotine seeking and taking.

Data support that individual variations in the mechanisms of nicotine seeking contribute to the heterogeneity of the population of tobacco smokers and may explain in part the limited efficacy of current therapeutic strategies for smoking cessation. Conditions are met to study these individual variations and identify their underlying neurobiological mechanisms using preclinical models in rodents. This strategy could increase our ability to develop individual-based therapeutic strategies for tobacco dependence.

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The authors declare no conflict of interest.

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Chapter 2

EXPERIMENTAL OBJECTIVES

Experimental Objectives

Given this general context, and in particular the preclinical experimental approach proposed in our perspective review (Garcia-Rivas et al., 2017), the objectives of this doctoral study were to:

1. **Evidence individual differences in the contributions of nicotine, and its associated cue, in nicotine seeking**, through experimental strategies in a well-established model for nicotine self-administration in rats,
2. **Characterize and validate these observed individual differences** through external behavioral, metabolic, and neurobiological correlates, that could also shed light on the biological mechanisms through which these different behaviors occur,
3. **Determine whether varenicline**, the most efficacious pharmacotherapy against tobacco dependence, **targets the contributions of nicotine, its associated cue, or a combination of both, in nicotine seeking**, that could explain its limited efficacy in smokers attempting to quit, and that could evidence the need for better tailored therapies based on the individual mechanisms of nicotine seeking.

Objectives 1 and 2 will be covered in Chapter 3, while Objective 3 will be covered in Chapter 4.

Chapter 3

EVIDENCE AND CHARACTERIZATION OF INDIVIDUAL DIFFERENCES IN THE MECHANISMS OF NICOTINE SEEKING

Introduction

We have recently proposed that the predictive validity of animal models may increase if individual variations are taken into account (Garcia-Rivas et al., 2017). This implies determining, within the same individuals, how nicotine seeking is controlled by (1) the pharmacology of nicotine in and of itself, and (2) the pharmacological effects of nicotine on surrounding environmental stimuli (Garcia-Rivas et al., 2017). While preclinical studies that have sought to understand the mechanisms of nicotine seeking, they have done it through what is inferred from the mean observations in their study populations. Since the classical, and most widely used, preclinical model of nicotine intravenous self-administration involves the contingent delivery of nicotine with a visual stimulus ('cue') (Rose and Corrigall, 1997), we sought to capture and bring into evidence such individual differences using this classical approach, by identifying subgroups of rats that differed in the particular contributions of nicotine and its associated cue in their habitual nicotine self-administration.

A key advantage of our individual-based approach of nicotine is that it is suitable for exploration of the neurobiology of individual differences. It is widely known that nicotine reinforcement involves the activation of $\alpha 4\beta 2$ -nAChRs in the ventral tegmental area (VTA), where they determine dopaminergic input into the nucleus accumbens (NAcc) and the prefrontal cortex (PFC) (Picciotto et al., 1998; Klink et al., 2001; Pidoplichko et al., 2004). However, other structures, including the basolateral amygdala (BLA), also express nAChRs, and can powerfully modulate dopamine transmission at the NAcc (Everitt et al., 1999; Chiamulera, 2005; Everitt and Robbins, 2005). The BLA, in addition, has been linked to conditioned reinforcement of drug-paired cues (Everitt and Robbins, 2005; Sharp, 2018). Since the balance between nicotine reinforcement and aversion plays a role in nicotine seeking, another structure of interest includes the medial habenula (mHb), which relays sensory information about nicotine aversive effects downstream towards the VTA (Antolin-Fontes et al., 2015).

In this chapter, we present in successive fashion, two experiments that aimed at characterizing these individual differences in nicotine-cue interactions during nicotine self-administration, using external behavioral, metabolic and neurobiological variables to validate such characterization.

Materials and Methods

SUBJECTS

Male Sprague–Dawley rats (n=124) weighing 280–300 g at the beginning of the experiments were single housed under a 12 h reverse dark/light cycle. In the animal facility, temperature ($22 \pm 1^\circ\text{C}$) and humidity ($60 \pm 5\%$) were controlled. Rats were habituated to environmental conditions and handled every day for 15 days before the start of experimental procedures. Standard chow food and water were provided ad libitum. All procedures involving animal experimentation and experimental protocols were approved by the Animal Care Committee of Bordeaux (CEEA50, N° 50120168-A) and were conducted in accordance with the guidelines of the European Union Directive 2010/63/EU regulating animal research.

SURGERY

A silastic catheter (internal diameter = 0.28 mm; external diameter = 0.61 mm; dead volume = 12 μl) was implanted in the right jugular vein under ketamine (80 mg/kg) / xylazine (16 mg/kg) anesthesia. The proximal end reached the right atrium through the right jugular vein, whereas the back-mount passed under the skin and protruded from the mid-scapular region. Immediately after surgery, rats received a single antibiotic injection (gentamicine 1 mg/kg i.p.). Rats were given a minimum of 5 days recovery before nicotine self-administration training began.

DRUGS

-(-)Nicotine-Hydrogen-Tartrate (Glentham, UK) was dissolved in sterile 0.9% physiological saline, for an initial training dose of 0.04 mg/kg free base. Nicotine solutions with concentrations different to the training dose (0.02mg/kg, 0.005mg/kg and 0.06mg/kg free base) were prepared afresh, and used instead of the training dose where indicated. All nicotine solutions were adjusted to pH =7.0. Nicotine, as well as sterile 0.9% physiological saline in control groups, was self-administered via intravenous (IV) route in a volume of 40 μl per self-infusion.

For the Pavlovian Conditioned Approach (PCA), sodium saccharin salt (Glentham Life Sciences, UK) was dissolved in drinking tap water, to a final concentration of 0.2% w/v free acid.

PAVLOVIAN CONDITIONED APPROACH (PCA)

Pavlovian Conditioning Apparatus

The setup used for Pavlovian Conditioned Approach consisted of 20 operant chambers made of plexiglas and metal (Imetronic, France). Each chamber (40 cm long x 30 cm wide x 36 cm high) was located in an opaque sound-attenuating cubicle equipped with an exhaust fan to assure air renewal and mask background noise. They were equipped with a retractable metal lever on the right side panel, 7 cm above the grid floor, and a drinking cup, located 6.5cm to the side of each retractable lever, on the same panel, and 8 cm above the grid floor (**Figure 1**). A lickometer circuit allowed for monitoring and recording of cup contacts. A pump attached to the cubicle delivered 0.12ml of 0.2% saccharin solution to the drinking cup via Silastic tubing (Dow Corning Corporation, MI, USA)

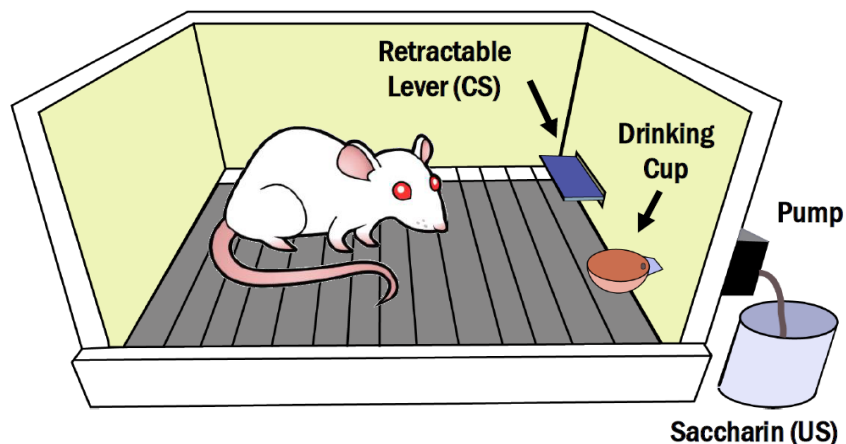


Figure 1 - Pavlovian Conditioning Apparatus – Operant chambers were equipped with a retractable lever and a drinking cup, placed on the same side of the operant chamber. An attached pump would deliver a small quantity of a solution containing saccharin to the drinking cup.

Pavlovian Conditioned Approach

Rats (n=59) were first habituated to drink the saccharin solution over three consecutive sessions. In these sessions, each rat was placed in one chamber, and once the session was initiated, 0.12ml of 0.2% saccharin solution was dispensed to the drinking cup, for a total of 20 presentations, at a fixed interval of 4 minutes and 30 seconds. Following habituation, rats were trained for Pavlovian conditioned

approach to the saccharin sweet reward. Every 4 minutes and 30 seconds, the retractable lever was deployed for a total of 10 seconds. Lever deployment made a brief and easily localizable noise. After 10 seconds, the lever was retrieved, coinciding with the activation of the pump, delivering 0.12ml of 0.2% saccharin to the adjacent drinking cup. The activation of the pump reinitiated the timer. Saccharin delivery was not dependent on responses at the lever or contacts with the cup, i.e. it remained non-contingent to the animal behavior. The retractable lever thus served as the conditioned stimulus (CS) to the delivery of the sweet reward, which acted as the unconditioned stimulus (US). A retractable lever was used as a CS, instead of a cue light, in order for it to be sufficiently different from the visual stimulus associated with nicotine delivery in the Self-Administration experiment described below. Every daily session consisted of 20 CS-US pairings, for a total of 11 sessions. Cup contacts (or “licks”) were measured for the 10 second period during lever deployment. Data was collected with a PC-Windows-compatible SK_AA software (Imetronic, France). The mean amount of licks during CS presentation, as well as the probability to lick and latency to first lick, were calculated, per rat, for sessions 10 and 11, and used as an individual variable for correlational studies with subsequent nicotine self-administration measures.

INTRAVENOUS SELF-ADMINISTRATION

Self-administration Apparatus

The self-administration setup consisted of 48 self-administration chambers made of Plexiglas and metal (Imetronic, France), and equipped with holes as operant manipulanda. These chambers were different, and located in a separate room, from those used in the PCA described above. Each chamber (40 cm long x 30 cm wide x 36 cm high) was located in an opaque sound-attenuating cubicle equipped with an exhaust fan to assure air renewal and mask background noise (**Figure 2**). For self-administration sessions, each rat was placed in one chamber, where its chronically implanted intracardiac catheter was connected to a pump-driven syringe (infusion speed: 20 μ l /sec) via Silastic tubing. Two holes, located at opposite sides of the chamber, at 5.5 cm from the grid floor, were used to record instrumental responding. One hole was associated with infusion delivery and designated as the

active hole, while the other was designated as the inactive hole and served as control. A white cue light (white LED, Seoul Semiconductor, South Korea), 1.8 cm in diameter, located 11.5 cm above the active hole, was used as nicotine (or saline) delivery-associated discrete visual cue, and is named thereafter ‘cue light’ or ‘cue’. It produced 5 Lux. Experimental contingencies were controlled and data was collected with a PC-Windows-compatible SK_AA software (Imetronic, France).

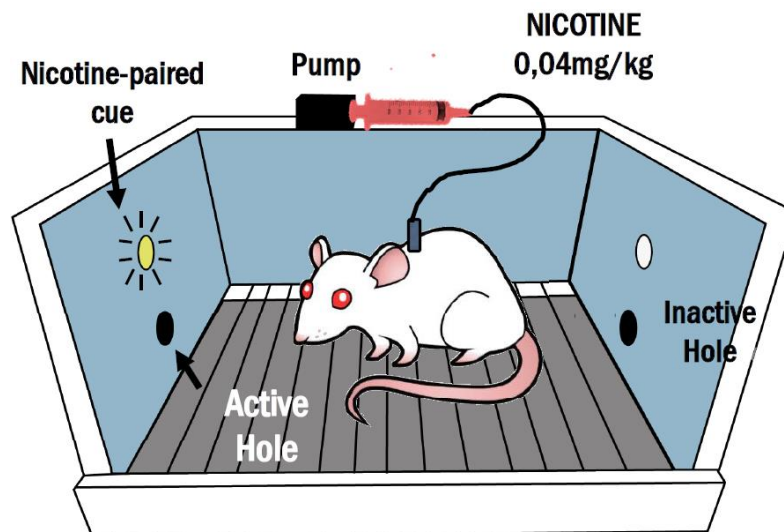


Figure 2 - Self-Administration Apparatus - Operant chambers were equipped with two holes at opposing sides of the chamber, with one cue light above the active hole. Contingent IV delivery of nicotine (or saline) was ensured by the activation of a pump loaded with a syringe loaded with the solution for IV delivery. The pump was connected to the rat via Silastic tubing, which would be attached to the back mount of the catheter on each rat.

Self-administration Procedures

Basal Training Protocol: Rats were separated into two groups: the “nicotine” group was trained to self-administer 0.04mg/kg infusions of nicotine, while the “saline” group was self-administering 0.9% physiological saline infusions. Group allocation was randomized to ensure homogenous groupings. For those rats that underwent prior characterization via PCA, it was ensured that both groups in mean did not differ significantly in their PCA variables, to minimize the potential of bias. Rats in both groups performed self-administration (SA) training 5 days a week, i.e. Monday to Friday. Sessions began two hours after the onset of the dark phase. Sessions lasted for three hours, during which, following nose-poking in the active hole under a fixed ratio 3 schedule of reinforcement, the white cue light was illuminated at the same time as the infusion pump was activated (40 μ l/infusion over 2s).

The white cue light remained on for 4 s in total; no further time out was applied. Nose-pokes at the inactive hole were recorded but had no scheduled consequences. To maintain catheter patency, catheters were flushed with ~10µl of heparinized saline (30IU/ml) after each self-administration session and before the self-administration sessions run on Monday. To define a significant self-administration behavior at the individual level, we used a discrimination index between active and inactive holes $[\text{active nose-pokes}/\text{total nose-pokes}] * 100$ strictly superior to 50% together with a minimal number of at least 6 self-infusions per session over the three consecutive sessions.

Response to Cue Omission Test: Nicotine and Saline rats performed a session similar to a standard SA session, except that infusions were not paired with the presentation of the contingent white cue-light for the whole duration of the session. Individual response to cue omission was expressed as percent change respect to baseline infusions.

Response to Nicotine Omission: Rats performed a session similar to a standard SA session, except that nicotine solutions were replaced with 0.9% physiological saline for the whole duration of the session. These infusions were still delivered with the contingent white cue-light. Saline rats kept self-administering saline during this test session. Individual response to cue omission was expressed as percent change respect to baseline infusions.

Response to Changes in SA Dose: Dose responsiveness was tested in all rats by changing the dose of nicotine to 0.02 mg/kg, 0.005mg/kg or 0.06mg/kg during the whole duration of the session. In sessions of Change in Dose (CD), new pump-driven syringes containing the new nicotine concentration were loaded before start of session. Infusions in all CD sessions were delivered with the contingent white cue-light, just as baseline protocol. Saline rats kept self-administering saline during these test sessions. Individual response to the first session after change in dose was expressed as percent change respect to baseline infusions. For comparison of individual responses to changes in different doses, a compensation index was calculated, according to the mathematical formula proposed by Harris et al., 2009: $1 - (\% \text{drop in nicotine intake followed by change in dose} / \% \text{drop in nicotine unit dose})$. A compensation index of 1.0 means that nicotine seeking behavior has adjusted to maintain the same

level of nicotine intake per session, i.e. intake has been completely compensated despite change in dose, while a compensation index close to zero means that rats had not attempted to change their responding from baseline parameters, despite the change in dose.

PLASMA COTININE QUANTIFICATION

The following day after completion of the self-administration schedule, rats were returned to their SA chambers, and placed for one last session, in which they non-contingently received five IV nicotine infusions, at the training dose of 0.04mg/kg. Saline rats received saline infusions instead. Upon starting the session, the pump was activated non-contingently, following the average spacing of the first 5 infusions during a habitual Monday session, in such a way that all nicotine rats received the same amount of nicotine at the same time. Rats remained undisturbed in the SA chamber for a total of 45 minutes after their last non-contingent infusion received, allowing enough time for nicotine to be partially metabolized to cotinine to detectable levels (Guillem et al., 2005). Rats were then anesthetized with 3% isoflurane and immediately euthanized by terminal decapitation. Trunk blood was collected in 9ml Vacuette® K2-EDTA tubes (Greiner Bio-One, France) and immediately placed in ice until centrifugation. To allow for plasma separation, samples were centrifuged at 3500 rpm for 10 minutes at 4°C, after which plasma was collected and stored at -80°C for later analysis. Cotinine was quantified in the plasma samples using a rat/mouse cotinine ELISA kit (Calbiotech, CA, USA), according to the manufacturer's protocol.

BRAIN MICRODISSECTION

Upon terminal decapitation, described above, brains were quickly isolated and snap-frozen by immersion in cold isopentane (-45°C), and stored at -80°C. Selected frozen brains were thawed to -20°C in a cryostat chamber (CM3050 S, Leica Microsystems, Wetzlar, Germany). Whole brain tissue was sectioned at 30 µm using a Leica cryostat and mounted in series with 8-10 sections per slide on polyethyl-ene-naphthalate membrane 1mm glass slides (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) that have been pretreated to inactivate RNases. Series were created from distinct coronal sections (bregma positions based on a reference brain atlas by Georges Paxinos and Charles Watson) and individual regions were matched across section and harvested by Laser Capture

Microdissection (LCM). The accumbens core (cNAcc) and shell (shNAcc) series were collected from bregma 3.00 mm to 0.48 mm, the basolateral amygdala (BLA) series were collected from bregma -1.60 mm to -3.00 mm and finally, the medial habenular nucleus area (MHb) series were collected from bregma -1.88 mm to -4.30 mm. Subsequently, the sections were immediately fixed for 30 seconds with 95% ethanol, followed by 75% ethanol for 30 seconds and by 50% ethanol for 30 seconds to remove the OCT. Sections were stained with 1% cresyl violet in 50% ethanol for 30 seconds and dehydrated in 50%, 75% and 95% ethanol for 30 seconds each, 2x in 100% ethanol for 30 seconds. Laser Pressure Catapulting microdissection (LPC) of samples was performed using a PALM MicroBeam microdissection system version 4.6 equipped with the P.A.L.M. RoboSoftware (P.A.L.M. Microlaser Technologies AG, Bernried, Germany). Laser power and duration were adjusted to optimize capture efficiency. Microdissection was performed at 5X magnification. The microdissected brain structures were collected in adhesive caps and re-suspended in 250µl guanidine isothiocyanate-containing buffer (BL buffer from ReliaPrep™ RNA Cell Miniprep System, Promega, WI, USA) with 10 µl 1-Thioglycerol, and stored at -80°C until extraction was done. Total RNA was extracted from microdissected tissues using the ReliaPrep™ RNA Cell Miniprep System (Promega, WI, USA) according to the manufacturer's protocol. The integrity of the RNA was checked by capillary electrophoresis using the RNA 6000 Pico Labchip kit and the Bioanalyser 2100 (Agilent Technologies, Massy, France), and quantity was estimated using a Nanodrop 1000 (Thermo Scientific, MA, USA). The RNA integrity number (RIN) was between 9.8 to 7.9.

QUANTIFICATION OF GENE EXPRESSION

Preparation of cDNA

The cDNA of each microdissected structure mentioned above were prepared from 105ng of total RNA, with the help of the qScript™ cDNA Super Mix kit (Quanta Biosciences, MA, USA). The reverse transcription reaction was done at 42°C for one hour, followed by an inactivation step at 85°C for 5 minutes. The corresponding cDNA solutions were stored at -80°C.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

For the qPCR, each well plate contained 3 μ l of primers (at a concentration of 2 μ M), 2 μ l of previously synthesized cDNA and 5 μ l of the 2X LightCycler [®] 480 SYBR Green I Master Mix (Roche, Basel, Switzerland), for a total reaction volume of 10 μ l. The first step of the qPCR consisted in activation of the DNA polymerase for 5 minutes, at 95 °C. After this, 45 cycles were performed, consisting in a denaturation step at 95 °C for 15 seconds, then a step at 61 °C for 30 seconds combining the hybridization of the primers and the elongation. The measurement of the fluorescence was carried out at the end of the elongation step in every cycle. An amplification curve obtained by plotting the fluorescence as a function of the number of cycles allowed for the quantification of the qPCR product.

Selection and validation of primers for qPCR

Genes of interest in the microdissected structures were of those encoding relevant nAChR subunits: α 2 (CHRNA2), α 4 (CHRNA4), α 5 (CHRNA5), α 7 (CHRNA7), β 2 (CHRNB2), β 3 (CHRNB3) and β 4 (CHRNB4), as well as those encoding for dopamine receptors: D1 (Drd1), D2 (Drd2) and D3 (Drd3).

The choice of primers was made by Primer Express 2.0 software (Thermo Fischer Scientifics, MA, USA). Primers were selected according to their specificity and efficacy. Specificity was verified by means of the melting curve representing the fluorescence emission as a function of temperature. This step was performed at the end of the qPCR by incrementing the temperature by 0.3 °C every 3 seconds between 65 °C and 95 °C, which dissociated the amplification product. Efficacy of the reaction was measured by performing a standard curve, plotting the number of cycles as a function of the decimal logarithm of the amount of cDNA on different concentrations (20, 10, 4, 0.8 and 0.16 ng per well). Only the pairs of primers with a single amplification product and efficiency close to 100% were retained. The sequences of the pairs of primers are reported in **Table 1**, below.

Selection of housekeeping genes

The expression of the 10 genes of interest, and 2 potential housekeeping genes, was analyzed first by qPCR on all individuals. The analysis of these results was carried out using the geNorm software

which classifies all the genes tested according to the different expressions between all the individuals. The gene with the lowest geNorm value was selected as housekeeping gene, suggesting that its expression is independent of the experimental conditions (Livak and Schmittgen, 2001; Bustin et al., 2009). This housekeeping gene, Eef1a1 (eukaryotic translation elongation factor 1 alpha 1), was common to the different structures studied.

Gene	GenBank ID	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
Eef1a1	NM_175838	ACCCTCCACTTGGTCGTTTTG	AGCTCCTGCAGCCTTCTTGTC
Drd1	NM_012546	CGCGTAGACTCTGAGATTCTGAATT	GAGTTAAGGAGCCACCACATCAGT
Drd2	NM_012547	GGGTGCTGGGACTGCTGATA	GAACCCTCCAAACTGCAGCTT
Drd3	NM_017140	CGTGGAAGGACTCGGAACTC	GTGGATAACCTGCCGTTGCT
Chrna2	NM_133420	CCCAGTGTACCTACTACTTTGTG	GTCATGGGATGATGAGGTTGAT
Chrna4	NM_024354	GGCAGTAGAAGGCGTCCAGTAC	CCTCCTTCACCGAGAAGTCAGT
Chrna5	NM_017078	TGTCTGGGCTAGCACAAAACC	ATACAGAACAGGGCAAAGTGGAG
Chrna7	NM_012832	CCTGGGCTCACAAGAATTCG	GACTGCTCTGCATTGGTTTCAG
Chrbn2	NM_019297	TCACACCTCCGTTACACATAGT	GGAGAAGGCTCGACCACAAG
Chrbn3	NM_133597	GCTCGGCAGATACGGTGCTA	CCCAGGCCAGTCTCTCTCTTC
Chrbn4	NM_052806	AAAGTGTCATCGAGGACTGGAAG	AAACACGAACACCCACAGGAA

Table 1 – Primers for Polymerase Chain Reaction (PCR). Listed are the forward and reverse nucleotide sequences used for the 10 genes of interest, and the housekeeping gene Eef1a1.

EXPERIMENTAL PROCEDURES

Experiment 1 – Exploration of Individual Differences in Nicotine-Cue Interactions

As a first step, we launched an exploratory experiment to assess whether individual differences in nicotine-cue interactions could be evidenced during a classical nicotine self-administration paradigm. For this experiment, 59 rats self-administered nicotine while 6 self-administered saline. After acquisition of self-administration, a first cue-omission test was done. Upon returning to baseline responding, the dose was then changed to 0,02mg/kg, followed by a second cue-omission test at this new dose. Sensitivity to nicotine was further assessed by testing responsiveness to 0,005mg/kg and 0.06mg/kg. To test whether nicotine metabolism could be related to nicotine-cue interactions, plasma was collected and cotinine quantified as described above. The precise timeline of this experiment is detailed in **Figure 3**.

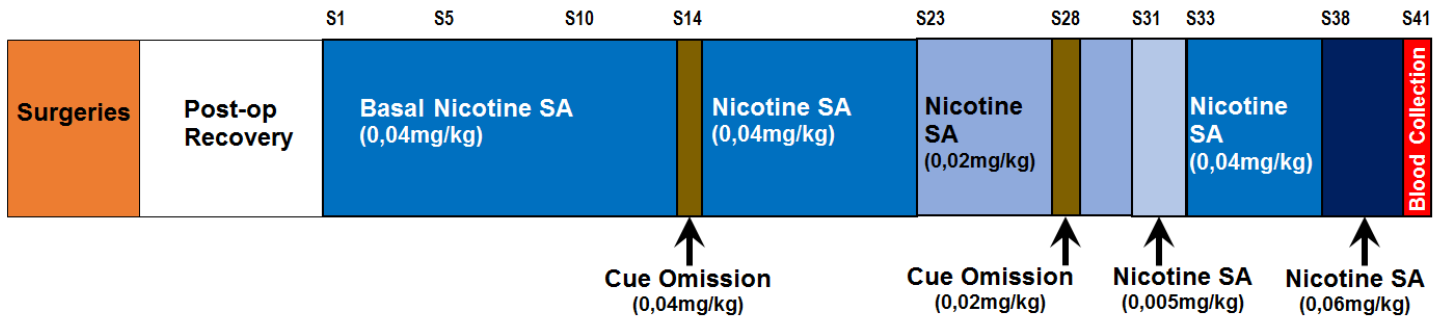


Figure 3 – Experiment1 – Timeline. After arrival and handling, rats were first catheterized and allowed for recovery before entering basal nicotine SA. A first cue omission test was done on session 14, before returning to basal conditions. On session 23, the training dose was changed to 0.02mg/kg, and a second cue omission test done on session 28. To further assess dose responsiveness, the dose was then lowered further to 0.005mg/kg, then returned to basal conditions, before a final dose change to 0.06 mg/kg. At the end of the experiments, blood was collected for quantification of plasma cotinine.

Experiment 2 – Refined Identification and Characterization of Individual Differences in Nicotine-Cue Interactions

Having evidenced individual differences in nicotine-cue interactions in the previous experiment, we launched a second experiment, to further refine the identification and characterization of these individual differences, as well as validating them through external variables. For this experiment, 59 rats were first characterized according to the Pavlovian conditioning to a saccharin-predicting cue, using the PCA approach. Rats were then put into self-administration, with 51 rats self-administering nicotine while 8 self-administered saline. The self-administration schedule was similar to Experiment 1, with three exceptions: (1) a nicotine-omission test was introduced after the first cue-omission test, (2) only one change in dose (0.02mg/kg) was tested and (3) gene expression in selected brain areas was quantified, as described above. The precise timeline of this experiment is detailed in **Figure 4**.

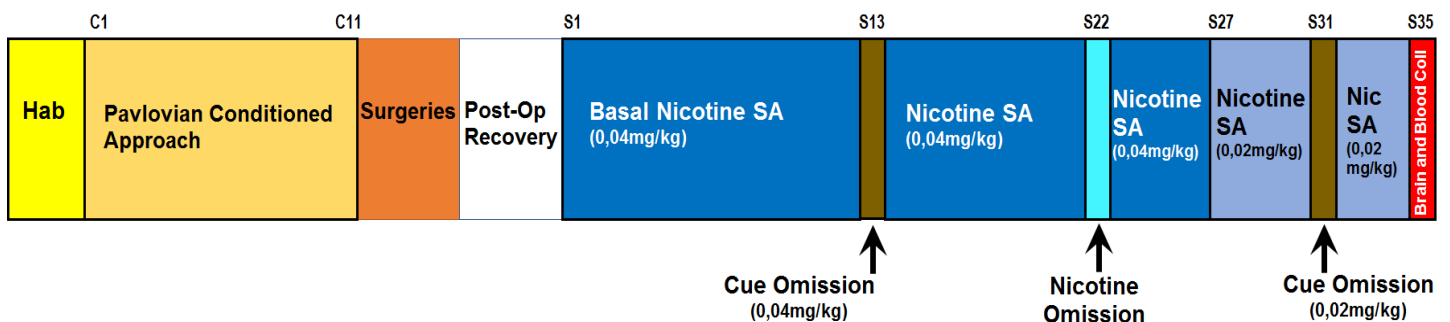


Figure 4 – Experiment2 – Timeline. After arrival and handling, rats were first habituated to saccharin drinking for three days prior to Pavlovian Conditioned Approach, which lasted for 11 sessions. Surgeries were done immediately after, and a post-op recovery period set in place before launching basal nicotine SA. The first cue omission test was done on session 13, before returning to basal conditions, after which a nicotine omission test was performed on session 22. On session 27, the dose was changed to 0,02 mg/kg. A last cue omission test was done on this dose on session 31. Blood and brains were collected at the end of self-administration schedule, as described above.

DATA ANALYSIS

General Self-Administration Behavior

Self-administration behavior was analyzed using repeated measures ANOVA with Time (number of sessions), Hole (active vs inactive) as within-subject factor, and experimental group (saline vs nicotine) or subgroups (subgroup1 vs subgroup 2 vs subgroup 3; cluster 1 vs cluster 2 vs cluster 3) as between-subject factor.

One sample t-tests were used to compare baseline self-infusions with those obtained after omission tests or changes in dose, or to compare omission scores obtained at two different doses. One way ANOVAs were performed to compare multiple variables in the nicotine groups (compensation indexes to three changes in dose), or to compare one variable across identified subgroups or clusters (omission scores, extinction indices, compensation indices, effects of change in dose baselines, inter-infusion intervals, cotinine levels, fold expression of genes). Significant main effects or interactions were explored by pairwise comparisons of means using the Tukey post hoc test.

To identify statistical differences in the temporal evolution of cumulative infusions, repeated unpaired t-tests were performed, using GraphPad Prism version 7.00 data analysis software system (La Jolla, CA, USA), comparing the mean infusions, achieved every minute, in baseline vs omission tests. In this case, to correct for multiple testing, we used a false discovery rate (FDR) approach, namely the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with a $Q = 1\%$, taking the q-value as a corrected p-value.

Pearson's correlation analyses were used to investigate correlations between continuous variables of interest.

All graphs were done using GraphPad Prism. The results are presented as mean \pm SEM. Differences were considered significant at $p < 0.05$.

Unless otherwise specified, the statistical analyses were performed using the STATISTICA 13.3.0 (2017) data analysis software system (TIBCO Software, CA, USA)

Criteria for Identification of Subpopulations

Experiment 1 – Cue Omission as Function of Nicotine Dose

Cue omission responses at both doses were expressed as percent change respect to their respective baseline infusions (%CueOm-004 and %CueOm-002). In order to assess the contributions of the cue as a function of nicotine dose, a *cue omission ratio* was individually determined by dividing %CueOm-004 by %CueOm-002 per rat. Since a ratio of 1.0 meant an equivalent cue omission regardless of dose, a threshold was defined at $\pm 25\%$ of ratio=1.0 (threshold points: ratio=0.75 and ratio=1.25), in order to form three distinct groups, differing in their cue omission as a function of nicotine dose.

Experiment 2 – Cluster Analysis based on Cue and Nicotine Omission Tests

A *k-Means* cluster analysis was run, taking into consideration two variables from cue (0,04mg/kg) and nicotine omission test, taken individually for each rat: (1) the *global omission effect*, expressed as the percent change of total infusions respect to their respective baseline infusions and (2) an *extinction index*, which was calculated by subtracting the relative amount of cumulative infusions achieved after 60 mins into the omission test session, from the same relative amount of cumulative infusions achieved after 60 mins on the corresponding baseline sessions (**Figure 5**). The 60 mins threshold was used, as preliminary data had shown that responding is generally stabilized after that time-point in the session. This extinction index variable provides temporal information about how quickly an extinction-like profile, following an omission test, is evident in the session

For the clustering analysis, only the first cue omission test was used (CueOm-004), as it was the cue omission done at the training dose, allowing a clear head to head comparison with the nicotine omission test, done also after a history of self-administration at the training dose. The *k-Means* cluster analysis was run using a *v*-fold cross-validation algorithm provided by the STATISTICA software, which allows for an automatic determination of cluster numbers. This allows for clusters to be formed from subpopulations arising naturally from the data, without any *a priori* assumptions on the number of clusters to be formed.

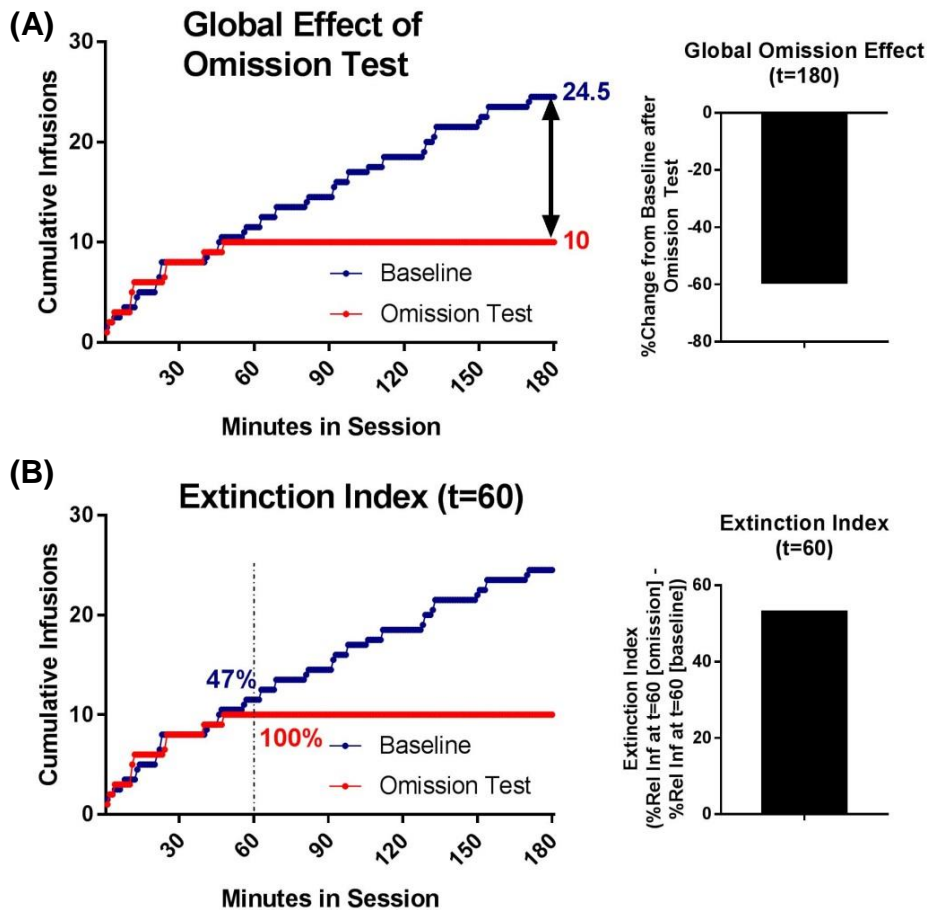


Figure 5 – Variables for Cluster Analysis. Two variables were chosen per omission test: **(A) Global Effect of Omission Test**, defined as the percent change of total infusions respect to their respective baseline infusions. In this example, this rat achieved 24.5 infusions in mean during baseline sessions, but only 10 in the omission test. This represents a -59.18% change in self-infusions. **(B) Extinction Index**, defined as the difference in the relative amount of infusions reached at t=60, between omission test day and baseline sessions. In this example, this rat achieved 47% of all baseline infusions by the 60th minute, but during the omission test, it had already reached its maximal infusions to be earned on that session (100%), leaving an extinction index of +53. The higher the extinction index, the strongest the extinction profile for the rat.

Analysis of gene expression

The analysis of gene expression was based on relative quantification, which results from the calculation of the variation factor (Fold Change, FC) between the different conditions, in this case, nicotine vs saline conditions. This calculation was based on the formula proposed by Livak & Schmittgen: $FC = 2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). For this, it was first necessary to calculate ΔCt , equal to the difference in expression between the gene of interest and the selected housekeeping

genes: $\Delta Ct = Ct (\text{gene of interest}) - Ct (\text{reference gene})$. Then, the value $\Delta\Delta Ct$ was calculated through the difference of expression between the nicotine and the saline conditions: $\Delta\Delta Ct = \Delta Ct (\text{nicotine}) - \Delta Ct (\text{saline})$. Since the aim of the study was to characterize individual differences, the $\Delta\Delta Ct$ was calculated by subtracting the *mean* ΔCt of the saline samples for that gene and structure, from the *individual* ΔCt obtained for *each* nicotine rat, for that gene and structure.

Experiment 1 - Exploration of Individual Differences in Nicotine-Cue Interactions

Results

Acquisition of Nicotine Self-Administration

Rats in both saline and nicotine groups successfully acquired self-administration after the first 13 days of training (**Figure 6**). The self-infusion rate increased progressively for the nicotine group, until stable, remaining significantly higher than the saline group [Session effect, $F(12, 756)=3,2170$, $p=0,0001$; Session x Group effect, $F(12, 756)=14,713$, $p<0,0001$]. By the last two sessions of the acquisition period, both groups had learned to significantly discriminate between active and inactive holes, demonstrating self-administration behavior [Hole effect, $F(1, 63)=35,223$, $p=<0,0001$; Hole x Session effect, $F(1,63)=0,59064$, $p= ns$; Hole x Session x Group effect, $F(1, 63)=0,03834$, $p= ns$]. Peaks at sessions 6 and 11 in both groups correspond to Mondays, when rats would return to the self-administration chambers after two days in the vivarium.

General Effects

Response to Cue Omission

Removal of the cue during the first cue omission test was associated with a significant drop in self-infusions, in both nicotine ($p<0.0001$) and saline ($p=0.04$) groups, revealing the contributions of the cue in their self-administration (**Figure 7**).

In the nicotine group, removal of the cue was nicotine-dose dependent (**Figure 8A**), with an overall stronger effect at the lower of dose of 0.02mg/kg ($p=0.008$). In the saline group, the second cue omission test resulted in a reversal of the effect seen at the first test ($p=0.046$), possibly due to a habituation effect of cue removal, or a progressive decrease in the reinforcing effects of the cue over time. Interestingly, in the nicotine group, the extent of cue omission effect at 0.04mg/kg was not correlated to the extent of cue omission at 0.02mg/kg (**Figure 8B**).

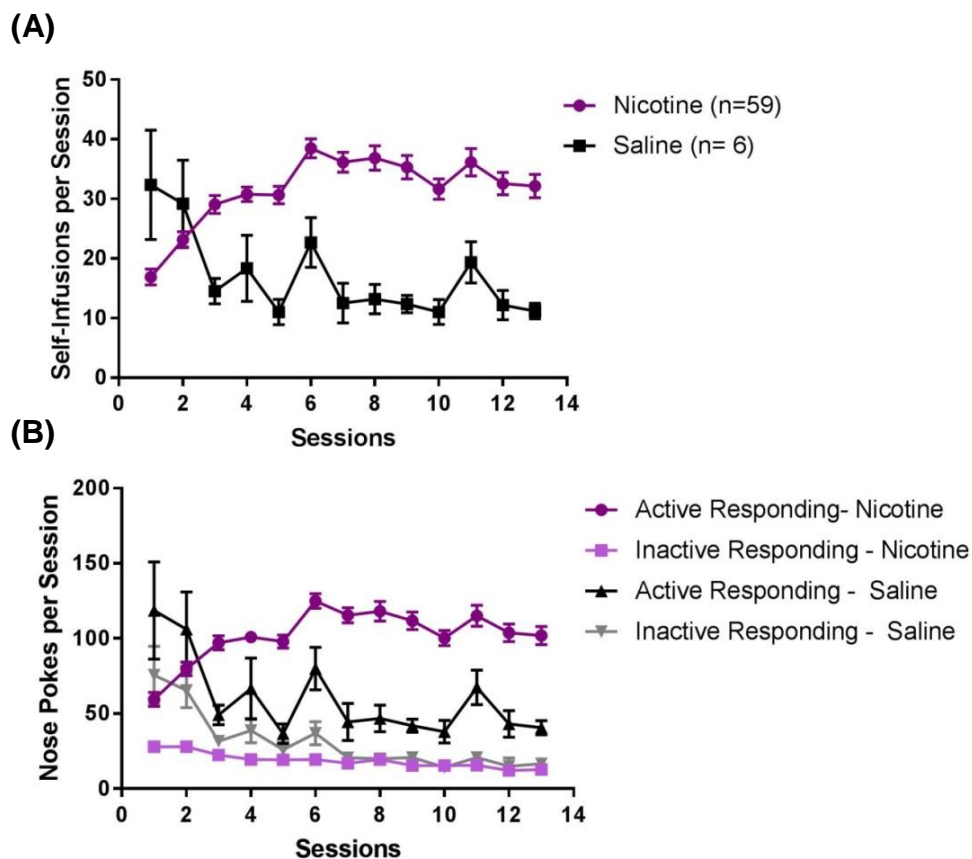


Figure 6 – Acquisition of Self-Administration – Both nicotine and saline rats acquired self-administration behavior by the end of the acquisition period. **(A)** Progression of saline vs nicotine self-infusions across time. By the end of the acquisition period, nicotine rats had a higher self-infusion baseline compared to saline rats. The relatively low infusion number of the nicotine rats in the first two sessions reveals a potential limiting effect of nicotine upon first encounter **(B)** Progression of active vs inactive nose pokes in both nicotine and saline groups across time. By the end of the acquisition period, rats in both groups could significantly discriminate active vs inactive responding. Symbols represent means and error bars represent SEM.

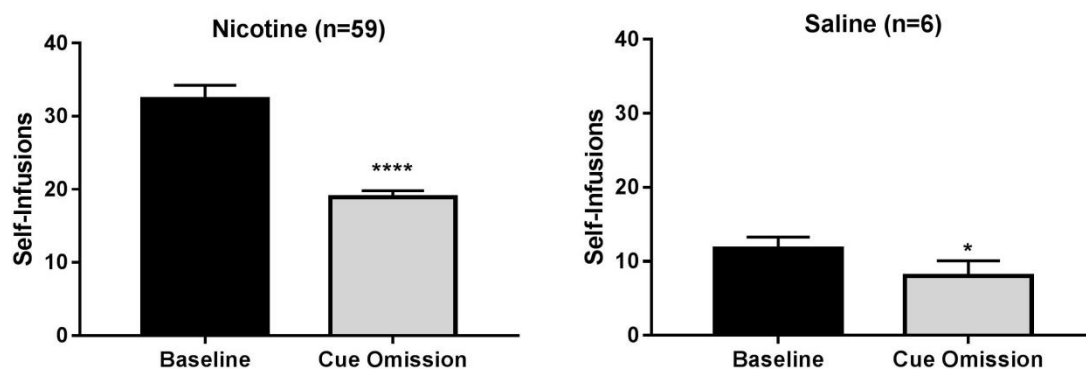


Figure 7 – Cue Omission Effect (0.04mg/kg) – Omission of the cue resulted in a drop in self-infusions, in both nicotine and saline groups. **** = $p < 0.0001$; * = $p < 0.05$, respective to their corresponding baseline. Bars represent means and error bars represent SEM.

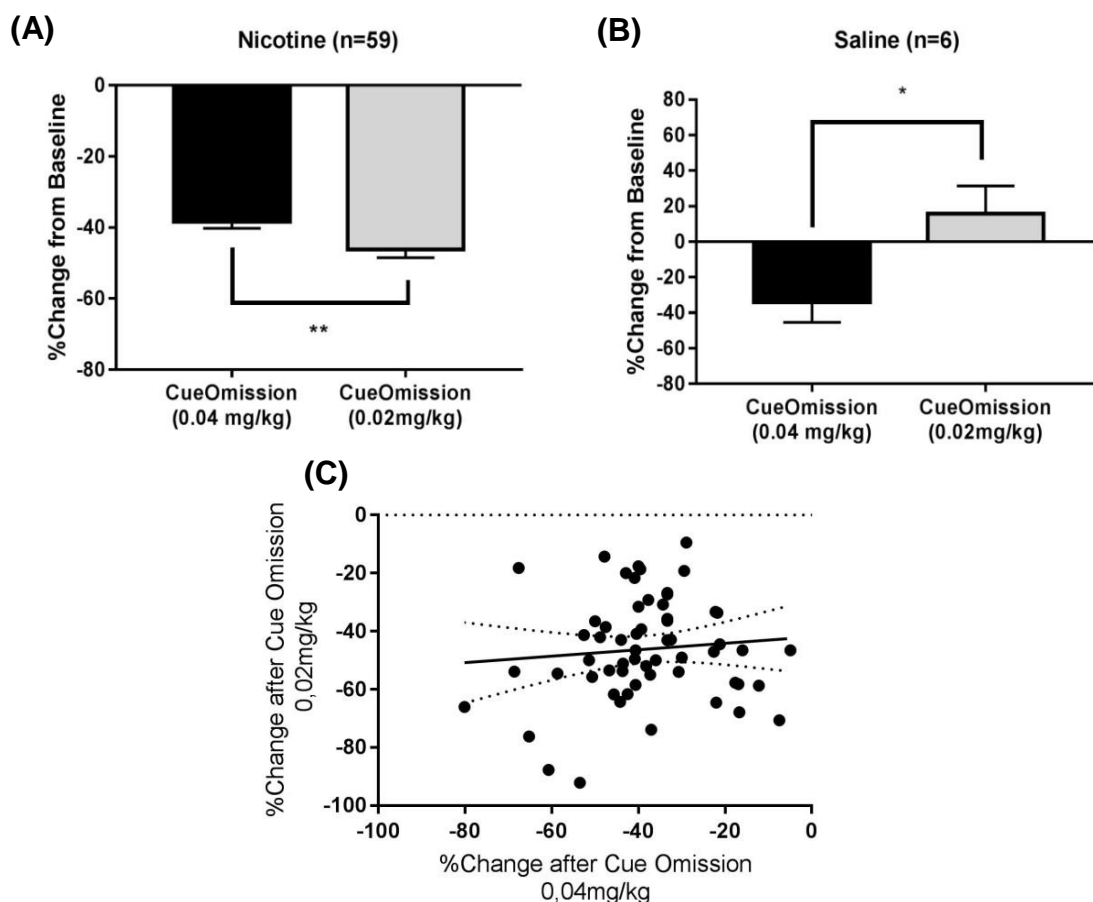


Figure 8 – Dose Dependency of Cue Omission Effect – (A) For the nicotine group, cue omission was stronger at the lower dose of 0.02mg/kg. **(B)** For the saline group, the second cue omission test resulted in the reversal of the first observed cue omission. ** = $p < 0.01$; * = $p < 0.05$, respective to the first cue omission test. Bars represent group means, and error bars represent SEM **(C)** For the nicotine group, the percent changes in self-infusions after the respective cue omission in either dose were not correlated. Symbols represent individual nicotine rats, solid black line represents the linear regression line, and dotted curves represent the 95% confidence bands.

Response to Changes in Nicotine Doses

A sudden reduction of 50% in nicotine dose, from 0.04 to 0.02 mg/kg, resulted in a drastic increase in self-infusions ($p < 0.0001$) with respect to its corresponding baseline (**Figure 9A**). A further reduction of 75% in nicotine dose, from 0.02 to 0.005 mg/kg, further increased responding (**Figure 9B**). Upon returning to the 0.04 mg/kg baseline, a 50% increase in nicotine dose resulted in a significant decrease in self-infusions ($p < 0.0001$) (**Figure 9C**). As expected, saline groups had no changes in their responding during these test days (**Figure 9D**).

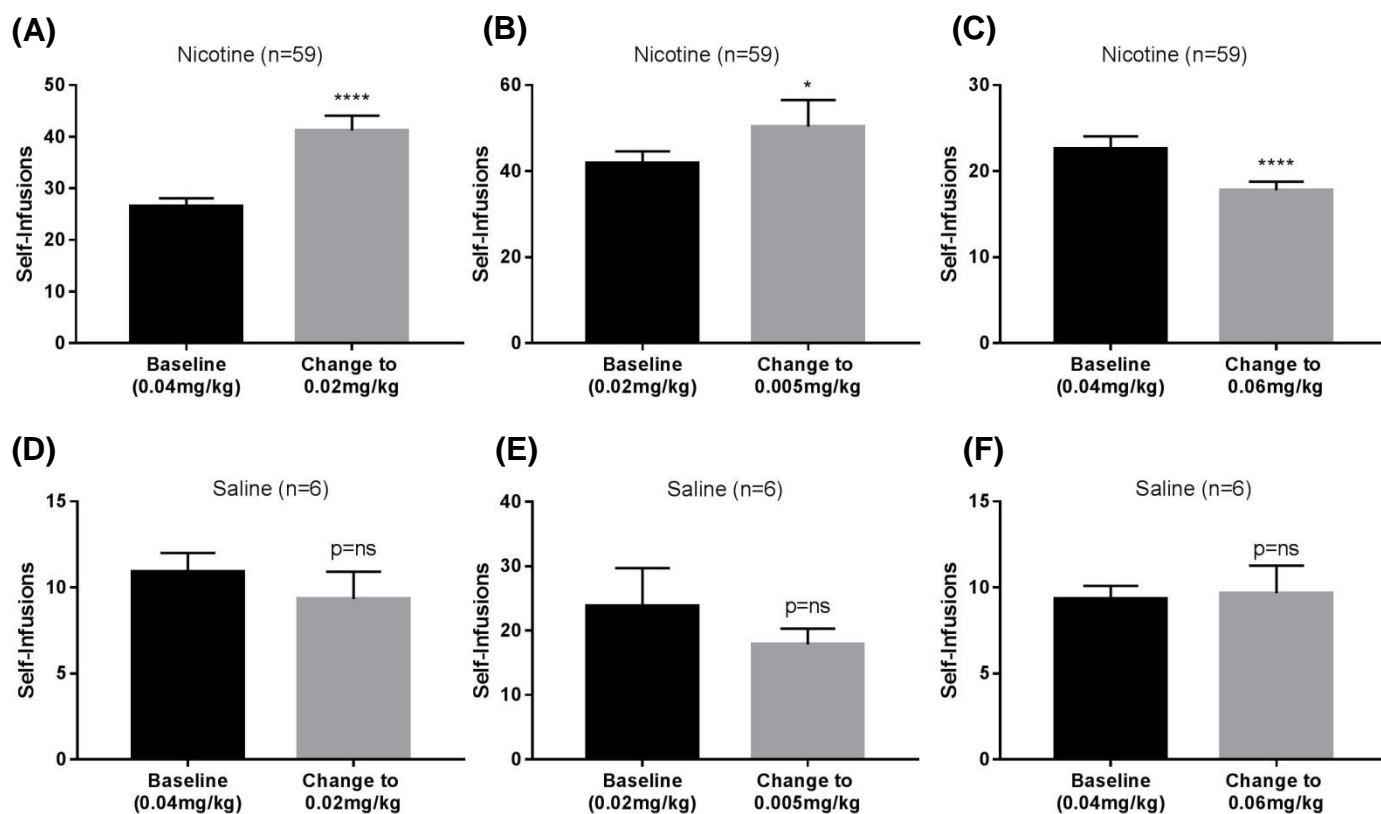


Figure 9 – Effects of Nicotine Dose Change - (A) A 50% reduction in nicotine dose, from 0.04 to 0.02mg/kg, resulted in an increase in self-infusions. **(B)** A further 75% reduction leads to a further increase in self-infusions. **(C)** Upon returning to training dose, rats were given a 50% increase in nicotine dose, leading to a significant decrease in self-infusions. **(D-F)** Saline rats continued to receive saline infusions during these test days, thus the responses remaining statistically equivalent to their respective baselines. **** = $p < 0.0001$; * = $p < 0.05$, respective to the corresponding baseline. Bars represent group means, and error bars represent SEM.

Since the compensation index assesses change in nicotine seeking according to the proportion of dose changed, it allows for a clear comparison between changes in behavior after several changes in dose. In the nicotine group, a 50% decrease in nicotine resulted in an incomplete compensation in mean **(Figure 10A)**, which is consistent with previous studies (Harris et al., 2009), although rats differed in the individual degree of compensation. A shift to 0.005mg/kg resulted overall in strong reduction in compensatory attempt, probably due to the futility of attempting complete compensation with such a low dose of nicotine. A shift to 0.06mg/kg shows in mean the same degree of compensation as with a shift to 0.02mg/kg, although with a wider amplitude of individual responses.

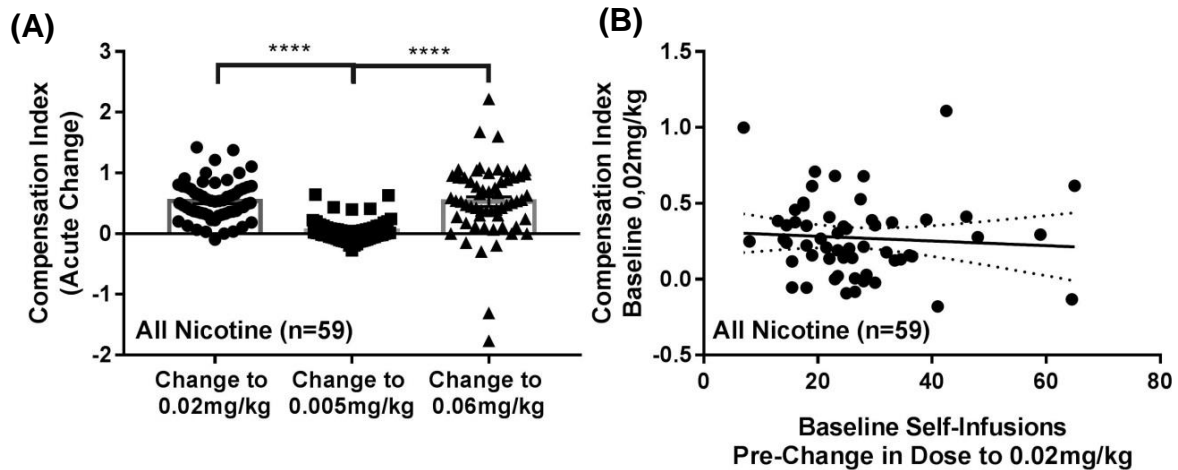


Figure 10 – Compensatory Attempts following Nicotine Dose Change – (A) After a change to 0,02mg/kg and to 0.06mg/kg, nicotine rats have a mean compensatory index of around 0.50, meaning that compensation is not complete. Noteworthy are, however, the individual variations in compensatory attempts. A shift to 0.005mg/kg resulted overall in strong reduction in compensatory attempts, probably due to the futility of attempting complete compensation with such a low dose of nicotine. **** = $p < 0.0001$. Bars represent the mean responses, and error bars represent the SEM. Symbols represent individual rats. **(B)** Baseline self-infusions before change in dose did not correlate with compensation index resulting in a new baseline after a 50% reduction in nicotine dose. Compensation index reported in this graph corresponds to the new stable baseline post-dose change (sessions 24-25-27) and not to the acute change (session 23), in order to compare it with results obtained by Harris et al (2009). Symbols represent individual rats, solid black line represents the linear regression line, and dotted curves represent the 95% confidence bands.

Based on previous studies, we sought to explore whether a compensatory attempt to a decrease in nicotine dose could be correlated negatively to the baseline pre-change in dose (Harris et al., 2009). In our nicotine population, the baseline pre-change in dose did not correlate to the compensation index calculated for the new baseline at 0.02mg/kg (**Figure 10B**).

Response to Cue Omission as a Function of Dose Change

Interestingly, at the population level, the compensation index after change to 0.02mg/kg correlated negatively with response to cue omission at the new dose of 0.02mg/kg (**Figure 11**), suggesting that the second cue omission is affected by how much they had compensated for the change in dose, at least in some of the individuals.

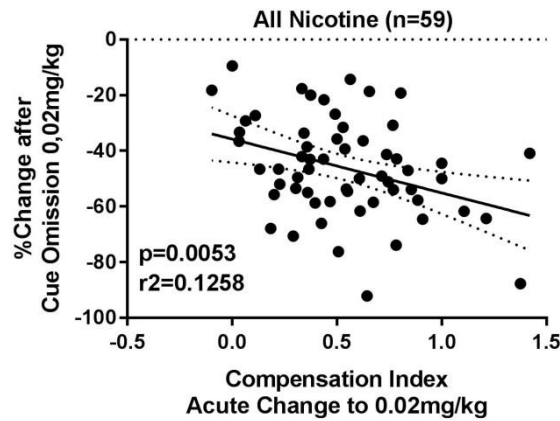


Figure 11 – Compensation to New Dose and Cue Omission at New Dose – Compensation index after an acute change to 0.02mg/kg correlates negatively with cue omission score at the same dose. This suggests that for some individuals, the second cue omission score is affected by the amount of compensation after change in dose. Symbols represent individual nicotine rats, solid black lines represent linear regression lines, and dotted curves represent the 95% confidence bands.

Nicotine Metabolism

At the population level, plasma cotinine levels were positively correlated with infusions at the end of the acquisition period ($p=0.011$) (**Figure 12**), and inversely correlated with percent change in infusions after cue-omission ($p=0.014$), suggesting that nicotine metabolism may be modulating aspects of nicotine self-administration, in particular involvement of cue, at least in some individuals.

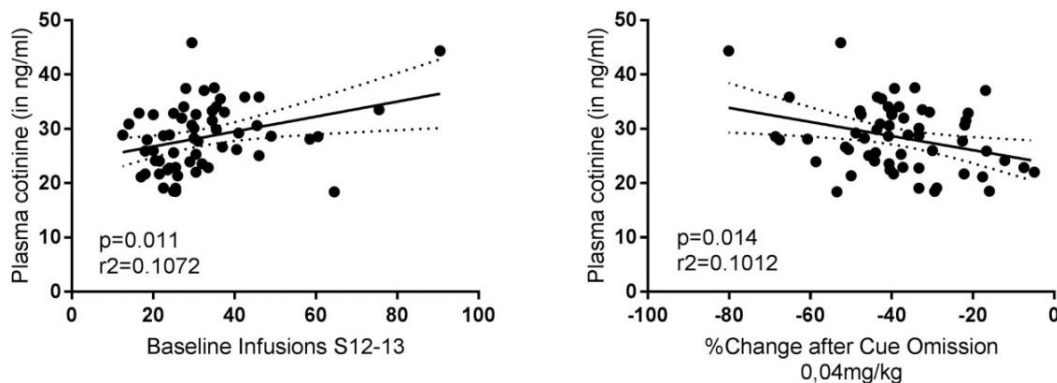


Figure 12– Nicotine Metabolism and Nicotine Self-Administration – Plasma cotinine levels correlated positively with baseline infusions at the end of the acquisition period, while also correlating negatively with cue omission effect at 0.04 mg/kg. Symbols represent individual nicotine rats, solid black lines represent linear regression lines, and dotted curves represent the 95% confidence bands.

Individual Differences in Nicotine-Cue Interactions

Definition and Characterization of Subgroups

Our first approach in studying individual differences in nicotine-cue interactions was to characterize individuals according to their cue omission effects as a function of nicotine dose. Rats that have the same cue omission effect regardless of nicotine dose can be argued to have a stable cue contribution in their nicotine self-administration, compared to rats that have a dose-dependent effect of cue omission. The lack of correlation between cue omission effects at 0.04 and 0.02 mg/kg (**Figure 8**) further suggested that this may be a potential source of individual differences in nicotine-cue interactions. We thus calculated a cue omission ratio (as explained in the Methods section), and separated animals according to a predefined threshold of ± 0.25 around a cue omission ratio of 1.0 (**Figure 13A**). Subgroup 1 (n=22) was selected for the effect of cue omission irrespective of dose; Subgroup 2 (n=24) had a lower cue omission effect at 0.04 mg/kg, and a stronger effect at 0.02 mg/kg, driving the observed general trend (**Figure 8A**). Subgroup 3 (n=13) had a stronger effect at 0.04 mg/kg compared to 0.02 mg/kg.

Baseline Self-Administration

During acquisition, Subgroup 1 appeared to have had higher responding than Subgroup 3 (**Figure 14**), although the differences are not statistically significant [Session effect, $F(12, 672)=31,013$, $p<0,0001$; Subgroup effect, $F(2, 56)=2,2519$, $p=ns$; Session x Subgroup effect, $F(24,672)=1,0501$, $p= ns$]. There were also no differences in active vs inactive hole discrimination by the end of acquisition [Hole effect, $F(1, 56)=192,76$, $p<0,0001$; Hole x Session effect, $F(1, 56)=1,3648$, $p= ns$; Hole x Session x Subgroup effect, $F(2, 56)=,60976$, $p= ns$].

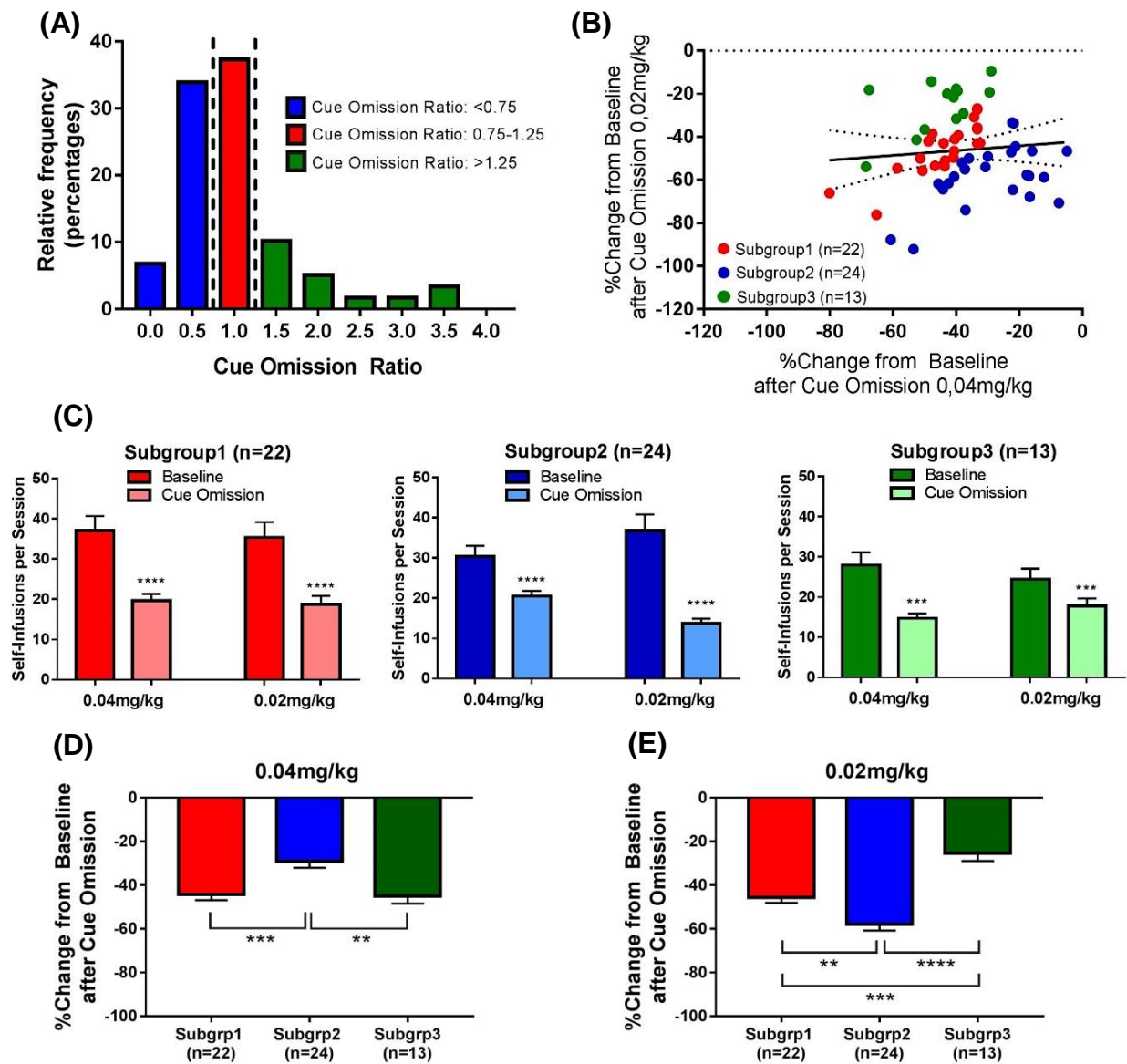


Figure 13 – Subgroup Selection – (A) Histogram of cue omission ratios in the nicotine group. Ratios of 0.75 and 1.25 were used as thresholds for group selection **(B)** Situation of individual groups in the graph plotted on Figure 09C. Subgroup 1 (n=22) had a cue omission ratio between 0.75 and 1.25; Subgroup2 (n=24) had a cue omission ratio of less than 0.75; Subgroup 3 (n=13) had a cue omission ratio greater than 1.25. Symbols represent individual nicotine rats. **(C)** Subgroup differences in self-infusions between baseline and cue omission, for two different doses. Subgroup 1 has the same effect regardless of dose; Subgroup 2 has a higher drop at 0.02mg/kg, while Subgroup 3 has a lower drop at 0.02mg/kg. ****=p<0.0001, ***=p<0.001, respective to the corresponding baseline. **(D-E)** Subgroup2 had the weakest cue omission effect at the 0.04mg/kg dose, but the strongest effect at the 0.02mg/kg dose. ****=p<0.0001, ***=p<0.001; **=p<0.01. Bars represent mean of subgroups, and error bars represent SEM.

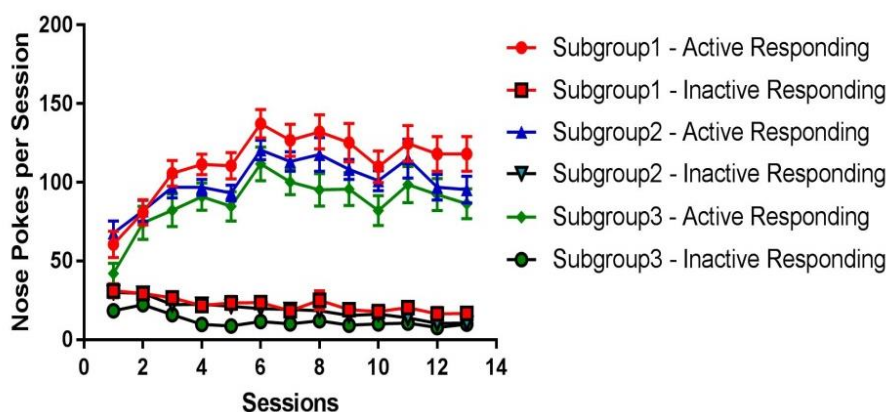


Figure 14 – Acquisition of Nicotine Self-Administration per Subgroup – Progression of active and inactive nose pokes during the 13 sessions of acquisition, per subgroup. Subgroup1 appears to have a higher acquisition baseline than subgroup 3, although differences are not significant. Symbols represent the mean of each subgroup and error bars represent SEM.

Relationship between Baselines and Cue Omission Scores

For the first cue omission test (0.04mg/kg), Subgroups 1 and 2 showed an inverse correlation between baseline infusions and the extent of cue omission effect (**Figure 15A-B**), suggesting that baseline responding could be correlated with the contributions of the cue in their self-administration. However, at the second cue omission test (0.02mg/kg), the relationship is only maintained in Subgroup2 (**Figure 15E**).

Response to Changes in Nicotine Doses

Subgroup2 appears to have stronger sensitivity to the first change in dose when compared to Subgroup3, although this difference is not statistically significant (**Figure 16A**). Notably, Subgroup3 had the weakest increase in infusions after change to 0.02mg/kg, and the weakest decrease in infusions after change to 0.06mg/kg (**Figure 16A and C**).

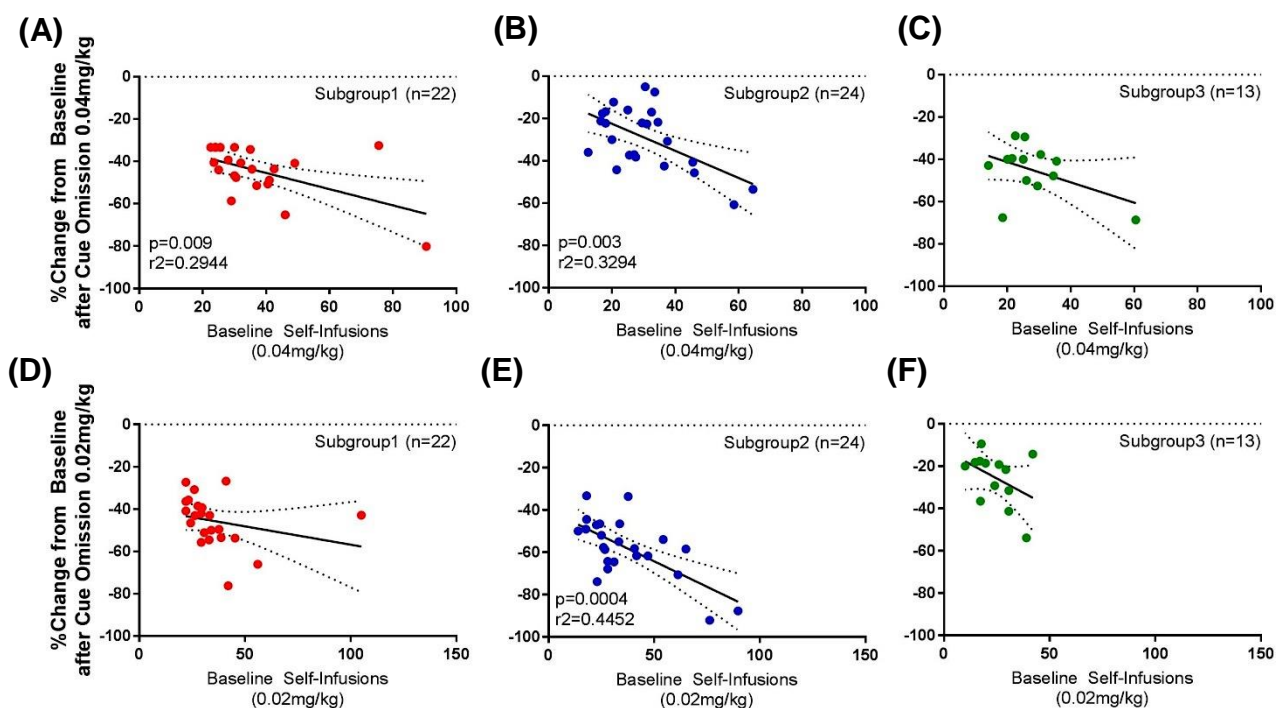


Figure 15 – Inverse Correlations between Baseline Infusions and Cue Omission Scores – (A-C) For cue omission at 0.04mg/kg, Subgroups1 and 2 showed negative correlations between baseline infusions and cue omission scores, while Subgroup3 showed no correlation. (D-F) For the second cue omission (0.02mg/kg), the inverse correlation is only observed in Subgroup2.

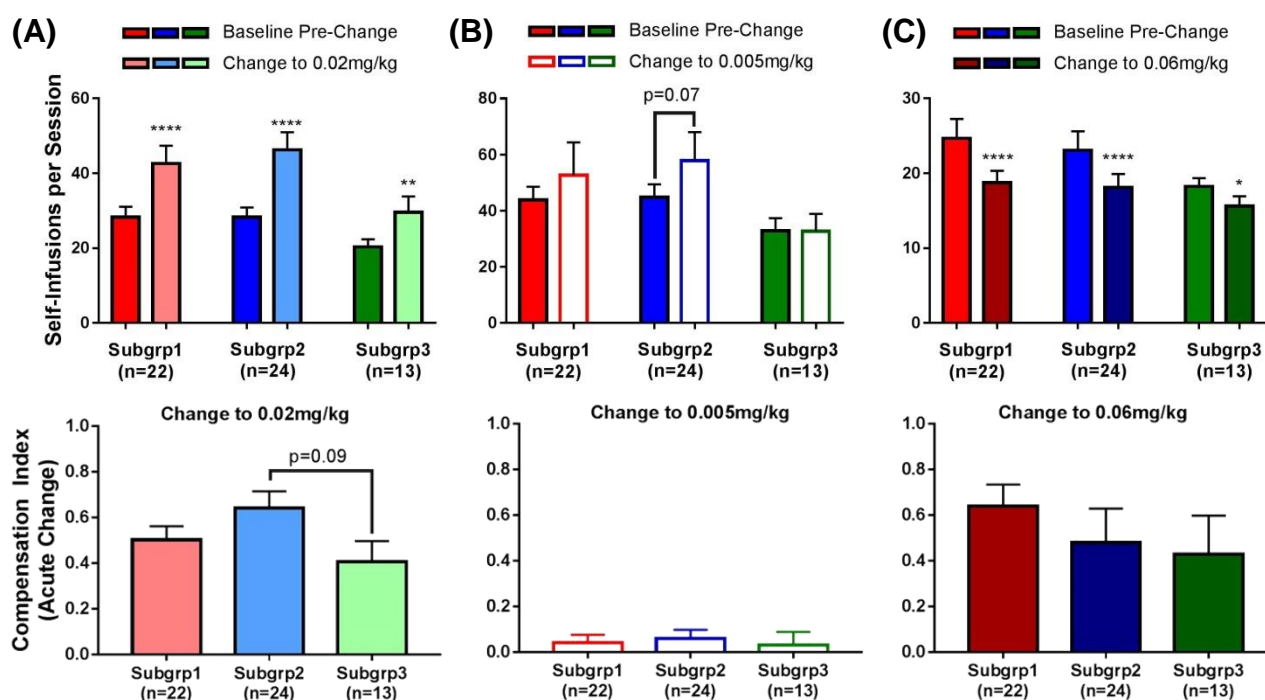


Figure 16 – Differential Nicotine Sensitivity per Subgroup – (A) Rise in self-infusions after first change in dose to 0.02mg/kg. Upper panel: All subgroups have a significant increase in self-infusions, although the effect is weaker in Subgroup 3.. Bottom panel: Compensation index at 0.02mg/kg appears higher, although differences are not statistically different (B) Rise in self-infusions after change in dose to 0.005mg/kg. Upper panel: Only mild increases in self-infusions are reported across all subgroups. Increase in Subgroup2 approaches statistical significance. Bottom panel: No changes among groups. (C) Decrease in self-infusions after change in dose to 0.06mg/kg. Upper panel: Subgroup3 had the weakest decrease in self-infusions. Bottom panel: no changes among groups. ****= $p < 0.001$; **= $p < 0.01$; *= $p < 0.05$, respective to the corresponding baseline. Bars represent the mean of each subgroup and error bars represent SEM.

Response to Change in Dose to 0.02mg/kg

Identified subgroups also did not have correlations between pre-change baseline and the compensation index at the new baseline of 0.02mg/kg (**Figure 17A-C**), mirroring the observation at the general population (**Figure 10B**)

Although not statistically significant, Subgroup2 had a negative trend between compensation index after change to 0.02mg/kg and response to cue omission at the new dose of 0.02mg/kg (**Figure 17E**), observed in the general population (**Figure 11**).

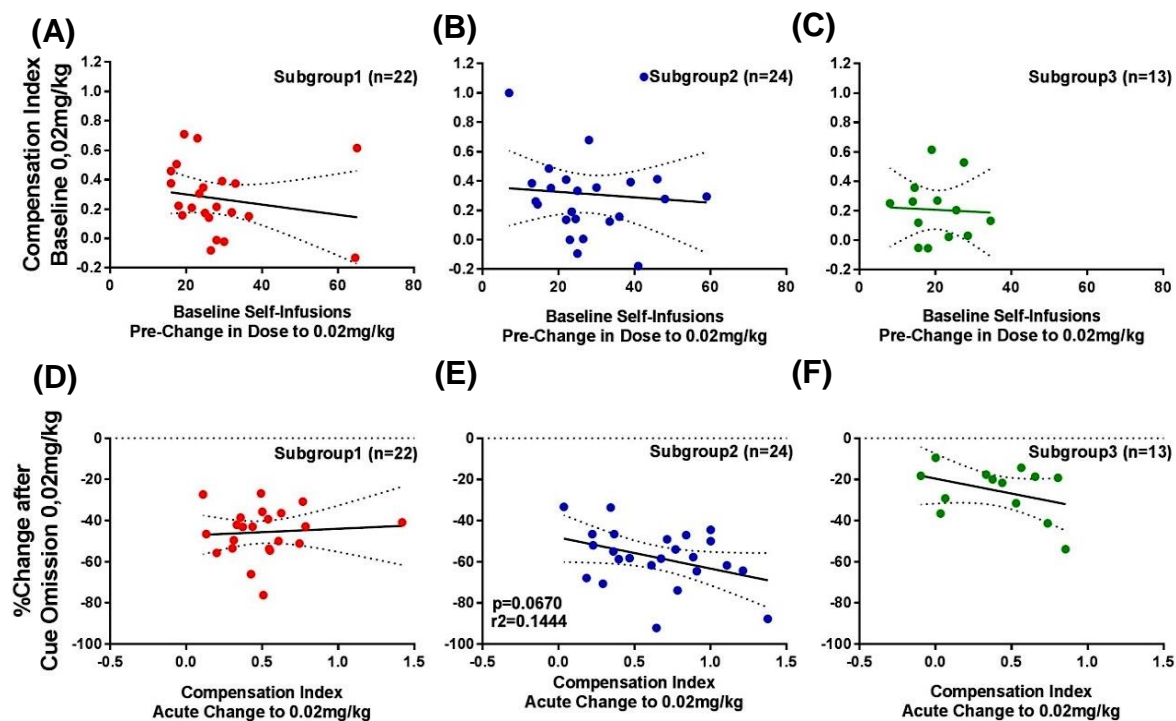


Figure 17 – Correlations of Compensation to 0.02mg/kg with Corresponding Baseline, and Cue Omission Effect at 0.02mg/kg – (A-C) Mirroring the general population trend, there was no correlation between the baseline pre-change in dose, and the compensation index at the new baseline of 0.02mg/kg. **(D-F)** Only in Subgroup2 there is a negative trend between compensation index after an acute change to 0.02mg/kg and cue omission score at the same dose. Symbols represent individual nicotine rats, solid black lines represent linear regression lines, and dotted curves represent the 95% confidence bands.

Nicotine Metabolism

Even though subgroups do not differ in their plasma cotinine levels (**Figure 18B**), only Subgroup 1 retains the positive correlation between cotinine and baseline infusions (**Figure 18B**) and the negative correlation between cotinine and cue omission (**Figure 18E**).

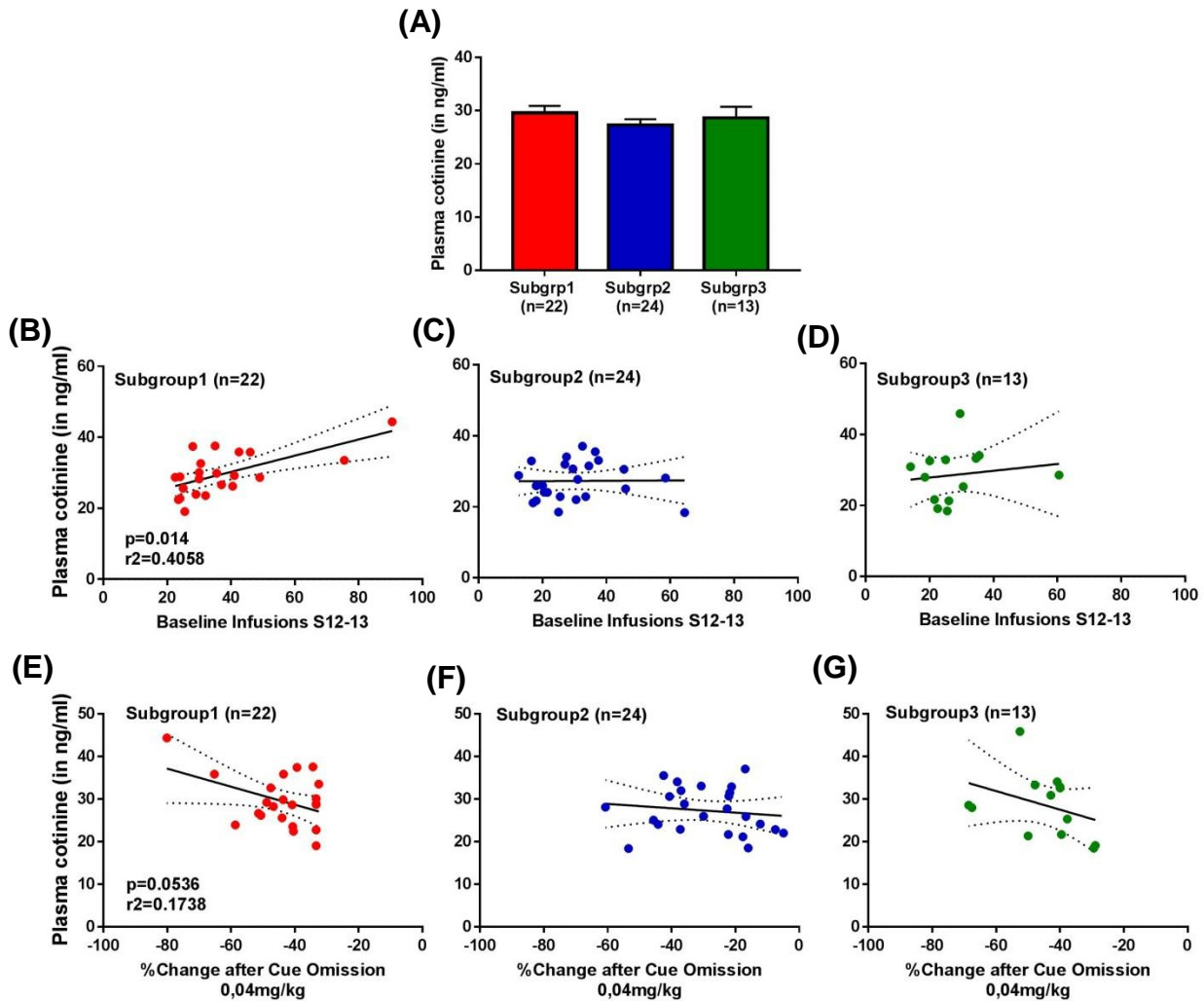


Figure 18 – Differential Role of Nicotine Metabolism in the Contribution of the Cue to NSA
(A) Subgroups did not differ in the amount of cotinine in their plasma. Bar represent the mean of each subgroup and error bars represent SEM. (B-D) Correlations between baseline infusions at the end of acquisition and plasma cotinine. Positive correlation observed only in Subgroup1. (E-G) Correlations between cue omission at 0,04mg/kg and plasma cotinine. Correlation for Subgroup1 approaches statistical significance, not so for the other Subgroups. Symbols represent individual rats.

It is noteworthy that for subgroup1, the positive correlations between cotinine and baseline infusions occur also with the other 0,04 mg/kg baselines (before changes to 0,02 and 0,06 mg/kg, respectively)

(Figure 19A-B). Furthermore, the spacing of infusions loaded during their first baseline sessions is negatively correlated to cotinine levels, only in this subgroup (Figure 19C).

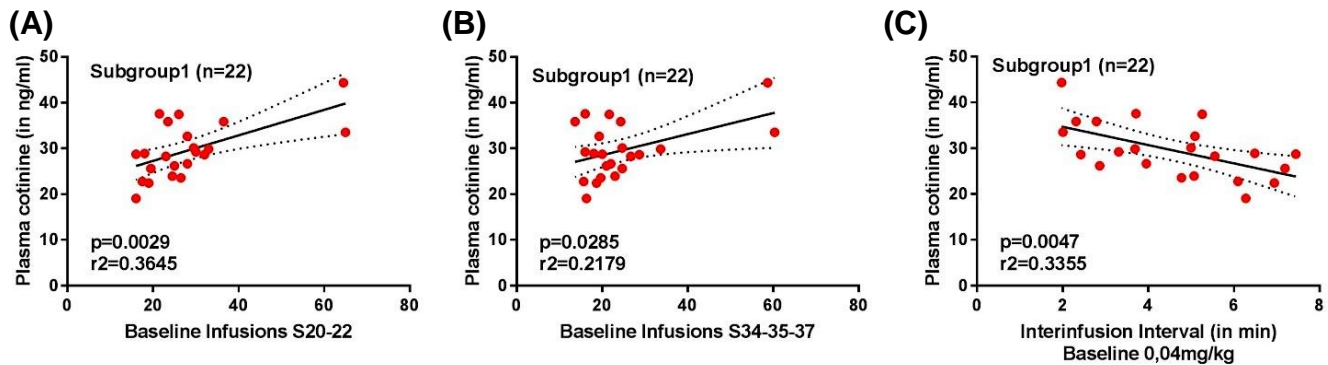


Figure 19– Nicotine Metabolism Predicts NSA at Training Dose in Subgroup 1 – (A) Positive correlation between baseline infusions prior to change in dose, and plasma cotinine. **(B)** Positive correlation between infusions obtained after return to 0.04mg/kg (session 34-35-37) and plasma cotinine. **(C)** Negative correlation between the interval between infusions, and plasma cotinine, on the first 0.04 mg/kg baseline after acquisition. Symbols represent individual rats.

Discussion

Experimental Approaches for Identification of Individual Differences in Nicotine Seeking

We have recently proposed that the predictive validity of animal models may increase if individual variations are taken into account (Garcia-Rivas et al., 2017). This implies determining, within the same individuals, how nicotine seeking is controlled by (1) the pharmacology of nicotine in and of itself, and (2) the pharmacological effects of nicotine on surrounding environmental stimuli (Garcia-Rivas et al., 2017). In this first experimental approach, we sought to capture and bring into evidence such individual differences in the classical model of nicotine IV self-administration. In our study, we identified three populations, which differed in the extent through which nicotine dose affected the relative contributions of the cue in their self-administration.

Sensitivity to Changes in Dose as Source of Individual Differences

It has been widely established that nicotine has only limited primary reinforcing actions (Caggiula et al., 2001; Rose, 2006), although there is evidence suggesting that some individuals are more sensitive to the primary reinforcing effects of nicotine than others (Tapper et al., 2004; Hutchison et al., 2007). Furthermore, even though it is widely recognized that changes in nicotine dose allows for little compensation (Rose and Corrigall, 1997), there is evidence of individual differences in the extent of compensation in nicotine seeking behavior following nicotine dose reduction, both in humans (Hecht et al., 2004) and animals (Harris et al., 2009; Grebenstein et al., 2015).

In the general population, we show that following a switch to a lower (0.02 mg/kg) and higher (0.06 mg/kg) nicotine resulted in incomplete compensation (**Figure 10A**), as expected, although rats differed in the extent of individual compensatory attempt, consistent with the findings of previous studies (Harris et al., 2009; Grebenstein et al., 2015). Contrary to the study by Harris and colleagues, however, we found no inverse correlation between baseline infusions pre-change in dose and compensation index for the baseline at the new dose (**Figure 10B**). This discrepancy could be due to differences in experimental protocol, namely rat strain (Holtzmann vs Sprague-Dawley), amount of animals per study (n=27 vs n=59) and exposure to nicotine (23-hr daily long-access sessions vs 3hr-daily short-access sessions) (Harris et al., 2009). Since long access to nicotine allows for intake escalation conducive to exploration of nicotine withdrawal (O'Dell et al., 2007; Cohen et al., 2012), it remains a possibility that individual differences in nicotine seeking may change as a function of drug exposure (short vs extended) or drug state (withdrawal vs non-withdrawal), and this could explain the differences between our study and that of Harris et al (2009). In fact, some humans studies have suggested that protracted experience with nicotine can shift the control of nicotine seeking, from a predominant cue-controlled behavior, to one more reliant on prevention or alleviation of pharmacological withdrawal to nicotine (Shiffman et al., 2012, 2015; Bani et al., 2014; Piasecki et al., 2014; Roberts et al., 2015; Ferguson et al., 2016; Shiffman and Terhorst, 2017). However, this shift is incomplete, and seen only in some individuals (Baker et al., 2012; Shiffman et al., 2015; Garcia-Rivas

and Deroche-Gamonet, 2018). Further exploration of individual differences in nicotine seeking, as a function of the length of nicotine exposure, is thus warranted (Garcia-Rivas et al., 2017).

Cue Omission as an Exploration of Cue Contribution in Self-Administration

The premise behind a cue omission test is to reveal the contributions of the cue in the acquired self-administration behavior. In their seminal work, Caggiula et al (2001) showed that omission of the nicotine-paired cue after 25 training sessions of IV nicotine delivery paired with a cue resulted in a drastic reduction in operant responding, despite that nicotine was still available for rats to self-administer. This revealed that the nicotine-paired cue is an important component of the behavior observed classically in nicotine self-administration (Caggiula et al., 2001, 2002). Our results are consistent with these findings, demonstrating that after 13 sessions of self-administration, the nicotine-paired cue contributed to the overall nicotine self-administration behavior observed during and immediately after acquisition (**Figure 7**).

The contributions of the cue in classical nicotine self-administration could be through two different mechanisms: (A) nicotine transforming the cue into a conditioned reinforcer, through repeated associations or (B) the potentiation of the reinforcing actions of the cue through a nicotine-induced enhancement of reinforcement (Caggiula et al., 2009). Indeed, the self-administration pattern of our saline group (**Figure 6**), and the effect of cue omission experienced in this group (**Figure 7**), clearly suggest that the cue is itself reinforcing, and capable of driving a sustained self-administration behavior. It is thus possible that nicotine is potentiating this reinforcing effect of the cue alone.

Since there is evidence that the nicotine-induced increase in cue reinforcement is dose-dependent (Liu et al., 2007), we decided to test whether cue omission responding varied as a function of nicotine dose, in a manner that could help clarify its dependency, or not, on nicotine dose. Contrary to our initial expectation, at the population level we saw an increase in cue omission effect at a lower dose, compared to cue omission at the training dose (**Figure 8A**). However, both cue omission effects were not correlated (**Figure 8C**), suggesting that rats differed individually in the extent to which dose

affected their cue omission response. We thus selected three subpopulations, based on how their cue omission response varied after a change in dose (Figure 13A).

Subgroup 1: Dose-Independency of Nicotine-Cue Interactions – Cue as Conditioned Reinforcer?

In our first subgroup (n=22), the number of self-infusions during cue omission at either dose was drastically lower from the corresponding baseline sessions (Figure 13C), suggesting that the cue was an important component of their nicotine self-administration, enough to decrease behavior if absent. Despite a strong increase in responding when the dose was changed to 0.02mg/kg (Figure 16A), and responsiveness to further changes in nicotine doses (Figure 16B-D), the response to cue omission at the training dose of 0.04mg/kg was the same, and proportional to, the response to cue omission at a lower dose of 0.02mg/kg (Figure 13B). Importantly, the degree of response to a change to 0.02mg/kg did not impact the degree of response to cue omission at the same dose (Figure 17). These results suggests that for these rats, the nicotine-associated cue has become an important component in their self-administration, remaining stable across time, and not being affected by changes in nicotine dose.

The dose-independency of cue omission effects in this subgroup could suggest that the contributions of the cue in their basal self-administration could be more related to the cue having become a conditioned reinforcer, rather than nicotine potentiating the reinforcing actions of the cue. It has been observed that, once established, the visual cue as a conditioned reinforcer to nicotine will maintain responding even in the absence of nicotine (Cohen et al., 2005).

Interestingly, nicotine metabolism in this subgroup predicted the baseline responding at the training dose (Figure 18B and 19), as well as the cue omission response (Figure 18E), even if mildly. Even if causality is not clear, it is possible that in these rats, nicotine metabolism determines their baseline intake at the training dose (the faster the metabolism, the higher the baseline) (Figure 19C). Then, in turn, the higher their baseline, the higher the likelihood to associate nicotine with its associated cue at this dose (Figure 15A), and thus, the greater its contribution as conditioned reinforcer. Indeed, human studies have revealed that smokers with faster metabolism tend to have greater reactivity to smoking-

related cues, compared to smokers with slower metabolism (Tang et al., 2012; Falcone et al., 2016), a phenomenon argued to be due to frequency of nicotine-cue associations (Tang et al., 2012).

Subgroup 2: Dose-Dependent Nicotine-Cue Interactions – Nicotine Effects on Cue?

The defining characteristic of our second subgroup (n=24) was the strongest cue omission effect at the lower dose of 0.02mg/kg (**Figure 13**), a phenomenon that drove the trend observed in the general population (**Figure 8A**). Interestingly, and despite not reaching statistical significance, subgroup2 had the highest compensation index for the first change in dose to 0.02 mg/kg (**Figure 16B**). Importantly, we observed a negative trend between this compensation index and the cue omission effect at the second dose of 0.02mg/kg (**Figure 17**). This suggests that degree of cue omission at 0.02mg/kg appears dependent on how much they attempted to compensate for an earlier change in dose.

One possible explanation for this phenomenon could be a delay in the speed in which the cue was becoming a conditioned reinforcer. It is not only that the cue omission effect at 0.02mg/kg is stronger, but also that the first cue omission effect at 0.04mg/kg is the weakest among the three subgroups (**Figure 13D-E**). Thus, it is possible that at the moment of the first cue omission test, the contributions of the cue in their self-administration were still weaker. It was necessary a boost in self-infusions, brought about through a change in dose (**Figure 16A**), to increase nicotine-cue pairings, translating itself into a better correlation between baseline and cue omission effect (**Figure 15B-E**), and probably converting the cue into a better conditioned reinforcer. An alternate explanation could be that rats in this subgroup were more sensitive to nicotine, in particular to its aversive effects. Indeed, there is neurobiological evidence suggesting that some individuals may be more sensitive to the aversive effects of nicotine, even at doses that are habitually reinforcing for others (Sartor et al., 2010; Hoft et al., 2011; Svyryd et al., 2016). It could thus be that for these animals, the balance between nicotine reinforcement and nicotine aversion at 0.04mg/kg training dose was shifted towards a stronger aversion profile, explaining why the nicotine-cue association was stronger at a lower dose. However,

this latter explanation is unlikely, as a switch to the highest dose of 0.06mg/kg was accompanied by decrease in self-infusions in a magnitude similar to Subgroup1.

Subgroup 3: Dose-Dependent Nicotine-Cue Interactions – Possible Effect of Nicotine on Cue?

The defining characteristic of our third subgroup (n=13) was a strong cue omission effect at the first dose of 0.04mg/kg, and a weaker effect at 0.02mg/kg (**Figure 13**). This trend is consistent with what it would be expected for a dose-dependent, reinforcing-enhancing effect of nicotine on a cue (Liu et al., 2007), in which the effect of nicotine on the cue would be reduced if the dose is reduced. Intriguingly, however, total infusions were consistently lower in this subgroup during acquisition period (**Figure 14**), and the compensation to a decrease in dose to 0.02mg/kg was the lowest among all groups (**Figure 16A**). This is against what would be expected for the phenotype of nicotine effects on the cue, as the reinforcer-enhancing effect of nicotine would have increased the rate of responding for the cue. Furthermore, the saline group had also a weaker effect at the second cue omission test (**Figure 8B**), making interpretation problematic. An alternative possibility is that animals in this subgroup were sensitive to the aversive effects of nicotine that occluded the formation of any meaningful nicotine-cue interactions, thus following the saline trend in regards to cue involvement in their self-administration. However, a switch to a very low dose of nicotine (0.005mg/kg) reveals no differences in responding (**Figure 16B**), and switch to a high dose of nicotine represents the weakest decrease in responding among all subgroups (**Figure 16C**), thus making this explanation unlikely.

Limitations

Our first experimental approach allowed us to evidence how nicotine-cue interactions varied in a population of outbred rats trained for a classical protocol of nicotine self-administration, which involves intravenous delivery of nicotine paired with a visual cue. We were able to reveal three subgroups of rats that differed in their dose-dependency of cue omission responses, and notably, for Subgroup1, we found a metabolic correlate that could partially explain their behavior.

However, even as subgroups were identified, there are difficulties in clearly disentangling the mechanisms behind their nicotine seeking behavior. Classification of the groups based on their differential cue omission score can give a clear picture for the group with the same cue omission, but the interpretation of Subgroups 2 and 3 is less evident. For Subgroup 3, we have insufficient evidence in the variables tested to draw meaningful conclusions as to the putative mechanism behind their behavior.

Perspectives into Experiment 2

Based on these limitations, we proposed a refined experimental approach, with better grouping criteria, and the introduction of more external variables that could help better characterize subpopulations of rats according to their mechanisms of nicotine seeking.

1. *Nicotine Omission Test*

We decided to introduce a nicotine omission test. In this session, rats would receive saline instead of nicotine, while still receiving cue presentation. Comparing how rats behave in both cue and nicotine omission tests would allow for a better head-to-head comparison of the involvement of nicotine, and cue, in basal nicotine self-administration. Even though we did have a near-nicotine-omission test (the switch to the very low dose of 0.005mg/kg), the accumulation of nicotine within the session presented a problem of interpretation, especially for slow metabolizers. A complete removal of nicotine was thus needed to remove this potential bias. We proposed to make the responses to nicotine and cue omissions test the defining criteria for subgroups to be formed within the population.

2. *Temporal Evolution of Omission Effects Within a Session*

In this experiment we had only observed the global change in behavior after cue omission or change in dose, i.e. the effect on total infusions per session. However, in order to better identify rats who respond quickly, or slowly, to a change in dose, a cue omission, or a nicotine omission test, we needed to analyze the individual behavior within the test session. This would be critical for the nicotine omission test, as any increase in global responding could be due to a

pure extinction-like profile, or due to a heightened self-administration of the cue in the absence of nicotine. We thus decided to extend our analysis into changes in responses within the test sessions, and include one of such ‘in-session’ parameter as a defining characteristic for our subpopulations, namely the ‘Extinction Index’, as explained in the Methods section (Figure 5B).

3. *Cluster Analysis*

In order to increase the statistical validity of our model, we decided to move towards a mathematical approach that could help us identify clusters of individuals that arise naturally from the population, with no arbitrary thresholds, and that significantly differ in the input variables we were interested in. For the specific details, refer to the Methods section.

4. *External Variables to serve as correlates.*

Finally, to bring validity to our behavioral observations within nicotine self-administration, we decided to expand the ‘toolbox’ of external variables that could help better characterize our groupings, beyond nicotine metabolism.

- ***Cue as Conditioned Reinforcer:*** to assess whether the contributions of the nicotine-paired cue as conditioned reinforcer could be predicted by an external, unrelated, and visually-different conditioned reinforcer, we decided to introduce a prior test to nicotine self-administration. Through Pavlovian Conditioned Approach, rats would be characterized in the extent of conditioning to a saccharin-predicting cue. For the specific details, refer to the Methods section.
- ***Neurobiological Targets:*** As a first step to understand the neurobiological underpinnings of the behavioral profiles identified, we decided to assess gene expression in key brain areas related to nicotine reinforcement, nicotine-cue interactions and nicotine aversion. For the specific details, refer to the Methods section.

Experiment 2 - Refined Identification and Characterization of Individual Differences in Nicotine-Cue Interactions

Results

General Effects

Pavlovian Conditioning

As sessions progressed, rats increased cup contacts ('licks'), during the 10-sec period in which the CS was deployed [Session Effect: $F(10, 580)=22,809, p<0,0001$] (**Figure 20A**). Additionally, as session progressed, rats were more likely to interact with the cup [Session Effect: $F(10, 580)=46,526, p<0,0001$] (**Figure 20C**), and with a faster speed [Session Effect: $F(10, 580)=33,346, p<0,0001$] (**Figure 20E**). These general measures indicated that rats learned that the CS predicted the delivery of the US, and initiated conditioned approach to the cup upon CS presentation. However, the extent of this Pavlovian conditioning was subject to individual differences, with some rats conditioning better than others, as seen from the respective histograms (**Figure 20A-C**, lower panels).

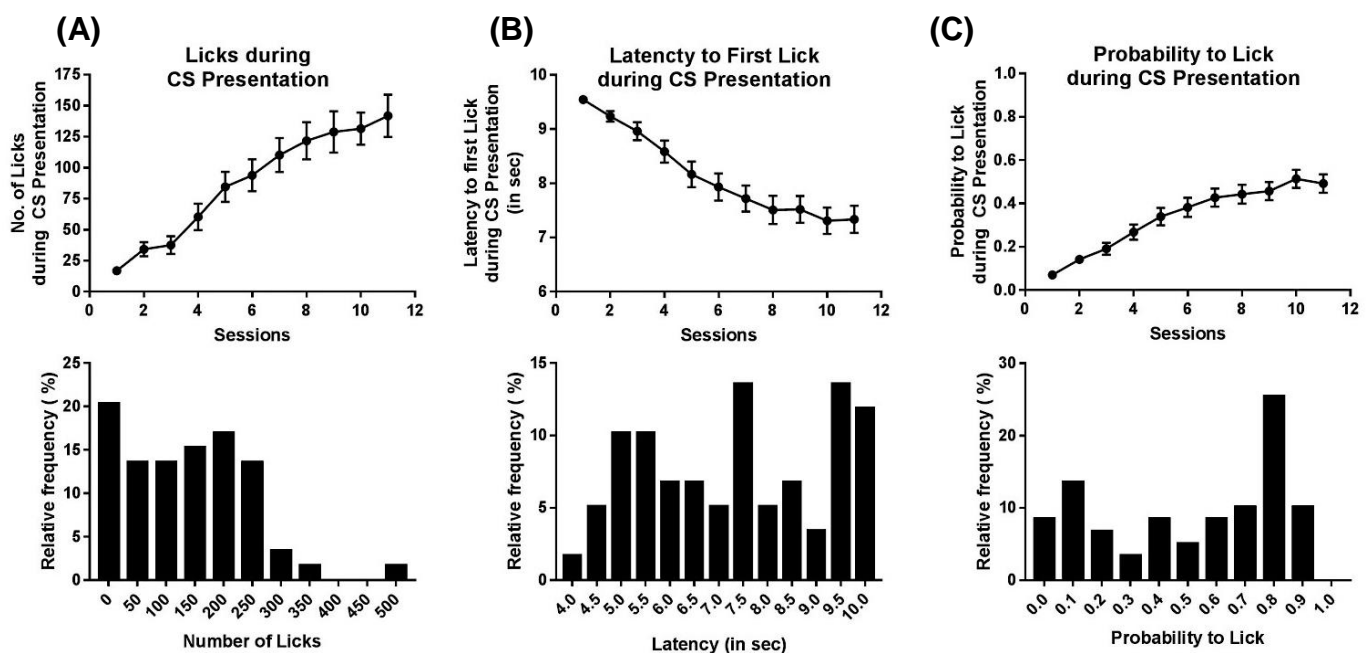


Figure 20 – Individual Differences in Conditioning to a Saccharin-Predictive Cue – (A) Licks during CS presentation. Upper panel: progression across sessions. Stabilization of rising trend was evident after session 9. Lower panel: histogram of mean licks during CS presentation in sessions 10 and 11. **(B)** Latency to first lick during CS presentation: Upper panel: progression across sessions. The latency also plateaued after session 9. Lower panel: histogram of mean latency in sessions 10 and 11. **(C)** Probability to Lick during CS Presentations: Upper panel: progression across sessions, also plateauing after session 9. Lower panel: histogram of the mean probability to lick in sessions 10 and 11. Symbols represent mean responses, error bars represent SEM.

The mean values for session 10 and 11 per variable, per rat, were taken into consideration as correlation variables for nicotine self-administration analysis. In preparation for nicotine self-administration, rats were randomly allocated into two groups: one group for nicotine self-administration (n=51) and another for saline self-administration (n=8). Rats in both groups did not differ significantly in their Pavlovian conditioning variables (**Figure 21**)

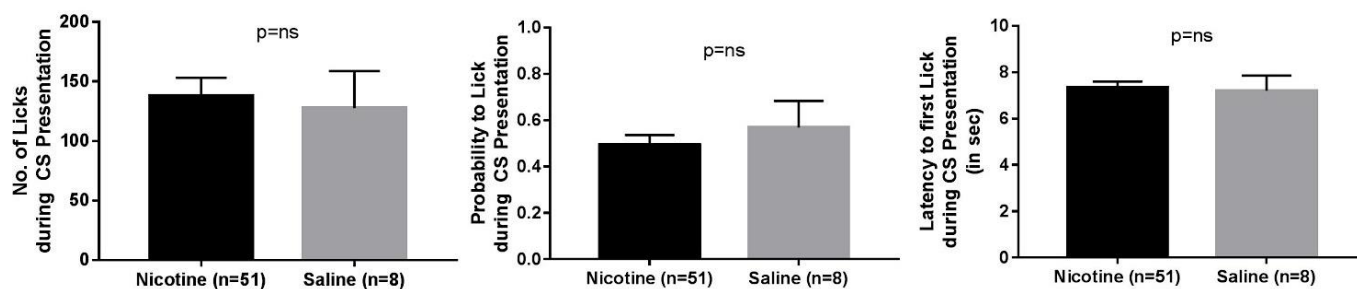


Figure 21 – PCA Variables of Allocated Groups for Self-Administration. Rats allocated in nicotine and saline groups had homogenous mean values for the three Pavlovian conditioning parameters studied. Bars represent mean responses, error bars represent SEM.

Acquisition of Nicotine Self-Administration

Rats in both saline and nicotine groups successfully acquired self-administration after the first 12 days of training (**Figure 22**). The self-infusion rate increased progressively for the nicotine group, while for the saline group it decreased, and stabilized [Session effect, $F(11,627)=26,140$, $p<0,0001$; Session x Group effect, $F(11,627)=48,384$, $p<0,0001$] (**Figure 22A**). By the last two sessions of the acquisition period, both groups had learned to significantly discriminate between active and inactive holes (**Figure 22B**), demonstrating self-administration behavior [Hole effect, : $F(1,57)=63,569$, $p<0,0001$; Hole x Session effect, $F(1,57)=1,6238$, $p=ns$; Hole x Session x Group effect, : $F(1,57)=0,01439$, $p=ns$].

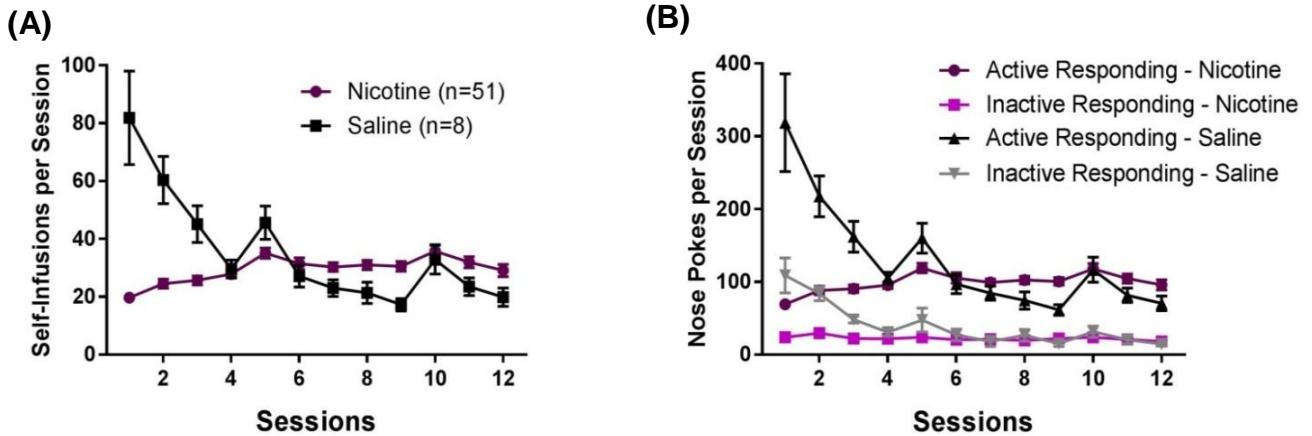


Figure 22– Acquisition of Self-Administration - Both nicotine and saline rats acquired self-administration behavior by the end of the acquisition period. **(A)** Progression of saline vs nicotine self-infusions across time **(B)** Progression of active vs inactive nose pokes in both nicotine and saline groups across time. By the end of the acquisition period, rats in both groups could significantly discriminate active vs inactive responding. Symbols represent means and error bars represent SEM

Intriguingly, saline self-administration was much higher in Experiment 2 compared to Experiment 1, notably during the first three sessions of self-administration (**Figure 23A**). By contrast, the nicotine groups acquired self-administration at an equivalent level in both experiments (**Figure 23B**). This observation could suggest that pre-training with Pavlovian conditioning to a saccharin cue may have sensitized responding to visual cues, an effect masked by the presence of nicotine, which may have limited the amount of visual reinforcers the rat would otherwise self-administer.

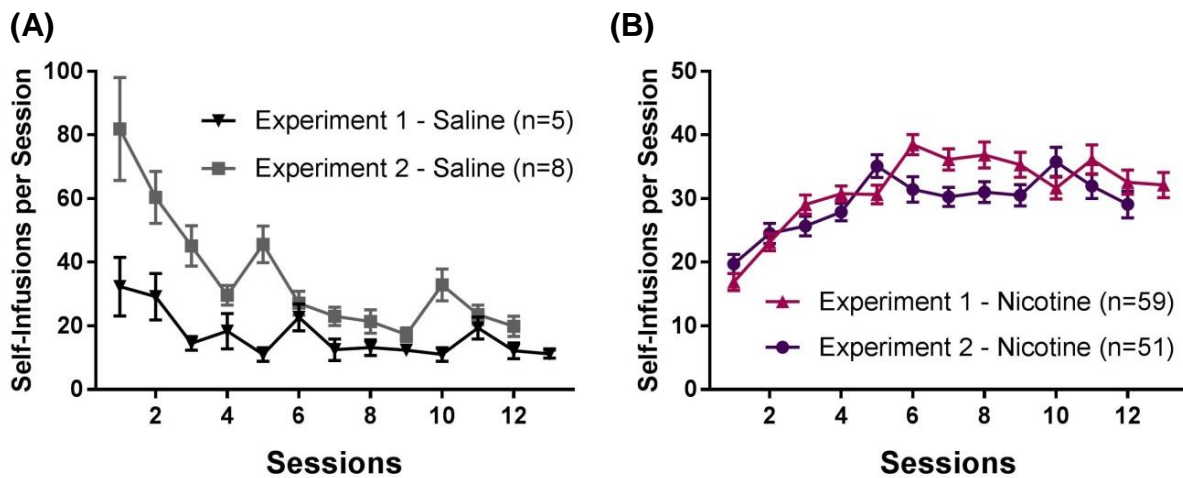


Figure 23– Comparison of Nicotine and Saline Self-Administration in Experiments 1 and 2 – **(A)** Compared to Experiment 1, the saline self-administration was much higher in Experiment 2, especially in the first few sessions in self-administration **(B)** By contrast, nicotine self-administration progressed in a similar way between Experiments 1 and 2. Symbols represent mean responses in each group, and error bars represent SEM.

Response to Cue Omission

Removal of the cue during the first cue omission test was associated with a significant drop in self-infusions, in both nicotine ($p < 0,0001$) and saline ($p = 0,002$) groups, revealing the contributions of the cue in their self-administration (**Figure 24**), in a manner consistent to Experiment 1.

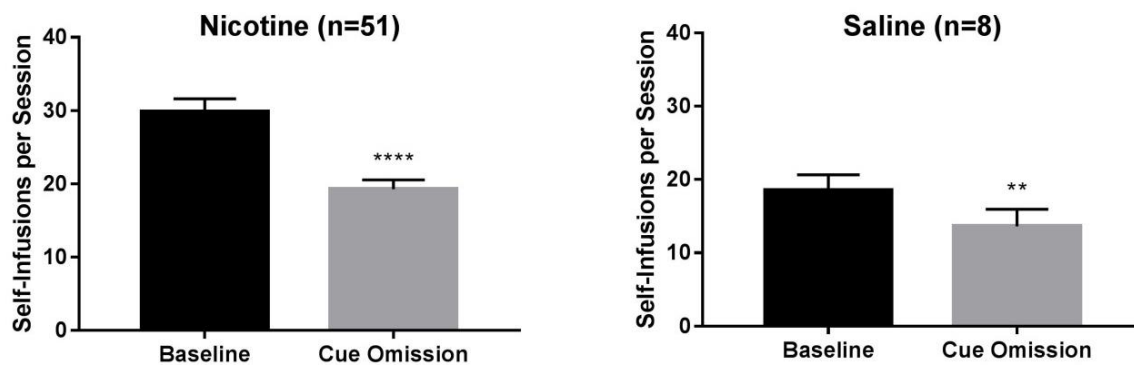


Figure 24– Cue Omission Effect - Omission of the cue resulted in a drop in self-infusions, in both nicotine and saline groups. **** = $p < 0,0001$; ** = $p < 0,01$, respective to their corresponding baseline. Bars represent means and error bars represent SEM

In the nicotine group, the effect of cue omission appears stronger at the lower dose of 0,02mg/kg (**Figure 25A**), although the effect is not statistically significant, probably due to three rats, in which the effect is in the opposite direction. In a manner similar to Experiment 1 (**Figure 8C**), in the nicotine group, the extent of cue omission effect at 0.04mg/kg was not correlated to the extent of cue omission at 0.02mg/kg (**Figure 25B**).

To assess whether pre-training with Pavlovian conditioning had impacted nicotine seeking behavior between Experiments 1 and 2, in particular in nicotine-cue interactions, we performed the same grouping analysis as done in Experiment 1, namely, separating them into three groups according to the ratio between their cue omission scores (**Figure 26**). There were no significant changes in the proportion of individuals allocated in Subgroups 1, 2 and 3 [Chi-Square = 0,4944, $df = 2$, $p = ns$]. This finding, together with the same acquisition curve in both experiments (**Figure 22**) suggests that, even

though pre-training had affected responding in the saline group, it had not made a significant impact on nicotine self-administration.

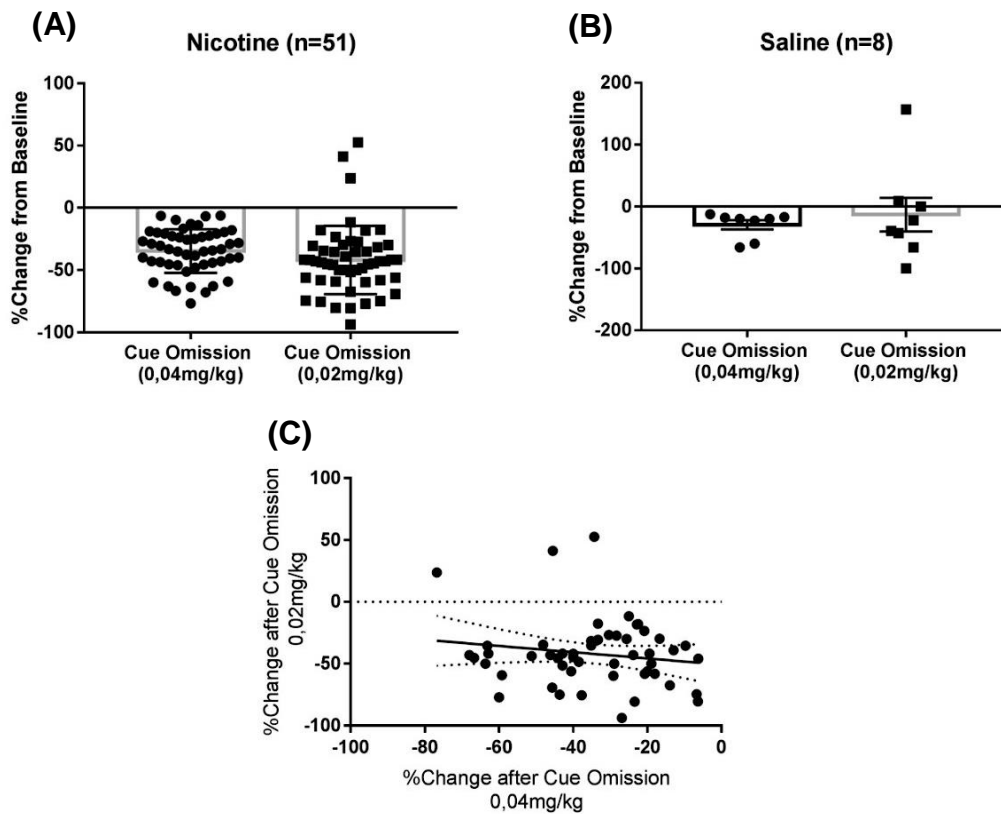


Figure 25– Cue Omission Effects at 0,04 and 0,02mg/kg - (A) For the nicotine group in general, cue omission was slightly stronger at the lower dose of 0.02mg/kg, although not statistically different. (B) For the saline group, in mean the second cue omission test was not different from the first test. Bars represent group means, and error bars represent SEM (C) For the nicotine group, the percent changes in self-infusions after the respective cue omission in either dose were not correlated. Symbols represent individual nicotine rats, solid black line represents the linear regression line, and dotted curves represent the 95% confidence bands.

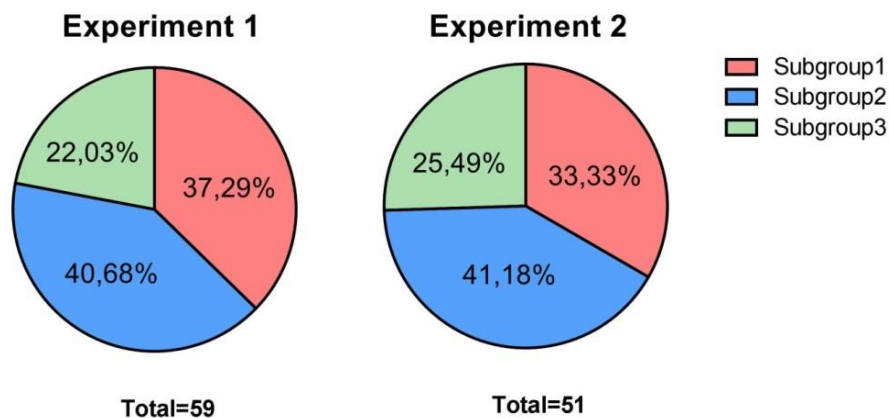


Figure 26– Comparison of Subgroup Proportions between Experiments 1 and 2. – Performing the same grouping strategy as Experiment 1, reveals similar proportions of subgroup composition in Experiment 2. A Chi-Square comparison of each Subgroup between Experiments reveals no statistical differences.

Response to Nicotine Omission

Nicotine substitution with saline in the nicotine group was associated with a significant increase in self-infusions ($p < 0.0001$), revealing the contribution of nicotine in their self-administration (**Figure 27A**). Since the saline group continued to receive saline infusions, their self-administration behavior did not change during the nicotine omission test (**Figure 27B**). Importantly, nicotine rats differed in the extent of nicotine omission effect at the individual level, with some rats increasing self-infusions to +200%, while some other remaining close to 0% change, or even slight decrease in infusions (**Figure 27C**).

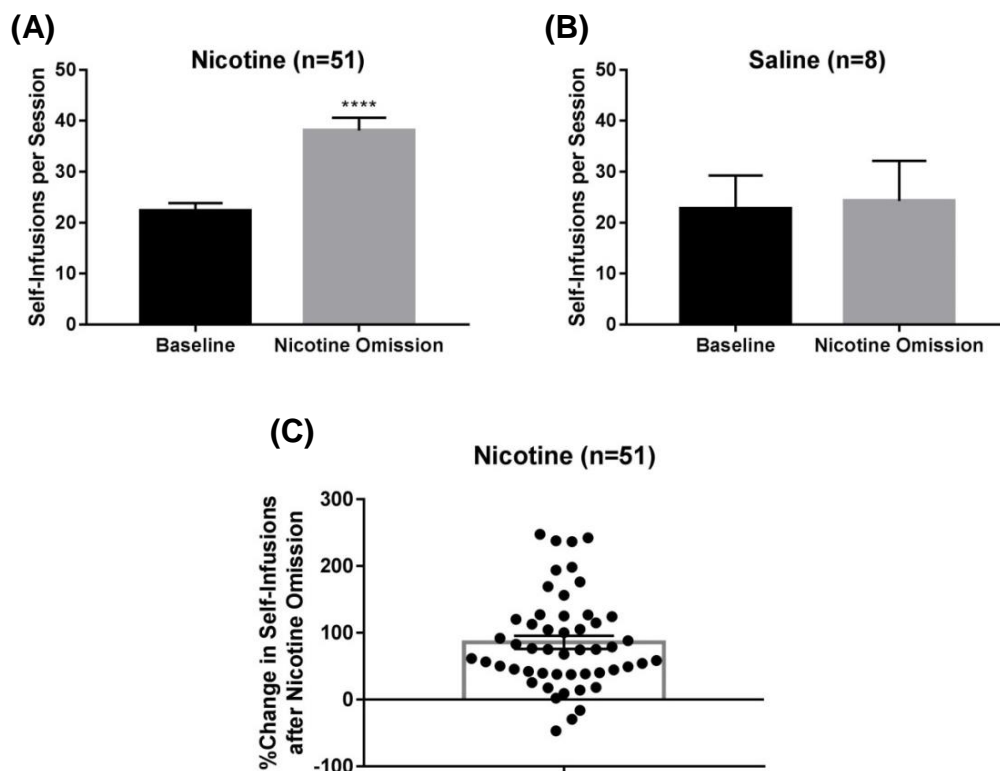


Figure 27 – Nicotine Omission Effects - (A) In the nicotine group, nicotine omission test resulted in a significant increase in self-infusions. ****= $p < 0.001$, respective to the corresponding baseline. **(B)** In the saline group, no difference in their behavioral response between baseline and test day. **(C)** Amplitude in nicotine omission responses reveals individual differences. Bars represent mean of respective groups, error bars represent SEM, and symbols represent individual rats.

Individual Differences in Nicotine-Cue Interactions

Definition and Characterization of Subgroups

In a refined approach to characterize rats according to their individual nicotine-cue interactions, we ran a cluster analysis taking into consideration two input variables, obtained from the first cue omission test and nicotine omission test, as explained in the Methods section. We identified 3 clusters of nicotine individuals that differed in the cluster input variables: Cluster1 (n=18), Cluster2 (n=21) and Cluster 3 (n=12) (**Figure 28**). A table summarizing how each cluster differed in the variables studied in this experiment is provided at the end of the Results section (**Table 2**).

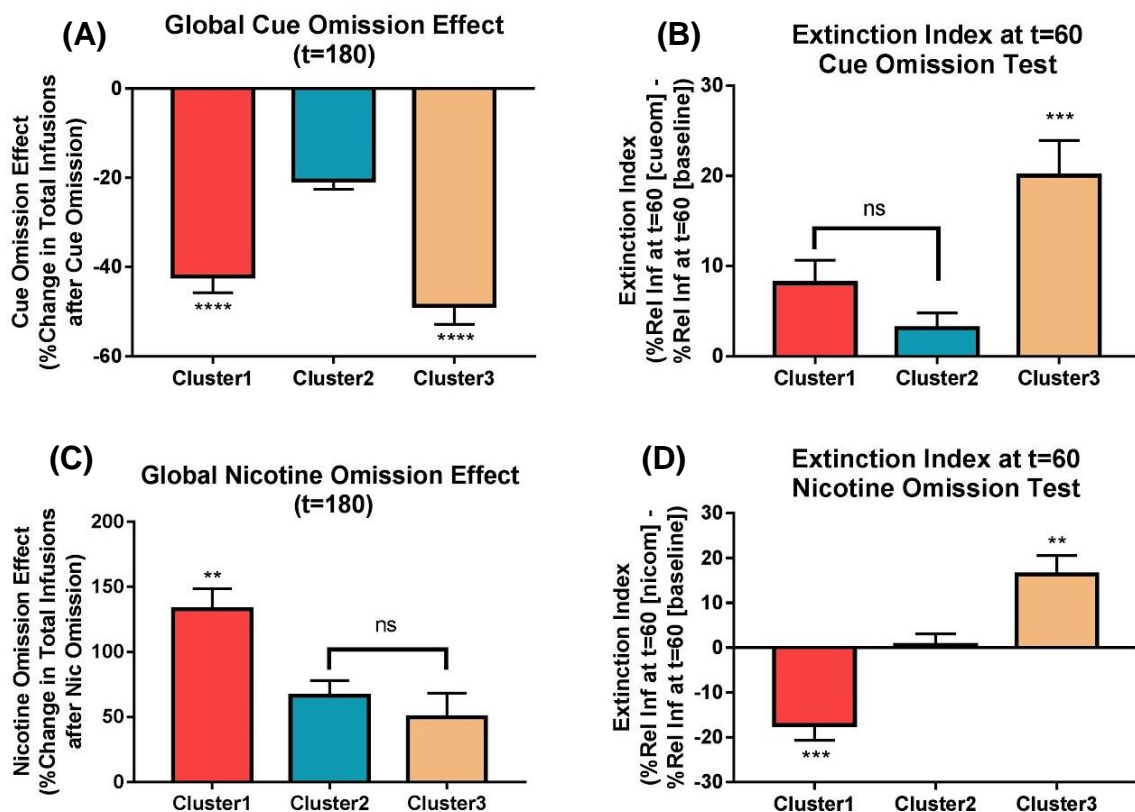


Figure 28 – Differences in Cluster Variables among Identified Clusters in Nicotine Rats
(A) Global Cue Omission Effect. Clusters1 and 3 had the strongest cue omission effect. **(B)** Extinction Index for Cue Omission Test. Cluster 3 had the highest extinction index for the cue omission test. **(C)** Global Nicotine Omission Effect. Cluster 1 had the highest nicotine omission effect. **(D)** Extinction Index for Nicotine Omission Test. Cluster 3 had the highest extinction index, also for the nicotine omission test. Cluster1 had a strongly negative extinction index, suggesting that its pattern of self-infusions during nicotine omission was higher than baseline. Bars represent mean of respective clusters, error bars represent SEM. ****= $p < 0.0001$, ***= $p < 0.001$, **= $p < 0.01$, respective to Cluster2.

Rats in Cluster 1 had a strong drop in self-infusions after cue omission (**Figure 28A**), but did not score high in its associated extinction index (**Figure 28B**). Furthermore, a timeline of cumulative self-infusions of baseline and cue omission test sessions shows a significant difference between both sessions at $t=64\text{m}$ and sustained for the remainder of the session (**Figure 29A**). In terms of nicotine omission, Cluster1 had the highest nicotine omission score of all clusters (**Figure 28C**), but also a negative extinction index profile during nicotine omission (**Figure 28D**). A timeline of cumulative self-infusions shows a very rapid separation between baseline and the nicotine omission test, with statistical significance achieved already at $t=2\text{m}$ and sustained for the rest of the session (**Figure 29B**). Importantly, an analysis on the inter-infusion interval during the middle of the nicotine omission test reveals that rats in Cluster 1 had drastically diminished the spacing between their infusions (**Figure 30**), compared to their baseline, suggesting a much rapid self-administration in the absence of nicotine.

Rats in Cluster 2 reported the weakest effect in both cue and nicotine omission tests (**Figure 28A and C**). In addition, elevations in extinction indices in both cue and nicotine omission were minimal (**Figure 28B and D**). Comparison of cumulative self-infusions show no differences between baseline and cue omission test (**Figure 29A**), but nicotine omission test reveals a significant increase in cumulative self-infusions to its corresponding baseline at $t=4\text{min}$, sustained for the remainder of the session (**Figure 29B**). However, an analysis of the interfusion interval during the mid-session of the nicotine omission test reveals that, at least between $t=60$ and $t=120$, rats in this cluster were self-administering at the same speed as during baseline sessions (**Figure 30**).

Rats in Cluster 3 have the strongest drop after cue omission (**Figure 28A**), accompanied by a drastic increase in its extinction index (**Figure 28B**), indicative that behavior is extinguishing in the absence of the nicotine-associated cue. Significant differences between baseline and cue-omission self-infusions are first evident at $t=91\text{min}$, and sustained for the remainder of the session (**Figure 29A**).

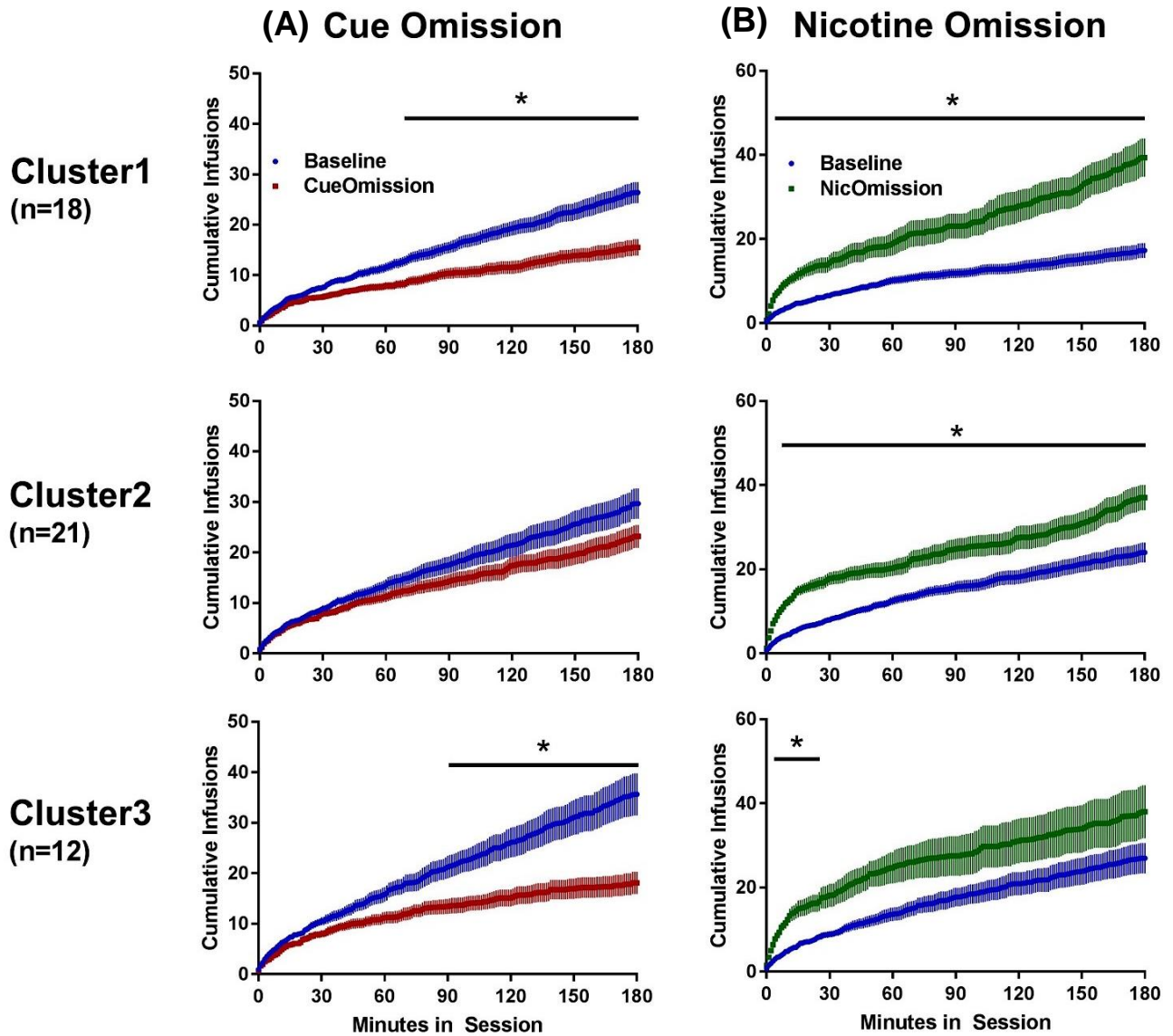


Figure 29 – Profiles of Cue and Nicotine Omission Tests from Identified Clusters - (A)

Effect of Cue Omission on Cumulative Infusions. Cue omission had a global effect of reducing self-infusions with respect to baseline, although clusters differed in the speed of such decrease. For Cluster1, cue omission had the fastest observable effect, with statistical difference reached at 64 minutes into the session, while for Cluster 3, at the 91th minute. The curve of self-infusions in Cluster 2 was not statistically different between baseline and cue omission tests at any point during the session. **(B)** Effect of Nicotine Omission on Cumulative Infusions. For all clusters, a sharp increase in responding was observed at the start of the nicotine omission session. However, statistical difference between cumulative infusions reached during baseline and nicotine omissions, for the remainder of the session, was only maintained in Clusters 1 and 2. Points represent the mean of each cluster, while error bars represented SEM. *= corrected p value of *at least* 0.05, comparing cumulative infusions, between baseline and omission sessions, minute by minute.

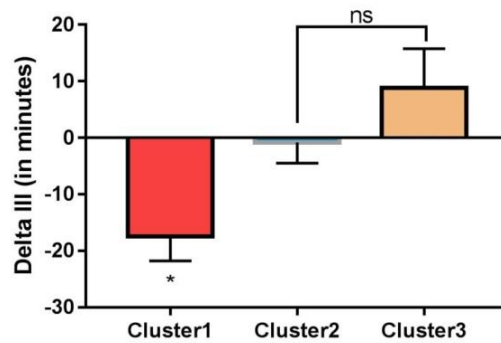


Figure 30 – Differences in Mid-Session Inter-infusion Intervals between Baseline Sessions and Nicotine Omission Test. Inter-infusion intervals (III) represent the spacing between self-infusions during a session of self-administration. Differences in III during the mid-session (between t=60 and t=120) indicates the effect of the omission test of the kinetics of infusion loading after stabilization. Cluster1 reported a substantial decrease in III during the nicotine omission test, indicating that rats had increased the frequency of self-infusions. Cluster3 had an elevation in III, although not statistically different from Cluster2, which had a minimal effect in III. *= $p < 0.05$, respective to Cluster2. Bars represent mean value of each cluster and error bars represent SEM.

In terms of nicotine omission, rats in Cluster3 have a weak global effect (**Figure 28C**), accompanied by an increase in its extinction index (**Figure 28D**), indicative that behavior is extinguishing in the absence of nicotine (**Figure 29B**). The extinction profile, however, is less clear than for cue omission, with only a limited elongation in the spacing between infusions during the mid-session period of the nicotine omission test (**Figure 30**). Interestingly, cumulative infusions between baseline and the nicotine omission test differ significantly only between t=4min and t=25min, after which significance is lost, suggesting that a compensation effort was attempted, but later abandoned (**Figure 29B**).

Differences in Acquisition of Self-Administration

During acquisition, Cluster1 appeared to have had lower active responding than Cluster3 (**Figure 31A**), although the differences are not significant [Cluster effect, $F(2, 48)=1,8159$, $p=ns$; Session effect, $F(11, 528)=13,452$, $p < 0,0001$; Cluster x Session effect, $F(22, 528)=0,85325$, $p=ns$]. Clusters also did not differ in active vs inactive hole discrimination by the end of acquisition [Cluster effect, $F(2, 48)=1,3292$, $p= ns$; Cluster x Hole effect, $F(2, 48)=1,8104$, $p=ns$; Cluster x Hole x Session effect, $F(2, 48)=1,5649$, $p=ns$]. Groups also did not differ in the baseline self-infusions (**Figure 31B**) or in the spacing of infusions in the midsession by the end of acquisition (**Figure 31C**).

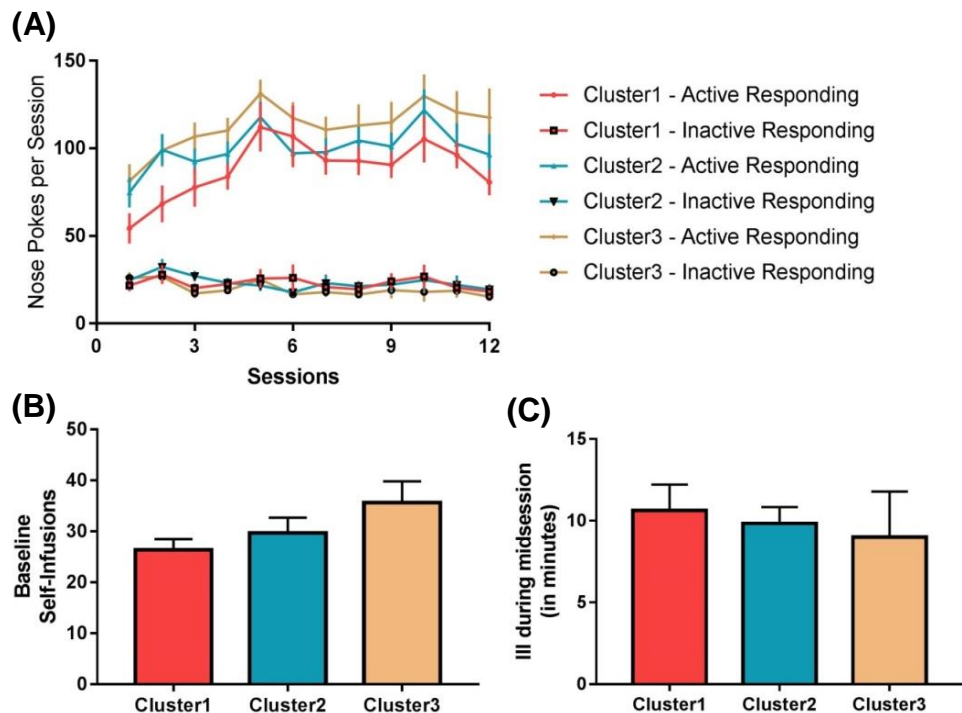


Figure 31 – Differences in Acquisition of Nicotine Self-Administration among Clusters - (A) Acquisition of self-administration (active vs inactive responding). Cluster 1 appears to have a lower acquisition curve, compared to cluster 3, although differences are not significant. **(B)** Baseline self-infusions at the end of acquisition. Cluster 1 appears to have a lower baseline, compared to cluster 3, although differences are not significant. **(C)** Midsession Ill at the end of acquisition. No differences among clusters. Symbols and bars represent mean cluster responses, and error bars represent SEM.

Pavlovian Conditioning and Cue Omission

To assess whether the extent of Pavlovian conditioning to a saccharin-predicting cue could predict the extent of cue omission effects, we ran a correlational analysis between the three parameters obtained from PCA and the individual cue omission score. At the population level, the extent of saccharin conditioning predicted the contribution of the cue in nicotine self-administration (**Figure 32**), although the low coefficient of determination (r^2) from the correlations suggests that the relationships are not strong at the population level.

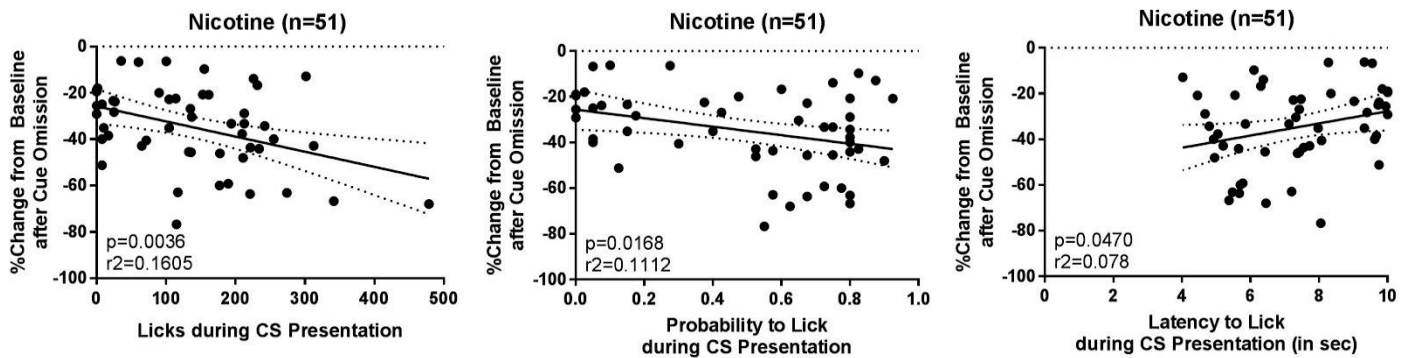


Figure 32– Correlates of Pavlovian Conditioning and Cue Omission – Cue omission scores in nicotine self-administration correlated with amount of licks, probability to licks, and latency to lick during CS presentation in Pavlovian Conditioned Approach. Individual symbols represent nicotine rats.

The correlations between Pavlovian Conditioning parameters and Cue Omission observed in the general population, are only maintained in Cluster1 (**Figure 33**). There are no correlations with any of the other clusters, explaining the low coefficient of determination in the general population, except for a trend between cue omission effect and licks during CS presentation in Cluster3, but which is not maintained with probability or latency to lick.

Response to 50% Reduction in Nicotine Dose

At the population level, in the nicotine group, a sudden reduction of 50% in nicotine dose, from 0,04 to 0,02 mg/kg, resulted in a drastic increase in self-infusions ($p < 0,0001$) with respect to its corresponding baseline (**Figure 34A**). As expected, saline rats did not experience any significant change in their responding on this test day (**Figure 34B**). Individual differences in sensitivity to a change in nicotine dose are also apparent (**Figure 34C**). Interestingly, responding to nicotine omission did not correlate with responding after dose reduction (**Figure 34D**).

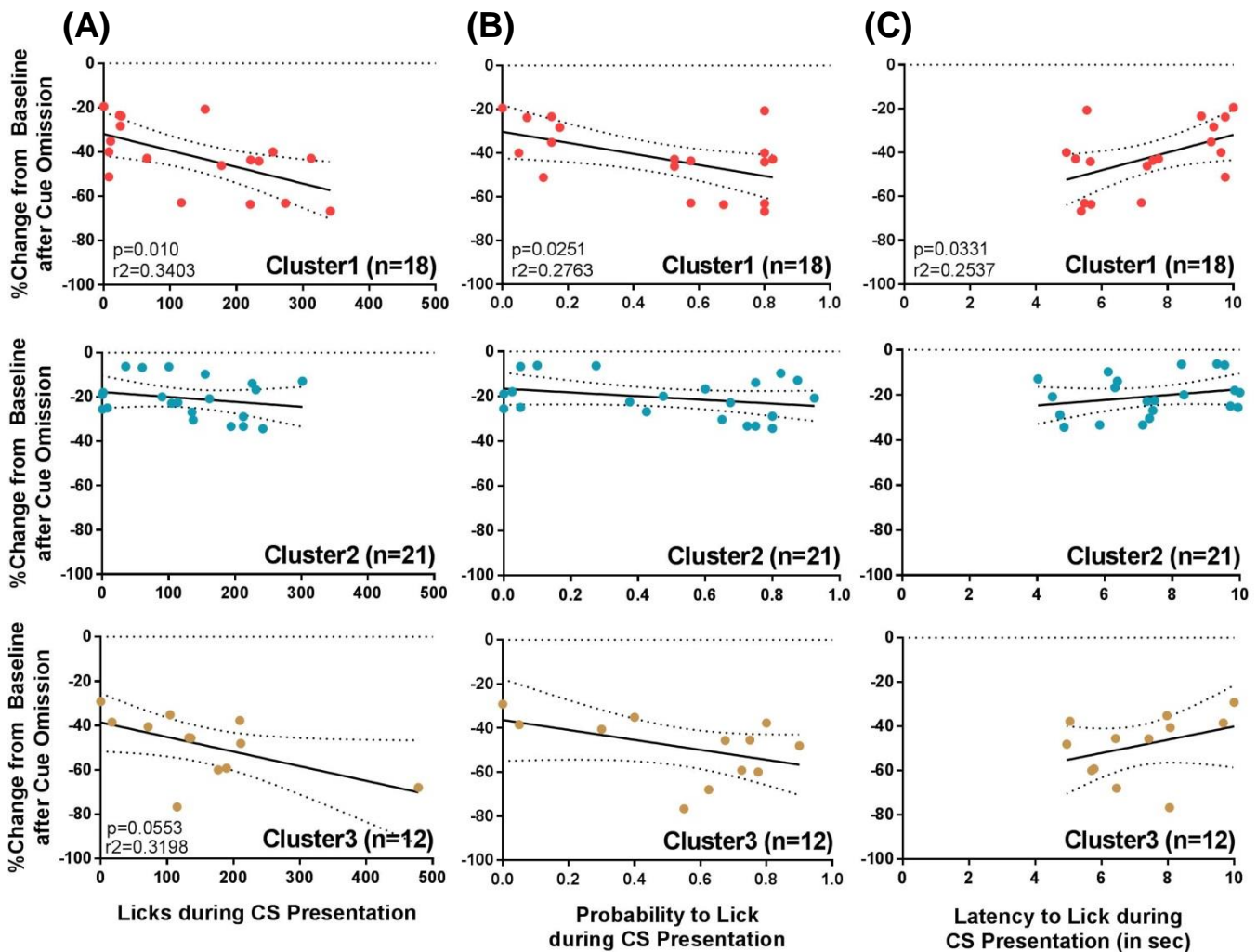


Figure 33 – Differences in Correlation between Pavlovian Conditioned Approach Variables and Cue Omission – Correlations between Cue Omission score and (A) Licks during CS Presentation, (B) Probability to Lick during CS Presentation and (C) Latency to Lick during CS Presentation, per Cluster. Correlations are only evident in Cluster 1. Symbols indicate individual rats.

At the cluster level, rats in Cluster1 reported in mean the highest percent increase in respect to baseline, to an almost complete compensation, although this percent increase was not statistically different from the other clusters (**Figure 35A**). However, when comparing actual infusion numbers between baseline and change in dose, only Clusters 1 and 2 have significant increases in responding, compared to Cluster 3, which reported a weak effect (**Figure 35B**). Furthermore, a minute to minute comparison of cumulative infusions shows a faster elevation of responding in Cluster1, with statistical difference between baseline and change in dose first achieved at t=61m and sustained for the rest of

the session (**Figure 35C**). Cluster3 responding was not statistically different from baseline at any point during the change in dose session (**Figure 35C**).

Interestingly, Cluster1 reports a negative trend in the relationship between cue omission response and change in dose response (**Figure 36**) while Cluster 2 reports the exact opposite trend.

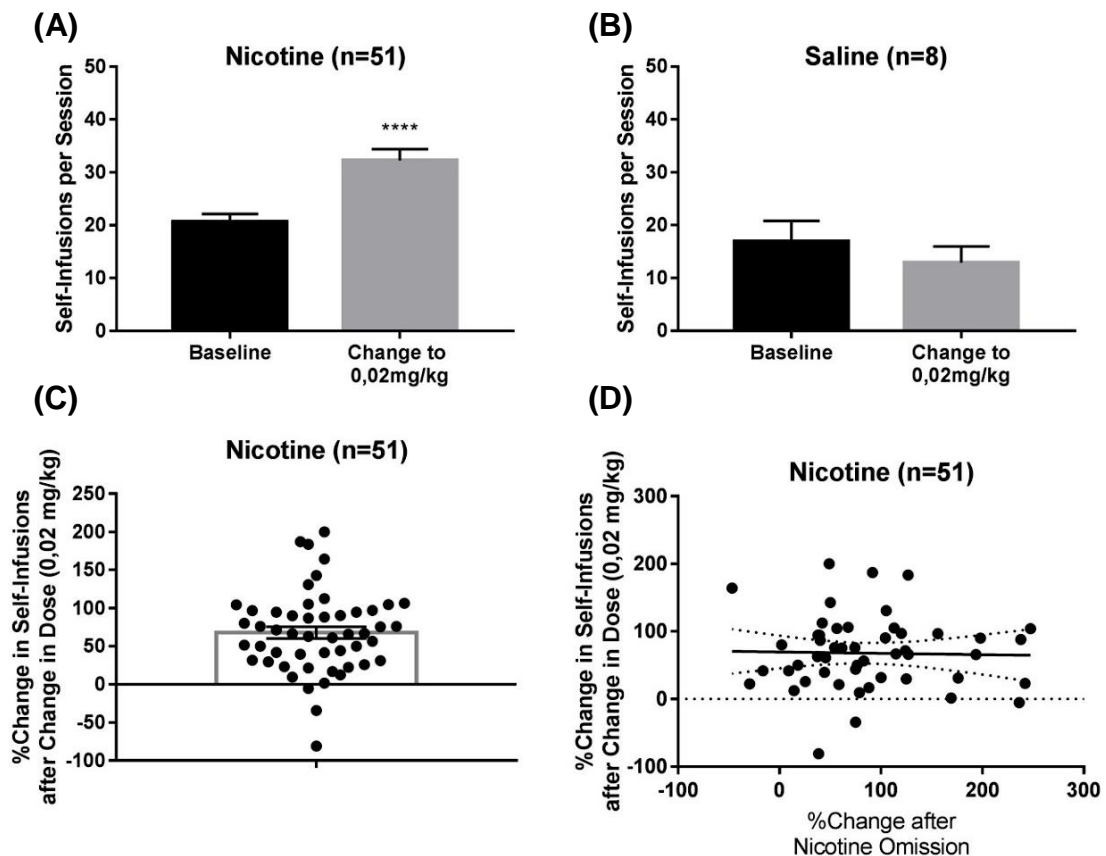


Figure 34 – Effect of a 50% Reduction in Nicotine Dose - (A) In the nicotine group, a switch to 0.02mg/kg resulted in a significant increase in self-infusions. ****= $p < 0.001$, respective to the corresponding baseline. **(B)** In the saline group, no difference in their behavioral response between baseline and test day. **(C)** Amplitude in nicotine omission responses reveals individual differences. **(D)** Extent of nicotine omission did not correlate with extent of compensation after change in dose. Bars represent mean of respective groups, error bars represent SEM, and symbols represent individual rats.

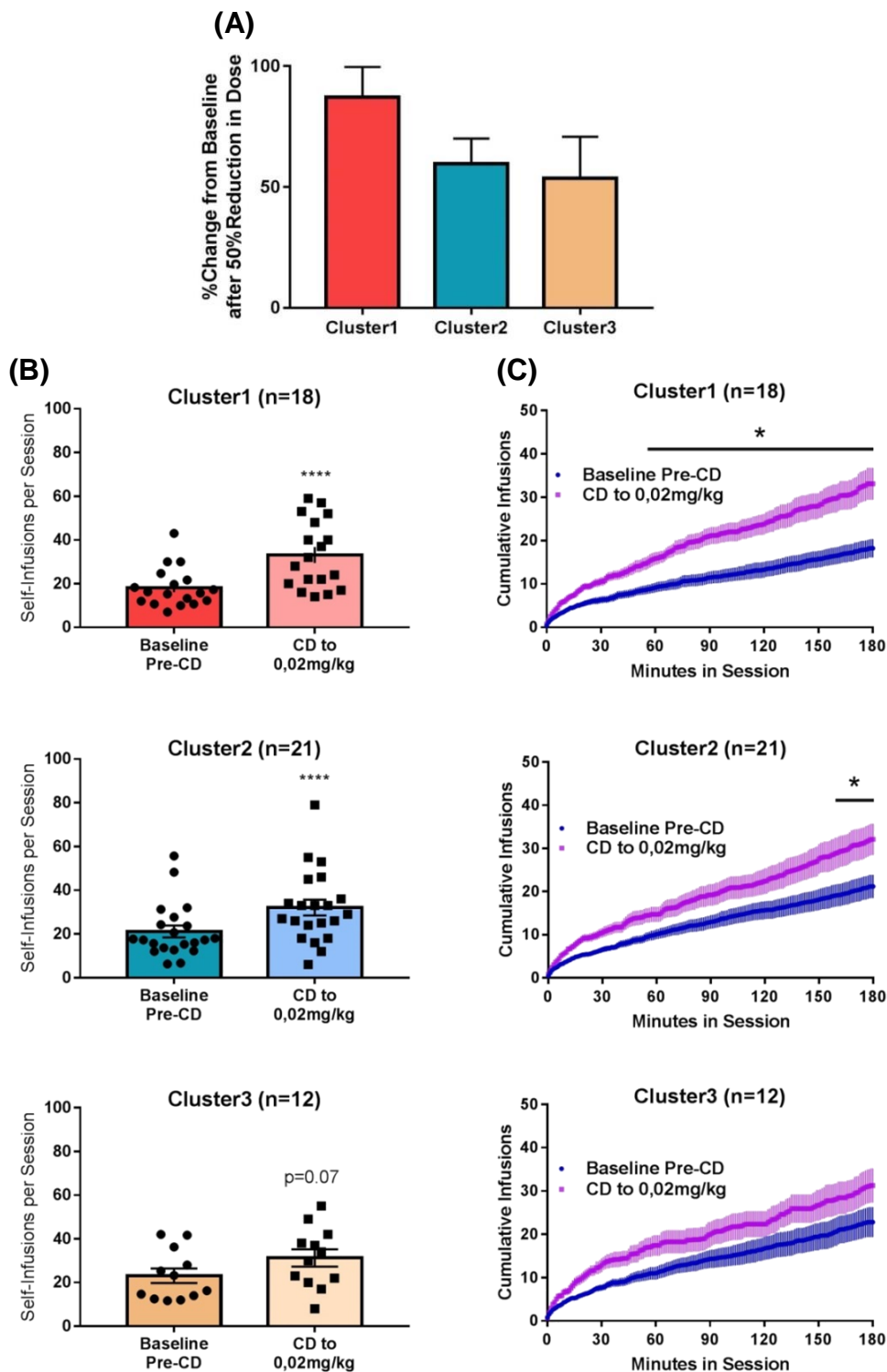


Figure 35 – Differences in Response to Dose Reduction among Clusters – (A)

Differences in percent change in self-infusions after change in dose. Cluster1 reports the highest percent increase, although not significantly different to the other clusters. **(B)** Differences in self-infusions between baseline and change in dose. Only Clusters1 and 2 report significant increases in self-infusions after change in dose. Bars represent mean cluster response, and error bars represent SEM. ****= $p < 0.0001$ respective to baseline. **(C)** Differences in cumulative infusions after change in dose. Cluster1 has the fastest increase in self-infusions after change in dose, with a significant difference to baseline self-infusions evident at $t = 61$ min. For Cluster2, curves are only significantly different at the end of the session. For Cluster3, there are no statistical differences between cumulative infusions. Points represent the mean of each cluster, while error bars represented SEM. *= corrected p value of *at least* 0.05, comparing cumulative infusions, between baseline and change in dose sessions, minute by minute.

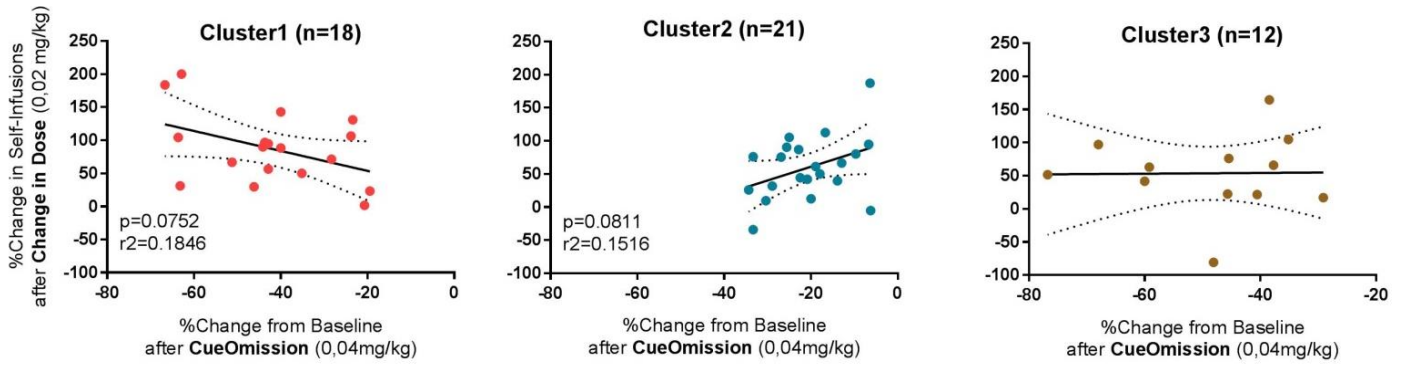


Figure 36 – Correlates between First Cue Omission (0.04mg/kg) and Change in Dose –

Despite not being statistically significant, Clusters 1 and 2 have opposing trends in the relationship between response to cue omission at 0.04mg/kg, and response to change in dose (0.02mg/kg). Symbols represent individual rats.

Differences in Responsiveness to Cue Omission (0,02mg/kg)

A second cue omission test at 0.02mg/kg revealed no differences in the percent decrease in self-infusions with respect to the corresponding baseline (**Figure 37A**). However, when comparing actual infusion numbers between baseline and change in dose, Clusters 1 and 2 have a stronger decrease in infusions (**Figure 37B**). Cluster 3 had a weaker effect (**Figure 37B**). Furthermore, a minute to minute comparison of cumulative infusions shows a faster decrease of responding in Cluster1, with statistical difference between baseline and cue omission test first achieved at t=14m and sustained for the rest of the session (**Figure 37C**). The effect of cue omission on the timeline of self-infusions for Cluster 3 was only significant for the last 45 minutes of the session (**Figure 37C**). Interestingly, the extent of responsiveness to a change in dose (to 0,02mg/kg) correlated with cue omission at 0,02mg/kg, but only for Cluster 3 (**Figure 38**), suggesting that in this cluster, sensitivity to the change in dose predicted the role of the cue in the self-administration at 0,02mg/kg.

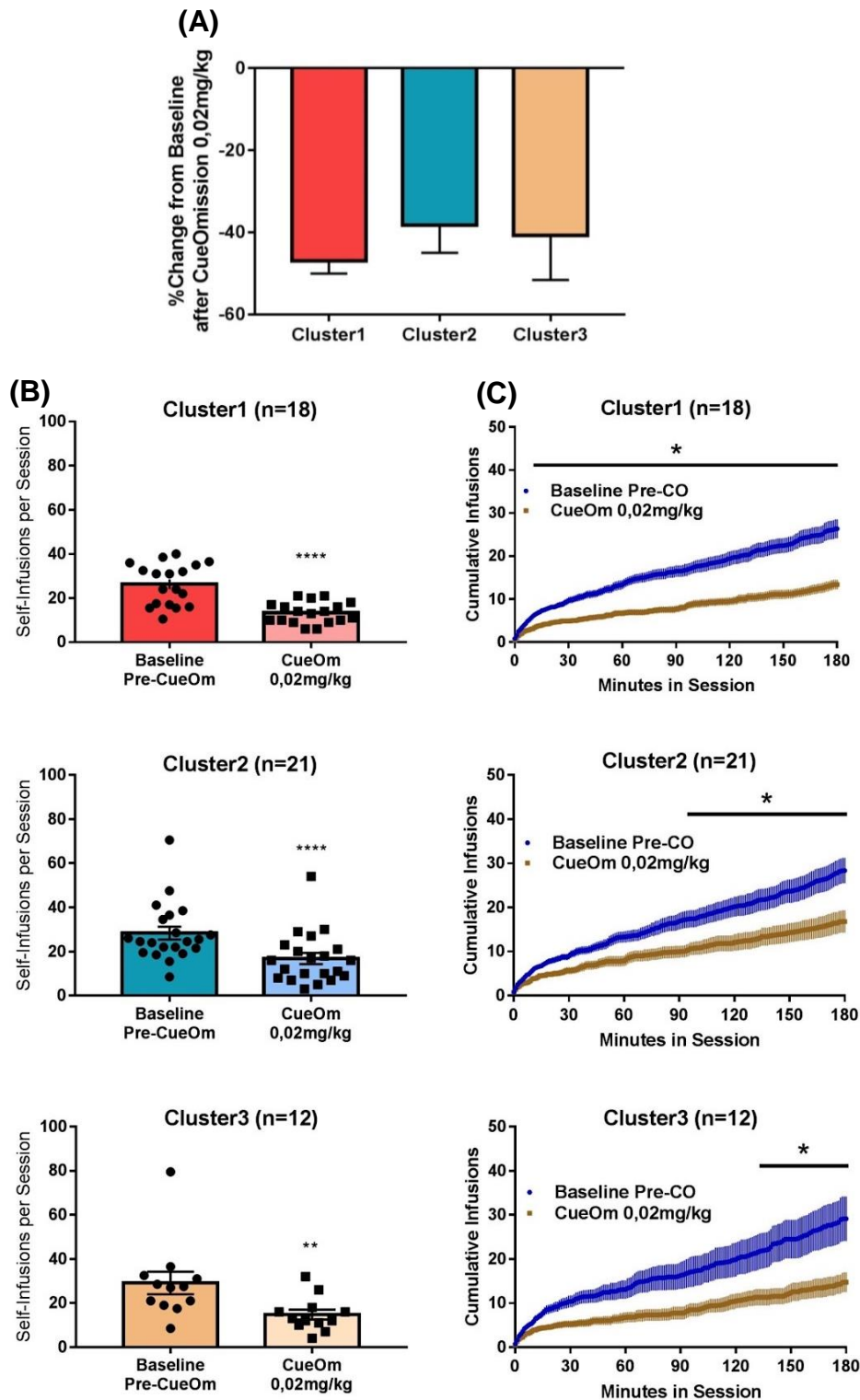


Figure 37 – Differences in Response to Cue Omission at 0,02mg/kg per Cluster - (A)

Differences in percent change in self-infusions after cue omission (0.02mg/kg). No differences among

clusters. **(B)** Differences in self-infusions between baseline and cue omission. All clusters report significant decreases in self-infusions, although Cluster3 had the weakest decrease. Bars represent mean cluster response, and error bars represent SEM. ****= $p < 0.0001$ relative to baseline.

(C) Differences in cumulative infusions after cue omission. Cluster1 has the fastest decrease in self-infusions after cue omission, with a significant difference to baseline self-infusions evident at $t=14$ min. For Cluster2, curves are only significantly different at $t=90$ min, and for Cluster3, at $t=135$ min. Points represent the mean of each cluster, while error bars represented SEM. *= corrected p value of *at least* 0.05, comparing cumulative infusions, between baseline and change in dose sessions, minute by minute.

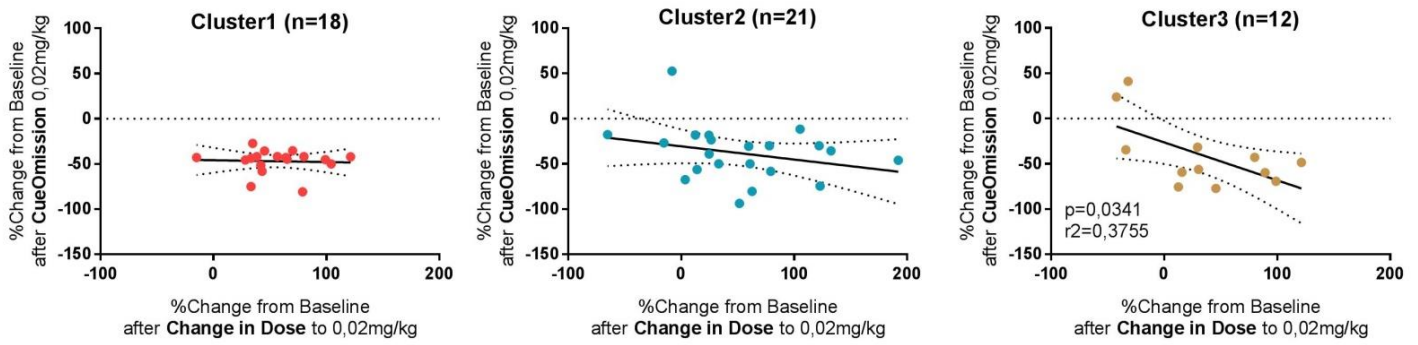


Figure 38 – Correlates between Change in Dose and Cue Omission (0,02mg/kg) – The behavioral response to change in dose to 0.02mg/kg was correlated to cue omission at that dose, but only for Cluster3. Symbols represent individual nicotine rats.

Nicotine Metabolism

At the population level, plasma cotinine levels were positively correlated with baseline infusions at the end of the acquisition period (**Figure 39A**), consistent with observations in Experiment 1. However, plasma cotinine was not correlated with percent change in infusions after cue-omission (**Figure 39B**), compared to Experiment 1.

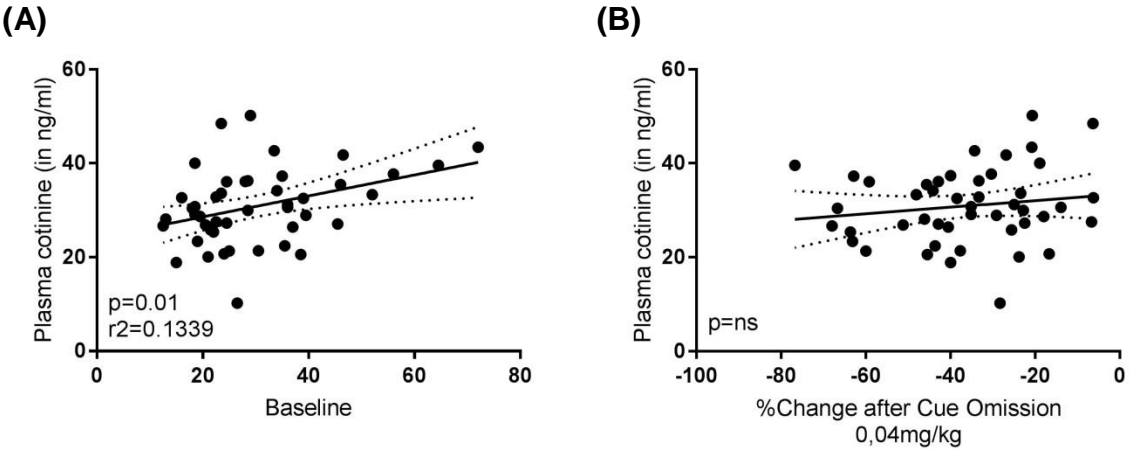


Figure 39 – Correlates of Nicotine Metabolism – (A) Plasma cotinine levels correlated positively with baseline infusions at the end of the acquisition period. (B) Plasma cotinine levels did not correlate with cue omission effect at 0.04 mg/kg. Symbols represent individual nicotine rats, solid black lines represent linear regression lines, and dotted curves represent the 95% confidence bands.

The aforementioned correlation between baseline infusions and plasma cotinine is not seen in the individual isolated clusters (**Figure 40**). Given the previous data obtained in Experiment 1, in which nicotine metabolism appeared to predict the first baseline in the group with stronger cue involvement in their self-administration, it remained a possibility that in our current clustering we are failing to capture that dimension, in isolation from nicotine responsiveness.

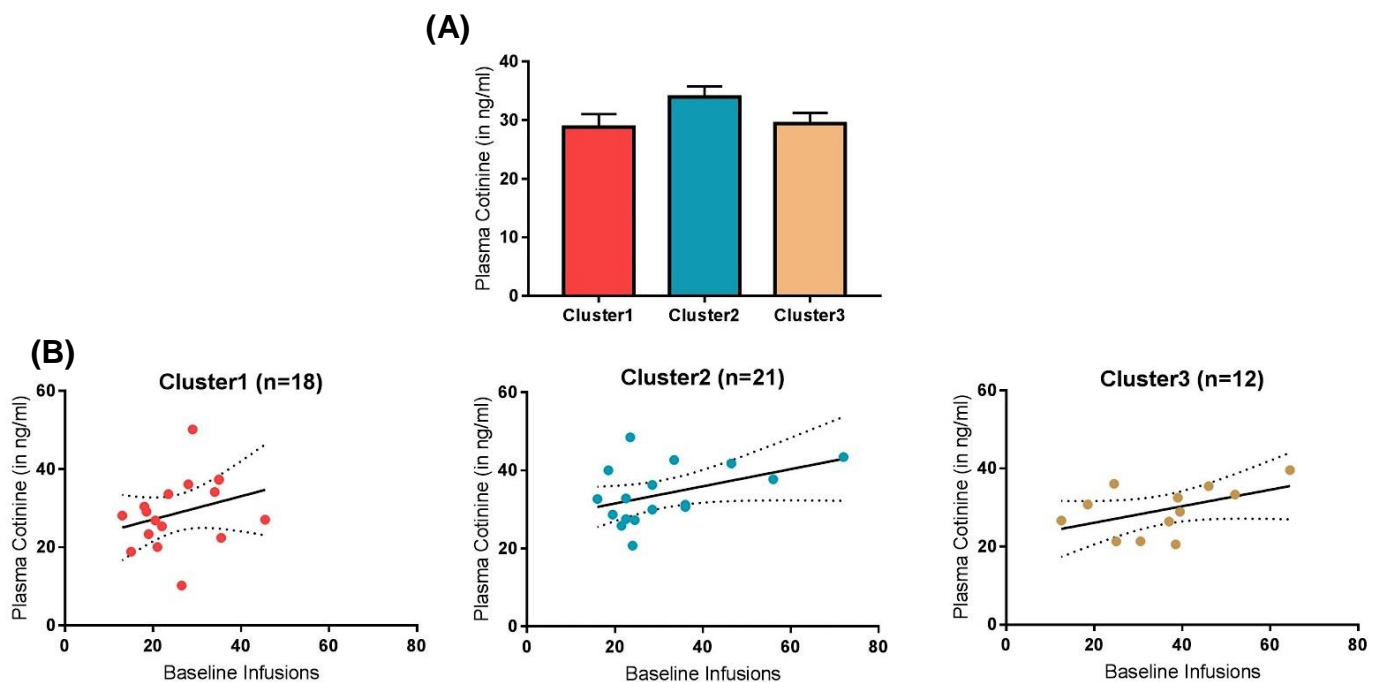


Figure 40 – Relationship between Nicotine Metabolism and Baseline Infusions per Cluster – (A) Comparison of plasma cotinine levels per cluster. There were no statistical differences between clusters. Bars represent mean cluster values, and error bars represent SEM. **(B)** Correlations between nicotine metabolism and baseline infusions after acquisition. Despite a correlation at the population level, no correlation at the individual cluster level. Symbols represent individual nicotine rats.

In order to assess this possibility, we ran a secondary clustering, in which all 51 nicotine rats were divided only based on their cue omission variables (global cue omission effect, and extinction index at $t=60$). We obtained two clusters (**Figure 41A**), a ClusterA ($n=24$), with the highest cue omission effect, and Cluster B ($n=27$), with the lowest cue omission effect. Notably, the positive correlation between baseline infusions and cotinine plasma levels were only observed in those rats with the highest cue contribution in their self-administration (**Figure 41C**).

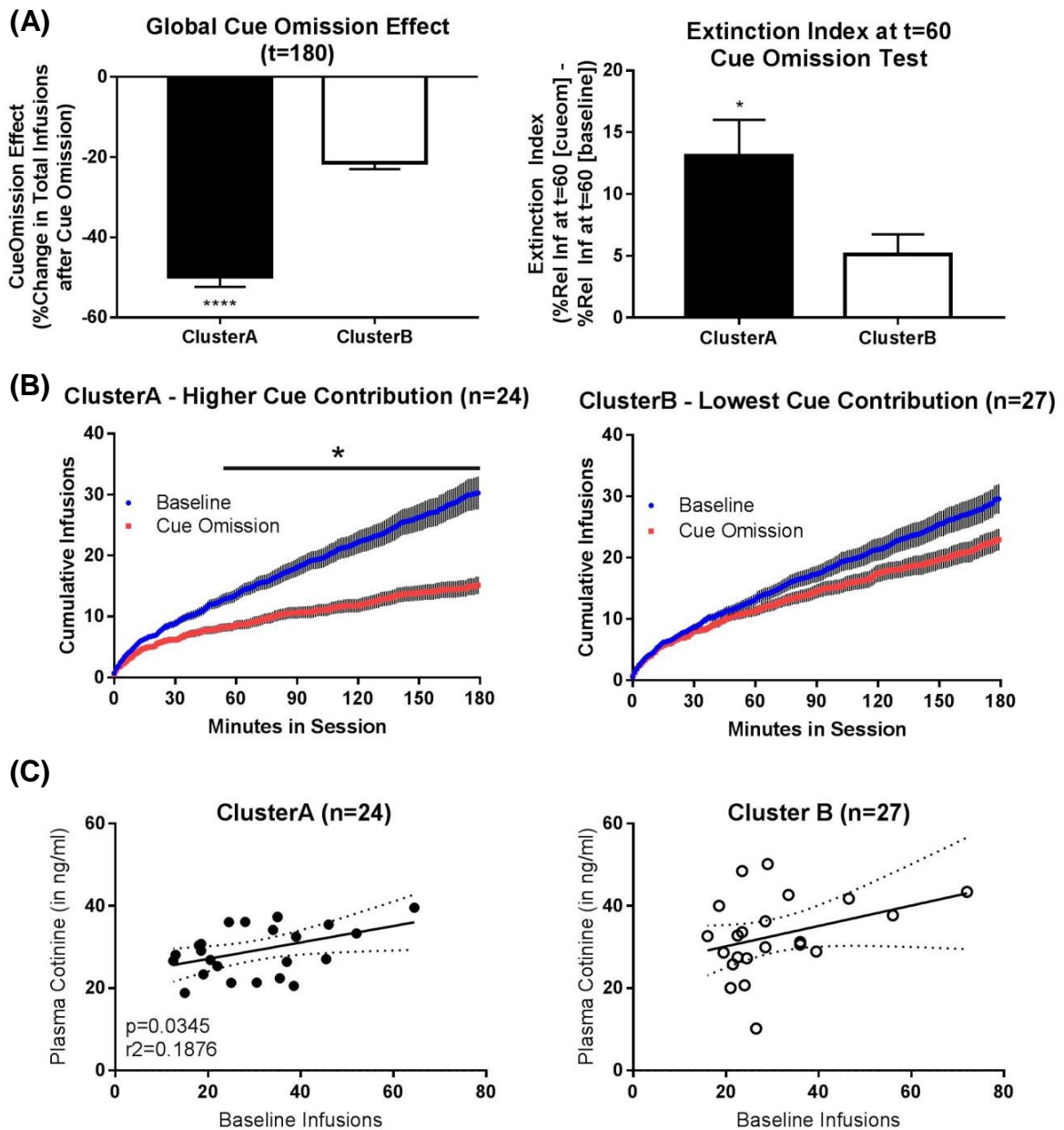


Figure 41 – Nicotine Metabolism predicts Baseline Nicotine Infusions, but only in Rats with Strong Cue Contribution – (A) Cluster analysis based only on Cue Omission parameters reveals two subpopulations: Cluster A, with the highest cue omission effect and highest extinction index at t=60, and Cluster B, with the lowest cue omission effect and lowest extinction index. Bars represent mean values per cluster and error bars represent SEM. ****= $p<0.0001$; *= $p<0.05$, respective to Cluster B. **(B)** Effect of Cue Omission on cumulative self-infusions. For Cluster A, the cue omission effect occurs fast, with significant differences in self-infusions at t=55min. No statistical differences in cumulative self-infusions for Cluster B. Points represent the mean of each cluster, while error bars represent SEM. * = corrected p value of *at least* 0.05, comparing cumulative infusions, between baseline and change in dose sessions, minute by minute. **(C)** Correlation between baseline infusions and plasma cotinine. Correlation is only evident in Cluster A. Symbols represent individual rats.

Gene Expression

General Effects

From all brains collected for gene expression (n=59), those of 22 nicotine and 3 saline rats were randomly selected for a first screening of gene expression. Due to time constraints, only the results of this first screening are shown.

At the population level, nicotine self-administration produced an upregulation of $\beta 2$ nAChR expression in both shell and core of the NAcc, although with a much more pronounced effect in the core (**Figure 42A**). In the shNAcc, the $\alpha 4$ nAChR was strongly upregulated, followed by $\alpha 7$ nAChR and D3 dopamine receptor. Expression of genes of interest in the BLA were all downregulated compared to saline controls. Gene expression at the mHb of genes of interest - $\alpha 5$ and $\beta 4$ nAChRs, involved in nicotine aversion- was not altered at the population level (**Figure 42A**).

At the cluster level, from all brains collected for gene expression (n=59), those of 22 nicotine and 3 saline rats were randomly selected for a first screening of gene expression. From these 22 nicotine brains, 9 belonged to Cluster 1, 8 belonged to Cluster2 and 5 belonged to Cluster 3. Expression of the housekeeping gene *Eef1a1* did not differ between clusters (data not shown). Cluster1 appeared to have the the highest upregulation of $\beta 2$ nAChR in the cNAcc, and $\alpha 4$ nAChR in the shNAcc, although not statistically significant (**Figure 42B**). However, expression of $\alpha 4$ nAChR in the BLA was the lowest in Cluster 1. Cluster 2 and Cluster 3 appear to have some downregulation of $\beta 4$ nAChR in the mHb compared to saline, although the trend is not statistically significant.

Comparison of these results with the DCts (**Figure 42C**) suggests that the observed effects, in particular the decreased expression of $\alpha 4$ nAChR in the BLA, are not due to a bias introduced by comparison to saline rats.

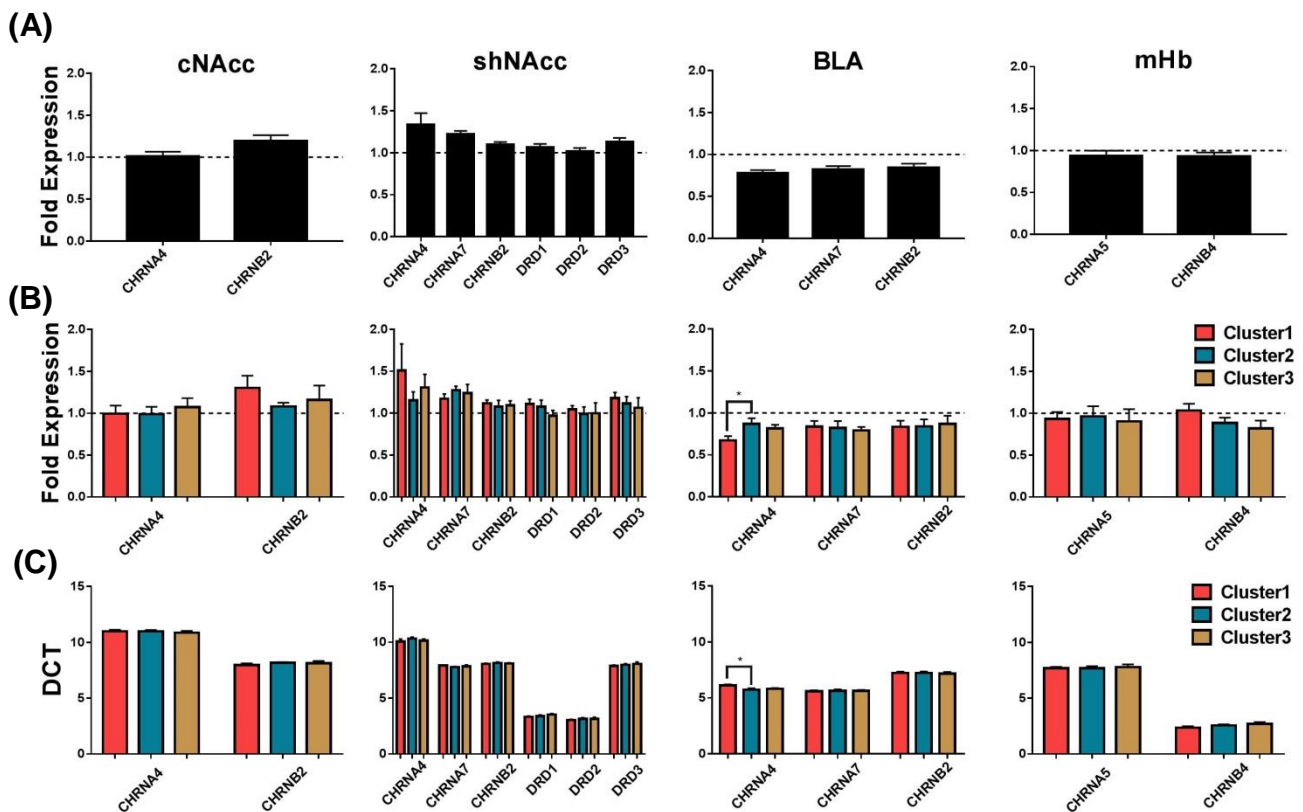


Figure 42 – Differences in Gene Expression in Brain Areas Related to Nicotine

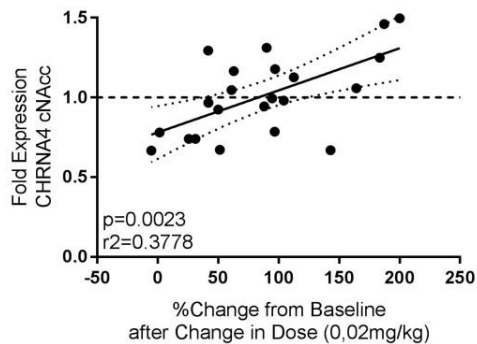
Reinforcement, Nicotine-Cue Interactions and Nicotine Aversion – (A) Fold expression of selected nAChR subunits, as well as dopamine receptors, in the core and shell of the nucleus accumbens (cNAcc and shNAcc), the basolateral amygdala (BLA) and the medial habenula (mHb) of nicotine rats (n=22). Nicotine treatment upregulated $\beta 2$ nAChR in the cNAcc, as well as $\alpha 4$ nAChR in the shNAcc, while decreasing gene expression of $\alpha 4$, $\alpha 7$ and $\beta 2$ nAChR in the BLA. **(B)** Fold expression of selected genes, per subcluster. Cluster1 had a trend of strong upregulation of $\beta 2$ nAChR in the cNAcc, as well as $\alpha 4$ nAChR in the shNAcc, while having the least gene expression of $\alpha 4$ nAChR in the BLA. **(C)** Same data as above, but expressed as Delta CTs prior to conversion to Fold Expression by comparison to saline rats. Bars represent mean responses and error bars represent SEM. $*=p<0.05$.

Correlates of Gene Expression and Behavioral Variables

(1) cNAcc

When comparing gene expression with nicotine self-administration, response to a 50% reduction in dose was positively correlated with fold expression of $\alpha 4$ nAChR expression in the cNAcc (**Figure 43A**). Cluster2 appears to be its major contributor (**Figure 43B**).

(A)



(B)

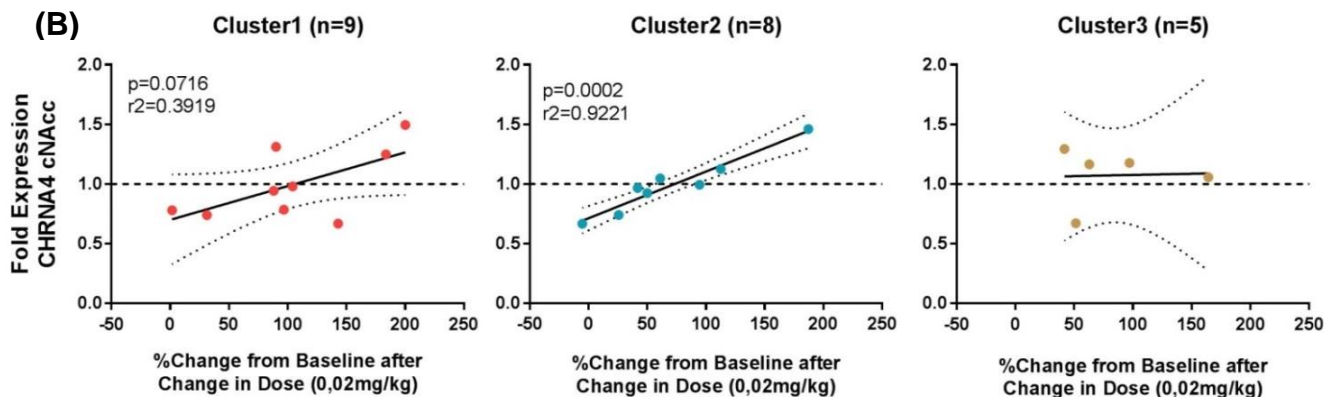


Figure 43 – Correlates between Gene Expression in the cNAcc and Nicotine Self-

Administration – (A) At the population level, change in responding after change in dose to 0.02mg/kg was positively correlated to fold expression of $\alpha 4$ nAChR in the cNAcc **(B)** At the cluster level, only Cluster2 maintained the correlation seen at the population level. Symbols represent individual rats.

(2) shNAcc

Response to a change in dose was correlated negatively to fold expression of $\alpha 7$ nAChR in the shNAcc at the population level **(Figure 44A)**. At the cluster level, this correlation was maintained in Clusters 1 and 2, but not in Cluster 3 **(Figure 44B)**. Interestingly, and despite no correlation at the population level **(Figure 44A)**, Cluster2 shows a negative trend between fold expression of dopamine D2 receptor and response to the cue omission test **(Figure 44B)**.

(3) BLA

Neither response to change in dose nor to cue omission was correlated with $\alpha 4$ nAChR expression in the BLA at the population level **(Figure 45A)**. However, at the cluster level, cluster 2 and 3 showed positive correlations between $\alpha 4$ nAChR expression and change in dose **(Figure 45B)**. Furthermore, Cluster 3 shows a positive correlation between fold expression of $\alpha 4$ nAChR and response to cue omission **(Figure 45C)**

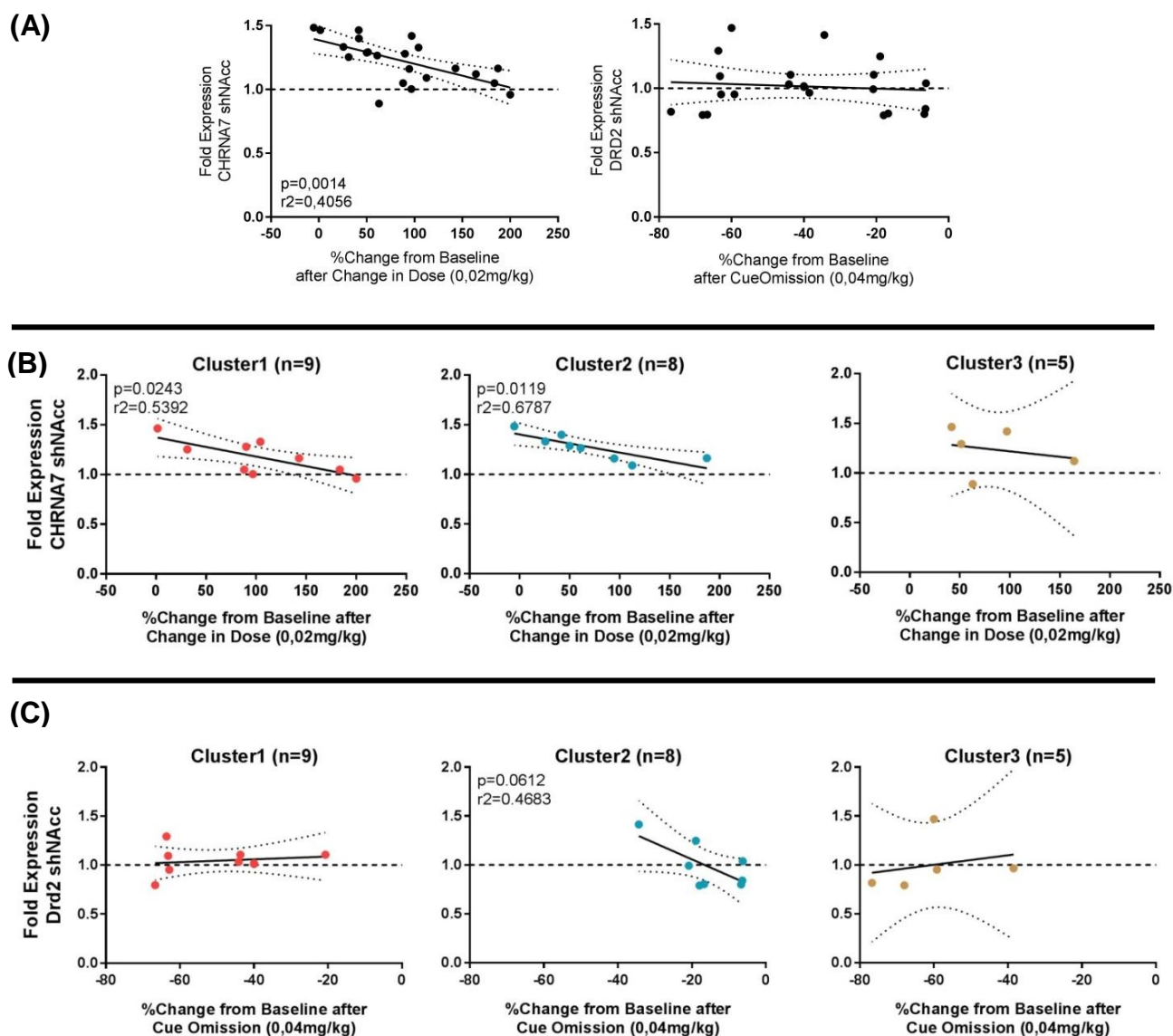


Figure 44 – Correlates between Gene Expression in the shNAcc and Nicotine Self-

Administration – (A) At the population level, change in responding after change in dose to 0.02mg/kg was negatively correlated to fold expression of $\alpha 7$ nAChR in the shNAcc; Response to cue omission at 0.04mg/kg was not correlated to fold expression of dopamine D2 in the shNAcc. **(B)** At the cluster level, only Cluster3 did not maintain the correlation between change in dose and $\alpha 7$ nAChR expression seen at the population level. **(C)** Cluster 2 reports a negative trend between cue omission response and expression of dopamine D2 receptor in the shNAcc. Symbols represent individual rats.

Response to change in dose was correlated with both $\alpha 4$ and $\beta 2$ nAChR expression in the BLA at the population level (**Figure 46A**). These relationships were only retained by Cluster 2 (**Figure 46-B-C**), while Cluster 3 only showed the correlation between change in dose and $\beta 2$ nAChR expression (**Figure 46C**). Cluster 1 reported none of these correlations.

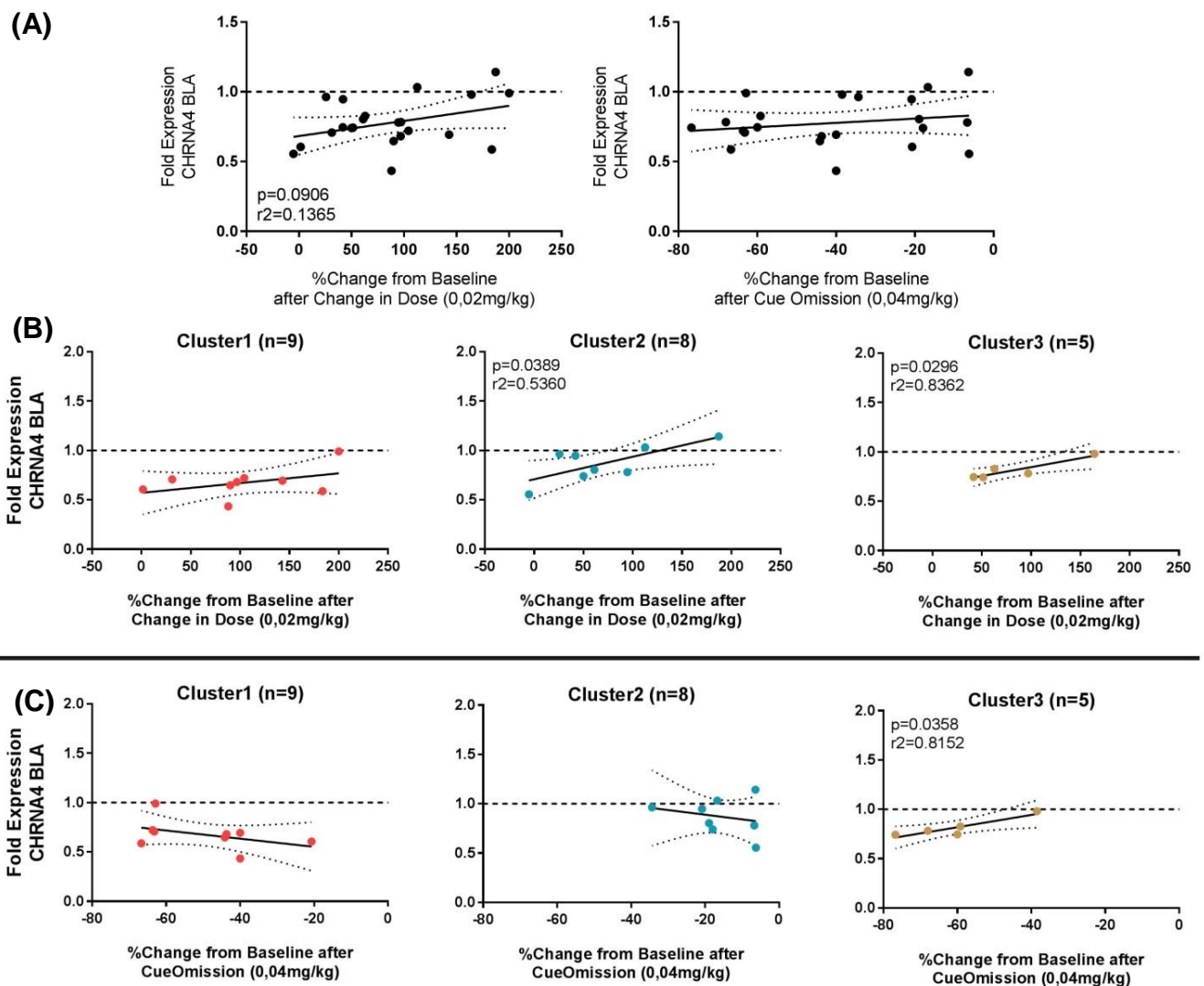


Figure 45 – Correlates between Gene Expression of $\alpha 4$ -nAChR in BLA and Nicotine Self-Administration - (A) At the population level, neither change in responding after change in dose to 0.02mg/kg nor response to cue omission at 0.04mg/kg were correlated to fold expression of $\alpha 4$ nAChR in the BLA. **(B)** At the cluster level, Clusters 2 and 3 had a positive correlation between change in dose and expression of $\alpha 4$ nAChR. **(C)** Cluster 3 had also a positive correlation between response to cue omission and expression of $\alpha 4$ nAChR. Symbols represent individual rats.

(4) mHb

Response to a nicotine omission test was correlated positively to fold expression of $\beta 4$ nAChR in the mHb at the population level (**Figure 47A**). At the cluster level, only Cluster1 maintained the same level of correlation (**Figure 47B**). Interestingly, and despite the lack of correlation at the population level, Cluster 1 showed a negative correlation between $\beta 4$ nAChR and response to change in dose (**Figure 47C**).

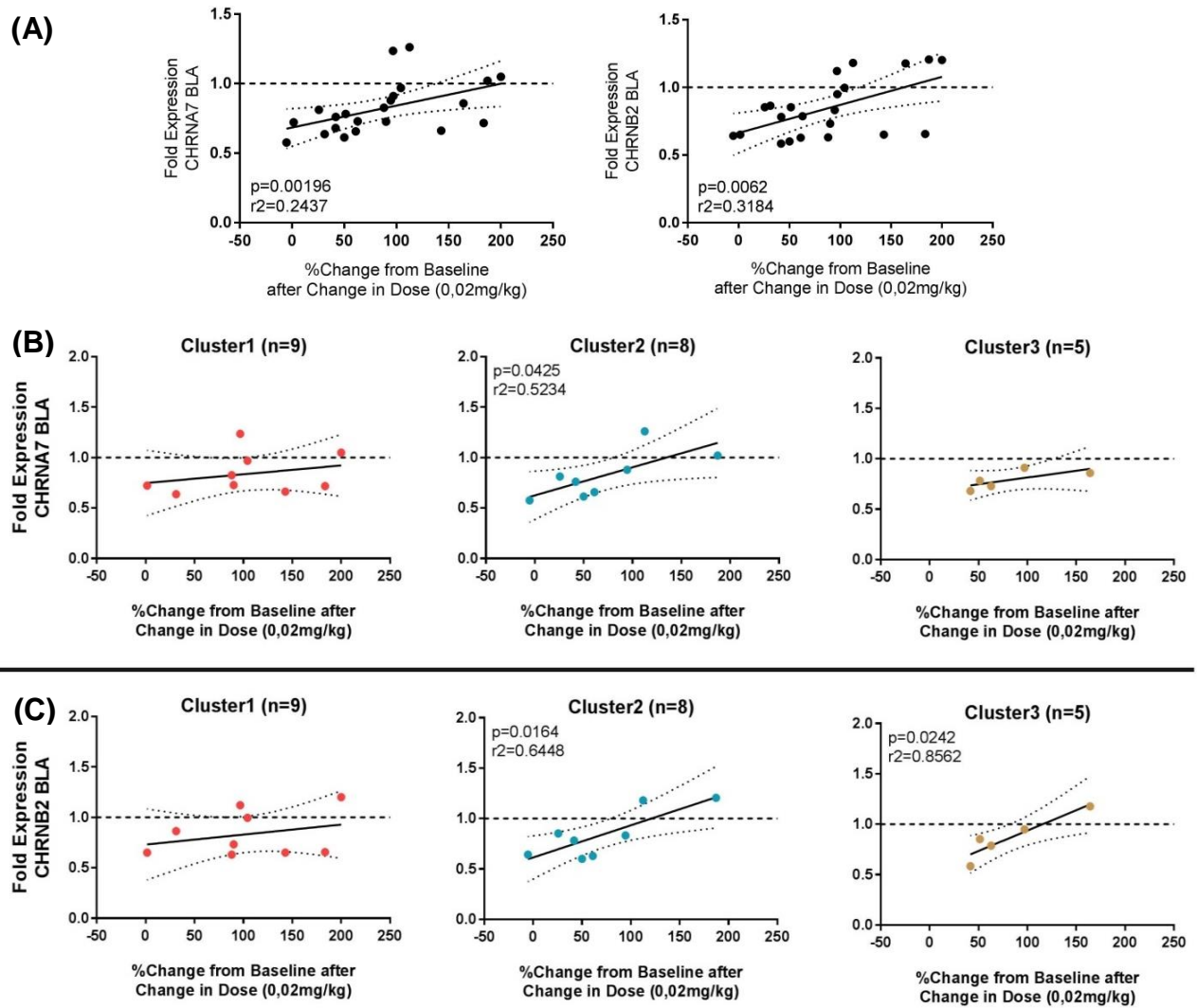


Figure 46 – Correlates between Gene Expression of $\alpha 7$ - and $\beta 2$ -nAChR in BLA and Nicotine Self-Administration - (A) At the population level, responding after change in dose correlated positively with both $\alpha 7$ and $\beta 2$ nAChR expression in the BLA. **(B)** At the cluster level, only Clusters 2 retained the correlation between $\alpha 7$ nAChR and change in dose observed at the population level. **(C)** Only Cluster 1 did not show the positive correlation between response to change in dose and expression of $\beta 2$ nAChR. Symbols represent individual rats.

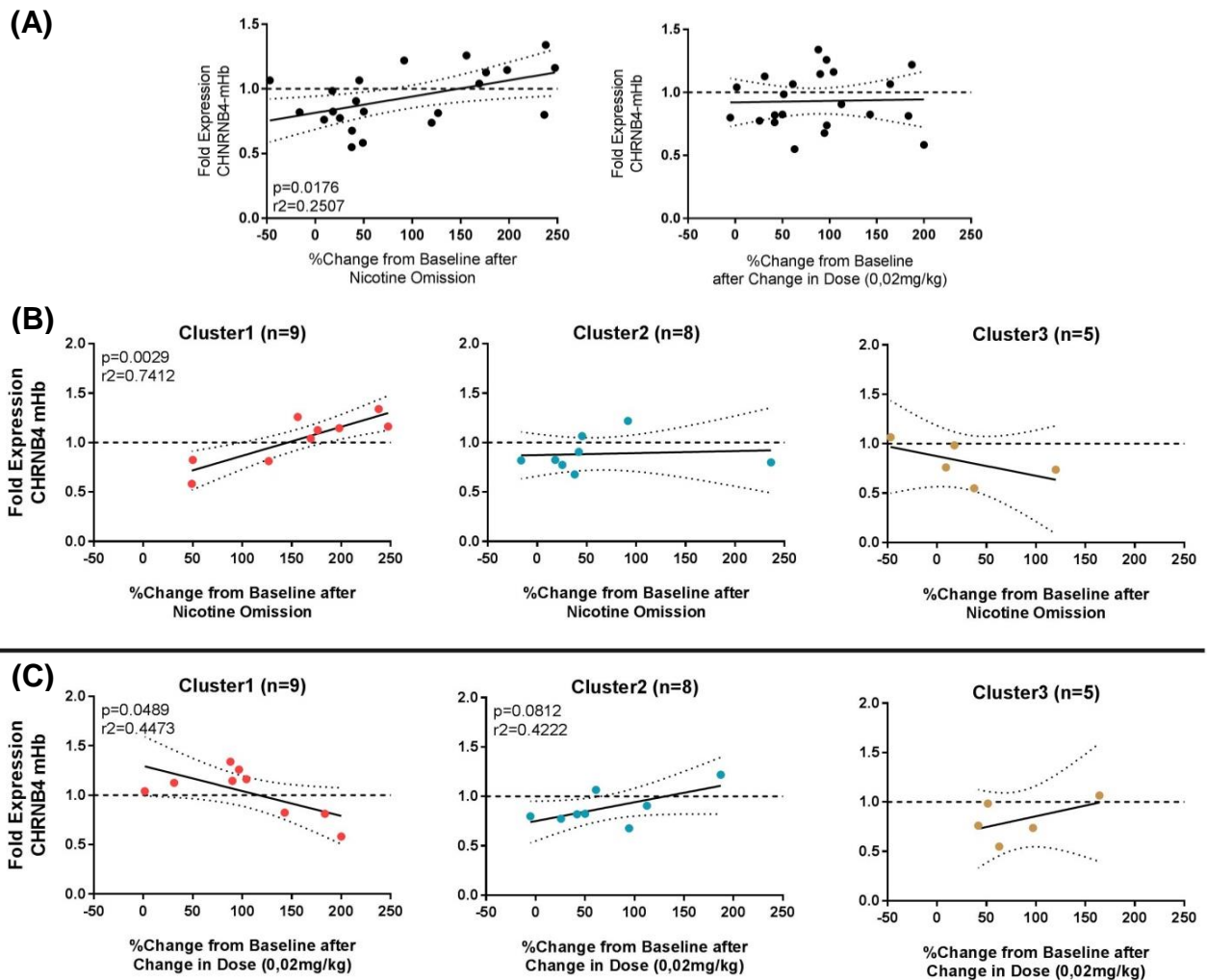


Figure 47 – Correlates between Gene Expression of β 4-nAChR in mHb and Nicotine Self-Administration - (A) At the population level, responding after nicotine omission correlated positively with both β 4 nAChR expression in the mHb. No correlations were observed between response to change in dose and β 4 nAChR expression **(B)** At the cluster level, only Clusters 1 retained the correlation between β 4 nAChR and nicotine omission observed at the population level. **(C)** Despite no correlations at the population level, Cluster 1 did not show negative correlation between β 4 nAChR and response to change in dose. Symbols represent individual rats.

Summary

To provide a global picture of the behavioral, metabolic and neurobiological differences per cluster, Table 2 summarizes their distinct profiles.

Characteristic	Cluster1 (n=18)	Cluster2 (n=21)	Cluster3 (n=12)
Cue Omission	Strong cue omission effect. Cue is needed for SA.	Weakest cue omission effect. Nicotine alone can drive most SA without cue.	Strongest cue omission effect, extinction profile. Cue is needed for SA.
Nicotine Omission	Strongest nicotine omission effect. Omission of nicotine elevates SA. Cue is needed for SA.	Weak nicotine omission, pattern of self-administration is similar to baseline. Cue alone can drive most SA without nicotine.	Weak nicotine omission effect, sharp early rise in infusions, but SA approaches an extinction profile. Nicotine is needed for SA.
Pavlovian Conditioning vs Cue Omission	Correlated, suggesting sensitivity to reward-paired visual cues.	No correlations	No correlations
Acquisition and Baseline Infusions	Slightly lower acquisition and baseline infusions, but not statistically different.	No differences	Slightly higher acquisition and baseline infusions, but not statistically different.
Change in Dose	Strongest, and fastest, increase in cumulative infusions after change in dose. The most sensitive cluster.	Increase in cumulative infusions after change in dose.	Weakest increase in cumulative infusions after change in dose. Least sensitive cluster.
Cue Omission (0,02 mg/kg)	Strongest, and fastest, decrease in cumulative infusions after cue omission at new dose.	Decrease in cumulative infusions after cue omission at new dose	Weakest decrease in cumulative infusions after cue omission at new dose.
Change in Dose vs Cue Omission (0,02 mg/kg)	No correlations	No correlations	Correlated, suggesting that cue experience is related to sensitivity to change in dose
Nicotine Metabolism	No differences	No differences	No differences
Gene Expression - cNAcc	$\alpha 4$ nAChR – trend positively with change in dose response (not significant)	$\alpha 4$ nAChR – correlated positively with change in dose response	No correlations
Gene Expression - shNAcc	$\alpha 7$ nAChR - correlated negatively with change in dose response	$\alpha 7$ nAChR - correlated negatively with change in dose response D2 dopamine - negative trend with cue omission effect	No correlations
Gene Expression - BLA	Lowest $\alpha 4$ nAChR in the BLA. No correlations	$\alpha 4$, $\beta 2$ and $\alpha 7$ nAChR – correlated positively with change in dose response	$\alpha 4$ and $\beta 2$ nAChR – correlated positively with change in dose response $\alpha 4$ nAChR – correlated positively with cue omission
Gene Expression - mHb	$\beta 4$ nAChR – correlated positively with nicotine omission, but negatively with change in dose response	No correlations.	No correlations.

Table 2 – Summary of Behavioral, Metabolic, and Neurobiological Correlates of Identified Clusters.

Discussion

Refined Approach at Identifying Individual Differences in the Mechanisms of Nicotine Seeking

In this second experiment, we sought to refine our experimental approach by improving our classification criteria, as well as expanding the external variables that could help validate the behavioral phenotypes identified. Through the combination of (1) adding a nicotine omission test that would allow direct comparison to cue omission test, (2) developing an ‘extinction index’ parameter, that would give us a temporal dimension of the omission effects and (3) using a clustering method widely used to identify individual differences (Newby and Tucker, 2004) without arbitrary thresholds, we evidenced three clusters of rats, which differed in the contributions of nicotine, cue, or their combination, in their nicotine self-administration, with external correlates that could confirm their behavioral phenotype.

Cluster1 – Strong Cue Sensitivity despite Nicotine Aversion Sensitivity

Rats in Cluster1 appeared to combine a strong sensitivity to the reinforcing effects of the visual cue, with sensitivity to the aversive effects of nicotine.

The strong involvement of the cue in the nicotine self-administration of Cluster 1 is evidenced by the strong cue omission response at the training dose of 0.04 mg/kg, with a sharp and sustained decrease in self-infusions (**Figure 29**), which was part of its defining criteria. Despite that the global effect of the cue-omission at 0.02mg/kg was not different from other groups (**Figure 37A**), a within-session analysis reveals that Cluster 1 had the most sustained reduction in self-infusions respective to its baseline, compared to the other clusters (**Figure 37C**).

Interestingly, Cluster1 was the only subgroup in which the cue omission effect at 0.04mg/kg was correlated with all variables obtained from Pavlovian Conditioned Approach (**Figure 33A-C**). This is noteworthy, as it reflects that the extent of conditioning to a saccharin-predicting cue (how many licks, how fast, and how likely) predicted the contributions of the nicotine-paired cue during nicotine self-administration. The extent of the conditioned response directed to the site where saccharin was to be

delivered (also known as ‘goal tracking’ response) is indicative of how strongly the CS has become predictive of the reward (Robinson and Flagel, 2009). This could be interpreted that for those rats in Cluster1 that were faster, and better, to learn the CS-US relationship during Pavlovian conditioning to saccharin, tended to have a faster, and better, learning capacity to associate nicotine with its associated cue, which was revealed through the cue omission test. In other words, rats in this cluster would retain the magnitude of reward-stimulus learning from saccharine, and apply it to nicotine.

However, this interpretation presents a key limitation for this cluster: rats in Cluster 1 appear to have sensitivity to nicotine aversion. Upon nicotine omission, rats not only self-administer the cue in the absence of nicotine, but they do it with a higher magnitude (**Figure 29B**), and with a much faster frequency (**Figure 30**) than their baseline conditions. Furthermore, the acquisition curve (**Figure 31A**), and the baseline infusions at the end of acquisition (**Figure 31C**) during nicotine self-administration is the lowest among the three clusters, suggesting a limited tolerance to nicotine. Finally, when switching to a lower dose, Cluster 1 had the strongest (**Figure 35A**) and fastest (**Figure 35C**) increase in self-infusions. Interestingly, the extent of cue omission response appears to predict the extent of response to a reduction in nicotine dose (**Figure 36**), i.e. those rats that tended to drop more self-infusions after cue omission tended to have the highest compensatory response when the dose was changed. This data suggests that nicotine was playing a limiting role in their self-administration, and that the most important component of their operant behavior was responding for the cue. Indeed, it is well known that nicotine is an irritant, and some individuals are protected from nicotine dependence through increased sensitivity to the aversive effects of nicotine (Sartor et al., 2010; Hoft et al., 2011; Haller et al., 2012; Svyryd et al., 2016).

Confirming a profile of nicotine aversion sensitivity, Cluster 1 was the only subgroup in which the expression of $\beta 4$ nAChR in the mHb is strongly correlated with nicotine omission score (**Figure 47-B**). The $\beta 4$ subunit is a key accessory subunit of nAChRs, linked to the efficacy of nicotine-evoked currents (Frahm et al., 2011; Slimak et al., 2014) and, in the mHb, it has been identified as one of the key players in mediating nicotine aversion (Salas et al., 2009; Frahm et al., 2011). The partnership of $\alpha 5$ and $\beta 4$ nAChR subunits in supporting evoked nicotine currents in the habenular-interpeduncular

pathway allows for negative regulation of nicotine intake (Antolin-Fontes et al., 2015). Indeed, mice that have a genetically-induced overexpression of $\beta 4$ nAChR in the mHb have reduced nicotine intake, and show strong place aversion to nicotine (Frahm et al., 2011; Slimak et al., 2014). Even if overall levels of $\beta 4$ in the mHb are not different among clusters (**Figure 42B**), the strong positive correlation between $\beta 4$ nAChR in the mHb and nicotine omission response in this cluster, suggests the involvement of habenular signaling in their nicotine omission response, adding strength to their nicotine aversion profile.

Interestingly, expression of $\beta 4$ nAChR in the mHb was also correlated with a change in dose response, but through a negative correlation (**Figure 47C**). This indicates that those rats in Cluster 1 with the highest expression of $\beta 4$ nAChR in the mHb were the most sensitive to nicotine aversive properties, with the highest increase in responding in a nicotine-free environment, but with the lowest compensatory increase when the dose of nicotine was lowered. It is possible that 0.02mg/kg was still too high a dose of nicotine for these rats with heightened $\beta 4$ expression, explaining their lack of compensatory increase in responding.

However, an increased sensitivity to nicotine reinforcement cannot be ruled out completely in rats of Cluster 1. Mice that have oversensitive $\alpha 5/\alpha 3/\beta 4$ nAChR have a shifted dose response curve, i.e., they are more sensitive to both the reinforcing and aversive effects of nicotine (Gallego et al., 2012). It can be argued that the lower baseline observed in our study could be explained by an increased sensitivity to nicotine, thus requiring less amount of nicotine to maintain nicotine self-administration. However, rats with hypersensitive $\alpha 5/\alpha 3/\beta 4$ nAChR tend to show a quicker acquisition curve, with a quicker discrimination between active and inactive holes (Gallego et al., 2012), which is not the case in our study (**Figure 31**).

In humans, the single nucleotide polymorphisms (SNPs) in $\beta 4$ nAChRs that provide hypersensitivity to nicotine aversion make smokers less likely to be nicotine dependent, with fewer number of cigarettes per day (Haller et al., 2012). Furthermore, these rare human $\beta 4$ nAChRs variants show increased currents to low doses of nicotine (Haller et al., 2014), and their forced expression in mice

reduced nicotine intake in a two-bottle choice protocol (Slimak et al., 2014), without abolishing it completely. In our Cluster 1, rats continued nicotine self-administration, even if at a low level. This suggests that their aversion to nicotine was not strong enough to prevent them from acquiring, and maintaining, self-administration, but it seems strong enough for the balance between nicotine reinforcement and aversion to be tipped towards the latter. In fact, rats in this cluster retain some level of reinforcement to nicotine, even if not heightened. Cluster1 reports the highest increase in $\alpha 4$ nAChR subunit expression in the shNAcc, and the highest increase in $\beta 2$ nAChR subunit expression in the cNAcc. The $\alpha 4\beta 2$ nAChR assembly in the nucleus accumbens plays key roles in nicotine reinforcement (Corrigall et al., 1994; Pontieri et al., 1996; McGranahan et al., 2011). In this regard, it is noteworthy that Cluster1 shows a positive trend between the expression of $\alpha 4$ nAChR subunit in the cNAcc and change in dose response (**Figure 43B**). Nicotine reinforcement is dependent on $\alpha 4\beta 2$ nAChR activation in both the VTA and the NAcc, in the latter through presynaptic modulation of dopamine release from incoming VTA axons (Dani et al., 2000), but also through activation of cholinergic interneurons in the NAcc (Aosaki et al., 1995). Activation of cholinergic interneurons in the NAcc is able to trigger release of dopamine from VTA axons terminating in the NAcc (Cachope et al., 2012; Threlfell et al., 2012), thus providing a complex interplay of the dopaminergic and cholinergic systems in the nucleus accumbens. This may suggest that compensation to a change in dose in this cluster may also involve a seeking for higher reinforcing effects of nicotine, rather than responding more for the cue in the midst of lower amounts of nicotine.

Furthermore, Cluster 1 and 2 showed negative correlations between $\alpha 7$ nAChR subunit expression in shNAcc and response to change in dose (**Figure 44B**), consistent with previous studies that have shown how $\alpha 7$ -nAChR-mediated signaling in the shNAcc is inversely correlated with motivation to self-administer nicotine (Brunzell and McIntosh, 2012; Harenza et al., 2014). Indeed, pharmacological blockade of $\alpha 7$ -nAChRs in the shNAcc greatly reduced nicotine self-administration using a progressive ratio approach (Brunzell and McIntosh, 2012), while full $\alpha 7$ -KO mice have been shown to have higher dopamine release in the NAcc (Besson et al., 2012), and show nicotine place preference at a lower dose compared to wild-types (Harenza et al., 2014). Furthermore, human studies have shown

that loss-of-function mutations in *CHRNA7*, the gene that encodes the $\alpha 7$ subunit, are a risk factor for tobacco dependence (Mexal et al., 2010; Araud et al., 2011; de Lucas-Cerrillo et al., 2011; Cameli et al., 2018).

Even if limited, this evidence suggests that rats in Cluster1 have some degree of reinforcement to nicotine, as shown by the involvement of $\alpha 4$ nAChR in the cNAcc, and that of $\alpha 7$ in the shNAcc, with the response to a change in dose. However, the balance between nicotine reinforcement and aversion may be tipped towards the latter.

In this sense, an alternative explanation for the correlation between conditioning to a saccharin-predicting cue and cue omission effect could be explained by the fact that ‘goal-tracking’ responses also tell us how strongly the reward itself is ‘wanted’, as rats with the highest goal-tracking response show incentive salience for the reward (Lee et al., 2018a). When considering the effect of saccharin conditioning to baseline saline self-administration (**Figure 23**), but not on that of nicotine, it is possible that conditioning to a sweet-reward, like saccharin, cross-sensitized responding to the visual cue, priming the dopaminergic system for a heightened response (Rada et al., 2005). In that sense, if Cluster 1 is more sensitive to nicotine aversion, then its true goal in nicotine self-administration is not much the reinforcement of nicotine or the effects of nicotine on the cue, but rather the reinforcing effects of cue alone, as revealed by the nicotine omission test. This response to the cue can be made equivalent to their ‘goal-tracking’ behavior during Pavlovian conditioning. Therefore, they retain a ‘saline-like’ heightened responsiveness for the cue, as the balance between nicotine aversion and reinforcement is tipped towards the former.

Cluster2 – Sensitivity to Nicotine and Nicotine-paired Cue as a Conditioned Reinforcer

Nicotine seeking in rats in Cluster 2, the biggest subgroup, appears driven by a combination of the primary reinforcing actions of nicotine, and the transformation of the nicotine-paired cue as a conditioned reinforcer, capable of driving self-administration even in the absence of nicotine.

Cluster2 had the defining characteristic of having the lowest effect of cue or nicotine omission (**Figure 28**), both in global effects, as well as extinction indexes. Removal of the cue did not greatly alter their self-administration profile (**Figure 29A**), while removal of nicotine caused an initial sharp increase in responding (**Figure 29B**), followed by a rate of self-infusions not different from their baseline rate (**Figure 30**).

The characteristic response to cue omission at the training dose could be explained by a heightened sensitivity to the reinforcing actions of nicotine, which, by themselves, appear enough to sustain self-administration. For example, rats in Cluster 2 show a substantial increase in their self-infusions after change in dose (**Figure 35**). When nicotine was omitted, the initial response was a sharp increase in self-infusions, possibly in an attempt to seek for the absent nicotine. The nicotine omission response of this Cluster2 is remarkable in that, after this brief initial response, the cue appears to have gained sufficient power as a conditioned reinforcer to drive a rate of self-administration that is not different from their baseline, even in the absence of nicotine. This is consistent with previous studies that have shown that nicotine can transform associated cues into conditioned reinforcers, which can sustain self-administration long after nicotine has been discontinued (Johnson et al., 2004; Cohen et al., 2005; Donny and Jones, 2009; Rupprecht et al., 2015).

In human studies, it has been shown that, in some individuals, the environmental stimuli that had become conditioned reinforcers due to their association with nicotine are major sources of craving (Tiffany and Hakenewerth, 1991; Cepeda-Benito and Tiffany, 1996), and thus contribute to relapse (Tiffany et al., 2000). Some smokers that have been switched to de-nicotinized cigarettes report lower cravings to smoke (Dallery et al., 2003; Donny and Jones, 2009; Barrett, 2010), suggesting that the conditioned stimuli associated with smoking, such as rolling a cigarette (Baker et al., 2006), or the oropharyngeal sensations of smoking (Rose et al., 1985; Brauer et al., 2001), have become strong reinforcers. On the same regard, some smokers report an increase in craving after observing friends smoking, or when visiting the places associated with smoking (Niaura et al., 1992; Conklin and Tiffany, 2002; Van Gucht et al., 2010; Shiffman et al., 2015).

Despite that in Cluster 2 both nicotine reinforcement and cue as conditioned reinforcer drive nicotine seeking, there seems to be some degree of individual variations within the same cluster, in which rats appear to form a continuum, from those who are predominantly more nicotine sensitive, to those who are predominantly more sensitive to the cue as a conditioned reinforcer. It is noteworthy that, despite not being statistically significant, there is a positive trend between cue omission and the response to an acute change in dose (**Figure 36**) seen only in this cluster, suggesting that those with the lowest cue omission score had the highest sensitivity to nicotine. Despite the general observation that nicotine is only poorly reinforcing (Caggiula et al., 2001; Rose, 2006), there is evidence that suggests that some individuals may have greater sensitivity to the reinforcing actions of nicotine than others, in particular those with key SNPs in $\alpha 4$ nAChRs (Tapper et al., 2004; Hutchison et al., 2007). Notably, the response to a change in dose was correlated positively with the expression of $\alpha 4$ nAChR subunit in the cNAcc (**Figure 43**), just like Cluster 1, albeit with a much stronger correlation.

It is interesting that response to a change in dose was also correlated to $\alpha 4$ nAChR, $\alpha 7$ and $\beta 2$ nAChR subunits in the BLA (**Figure 45B** and **46B**). Furthermore, expression of $\alpha 4$ nAChR in the BLA was the highest in Cluster 2 (**Figure 42**). The BLA is part of a wider network of brain structures involved in drug reinforcement (Everitt and Robbins, 2005), in particular in the establishment of contingent relationships between drugs and associated environmental cues (Sharp, 2018), the latter becoming conditioned reinforcers that can energize drug seeking. The BLA receives dopaminergic input from the VTA, as well as glutamatergic inputs from the thalamus and medial prefrontal cortex, and sends projections to the NAcc, where it plays a role in modulating dopamine neurotransmission (Everitt et al., 1999; Chiamulera, 2005; Everitt and Robbins, 2005). Activation of the BLA upon cue presentation precedes, and it is necessary to, cue-evoked firing at the NAcc (Ambroggi et al., 2008).

While an involvement of the BLA in the sensitivity to the reinforcing effects of nicotine per se has never been directly studied in the literature, the correlations observed in this study could simply evidence the complex involvement of the BLA-NAcc pathway in nicotine-cue interactions, in particular in updating the contingency of the behavior after a sudden decrease in dose.

Despite not being statistically significant, Cluster2 reports a trend between dopamine D2 receptor expression in the shNAcc, and the response to cue omission (**Figure 44C**), i.e. the highest D2 expression in this area was related to the highest drop in self-infusions after cue omission. Dopamine neurotransmission in the shell of the nucleus accumbens, through activation of both D1 and D2 dopamine receptors, has been long implicated with nicotine reinforcement (Corrigall and Coen, 1991; Corrigall et al., 1994), but also with the establishment of cues as conditioned reinforcers to nicotine (Liu et al., 2010). Pharmacological blockade of D1 or D2 dopamine receptors reduced cue-induced reinstatement to nicotine seeking (Liu et al., 2010), providing evidence that the dopamine neurotransmission at the D1-D2 receptors are important in nicotine-paired cues becoming conditioned reinforcers, in a similar way that it has been found for other food or drug reinforcers (Koch et al., 2000; Schmidt and Pierce, 2006; Wise, 2006; Di Chiara and Bassareo, 2007; Lex and Hauber, 2008).

Considering the role of dopamine in cue conditioning, our results suggest that, for Cluster2, the extent of cue omission response occurs as a function of how much the cue has gained reinforcement properties by its Pavlovian association with nicotine, most probably involving a dopamine at the shNAcc. This could explain why those rats with the lowest cue omission score had also the highest sensitivity to a change in dose to nicotine (**Figure 36**).

Thus, for Cluster 2, nicotine seeking is potentiated by sensitivity to nicotine primary reinforcement, as well as by a cue acting as conditioned reinforcer. Since the strength of a conditioned reinforcer depends, in part, on the strength of the primary reinforcer (Annau and Kamin, 1961; A. Rescorla and Wagner, 1972), including nicotine (Palmatier et al., 2008a), it comes as no surprise that in this subgroup of rats with an overall heightened sensitivity to nicotine reinforcement, the cue has also gained strength as a conditioned reinforcer, both factors contributing to the nicotine seeking profile of this subgroup.

Cluster3 – Nicotine-induced Enhancement of Cue Reinforcing Effects

Nicotine seeking in rats in Cluster 3, appears driven by the reinforcing-enhancing effects of nicotine on surrounding stimuli, and less by the primary reinforcing actions of nicotine, or cue, by themselves.

The defining characteristic of Cluster 3 is a strong decrease in cue omission, a relatively low increase in nicotine omission, but strong extinction indexes in both omission tests (**Figure 28**). Omission of the cue, or nicotine, leads to an extinction-like phenotype (**Figure 29**), revealing the necessity of both nicotine and cue to be present in order to drive the self-administration behavior

A closer observation should reveal that the extinction-like behavior after cue omission is much stronger than that after nicotine omission. In other words, if needed to be separated from each other, the contributions of the cue appear to be more well-defined than those of nicotine alone. In fact, Cluster 3 showed the poorest response to change in nicotine dose (**Figure 35C**). Furthermore, the response at cue omission at 0.02mg/kg was the weakest of all clusters (**Figure 37C**), and not comparable with the strong cue omission profile seen in this cluster at the training dose. This trend is consistent with what it would be expected for a dose-dependent, reinforcing-enhancing effect of nicotine on a cue (Liu et al., 2007), in which the effect of nicotine on the cue would be reduced if the dose is reduced. Critically, however, the contributions of the cue were dose-dependent, as the extent of cue omission response at 0.02mg/kg was negatively correlated to how much they had compensated in response to the change in dose from 0.04 to 0.02mg/kg (**Figure 38**). This adds further support to the notion that nicotine is directly determining cue reinforcement in this cluster.

The reinforcing-enhancing effects on cues exerted by nicotine have been proposed as one of the key mechanisms through which nicotine can be so addictive (Caggiula et al., 2009; Rupprecht et al., 2015), as it can powerfully increase the incentive salience of surrounding stimuli, making self-administration much more robust. It is noteworthy that Cluster 3 had greater number of self-infusions achieved during acquisition days, and had a slightly higher baseline before the first cue omission test (**Figure 37**), a trend that was maintained throughout the experiment (data not shown).

The behavioral profile of Cluster3 reminds the profile of those smokers who consume nicotine for its effect in enhancing the salience of stimuli in their surrounding environment. Even though the reinforcer-enhancing effects of nicotine were first documented in animals (Chaudhri et al., 2006; Liu et al., 2007; Palmatier et al., 2007), there is now substantial evidence of its existence in humans (Perkins and Karelitz, 2013, 2014; Perkins et al., 2015, 2017, 2018; Martin and Sayette, 2018). In addition, some studies have proposed a ‘self-medication’ hypothesis of nicotine seeking, in which individuals with socioeconomic or health conditions associated with poor opportunities for reward seek nicotine for its reinforcer-enhancing effects on environmental stimuli (Perkins, 2009; Audrain-McGovern et al., 2014; Leventhal, 2016; Lee et al., 2018b). It is now well documented that sensory anhedonia during nicotine withdrawal can be a strong factor for relapse (Pergadia et al., 2014; Cook et al., 2015; Piper, 2015; Piper et al., 2017).

For Cluster3, correlations between gene expression and selected variables in nicotine self-administration were only seen in the BLA. Expression of $\alpha 4$ and $\beta 2$ nAChR in the BLA was positively correlated with response to a change in dose in a manner similar to Cluster2 (**Figure 45** and **46**). Importantly, for Cluster3, $\alpha 4$ nAChR in the BLA was also correlated with cue omission effect (**Figure 45C**), although it is noteworthy that rats with the lowest $\alpha 4$ nAChR in the BLA had the highest cue-omission effect, but at the same time had the lowest response to change in dose. This suggests that $\alpha 4$ -containing nAChRs in the BLA of Cluster 3 may play opposing roles in the modulation of sensitivity to nicotine dose and response to cue omission.

The association of the BLA in the cue omission response in Cluster 3 is not surprising, considering that BLA is pivotal in stimulus-control of drug seeking behaviors (Whitelaw et al., 1996; Meil and See, 1997; Di Ciano and Everitt, 2004; Everitt and Robbins, 2005; Sharp, 2018), including nicotine (Everitt et al., 1999; Chiamulera, 2005; Kelsey et al., 2009; Koob and Volkow, 2010; Khaled et al., 2014). Although the nicotine-induced enhancement of cue reinforcement has been extensively studied in animal studies (Olausson et al., 2004; Chaudhri et al., 2006; Palmatier et al., 2007; Liu et al., 2007; Caggiula et al., 2009; Grimm et al., 2012; Palmatier et al., 2013), and is also evident in some human studies (Perkins and Karelitz, 2013, 2014), the precise neurobiology of these effects is poorly known.

The only studies that have attempted to discover the biological mechanisms of this phenomenon showed that the reinforcing-enhancing effects of nicotine are dependent on $\alpha 4\beta 2$ nAChR (Liu et al., 2007) and D1 and D2 dopamine signaling (Palmatier et al., 2014), but are not dependent on $\alpha 7$ nAChR (Liu et al., 2007) nor metabotropic glutamate receptor function (Palmatier et al., 2008b). The precise structures in the circuitry recruited in this phenomenon are, however, still unknown, although there are reasons to point at the BLA as a key structure in this enhancement of reinforcement. The BLA is rich in nAChRs, in particular those containing $\alpha 4\beta 2$ (Wada et al., 1989) and $\alpha 7$ (Klein and Yakel, 2006) subunits, which have been found to fine tune amygdalar synaptic transmission (Huang et al., 2008; Jiang and Role, 2008; Mansvelder et al., 2009; Feduccia et al., 2012; Pidoplichko et al., 2013), thus impacting behaviors like working memory (Addy et al., 2003; Barros et al., 2005) and conditioned place preference (Zarrindast et al., 2010). Dopamine signaling in the NAcc can be controlled by the BLA (Everitt et al., 1999; Chiamulera, 2005; Everitt and Robbins, 2005), and because of this, the BLA it is thought a major integrative hub for incoming information about sensory and motivational value of environmental stimuli, relaying such signals to the NAcc in preparation for a behavioral response to said stimuli (Chiamulera, 2005; Everitt and Robbins, 2005). It follows that the BLA would play a key role in the nicotine effects on cue observed in Cluster3.

Nicotine Metabolism and Baseline Self-Administration of Nicotine

The speed of nicotine metabolism has been identified as an often-overlooked factor in determining the eventual risk for nicotine dependence in humans, with slow metabolizers being at a reduced risk to being smokers (Mamoun et al., 2015), while fast metabolizers being more likely to transit into heavy smoking (Rubinstein et al., 2013). While nicotine metabolism in our Experiment 2 was not related to any parameter in our identified clusters, baseline self-infusions correlated with the speed of metabolism at the population level, (**Figure 39**). Interestingly, this correlation was maintained only in those rats with the strongest sensitivity to the cue (**Figure 41**). Although causality is unknown, a possible interpretation could be that a faster metabolism is translating itself into a quicker self-

administration to nicotine, and this in turns results in increased nicotine-cue presentations. A similar proposal has risen in human studies, which have evidenced that fast metabolizers tend to have higher cue-induced craving to smoking-related cues compared to slow metabolizers (Tang et al., 2012; Falcone et al., 2016). It thus follows that the metabolism-baseline relationship would be more evident in those where the cue has gained reinforcement properties in its pairing with nicotine. However, the low coefficient of variation (**Figure 41**) suggests that these relationships are weak, as nicotine-cue interactions are probably much more complex than simple Pavlovian CS-US pairings, as it has been explained in the description of our Clusters.

Limitations

In this study, we have sought to evidence, and characterize, individual differences in the mechanisms that drive nicotine seeking, in particular, those that involve different nicotine-cue interactions, as we had previously proposed (Garcia-Rivas et al., 2017).

There are specific limitations to our findings. First, the gene expression analysis presented is not complete, as due to time constraints, only 42% of all brains underwent microdissection and PCR. A first important step is to complete the validation of these neurobiological correlates that distinguish each cluster, and by so doing validating the conclusions discussed in this manuscript.

Second, an inherent limitation of correlational studies is that causation can only be inferred. While correlational studies provide us with useful information to support the involvement of structures and suggest possible mechanisms, further studies should assess causality, through interventions that prevent, generate, or reverse, the phenotypes observed in this study, through the use of techniques like opto- or chemogenetics, or through the plethora of genetic tools developed in mice.

Third, given the limited timeline of the experiment, only limited tests were done. Further tests could have provided complementary information about the contributions of the cue, and the nicotine reinforcement, in their nicotine seeking, for example, testing more dose changes (to better assess nicotine sensitivity), using a progressive ratio protocol (to evidence different degrees of nicotine

reinforcement) and cue-induced reinstatement (as complementary dimension to cue omission tests), as well as extending the length of nicotine exposure, to assess the temporal evolution of these nicotine-cue interactions

Conclusion

Despite the aforementioned limitations, which have the potential to be bypassed in further studies extending beyond this dissertation, here we have provided neurobiological, metabolic and behavioral correlates of individual differences in the mechanisms that drive nicotine seeking. We evidenced three clusters of rats that differ in the extent in which nicotine, and its associated cue, can drive their self-administration behavior. One of such groups (Cluster1) showed self-administration of the cue, despite an aversive sensitivity to nicotine. The other two groups (Cluster2 and 3) showed nicotine-cue interactions: for Cluster 2, the cue seemed a conditioned reinforcer, while for Cluster 3, the reinforcement of the cue appeared dependent on nicotine. These results are not only the first preclinical demonstration of different mechanisms that drive nicotine seeking, but also raise important implications in the therapeutic approaches against tobacco dependence.

Chapter 4

THE EFFECT OF VARENICLINE ON THE REINFORCEMENT-ENHANCING EFFECT OF NICOTINE ON ASSOCIATED CUES

TITLE PAGE

Varenicline targets the reinforcement-enhancing effect of nicotine on its associated cues during nicotine self-administration

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ABSTRACT

Varenicline (Champix® or Chantix®), a partial agonist of nicotine at the $\alpha 4\beta 2$ nicotinic receptor, is an approved pharmacotherapy against tobacco dependence, although with limited efficacy.

Nicotine seeking is strongly supported by complex interactions between nicotine and environmental cues. A key effect of nicotine on surrounding cues is its capacity to enhance the reinforcing properties of salient environmental stimuli, a phenomenon that is thought to play a prominent role in nicotine seeking. It is still not well understood whether the decrease of nicotine self-administration by acute Varenicline results from antagonism of the primary reinforcing effects of nicotine, of the reinforcement-enhancing effect of nicotine on cues, or of a combination of both.

Using an intravenous self-administration paradigm in rats, we confirmed that acute Varenicline decreases self-administration reinforced by nicotine accompanied by a salient visual cue light and demonstrated that it also decreases self-administration reinforced by nicotine alone, to a lesser extent.

Using a novel approach that allows for changing the visual salience of the nicotine-paired cue during self-administration, we demonstrate that nicotine enhances the reinforcing effects of a sudden increase in cue salience. Critically, we show that Varenicline specifically targets this potentiation by nicotine of increased cue salience.

Our results support individual variations in both nicotine reinforcing effects and nicotine-induced enhancement of cue reinforcing effects. Importantly, individual variations in the latter, but not in the former, would determine the amplitude of acute Varenicline-induced decrease in seeking.

Since it is known that smokers differ in the mechanisms that drive their drug seeking, these results suggest that Varenicline might be more beneficial as a clinical tool in those smokers who are more sensitive to nicotine effects on surrounding stimuli, and less for those who seek nicotine primarily for the primary reinforcing effects of nicotine.

INTRODUCTION

Tobacco dependence continues to be a worldwide major health burden, being responsible for as much as 7 million deaths per year (WHO, 2017). More than 70% of smokers wish to quit (U.S. Department of Health and Human Services, 2012), but less than 10% succeed without medical support (Rigotti, 2012). Even so, a major obstacle in ceasing to smoke is the limited efficacy of available treatments against tobacco dependence (Schuit et al, 2017). For instance, from all patients treated with Varenicline (Champix® or Chantix®), one of the most effective approved pharmacotherapies in supporting smoking cessation (Cahill et al, 2013; Hartmann-Boyce et al, 2014), only 40% remain abstinent at the end of a 12-week-long treatment, dropping to 20% in the following months after treatment cessation (Jordan and Xi, 2018; Niaura et al, 2008; Oncken et al, 2006).

Varenicline was specifically developed as a partial agonist for $\alpha 4\beta 2$ -containing nicotinic cholinergic receptors (Coe et al, 2005; Rollema et al, 2007b, 2007a), which mediate the primary reinforcement properties of nicotine, the major psychoactive compound of tobacco (Benowitz, 1992). Considering, however, that nicotine has a very complex psychopharmacology (Caggiula et al, 2001, 2002; Le Houezec and Benowitz, 1991; Palmatier et al, 2007, 2009, Perkins and Karelitz, 2013, 2014; Rupprecht et al, 2015; Yager and Robinson, 2015), the precise psychopharmacological targets of Varenicline remain poorly understood (Brandon et al, 2011; Gass et al, 2012).

Despite its strong addictive potential, nicotine has relatively poor direct primary rewarding and reinforcing properties by itself, compared to other drugs of abuse (Caggiula et al, 2001; Rose, 2006). In addition, and in a manner different to other psychostimulants, nicotine can enhance the reinforcing value of environmental cues that are primary reinforcers by themselves, or that have acquired reinforcing value through pairing with another reinforcer (Caggiula et al, 2009; Rupprecht et al, 2015). The interplay between nicotine and environmental cues is complex and difficult to disentangle, but plenty of evidence suggests it is a determinant factor in tobacco seeking (Caggiula et al, 2001, 2002; Garcia-Rivas and Deroche-Gamonet, 2018).

Understanding which psychopharmacological dimensions of nicotine seeking are being affected by Varenicline could clarify its limited efficacy, and could help better define future treatments against tobacco dependence. In this perspective, preclinical animal models can be useful. Studies using classical nicotine self-administration in rats have shown the interplay between nicotine and environmental cues: nicotine itself is poorly self-administered, while pairing it with a salient visual cue synergistically enhances nicotine seeking (Caggiula et al, 2001, 2002; Donny et al, 2003). In these conditions, it is well known that acute Varenicline decreases nicotine self-administration in rats (Funk et al, 2016; Le Foll et al, 2012; O'Connor et al, 2010; Rollema et al, 2007b) and that Varenicline would poorly target the sole nicotine reinforcing effects (Clemens et al, 2017). In these studies, however, varenicline effects on nicotine or its associated cue, have been demonstrated exclusively in rats trained for self-administration of nicotine paired with a cue (nicotine+cue), in experimental conditions which do not allow to clearly disentangle the psychopharmacology of Varenicline.

Some preclinical studies have aimed at specifically studying the effects of varenicline in nicotine-cue interactions. Varenicline has been shown to dose-dependently antagonize the reinforcement-enhancing effect caused by nicotine (Levin et al., 2012). Consistent with its nature as partial agonist, it has also been shown that varenicline can enhance the reinforcement value of surrounding stimuli in a dose-dependent manner, although with a much weaker effect than nicotine (Barrett et al., 2018). This last result is consistent with a previous study, which used self-administration of varenicline and a visual cue self-administered through two different levers, to reveal such reinforcement-enhancing effect of

varenicline (Schassburger et al., 2015). However, the studies by Levin and Barret assessed the effect of nicotine and varenicline in responding for a visual cue, after nicotine was administrated passively by the experimenter.

Thus, to date, whether varenicline affects nicotine self-administration through antagonizing the primary reinforcing effects of nicotine, the reinforcement-enhancing effect of nicotine on visual cues, or a combination of both, is still not well understood. Because a key determinant of the synergistic interaction between nicotine and a salient cue is the primary reinforcing effects of the cue (Caggiula et al, 2009; Chaudhri et al, 2006), we developed an experimental procedure that allows for increasing these primary reinforcing effects during self-administration and tested the effect of Varenicline while contingently manipulating the reinforcing-enhancing effect of nicotine on the cue.

METHODS AND MATERIALS

SUBJECTS

Male Sprague–Dawley rats, weighing 280–300 g at the beginning of the experiments, were single housed under a 12 h reverse dark/light cycle. In the animal house, temperature ($22 \pm 1^\circ\text{C}$) and humidity ($60 \pm 5\%$) were controlled. Rats were habituated to environmental conditions and experimental handling for 15 days before surgery. Standard chow food and water were provided *ad libitum*. All procedures involving animal experimentation and experimental protocols were approved by the Animal Care Committee of Bordeaux (CEEA50, N° 50120168-A) and were conducted in accordance with the guidelines of the European Union Directive 2010/63/EU regulating animal research.

SURGERIES

A silastic catheter (internal diameter = 0.28 mm; external diameter = 0.61 mm; dead volume = 12 μl) was implanted in the right jugular vein under ketamine (80 mg/kg) / xylazine (16 mg/kg) anesthesia. The proximal end reached the right atrium through the right jugular vein, whereas the back-mount passed under the skin and protruded from the mid-scapular region. Immediately after surgery, rats received a single antibiotic injection (gentamicine 1 mg/kg i.p.). Rats were given 5-7 days recovery before nicotine self-administration training began.

DRUGS

Ketamine hydrochloride (80 mg/kg) (Imalgène 1000; Rhône Mérieux, Lyon, France) and xylazine hydrochloride (16 mg/kg) (Rompun; Rhône Mérieux, Lyon, France) were mixed with saline and administered intraperitoneally in a volume of 2 ml/kg of body weight. (-)-Nicotine-Hydrogen-Tartrate (Glentham, UK) was dissolved in sterile 0.9% physiological saline for a final dose of 0.04 mg/kg free base. Nicotine, as well as sterile 0.9% physiological saline in control groups, was self-administered by the rats via intravenous (i.v.) route in a volume of 40 μl per self-infusion. Nicotine solution was adjusted to a pH of 7.

Varenicline or 7,8,9,10-Tetrahydro-6,10-methano-6H-pyrazino[2,3-h] [3]benzazepine tartrate (Tocris, UK) was dissolved in sterile 0.9% physiological saline for a final dose of 1 mg/kg free base.

Varenicline was administered intraperitoneally (i.p.) 30 min prior to self-administration, in a volume of 1 ml/kg.

INTRAVENOUS SELF-ADMINISTRATION

Self-administration Apparatus

The self-administration setup consisted in 48 self-administration chambers made of plexiglas and metal (Imetronic, France), and equipped with holes as operant manipulanda. Each chamber (40 cm long x 30 cm width x 52 cm high) was located in an opaque sound-attenuating cubicle equipped with an exhaust fan to assure air renewal and mask background noise. For self-administration sessions, each rat was placed in one chamber where its chronically implanted intracardiac catheter was connected to a pump-driven syringe (infusion speed: 20 μ l / sec). Two holes, located at opposite sides of the chamber at 5.5 cm from the grid floor, were used to record instrumental responding. In given experimental groups and experiments, a common white light (white LED, Seoul Semiconductor, South Korea), 1.8 cm in diameter, located 11.5 cm above the active hole, was used as nicotine (or saline) delivery-associated discrete visual cue, and is named thereafter 'cue light' or 'cue'. It produced 5 Lux. As well, in given experimental groups and experiments, a blue light (blue LED, Sloan Precision Optoelectronics, Switzerland), 1.8 cm in diameter, located on the opposite wall at 17 cm of the floor on the left side, was used as, and is named thereafter, Ambient light and abbreviated *AL* (**Supplementary Fig1**). It produced 15 Lux at a wavelength of 475nm, which is known to not affect vision in Sprague Dawley rats in similar exposure time as our experimental approach (Tosini et al., 2016). LED intensities were both measured in the middle of the cage with a Lux-meter (Moineau Instruments, France). Experimental contingencies were controlled and data was collected with a PC-windows-compatible SK_AA software (Imetronic, France).

Self-administration Procedures

In the three experiments presented below, self-administration testing is initiated on a Tuesday. Then rats performed self-administration training 5d/week (Monday to Friday). Self-administration sessions began two hours after the onset of the dark phase. Nose-poke in the active hole under a fixed ratio 3 schedule of reinforcement (FR3) produced the activation of the infusion pump (40 μ l over 2 seconds). Nose-pokes at the inactive hole were recorded but had no scheduled consequences. To maintain catheter patency, catheters were flushed with ~10 μ l of heparinized saline (30 IU/ml) after each self-administration session and before the self-administration sessions run on Monday.

In Experiment 1, to define a significant self-administration behavior at the individual level, we used a discrimination index between active and inactive holes [active nose-pokes/total nose-pokes]*100] strictly superior to 50%, together with a minimal number of at least 6 self-infusions per session over the three consecutive sessions and with stability in the number of self-infusions (\pm 10%) over the last two sessions.

EXPERIMENTAL PROCEDURES

A. Effect of Varenicline on self-administration behavior reinforced by either a discrete cue light, a nicotine infusion or a combination of both nicotine and cue light.

Experiment 1: Comparison of self-administration behavior reinforced by a discrete cue light, a nicotine infusion or a combination of both nicotine and cue light.

Rats were trained for intravenous self-administration, during 2-hour daily access to reinforcers. Nose-poking in the active hole at FR3 was reinforced either by a nicotine infusion (*nicotine*, n=25), a nicotine infusion plus a discrete cue light (*nicotine+cue*, n=8), or a saline infusion plus a discrete cue light (*saline+cue*, n=10). For the *nicotine* group, following nose-poking in the active hole at FR3, the infusion pump was activated for 2 sec. For the *nicotine+cue* and *saline+cue* groups, nose-poking in the active hole at FR3, turned on the white cue light located above the hole, simultaneous to the activation of the infusion pump. The white cue light remained on for 4 s in total.

Effect of Varenicline on self-administration behavior. After 27 daily basal sessions, rats, showing a significant self-administration behavior according to the acquisition criteria, were administered with Varenicline (1 mg/kg, ip) 30 min prior to a basal self-administration session. The average number of infusions over training sessions 26-27 was used as baseline.

B. Effect of Varenicline on the synergistic reinforcing effects of nicotine and a discrete cue light.

Experiment 2: A procedure to alter the primary reinforcing effects of the cue light.

A key determinant of the synergistic interaction between nicotine infusion and an associated discrete cue light relies on the primary reinforcing effect of the cue. Our goal here was to manipulate these reinforcing effects, and notably to provoke an increase in these effects.

Two groups of rats were trained for *saline+cue* self-administration, as described in experiment 1, except that for one group (*AL*, n=15), the *Ambient light (AL)* was on throughout the first 7 acquisition sessions. For the other group (*No Ambient light, No AL*, n=15) the *AL* was off during the same period. On the eighth session of self-administration, the *Ambient light* conditions were switched; turned off for the *AL* group and on for the *No AL* one. On sessions 9 and 10, the *No AL* group was split into two, with half of the rats switched back to their original *No AL* condition (*Single AL Insertion* subgroup, n=7), while the other half remaining under the new *AL* condition (*Sustained AL Insertion* subgroup, n=8). All rats from the *AL* group remained without the *AL* for sessions 9 and 10 (see protocol diagram on **Supplementary Fig 2**).

Experiment 3: Effect of Varenicline on the reinforcing-enhancing effects of nicotine on cues in nicotine+cue self-administration.

Effect of increasing (vs decreasing) the cue primary reinforcing effects on nicotine self-administration: Two groups of rats were trained for *nicotine+cue* self-administration, as described in experiment 1. As in experiment 2, the *AL* was on throughout the basal training self-administration sessions, for one group (*AL*, n=36), and was off for the other one (*No AL*, n=19). After 22 daily basal sessions, we tested the effect of: (1) suppressing, and adding, the *AL* on self-administration in the *AL* and *No AL* groups, respectively.

Effect of Varenicline on increasing (vs decreasing) the cue primary reinforcing effects during nicotine+cue self-administration: Back to respective basal conditions, we then tested the effect of: (1)

Varenicline (1 mg/kg, i.p.) administered 30 min prior to a basal session, (2) Varenicline (1 mg/kg, i.p.) administered 30 min prior to session during which the *AL* was manipulated, i.e. suppressed in the *AL* group and inserted in the *No AL* group.

Tests sessions were interspaced by 2 to 4 basal sessions. A new test was performed only if baseline was stable over 2 consecutive sessions and had return to the level of infusions of sessions 21-22.

DATA ANALYSES

Self-administration.

Total responses in the active and inactive holes and total number of infusions per self-administration session were considered.

Effect of Varenicline and/or AL manipulation.

To evaluate Varenicline and/or *AL* manipulation (*AL* removal or *AL* insertion), delta infusions from baseline (infusions at test – infusions at baseline) were calculated. Baseline infusions correspond to the mean infusions over the two sessions preceding a test.

STATISTICAL ANALYSES

Self-administration behavior was analyzed using repeated measures ANOVA with Time (number of sessions), Hole (active vs inactive), Test (Baseline vs Test), Condition (*AL*On to *AL*Off, *AL*Off to *AL*On, *AL*On to *AL*Off+Var, *AL*Off to *AL*On+Var), as within-subject factor, and experimental group (*saline+cue/nicotine+cue/nicotine*, *AL/ No AL*) as between-subject factor.

Significant main effects or interactions were explored by pairwise comparisons of means using the Newman Keuls post hoc test. Pearson's correlation analyses were used to investigate correlation between variables of interest. A t-test was used to compare the *AL* Removal effects (or of *AL* Insertion effects) on *saline+cue* and *nicotine+cue* self-administration.

The results are presented as mean±SEM. Differences were considered significant at $p < 0.05$.

The statistical analyses were performed using the STATISTICA 13.3.0 (2017) data analysis software system (TIBCO Software Inc., Palo Alto, CA, USA).

RESULTS

Nicotine and a discrete cue light contribute synergistically to self-administration (Fig 1a).

Rats were trained for intravenous self-administration reinforced by either saline infusions associated with a discrete cue light (*saline+cue*), nicotine infusions associated with a discrete cue light (*nicotine+cue*) or nicotine infusions alone (*nicotine*).

Over the first 15 self-administration sessions, the three groups differed significantly regarding both number [Group, $F(2,42)=10.77$, $p<0.001$] and pattern [Group x Session, $F(28,588)=6.7$, $p<0.0001$] of reinforcers earned (**Fig 1a**), and number and discrimination in responses (**Fig 1b**).

Nicotine first tended to compromise, but secondarily amplified, the reinforcing effects of a discrete cue light. Thus, *nicotine+cue* rats tended to show lower self-administration behavior than *saline+cue* rats during the first two sessions. Then *nicotine+cue* rats increased their self-administration behavior, while *saline+cue* rats decreased it. This increase in *nicotine+cue* self-administration was only partly paralleled by a slight increase in *nicotine* self-administration that never overcame the level of *saline+cue* self-administration. These self-administration patterns indicate that nicotine and cue synergistically enhanced each-other reinforcing values.

Nicotine and saline+cue are both mild, but different, reinforcers

Although the behavior of the *saline+cue* and the *nicotine* groups stabilize at a similar level from session 6, observations exclude that they are similar, and due to their common stimulus, i.e. intravenous infusion.

First, if the behavior of the two groups was exclusively supported by the intravenous infusion, the two groups would be similar. Now, up to session 6, the *saline+cue* group produced a higher number of self-infusions than the *nicotine* one [Group, $F(1,36)=8.5$, $p<0.01$] and the two profile of self-infusions differ with decrease and progressive increase up to stabilization, respectively [Group x Session, $F(5,180)=5.7$, $p<0.0005$].

Second, the intravenous infusion is most likely poorly involved in supporting the behavior. In the *saline+cue* group, the cue is a reinforcer. Indeed, in a preliminary experiment, 8 rats were trained for *saline+cue* for 13 sessions in conditions similar to the ones described in experiment 1. Omission of the cue on session 14 produced a significant decrease in self-administration (**Supplementary Fig 3**) supporting that the cue contributes to the reinforcing effects.

The mild reinforcing effects in *nicotine* and *saline+cue* rats, as compared to *nicotine+cue* rats, were further confirmed when using threshold criteria for discrimination, i.e. number of infusion and stability in behavior (see methods), to define a significant self-administration behavior at the individual level. While 100% of the *nicotine+cue* rats reached the criteria (8/8), only 40% of the *nicotine* rats (10/25) and 50% of the *saline+cue* rats did (5/10) (**Supplementary Fig 4a**).

Distribution of the individual scores of self-infusions in the rats showing self-administration based on these criteria (**Supplementary Fig 4b**) also further supports the difference in nature of the reinforcer acting in the *nicotine* and the *saline+cue* groups.

Supplementary Figs 4c-f show the self-infusions and responses in rats, which reached (Supplementary Fig4c-d) or not (Supplementary Fig4e-f) these criteria.

Varenicline decreases nicotine+cue and nicotine self-administration (Fig 2).

After 27 sessions, the effect of Varenicline on self-administration was tested in the *saline+cue* (n=6), *nicotine+cue* (n=8) and *nicotine* (n=11) rats that met self-administration criteria evaluated on behavior expressed on sessions 26 and 27. Varenicline decreased self-administration as measured by the number of self-infusions earned [Test effect, $F(1,24)=30.6$, $p<0.0001$]. This effect was function of the experimental group [Test x Group, $F(2,24)=4.71$, $p<0.05$] with a significant effect in rats self-administering *nicotine+cue* ($p<0.0001$) and *nicotine* ($p<0.05$) (**Fig 2a**). According to the effect on self-infusions, Varenicline decreased nose-poking in a group-dependent [Test effect, $F(1,24)=22.49$, $p<0.0001$; Test x Group, $F(2,24)=4.55$, $p<0.05$] and hole-dependent manner [Test x Hole, $F(1,24)=28.4$, $p<0.0001$], exclusively targeting the active hole (**Supplementary Fig 5**).

The effect of Varenicline, as measured by the delta-infusions from baseline [Group effect, $F(2,24)=3.29$, $p<0.05$], was higher in the *nicotine+cue* group than in the *saline+cue* ($p<0.05$) and *nicotine* groups ($p<0.05$), in which the delta-infusions were similar (**Fig 2b**). However, it was different from zero in the *nicotine* group ($p<0.0001$), but not in the *saline+cue* one. Also, in the *nicotine* group no correlation was observed between the number of basal self-infusions and the effect of Varenicline as measured by delta-infusions from baseline (**Fig 2c**). Hence, despite a low level of self-administration, Varenicline appears to exert a specific effect on *nicotine* self-administration, unrelated to the individual behavioral level.

Varenicline targets the reinforcing-enhancing effect of nicotine on its associated salient cue.

Because a key determinant of the synergistic interaction between nicotine and a salient cue is the primary reinforcing effects of the cue (Caggiula et al, 2009; Chaudhri et al, 2006), we developed an experimental procedure that allows for increasing these primary reinforcing effects during self-administration and tested the effect of Varenicline while contingently manipulating the reinforcing-enhancing effect of nicotine on the cue.

Results of experiment 1 supported that nicotine and the cue interact synergistically to produce reinforcing effects, but did not allow concluding whether Varenicline was able specifically targeting this interaction. To further explore this hypothesis, we aimed at testing the effect of Varenicline while manipulating this nicotine-cue synergy in the same individuals. As a first step, we aimed at developing a procedure that would allow to promoting (*vs* compromising) the nicotine-induced enhancement of the reinforcing properties of its associated cue. As this enhancement is depending on the primary reinforcing effects of the cue, we initially worked on a procedure allowing to increase (*vs* decrease) these reinforcing effects.

An interfering Ambient light (AL) alters the primary reinforcing effects of the discrete cue light.

As in experiment 1, rats self-administered *saline+cue*, as shown by a significant discrimination between active and inactive holes over the 7 sessions of self-administration [Hole effect, $F(1,28)=28.7$, $p<0.0001$]. However, this discrimination was function of the experimental group. The 15 Lux AL compromised the expression of the reinforcing effects of the discrete cue light [Group effect, $F(1,28)=10.4$, $p<0.01$]. In standard conditions (*No AL*), *saline+cue* induced self-administration behavior, while in the AL condition, with the same *saline+cue* reinforcer, rats did not discriminate

significantly between active and inactive holes [Group x Hole, $F(1,28)=18.7$, $p<0.0001$]. In the standard *No AL* condition, although behavior decreased over sessions, discrimination remained significant up to the last session ($p<0.005$) (**Supplementary Fig 6a-b**).

Not only *No AL* rats discriminated between the inactive control hole and the active hole associated with *saline+cue* delivery (**Supplementary Fig 6a-b**), but they also earned significantly more reinforcers than the *AL* rats [Group effect, $F(2,44)=8$, $p<0.01$] (**Fig 3a**).

Critically, it is unlikely that the absence of discrimination between active and inactive holes, as well as the decrease in the number of reinforcers earned result from a non-specific stress-like or aversive effect of the *AL*. First the number of inactive nose-poking is not affected (**Supplementary Fig 6b**) and the switch of the *AL* conditions on session 8 further attested that the *AL* compromises the cue light reinforcing effects. The *AL* was suppressed in the *AL* rats (*AL Removal*) and inserted in the *No AL* ones (*AL Insertion*). The switch altered self-administration behavior differently according to the group, increasing self-infusions in the *AL Removal* group while decreasing it in the *AL Insertion* group [Condition x Group, $F(1,28)=7.7$, $p<0.01$] (**Fig 3b**).

To better understand the effect of *AL* Removal and Insertion, *No AL* rats were split into two groups for the following two sessions (9 and 10): one group (*Sustained AL Insertion*, $n=8$), maintained the newly acquired *AL* condition, while the other (*Single AL Insertion*, $n=7$) returned to their *No AL* condition (protocol diagram on **Supplementary Fig 2**). *Sustained AL Insertion* further diminished self-administration in sessions 9 and 10, compared to sessions 6 and 7, while rats in the *Single AL Insertion* group appeared to compensate by increasing their mean infusions, when back to the initial *No AL* condition (**Supplementary Fig 7**). In the case of the *Sustained AL Removal* rats, for sessions 9 and 10 the removal of the *AL* was maintained, further increasing self-administration in comparison to sessions 6 and 7 (**Supplementary Fig 7**).

The interfering AL procedure allows revealing the reinforcement-enhancing effect of nicotine on its associated salient cue during nicotine self-administration.

Having established that it was possible to increase the reinforcing effects of the cue by *AL* Removal, we tested its effect on *nicotine+cue* self-administration, both on acquisition and once behavior was established.

During acquisition under the *No AL* condition, the number of *nicotine+cue* self-infusions was higher than under the *AL* condition [Group effect, $F(1,49)=5.36$, $p<0.05$], but the difference decreased over the 20 self-administration sessions [Group x Session, $F(19,331)=4.14$, $p<0.0001$] and the *AL* group reached and maintained the level of self-infusions of the *No AL* group by session 15 (**Fig 3c**). Supporting that the *AL* was affecting discrimination of the cue light, and was not playing as a stress factor, nose-poke responses (**Supplementary Fig 6c**) reveal that rats in the *AL* condition did not discriminate on the first session and as for the *saline+cue* self-administration, inactive nose-poking was similar in the *AL* and *No AL* conditions from session 2.

Once stabilized, removal of the *AL* increased self-administration behavior by the *AL* group [Test effect, $F(1,35)=47.9$, $p<0.0001$], while insertion of the *AL* decreased self-administration behavior by the *No AL* group [Test effect, $F(1,18)=24.46$, $p<0.001$] (**Fig 3d**).

Critically, as summarized on **Fig 3e**, the effect of the *AL* removal was much more pronounced in *nicotine+cue* conditions compared to *saline+cue* conditions (t-test, $p<0.01$), supporting that any increase in visual salience of the cue is magnified by nicotine.

By comparison, introduction of the *AL* had the same effect in both *nicotine+cue* and *saline+cue* conditions, suggesting a non-specific effect on visual perception, which is not potentiated by nicotine.

Varenicline targets the reinforcement-enhancing effect of nicotine on its associated salient cue.

Once stabilized, self-administration behavior by the *AL* group was altered by removal of the *AL*, by Varenicline or a combination of both [Test effect, $F(2,70)=64.8$, $p<0.0001$]. According to the condition tested, the test effect was different however [Test x Condition, $F(2,70)=76.3$, $p<0.0001$]. *AL* removal alone produced an increase (**Fig 4a red bar**) in *nicotine+cue* self-administration ($p<0.001$). When *AL* removal was combined with Varenicline administration, Varenicline abolished completely the effect of *AL Removal* and decreased *nicotine+cue* self-administration even below *AL Baseline* (**Fig 4a dashed red bar**, $p<0.01$ vs *AL Baseline*). However, this later effect was of a lower extent than when Varenicline was applied in the basal self-administration conditions, i.e. with maintenance of the *AL* ($p<0.001$) (**Fig 4a grey bar**). Critically, Varenicline and *AL Removal* effects were not simply additive. When evaluating the effect of *AL Remov + Var* to the effect of *AL Remov* alone, one yields an effect which is much higher than the one of Varenicline alone on basal self-administration, suggesting that Varenicline specifically abolishes the enhancing effects of the *AL Removal* (**Fig 4b**). Noteworthy, this interpretation is supported by the correlation analysis (**Fig 4c**) showing a high reverse, almost 1 to 1 correlation, between the effect of *Increased Cue Salience* by *AL Removal* ($\Delta ALRemov=ALRemov - AL$ baseline) and the calculated *Var* effect during *Increased Cue Salience* by *AL Removal* ($\Delta ALRemov+var - \Delta ALRemov$). Varenicline treatment during *Increased Cue Salience* by *AL Removal* appears to reduce infusions from an amount equivalent to the increase produced by the *Increased Cue Salience* alone. In other words, in these *AL Removal* conditions, Varenicline decreases specifically the individual increase produced by *AL Removal*, i.e. the individual potentiation of *nicotine+cue* self-administration produced by the *Increased Cue Salience*.

Self-administration behavior by the *No AL* group was decreased by insertion of the *AL*, by Varenicline or a combination of both [Test effect, $F(2,36)=4.4$, $p<0.05$] (**Fig 4d**). According to the condition tested, the test effect was different however [Test x Condition, $F(2,36)=9.3$, $p<0.001$]. Insertion of the *AL*, in rats trained in absence of it, produces a significant decrease in *nicotine+cue* self-administration (**Fig 4d blue bar**), which was similar in amplitude to the effect of Varenicline (**Fig 4d grey bar**). When combined to *AL Insertion*, Varenicline amplified the effect of the *AL Insertion* (**Fig 4d dashed grey bar**). Notably, the combined effect of *AL Insertion* and Varenicline were not synergistic but additive as shown on **Fig 4e**. When subtracting the *AL Insert* effect from the *AL insert+Var* effect, to get the *Var effect on decreased cue salience*, the result was similar to the effect of Varenicline alone (*Var effect alone*) (**Fig 4e**). Differently from what was observed for the effect of Varenicline on *Increased Cue Salience* by *AL Removal*, there were no 1 to 1 correlation between the decreased effect of *AL Insertion* on self-administration and the effect of Varenicline on this *AL Insertion* effect (**Fig 4f**), indicating that Varenicline had no specific effect on the *AL Insertion*-induced decrease in self-administration.

DISCUSSION

Varenicline is acknowledged as one of the most efficient therapeutic tools for tobacco dependence. However, its efficacy is limited both in time and to a portion of patients (Jordan and Xi, 2018; Niaura et al, 2008; Oncken et al, 2006). Even though the molecular pharmacology of Varenicline is well-known (Coe et al, 2005; Rollema et al, 2007a), its psychopharmacological actions are still poorly understood. Importantly, growing evidence supports a heterogeneity in the psychopharmacological mechanisms underlying tobacco dependence (Garcia-Rivas et al, 2016; Garcia-Rivas and Deroche-Gamonet, 2018). Hence, subpopulations of smokers might benefit from Varenicline less than others and contribute to its apparent limited efficacy.

In this study, we evidenced that acute Varenicline reduced nicotine-induced enhancement of the reinforcing properties of a nicotine-paired cue during intravenous self-administration. This effect was depending on how much nicotine-cue interactions was contributing to self-administration behavior at the individual level. Differently, the decrease by acute Varenicline of self-administration of nicotine alone was not related to individual basal levels of self-administration.

Nicotine alone is a poor primary reinforcer, but is strong enough to drive self-administration in certain individuals, but not in others.

Nicotine has weak primary reinforcement properties. Hence, classical nicotine self-administration has been developed to pair contingent nicotine IV delivery with the presentation of a salient visual cue light (Caggiula et al., 2001). In our study, we used the saline+cue condition as a control group evidencing the contribution of the cue in driving self-administration behavior. Comparison with the nicotine+cue group reveals the actual contribution of nicotine in nicotine+cue self-administration behavior. It is unlikely that the intravenous infusion, and not the cue (**Supplementary Fig 3**) or nicotine, supports the self-administration behavior. A discrete cue light alone can act as a primary reinforcer in drug naïve rats (Deroche-Gamonet et al., 2002) and previous studies have shown that nicotine alone at a similar dose as the one used here produced a self-administration behavior, while saline alone does not (Caggiula et al, 2002).

In our study, by session 15, 100% of all rats trained in nicotine+cue condition showed criteria of significant self-administration behavior, but only 40% of all rats trained in the nicotine alone condition reached the same criteria (**Supplementary Fig 4a**). These results not only confirm the well-known observation described by Caggiula and colleagues, but it extends it with the observation that some rats appear much more sensitive to the reinforcing properties of nicotine, thus driving nicotine self-administration despite the lack of salient environmental cues. To the best of our knowledge, this is the first time this is evidenced in a nicotine self-administration paradigm, supporting that individuals may vary in the mechanisms that drive their nicotine seeking (Garcia-Rivas and Deroche-Gamonet, 2018).

A novel procedure that allows targeting the reinforcing-enhancing effects of nicotine on its associated salient cue during nicotine self-administration.

In a previous study, Palmatier et al (2007) first compared two visual stimuli for the strength of their primary reinforcement in nicotine-naïve animals. Then they observed that nicotine, administered passively, powerfully increased the reinforcement of the visual stimulus with the strongest primary reinforcing effects, with a much weaker effect for the other visual stimulus. Thus, the authors concluded that the reinforcing-enhancing effects of nicotine are dependent on the strength of the

primary reinforcement of the visual cue in a nicotine-naïve state. Further studies have assessed the effect of varenicline on this nicotinic enhancement of cue reinforcement, but in conditions that are different from volitional nicotine intake (Levin et al., 2012; Barrett et al., 2018). Here, we developed a novel experimental approach that consists of a sudden increasing in the visual salience of the nicotine-paired cue, through the removal of an interfering Ambient light (AL). This approach allowed us to explore the observations by Palmatier et al (2007), but in the context of nicotine self-administration, and within the same individuals.

In our experiment, the presence of the *AL* reduced operant responding in both *saline+cue* and *nicotine+cue* conditions (**Fig 3**). A possible explanation for the interfering effect of the *AL* could be a non-specific aversive or stressful effect, rather than a reduction in the reinforcing effects of the cue. However, this explanation is unlikely. The aversive effect of an ambient stressor would have impacted both active and inactive responding, while this is not the case. While active nose pokes were higher in the *No AL* condition, inactive nose-pokes in both *AL* and *No AL* conditions remained at the same level, both during *saline+cue* and *nicotine+cue* (**Supplementary Fig 6**) self-administration. Importantly, in the *AL* condition, discrimination between active and inactive holes is greatly affected in the first sessions during acquisition (**Supplementary Fig 6**), compared to the *No AL* condition. It is noteworthy that the presence of the *AL* delayed the acquisition of self-administration of *nicotine+cue*, which became equivalent to that of the *No AL* condition starting session 17 (**Fig 3c**). Overall, this data strongly supports that the interfering effect of the *AL* is due to a reduction of the visual salience of the cue through visual interference, rather than a mere stress effect caused by the *AL*.

It is also noteworthy that the increase in self-administration due to removal of the visual interference was much more pronounced in nicotine+cue conditions compared to saline+cue conditions (**Fig 3e**), evidencing the magnifying effect by nicotine on a sudden increase in cue reinforcing effects. This is consistent with previous studies showing that nicotine can increase the reinforcement and incentive salience of cues that have already reinforcing value (Donny et al, 2003; Palmatier et al, 2007, 2013; Rupperecht et al, 2015). It thus follows that any increase in salience of nicotine-paired cues would be magnified even further by nicotine, as supported by our study. No other study to date has specifically addressed this possibility. By comparison, decreasing the cue salience by introduction of the *AL* has the same decreasing effect on both nicotine+cue and saline+cue self-administration, suggesting a non-specific decrease in visual perception, which is not altered by nicotine (**Fig 3e**).

Varenicline targets the reinforcing effects and reinforcing-enhancing effects of nicotine on its associated cue.

In accordance with the literature (Funk et al, 2016; Le Foll et al, 2012; O'Connor et al, 2010; Rollema et al, 2007b), we showed that Varenicline reduces nicotine+cue self-administration. We demonstrated that acute Varenicline also decreases behavior in rats self-administering nicotine alone, although to a lesser absolute extent. In the same conditions, acute Varenicline has no effect on the self-administration of the salient visual cue by itself.

A limitation in exploring Varenicline effects on the sole reinforcing effects of nicotine is that these are relatively weak, and even for those rats that acquired nicotine self-administration without the presence of a nicotine-paired cue, their baseline nicotine seeking behavior is substantially lower than in for nicotine+cue self-administration. This could compromise the detection of varenicline effects, as decreases in responding are less evident when the baseline responding is already low. However, it is critical that the effect of Varenicline was independent of baseline self-administration (**Fig 2c**).

Clemens et al. (2017) reported that after early training in nicotine+cue self-administration, acute Varenicline (1mg/kg) did not significantly impact self-administration of nicotine alone, 7 sessions after the cue had been removed from the protocol. Prior association with a cue together with a 7 sessions extinction-like procedure might be responsible for the difference between Clemens et al. (2017) and our study.

Our study also complement previous findings in clarifying the reinforcing-enhancing effects of varenicline on a visual cue: namely, that these effects are only observed when individuals have been previously exposed to nAChR agonists. Contrary to our study, Clemens et al. (2017) and Barrett et al. (2018) showed that acute Varenicline increased the self-administration of a visual cue alone in the absence of nicotine. Furthermore, Levin et al. (2012) briefly reports, in drug-naïve animals, the reinforcing-enhancing effects of varenicline on visual cues. However, and differently to our case, in these studies rats had been previously exposed to either nicotine or varenicline: In Clemens et al. (2017), rats had been previously trained for nicotine+cue self-administration and Varenicline tested after 7 self-administration of the cue alone, through a nicotine extinction-like procedure. In Barrett et al. (2018), varenicline was tested following a history of repeated passive exposure to nicotinic agonist (nicotine) administered after the cue self-administration sessions. In Levin et al. (2012), the authors make a brief comment that the reinforcing-enhancing effects of varenicline were evident in the first seven sessions of repeated varenicline exposure, although it remains unknown where such reported effects were already substantial on the first session. It is noteworthy that in these three cases, the reinforcing-enhancing effects of varenicline appear similar, regardless of whether the nicotinic agonist was present at the moment of cue self-administration (Clemens et al., 2017; Levin et al., 2012) or disconnected from it (Barrett et al., 2018). In our study, the lack of previous history with nAChR agonists in saline+cue rats could thus explain the lack of reinforcing-enhancing effects of varenicline described by Clemens, Levin and Barrett. This temporal requirement could most probably involve upregulation of alpha4beta2-containing nAChRs, caused by chronic exposure to both nicotine (Marks et al., 1983; Buisson and Bertrand, 2001; Staley et al., 2006) and varenicline (Marks et al., 2015). Nicotine, however, is known for its acutely reinforcing-enhancing effect of stimuli, even in drug-naïve individuals (Rupprecht et al. 2015; Perkins, Karelitz, & Boldry 2017). This supports that varenicline does not necessarily reproduce a nicotine-like increase in cue reinforcing effects, but requires a cholinergic system already sensitized by nicotinic agonists, making rats more sensitive to the reinforcing-enhancing effect of nicotinic agonists to cues. Further studies are needed to explore this possibility.

Varenicline targets the reinforcement-enhancing effect of nicotine on its associated cue during self-administration.

Using the visual interfering procedure described in the first paragraph, we evidenced a direct indication that Varenicline specifically reduces the reinforcement-enhancing effects of nicotine on surrounding cues during nicotine self-administration.

Varenicline effect on nicotine self-administration was magnified when the nicotine-paired cue gains salience during removal of the visual interference, in comparison with the nicotine-paired cue remaining undisturbed, or when its salience is decreased during AL insertion. Critically, Varenicline appeared to specifically target the enhanced response due to AL removal. Indeed large individual differences were observed in the increase of self-administration in response to AL removal (**Fig 3c, abscissa**). Varenicline appeared to precisely target this enhanced effect, at the individual level. Thus,

for example a rat showing an increase of 30 reinforcers in response to AL removal in control condition had a decrease of 30 infusions by Varenicline in the AL removal condition. To our knowledge, we are the first to report an effect of varenicline that depends on the strength of nicotine-cue interactions: when the interaction is stronger, the effect of varenicline is stronger, and it is less so when the interaction is weaker. This observation strongly supports the rationale for individual variations in the mechanisms of nicotine seeking (Garcia-Rivas and Deroche-Gamonet, 2018), with individuals being more sensitive than others to the influence of the reinforcement-enhancing effect of nicotine on environmental cues, and that could differently benefit from Varenicline treatment.

Levin et al. (2012) showed that Varenicline could antagonize the nicotine-induced enhancing of reinforcement of an unconditioned cue. However, rats were not self-administering nicotine+cue, but rather receiving non-contingent nicotine through a subcutaneous injection before every session of cue self-administration. Notwithstanding, altogether with our data, this strongly supports that Varenicline can reduce the nicotine-induced increase in cue reinforcement.

It has been previously shown that the reinforcement-enhancing effect of nicotine on cues is dependent on $\alpha 4\beta 2$ -containing nAChRs (Liu et al, 2007), but also on the dopaminergic system (Palmatier et al, 2014). Given the precise molecular pharmacology of Varenicline, a possible mechanism for Varenicline actions could be to antagonize nicotine at the $\alpha 4\beta 2$ -containing nAChRs located in the ventral tegmental area (VTA), thus reducing the nicotine-induced tonic firing of dopaminergic neurons, leading to decreased tonic release of dopamine in the nucleus accumbens (NAcc) (Crunelle et al, 2010). Such a mechanism could also be involved in the effect of varenicline on the primary reinforcing effects of nicotine, which are also thought to be dependent on VTA to NAcc signaling (Di Chiara, 2000; Picciotto and Corrigall, 2002). An alternative mechanism could involve other structures in the circuitry controlling nicotine-cue interactions, such as the basolateral amygdala, an area rich in $\alpha 4\beta 2$ -containing nAChRs (Feduccia et al, 2012) and also involved in drug-cue interactions (Janak and Tye, 2015).

In our study, we have investigated the psychopharmacological targets of Varenicline during early nicotine+cue self-administration. Future studies should address whether prolonged exposure to nicotine changes the way Varenicline affects nicotine and nicotine+cue self-administration. The differential effects of Varenicline in nicotine+cue self-administration in short vs prolonged exposure to nicotine might depend on the experimental approach: George et al. (2011) reports that Varenicline does not differently affect rats with long access to nicotine (23-hour sessions) compared to short access (1-hour session). The study by Clemens et al. (2017) on the other hand, shows that after an extended training (40 sessions) with a short access protocol, Varenicline seems to also target the reinforcing properties of nicotine alone, compared to early training (20 sessions). However, the specificity of this Varenicline effect is problematic, as the decrease in responding is seen both in active and inactive responding. These results warrant further exploration.

Furthermore, as a treatment for tobacco cessation, daily doses of Varenicline are recommended in the week leading up to a cessation attempt, with continuous daily administration over the following 11 weeks after cessation (Ebbert et al, 2010). While our study only assessed the effect of an acute exposure to 1mg/kg Varenicline, further studies need to assess if prolonged exposure to Varenicline affects the psychopharmacological dimensions of nicotine seeking during nicotine self-administration in a different way than those after acute exposure shown here. Studies with repeated varenicline administration have been performed but focused on the reinforcing effects of a visual cue either in rats never exposed to nicotine (Levin et al., 2012) or previously administered with passive nicotine injections (Barrett et al., 2018). In addition, within the same study by Levin et al (2012), varenicline

1mg/kg failed and succeeded to increase the reinforcing effects of a visual stimulus in two experiments within the same study, obscuring any consistent interpretation of the effect of varenicline at this dose.

Despite this, our results raise therapeutic implications. Increasing clinical and preclinical data suggests that smokers differ in the mechanisms that drive their nicotine seeking (Garcia-Rivas and Deroche-Gamonet, 2018), with some smokers having stronger sensitivity to the primary reinforcing actions of nicotine (Esterlis et al, 2016; Hutchison et al, 2007), while some others being more sensitive to the effects of nicotine on surrounding cues (Perkins, 2009; Perkins et al, 2017; Van Heel et al, 2017). Our results support individual variations in both nicotine reinforcing effects and nicotine-induced enhancement of cue reinforcing effects in the rat. Critically, our data suggest that individual variations in nicotine-induced enhancement of cue reinforcing effects, but not individual variations in nicotine reinforcing effects, would determine the amplitude of acute Varenicline-induced decrease in seeking. Altogether, Varenicline might be more beneficial for smoking cessation in those who are especially sensitive to nicotine effects on surrounding cues, and not for those who are more sensitive to the primary reinforcing effects of nicotine.

Further studies need to clarify more precisely the action of Varenicline, using a preclinical model that would allow for the fine exploration of individual differences in the mechanisms that drive nicotine seeking (Garcia-Rivas et al, 2016).

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The authors have no conflicts of interest to declare.

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FIGURES LEGENDS

Figure 1. Nicotine and infusion-associated discrete cue light contribute synergistically to self-administration behavior. Operant nose-poking at FR3 in active hole was reinforced by the delivery of an intravenous infusion of saline associated with the lighting of a salient visual cue above the active hole (*saline+cue*), of a nicotine intravenous infusion associated with the lighting of a salient visual cue above the active hole (*nicotine+cue*) or of the sole delivery of a nicotine intravenous infusion (*nicotine*). **a.** Infusions earned per session over the 15 first behavioral sessions. **b.** Responses in the active and inactive holes per session over the 15 first behavioral sessions. Symbols denote group mean and error bars denote SEM.

Figure 2. Varenicline targets nicotine primary reinforcing effects and the nicotine-cue synergistic interactions. **a.** Mean infusions earned in basal conditions (*Baseline*) and after Varenicline administration (1mg/kg i.p., 30 min prior to session) in rats self-administering *saline+cue*, *nicotine+cue* or *nicotine*. For *Baseline*, infusions are averaged over the two last sessions prior to Varenicline test. **b.** Effect of Varenicline as calculated by the delta between infusions earned in baseline and infusions earned under Varenicline effect, in rats self-administering *saline+cue*, *nicotine+cue* or *nicotine*. **c.** Correlation between basal self-infusions and acute Varenicline effect as measured by delta-infusions from baseline, in the *nicotine* group. Symbols and bars denote group mean and error bars denote SEM. *p = 0.05, ***p=0.001. \$p=0.05 and \$\$\$p=0.001 as compared to respective baseline. %p=0.05, %%p=0.01, as compared to zero.

Figure 3. An interfering ambient light (AL) alters of the primary reinforcing effects of a salient discrete cue light. **a.** Infusions earned per session over 7 behavioral sessions during which operant nose-poking in the active hole was reinforced at FR3 by the delivery of an intravenous infusion of saline associated with the lighting of a salient visual cue above the active hole. The presence of a 15 Lux *Ambient light (AL)* reduced self-administration behavior as compared to the control condition (*No AL*). **b.** Effect on infusions earned of *AL Removal* and *AL Insertion* in rats trained for *saline+cue* self-administration over 7 sessions in the *AL* and *No AL* conditions, respectively. Basal infusions are averaged over the two last sessions prior to *AL Insertion (or Removal)* test. **The interfering AL delays acquisition of nicotine+cue self-administration.** **c.** Infusions earned per session over the first 19 behavioral sessions during which operant nose-poking in the active hole was reinforced at FR3 by the delivery of an intravenous infusion of nicotine associated with the lighting of a salient visual cue above the active hole. **d.** Effect on infusions earned of *AL Removal* and *AL Insertion* in rats trained for *nicotine+cue* self-administration in the *AL* and *No AL* conditions, respectively. **The interfering AL procedure allows revealing the reinforcement-enhancing effect of nicotine on its associated salient cue.** **e.** Comparison of *AL Removal* and *AL Insertion* effects in rats trained for *saline+cue* or *nicotine+cue* self-administration. While *AL Insertion* in *No AL* rats produced a similar decrease in *saline+cue* and *nicotine+cue* rats (bottom), *AL Removal* produced a stronger increase in *nicotine+cue* rats (top). Symbols and bars denote group mean and error bars denote SEM. *p = 0.05; **p=0.01, ***p=0.001.

Figure 4. Varenicline targets the reinforcement-enhancing effect of nicotine on its associated salient cue. **a.** Infusions earned in rats trained for *nicotine+cue* self-administration in the presence of the interfering AL (*AL Baseline*), in response to Varenicline (*Var*), to AL Removal (*AL Remov*) or a combination of both (*AL Remov+Var*). **b.** Comparison of Varenicline effect in *AL Baseline* condition (Infusions *Var AL Baseline* – Infusions *AL Baseline*) and in *Increased Cue Salience* condition (by *AL Removal*) [calculated from the combined effect of *AL Removal* and *Varenicline* (Infusions *AL Remov+Var* – Infusions *AL Baseline*) minus the effect of *AL Removal* (Infusions *AL Remov* – Infusions *AL Baseline*)]. Varenicline absolute effect was amplified in the *Increased Cue Salience* condition (by *AL Removal*). **c.** Almost 1 to 1 negative correlation between the effect of *Increased Cue Salience* and the calculated effect of Varenicline on *Increased Cue Salience*. The individual increase in *nicotine+cue* infusions by *Increased Cue Salience* was antagonized by Varenicline. **d.** Infusions earned in rats trained for *nicotine+cue* self-administration in the absence of the interfering AL (*No AL Baseline*), in response to Varenicline (*Var*), to AL Insertion (*AL Insert*) or a combination of both (*AL Insert+Var*). **e.** Comparison of Varenicline effect in *No AL* baseline condition (Infusions *Var No AL* baseline – Infusions *No AL Baseline*) and in *Decreased Cue Salience* condition (by *AL Insertion*) [calculated from the combined effect of *AL Insertion* and *Varenicline* (Infusions *AL Insert+Var* – Infusions *No AL Baseline*) minus the effect of *AL Insertion* (Infusions *AL Insert* – Infusions *No AL Baseline*)]. Varenicline absolute effect was similar in the two conditions. **f.** Poor correlation between the effect of *Decreased Cue Salience* (by *AL Insertion*) and the calculated effect of Varenicline on *Decreased Cue Salience* (by *AL Insertion*). The individual alteration in *nicotine+cue* infusions by *Decreased Cue Salience* was not of comparable amplitude to the alteration produced by Varenicline on *Decreased Cue Salience*.

Bars denote group mean and error bars denote SEM. Data points reflect individual scores. * $p = 0.05$; ** $p = 0.01$, *** $p = 0.001$.

FIGURES

Figure 1

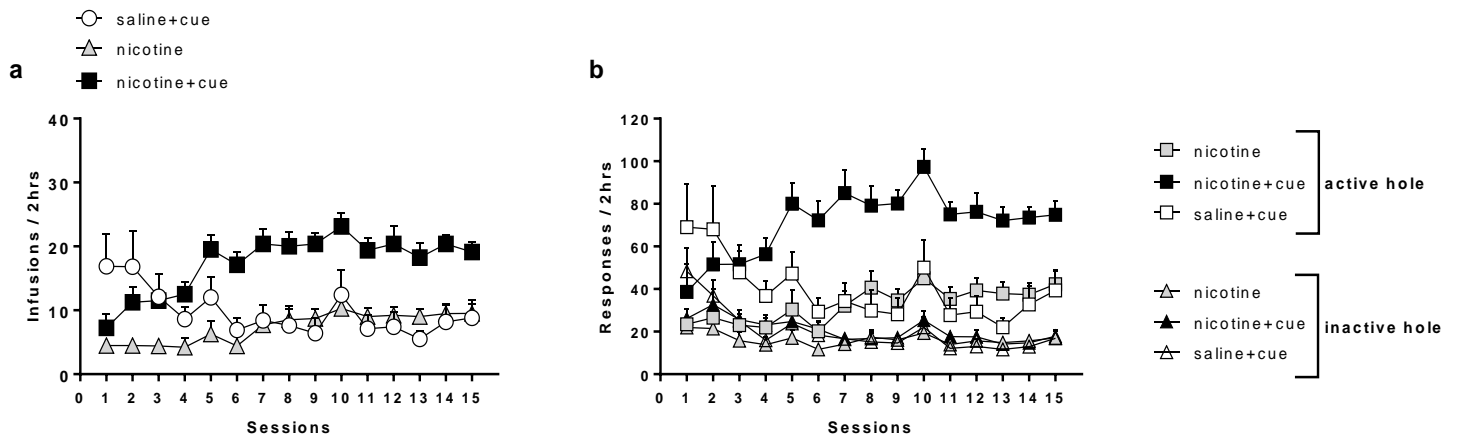


Figure 2

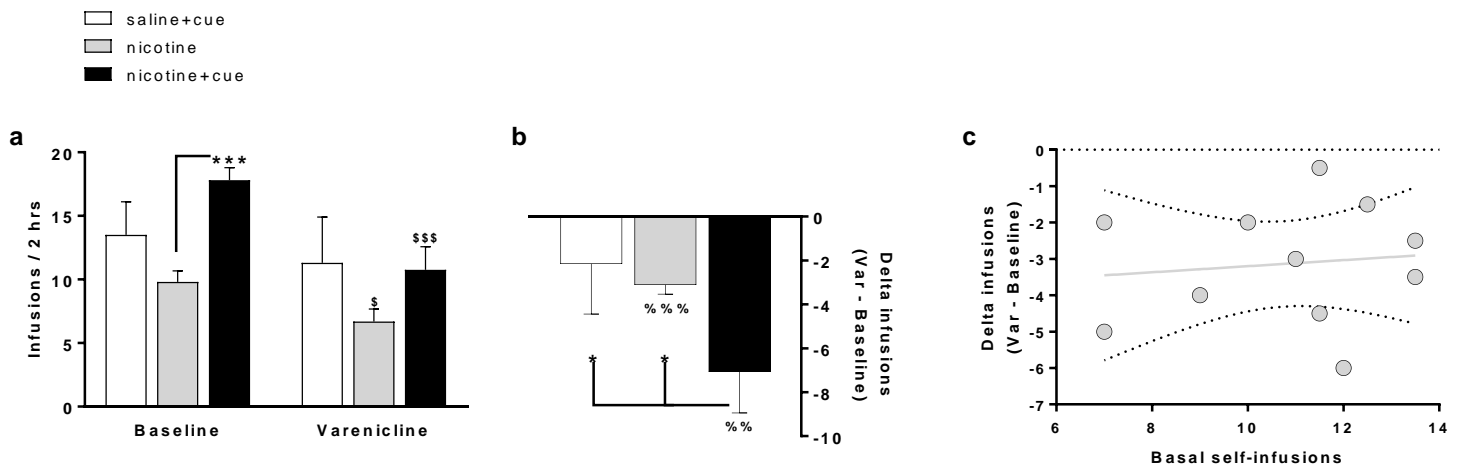


Figure 3

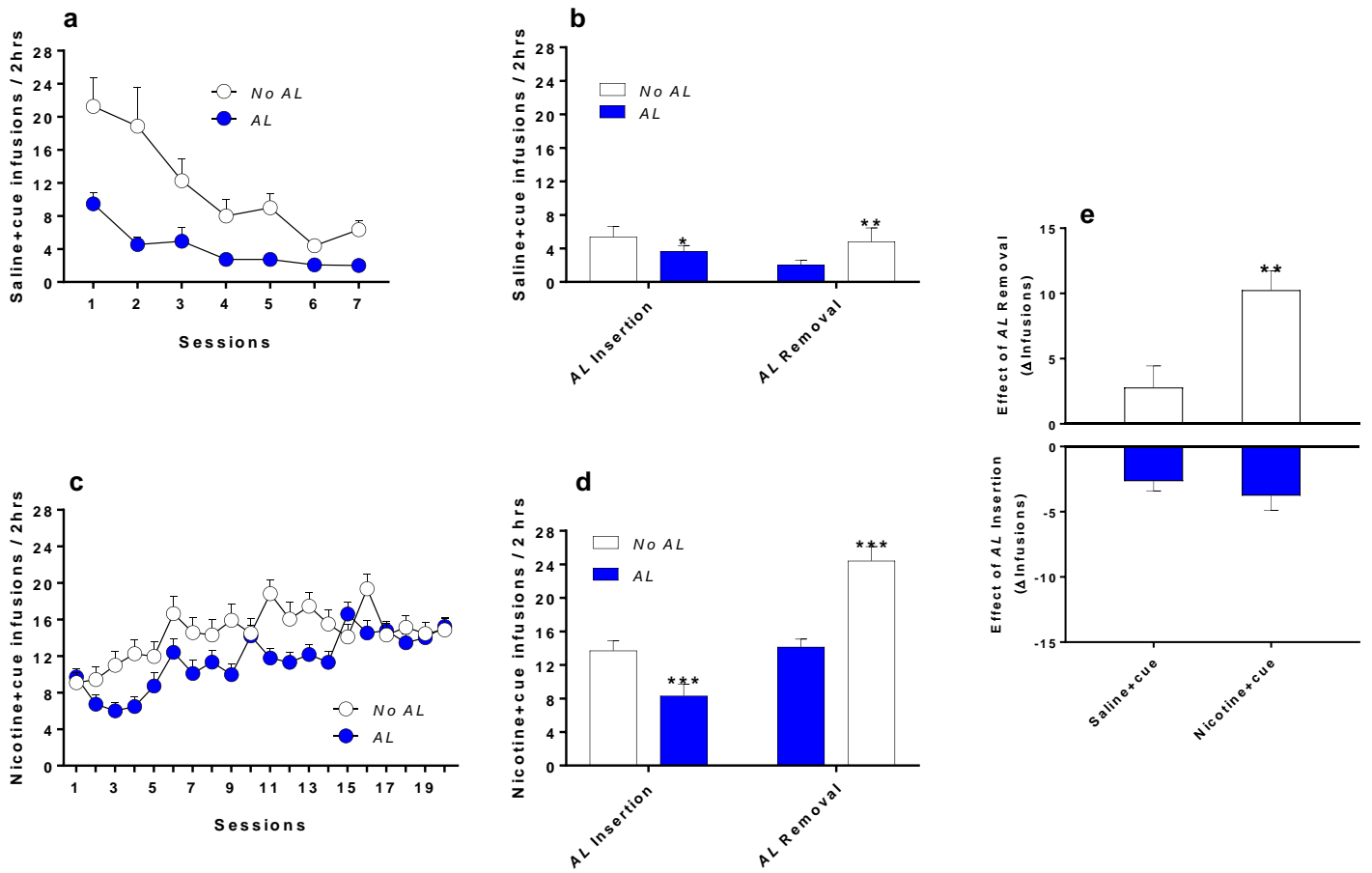
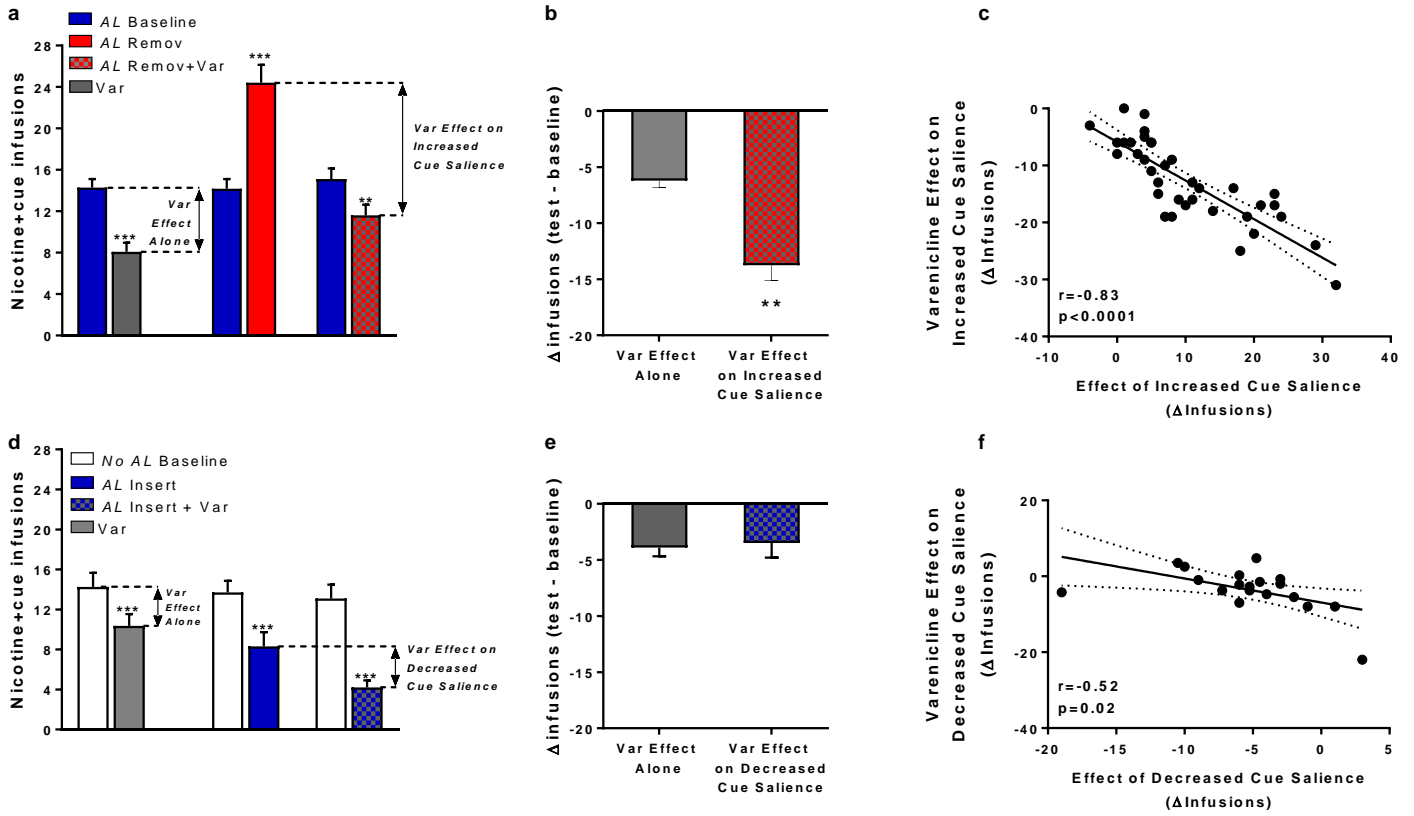
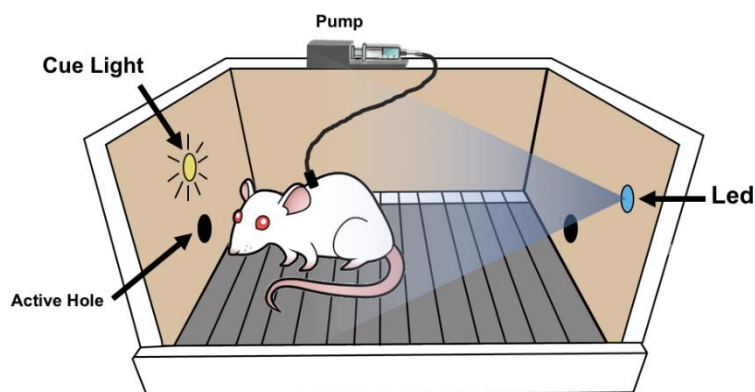


Figure 4



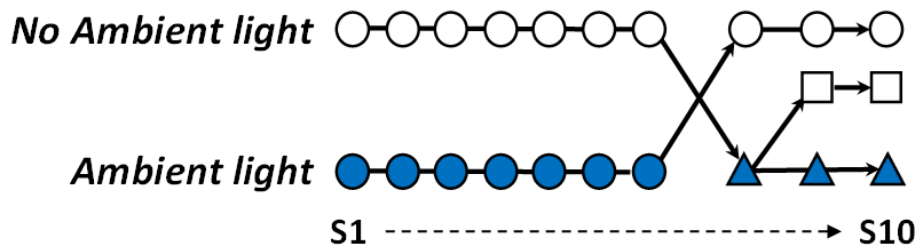
SUPPLEMENTARY MATERIAL

Supplementary Figure 1



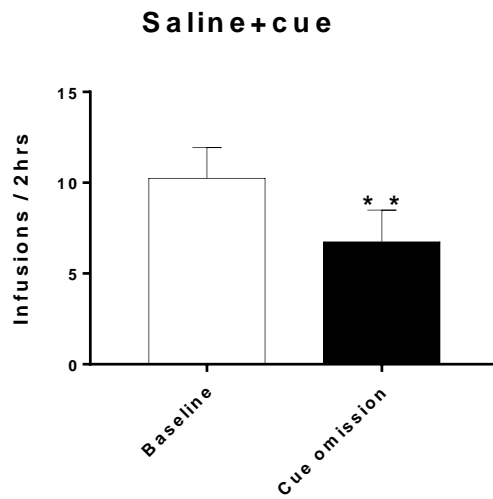
Scheme of the intravenous self-administration operant chamber. Each chamber (40 cm long x 30 cm width x 52 cm high) was located in an opaque sound-attenuating cubicle equipped with an exhaust fan to assure air renewal and mask background noise. For self-administration sessions, each rat was placed in one chamber where its chronically implanted intracardiac catheter was connected to a pump-driven syringe (pump). Two holes, located at opposite sides of the chamber at 5.5 cm from the grid floor, were used to record instrumental responding. In given experimental groups and experiments, a white light (white LED, Seoul Semiconductor, South Korea), 1.8 cm in diameter, located 11.5 cm above the active hole, was used as nicotine (or saline) delivery-associated discrete visual cue. A blue light (blue LED, Sloan Precision Optoelectronics, Switzerland), 1.8 cm in diameter, located on the opposite wall at 17 cm of the floor on the left side, was used as ambient light (*AL*). It produced 15 Lux at a wavelength of 470nm.

Supplementary Figure 2



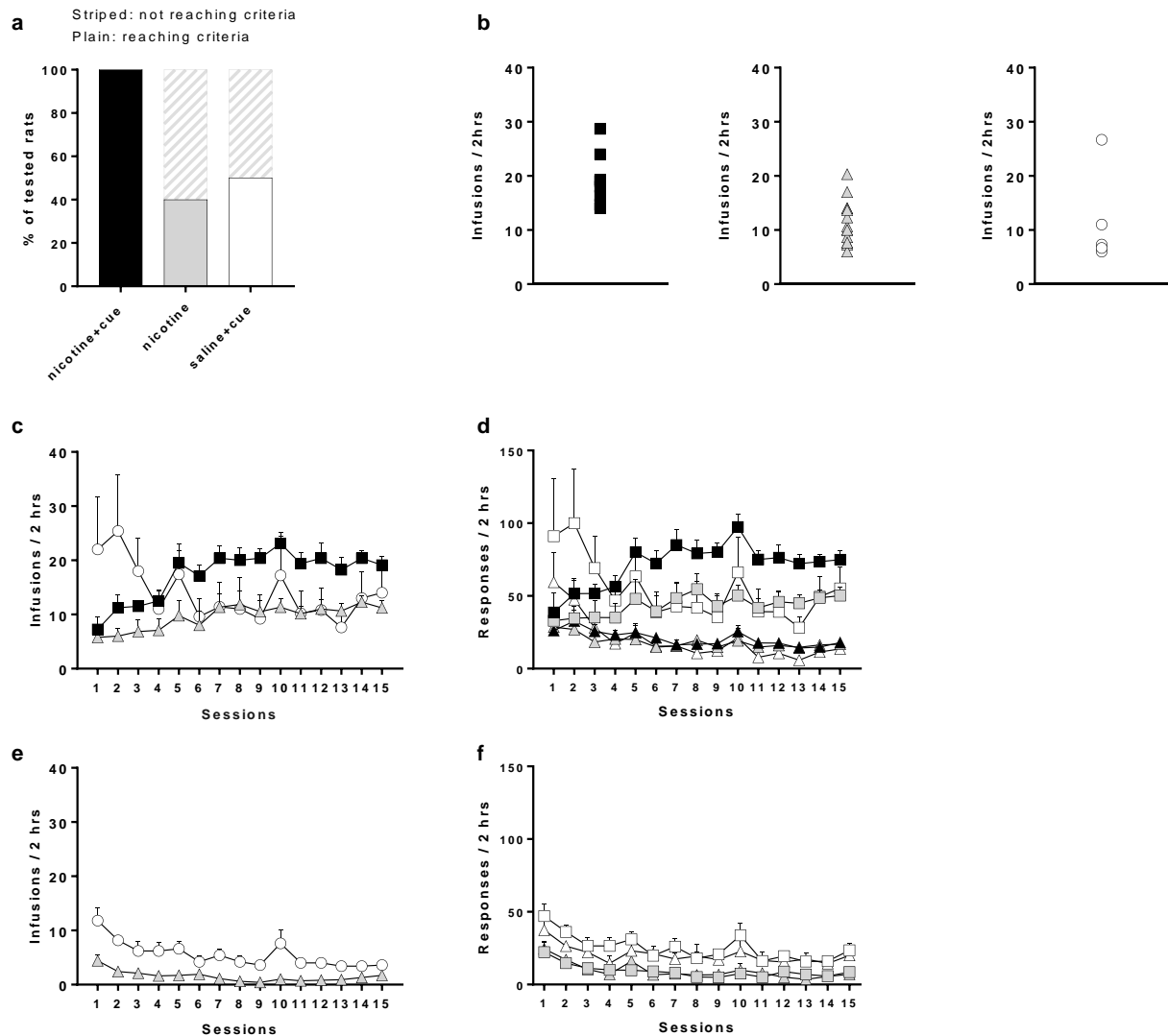
Protocol of experiment 2. Two groups of rats were trained for *saline+cue* self-administration. For one group (*Ambient light, AL*, $n=15$), the *Ambient light* was on throughout the first 7 sessions. For the other group (*No Ambient light, No AL*, $n=15$) the *AL* was off during the same period. On the eighth session of self-administration, the *AL* conditions were switched; turned off for the *AL* group and on for the *No AL* one. On sessions 9 and 10, the *No AL* group was split into two, with half of the rats switched back to their original *No AL* condition (white squares, *Single AL Insertion* subgroup, $n=7$), while the other half remaining under the new *AL* condition (blue triangles, *Sustained AL Insertion* subgroup, $n=8$). All rats from the *AL* group remained without the *AL* for sessions 9 and 10. Each symbol represents one self-administration session.

Supplementary Figure 3



Cue omission decreases *saline+cue* self-administration. Mean infusions earned in basal conditions (*Baseline*) and during a cue omission test in rats self-administering *saline+cue*. For *Baseline*, infusions are averaged over the two last sessions prior to omission test. ** $p=0.01$. Bars denote group mean and error bars denote SEM.

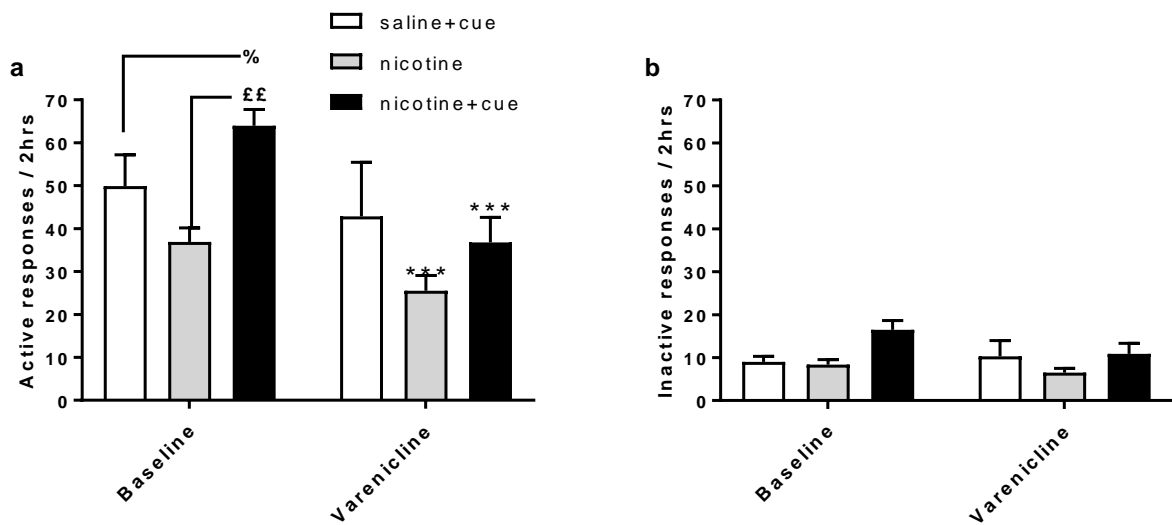
Supplementary Figure 4



Acquisition of self-administration behavior in rats self-administering *nicotine*, *nicotine+cue* or *saline+cue*. **a.** % of tested rats acquiring self-administration according to the defined criteria. **b.** Distribution of individual scores of mean self-infusions per session in rats meeting acquisition criteria, in *nicotine+cue*, *nicotine*, and *saline+cue* groups, from left to right. **c-d.** Mean self-infusions per session and hole responses (squares=active hole, triangles=inactive hole) in rats meeting self-administration criteria. **e-f.** Mean self-infusions per session and hole responses (squares=active hole, triangles=inactive hole) in rats not meeting self-administration criteria, in the *nicotine* and the *saline+cue* groups.

c-f: Symbols denote group mean and error bars denote SEM.

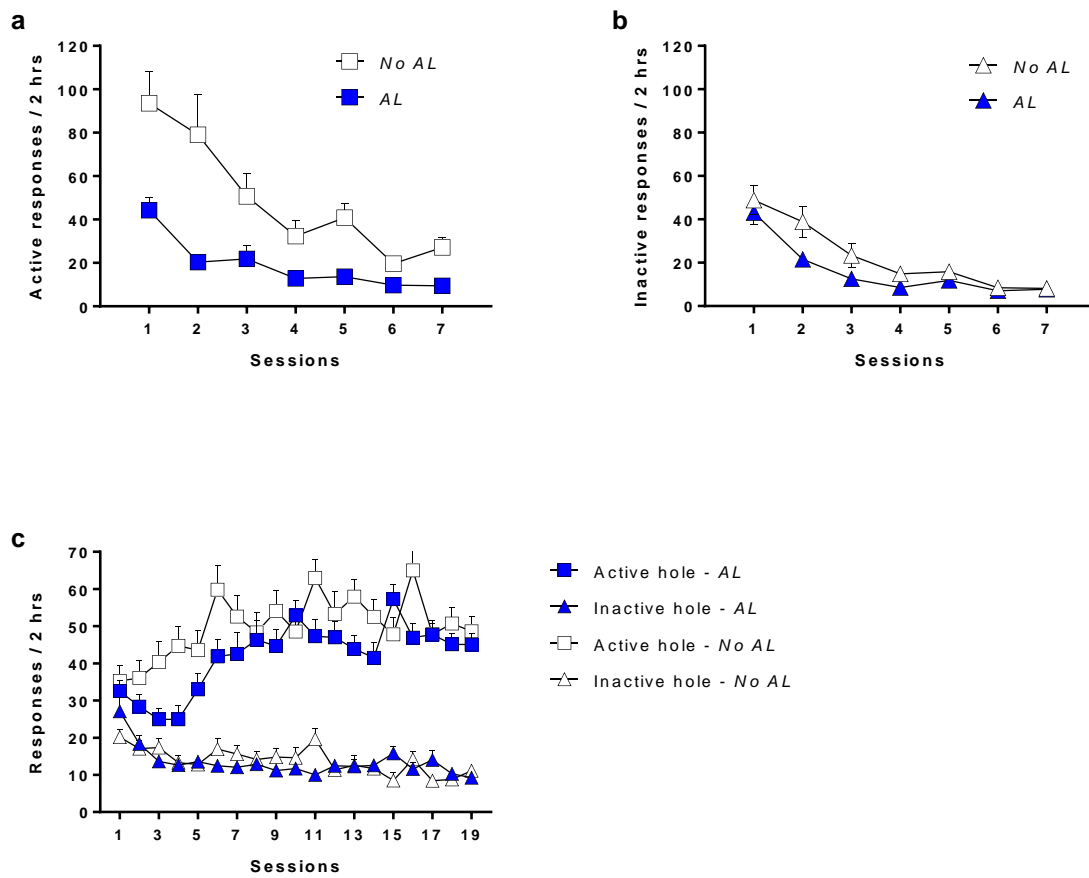
Supplementary Figure 5



Varenicline targets the nicotine-cue synergistic interactions, rather than nicotine or cue primary reinforcing effects. a. Mean active nose-pokes in basal conditions (baseline) and after Varenicline administration (1mg/kg i.p., 30 min prior to session) in rats self-administering *saline+cue*, *nicotine+cue* or *nicotine*. For Baseline, responses are averaged over the two last sessions prior to Varenicline test. **b.** same as **a.** for inactive nose-pokes.

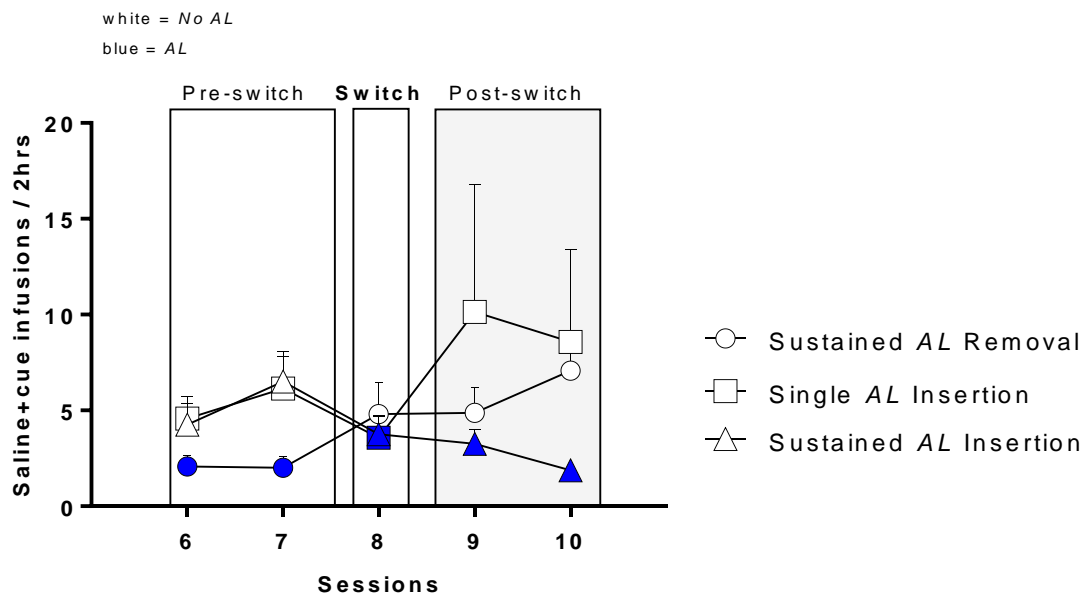
Bars denote group mean and error bars denote SEM. ***p=0.001 as compared to respective baseline. ££ p=0.01, %p=0.05.

Supplementary Figure 6



Impact of Ambient light (AL) on saline+cue and nicotine+cue self-administration. a. Mean active responses per session in rats self-administering saline+cue in presence or absence of AL. **b.** Same as **a.** for inactive responses. **c.** Mean active and inactive responses per session in rats self-administering nicotine+cue in presence or absence of AL. Symbols denote group mean and error bars denote SEM.

Supplementary Figure 7



An interfering Ambient light (AL) alters of the primary reinforcing effects of a salient discrete cue light.

Effect on *saline+cue* infusions per session of insertion or removal of an interfering AL. Sessions 6 and 7 represent the last 2 of 7 self-administration sessions during which active nose-poking was reinforced at FR3 by the delivery of an intravenous infusion of saline associated with the lighting of a salient visual cue (*saline+cue*), in the absence (control - *No AL* - white symbols) or presence (*AL* - blue symbols) of an interfering AL. At this point (session 8), according to Supplementary Fig 2, the AL conditions were switched; turned off for the *Ambient light* group and on for the *No AL* one (mean data shown on **Figure 3b**).

Then initial *No AL* rats were split into two groups for the following two sessions (9 and 10): one group (*Sustained AL Insertion*), maintained the newly acquired AL condition, while the other (*Single AL Insertion*) returned to their *No AL* condition. AL insertion effect was further amplified by *Sustained AL insertion*, while a rebound was observed by AL Removal in the *Single AL Insertion* subgroup. To show the similarity in baseline and in response to AL Insertion of the two subgroups of *No AL* rats, results of the two subgroups are shown separately on sessions 6, 7 and 8 (white squares and triangles).

In the initial AL rats, the removal of the AL was maintained (*Sustained AL Removal*), further increasing self-administration in comparison to sessions 6 and 7. Symbols denote group mean and error bars denote SEM.

Chapter 5

GENERAL DISCUSSION

General Discussion and Perspectives

While tobacco dependence remains a leading cause of preventable premature deaths worldwide (WHO, 2017), therapeutic strategies to help smokers quit have only limited efficacy (Schuit et al., 2017). In the introduction of this dissertation, we have summarized plenty of animal and human data, which suggest that the mechanisms that drive nicotine seeking may be different among individuals, and this could explain the limited efficacy of available treatments (Garcia-Rivas et al., 2017; Garcia-Rivas and Deroche-Gamonet, 2018).

Based on a preclinical strategy we recently proposed (Garcia-Rivas et al., 2017), we identified three clusters of rats that differed in the contributions of nicotine, and associated cue, in their nicotine seeking. One of such groups (Cluster1) showed predominant self-administration of the cue, despite showing some aversive sensitivity to nicotine. This is the first time, to our knowledge, that aversion to nicotine is evidenced in a classical model of nicotine self-administration using a dose within the standard range often used in the literature (Donny et al., 1998; Abrous et al., 2002; Adriani et al., 2003; Harris et al., 2009; Smith et al., 2014; Grebenstein et al., 2015). This raises interesting questions about the validity of the mean behavior observed in studies that use nicotine self-administration, as until now an increased sensitivity to nicotine aversion in subpopulations of individuals within outbred populations of rats has been widely overlooked. Further studies could explore the precise involvement of the $\beta 4$ nAChR subunit in the mHb (Haller et al., 2012; Slimak et al., 2014; Antolin-Fontes et al., 2015), in the aversive-like effects observed during a classical protocol of nicotine self-administration, as observed in our Cluster 1.

The other two groups (Cluster2 and 3) showed nicotine-cue interactions: for Cluster 2, the cue seemed to have become a conditioned reinforcer to nicotine, while for Cluster 3, the reinforcement of the cue appeared dependent on the presence of nicotine. We validated these behavioral profiles using external correlates that could also provide information about the possible biological mechanisms behind them. In Cluster3, notably, we saw consistent correlations of expression of nAChRs in the BLA in responses

to a change in dose, and to cue omission effects, suggesting that the nicotine-dependent reinforcement of the cue observed in this cluster could probably involve the BLA. This suggestion is not far from what the literature already shows about the involvement of the BLA in drug-cue interactions (Ambroggi et al., 2008; Chiamulera, 2005; Everitt and Robbins, 2005; Janak and Tye, 2015; Sharp, 2018).

The nicotine-dependent reinforcement of surrounding cues is thought to be a separate phenomenon from the classical observation of a drug-paired cue becoming a conditioned reinforcer upon repeated pairings with nicotine (Caggiula et al., 2009; Rupperecht et al., 2015). Indeed, various studies have evidenced that the reinforcer-enhancing effects of nicotine on surrounding cues is not dependent on any learning or associative mechanisms, as it can be evidenced upon non-contingent delivery of nicotine (Chaudhri et al., 2006; Palmatier et al., 2007; Levin et al., 2012; Perkins et al., 2015) and even at the first acute exposure to nicotine (Rupperecht et al., 2015; Perkins et al., 2017). Because of the powerful regulation that nicotine can thus exert on the incentive salience of surrounding environmental stimuli, this process of nicotine-induced enhancement of cue reinforcement is seen as a key component in nicotine seeking (Caggiula et al., 2009; Rupperecht et al., 2015).

Whether available therapies against tobacco dependence specifically target any of these psychopharmacological dimensions of nicotine seeking is still not well understood. Varenicline, the most efficacious of all pharmacotherapies against tobacco dependence, was developed specifically as a partial agonist of $\alpha 4\beta 2$ nAChRs (Coe et al., 2005; Rollema et al., 2007), and thus, combines an antagonist-like effect at these receptors in the presence of nicotine, thus reducing the reinforcement of tobacco products, while acting as partial agonist in the absence of nicotine, reducing tonic background craving thought to play a pivotal role in triggering smoking relapse. Up until now, only limited evidence exists as to its targeting of the aforementioned dimensions of nicotine seeking. In our chapter 4, we developed an experimental strategy conducive to evidence the nicotine-induced enhancement of nicotine reinforcement within a classical model of self-administration. Based on this model, we provided evidence that varenicline is directly affecting the reinforcing-enhancing effects of nicotine on surrounding cues, with only a very limited effect in the reinforcing actions of nicotine per se.

Importantly, the magnitude of the effect of varenicline was proportional to the magnitude of the reinforcing-enhancing effect of nicotine on the cue, indicating that varenicline may be more beneficial in those individuals that are most sensitive to such nicotine effects on the environment.

In Cluster 2, we observed nicotine seeking controlled by both nicotine, and its associated cue as a classical conditioned reinforcer. Despite plenty of evidence suggesting that varenicline can reduce background tonic craving (Ferguson and Shiffman, 2009; Ravva et al., 2015; Cui et al., 2018), its role in episodic cue-induced craving remains controversial, with some studies suggesting that varenicline reduces it (Brandon et al., 2011; Franklin et al., 2011; Ray et al., 2013; Versace et al., 2017; Wilcox et al., 2018), while others seeing no effect at all (Niaura et al., 2008; Ferguson and Shiffman, 2009; Gass et al., 2012; Hitsman et al., 2013; Ravva et al., 2015; Cui et al., 2018). Since we did not directly assess the acute effect of varenicline on the strength of a conditioned reinforcer to nicotine, further studies could assess whether, and how, varenicline treatment can disrupt this phenomenon in rats that have the same behavioral phenotype as Cluster2 in our study.

Given our findings in Chapter 4, varenicline could be of greater benefit for individuals that match the profile of our Cluster 3 rats, in which the main driver of their self-administration behavior appears to be the nicotine-induced enhancement of cue reinforcement. A potential experiment to test this possibility would be to train rats to self-administer nicotine, cluster them according to their differential responding to cue and nicotine cue omission tests as done before, and then test the extent of drop in their normal self-administration in the three clusters after an acute IP injection of varenicline. We hypothesize a greater drop in the percent of self-infusions of Cluster3, compared to the other clusters.

However, a deeper exploration of the neurobiology of the reinforcing-enhancing effect of nicotine, and its antagonism by varenicline, would be more useful in disentangling the precise mechanisms of this psychopharmacological phenomenon. An inherent limitation of correlational studies, like the one presented in this dissertation, is that causation can only be inferred. As important as correlational studies are for evidencing phenomena, further studies should assess causality, through interventions that prevent, generate, or reverse, the phenotypes observed in this study. In this regard, a potential

approach could be to turn to the plenty genetic tools developed for the exploration of mice neurobiology (Zelena et al., 2017). Although obvious species differences ought to be considered (Ellenbroek and Youn, 2016), both mice and rats have been extensively used in the study of the nicotine dependence (Malin and Goyarzu, 2009; O'Dell and Khroyan, 2009; Cohen and George, 2013). In particular, the recent development of light-controlled nAChRs (Tochitsky et al., 2012), which combine optogenetics with pharmacology, now gives the opportunity to alter, rapidly and reversibly, nicotinic cholinergic signaling in key brain areas relevant to nicotine seeking (Durand-de Cuttoli et al., 2018). Given our findings in Chapter 3, the role of $\alpha 4\beta 2$ nAChRs in the BLA could be of interest in these further studies. A potential experiment to test the role of $\alpha 4\beta 2$ nAChRs in the BLA, in the nicotine-induced enhancing of cue reinforcement, could be to train mice, which would have been genetically modified to express light-controlled $\alpha 4\beta 2$ -nAChRs in the BLA, to develop operant responding for a cue light, as others have done with wild-type mice (Contet et al., 2010). After acquisition, we would test for the reinforcing-enhancing effect of nicotine, by administering nicotine subcutaneously prior to their habitual cue operant session, in a manner similar to other studies in rats (Palmatier et al., 2007; Levin et al., 2012). The nicotine-induced enhancement of reinforcement would be quantifiable by an increase in visual reinforcers earned, compared to a control group pre-treated with saline. Then, by directing an optic fiber to the BLA, we would assess whether inactivation of the photo-sensitive $\alpha 4\beta 2$ nAChRs in this site would decrease the reinforcing-enhancing effect of nicotine on the visual reinforcer, and whether activation of the photo-sensitive $\alpha 4\beta 2$ nAChRs in the absence of nicotine would 'mimic' such reinforcing-enhancing effect. This would give us key evidence about whether nicotinic cholinergic transmission at the BLA is central to this phenomenon.

To complement these findings, and build a bridge towards individual approaches to therapeutics, later studies can also be done in rats to confirm these observations obtained from mice studies. These other studies could involve a classical pharmacological approach, directing $\alpha 4\beta 2$ antagonists or partial agonists, like varenicline, through bilateral cannulae directed to the BLA of rats trained to self-administer nicotine in conditions in which the reinforcing-enhancing effects can be identified. Such a protocol as the one we used in Chapter 5 could be of relevance in this experiment. If the effects of

intra-BLA varenicline are the same as those observed through systemic infusion of varenicline in Chapter 5, this would bring conclusive evidence of the involvement of the BLA in the reinforcing-enhancing effects of nicotine, as well as demonstrating the precise mechanism through which varenicline can antagonize these effects. These findings would give us interesting insights in the neurobiological mechanisms behind the reinforcement-enhancing effects of nicotine on surrounding cues, as well as raising important implications for a better, and tailored use, of varenicline, in those individuals more sensitive to the reinforcing-enhancing effects of nicotine.

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Résumé

Le tabac est une drogue de choix pour environ 1,1 milliard de personnes dans le monde. C'est la drogue dont l'abus a la prévalence la plus élevée, derrière l'alcool. L'usage du tabac est particulièrement élevé dans l'Union européenne, où 24% des personnes de plus de 15 ans sont des fumeurs, dont 80% sont des utilisateurs quotidiens. Les conséquences sanitaires sont importantes, car le tabagisme, ainsi que l'exposition indirecte au tabac, sont des causes directes de mortalité prématurée, causant 6 millions de décès chaque année dans le monde. En effet, en Europe, le tabac est responsable de 26% des décès chez les adultes âgés de 30 à 69 ans. Les maladies attribuées au tabagisme représentent environ 6% des dépenses mondiales de santé et environ 2,5% du produit intérieur brut de l'Union Européenne. En dépit des mesures sociales et politiques développées au cours des quinze dernières années pour réduire le tabagisme, il continue d'être la principale cause de décès évitables dans le monde entier.

Entre 30 et 50% des fumeurs répondent aux critères de diagnostic de dépendance au tabac. Bien que 70% des fumeurs souhaitent arrêter de fumer, seuls 6% d'entre eux seront en mesure de le faire sans intervention médicale. Les fumeurs occasionnels ne sont pas épargnés par les propriétés addictives du tabac et présentent également des taux élevés d'échec au sevrage tabagique. En fait, les fumeurs occasionnels peuvent éprouver de fortes envies de consommer qui ne peuvent être expliquées que par des facteurs pharmacocinétiques, mais qui prédisent néanmoins fortement l'échec des tentatives de sevrage. Exacerbant ce problème, les pharmacothérapies approuvées contre la dépendance au tabac ne sont efficaces que chez un nombre limité de personnes désireuses d'arrêter de fumer. Par exemple, la varénicline, bien qu'elle soit le traitement le plus efficace contre la dépendance au tabac, ne maintient l'abstinence au-delà de six mois que chez environ 20% des utilisateurs. Les efforts de recherche biomédicale dans la lutte contre le tabagisme ont donc focalisé leur attention sur la compréhension des mécanismes biologiques de la dépendance au tabac, qui pourraient améliorer l'utilisation et le développement de thérapies et contribuer à améliorer le taux de succès du sevrage.

La nicotine est le principal composant du tabac responsable de ses fortes propriétés addictives. De nombreuses études ont montré que deux principaux mécanismes contribuent au maintien de la prise de nicotine : (1) les effets pharmacologiques de la nicotine impliqués dans la dépendance physique, rechercher pour leurs effets renforçants primaires ou pour l'atténuation des effets du sevrage nicotinique, (2) les effets de stimuli environnementaux neutres qui ont acquis des propriétés de la nicotine par conditionnement pavlovien et produisent une envie de fumer, (3) la recherche des effets de la nicotine sur les renforçateurs naturels (le café ressenti « meilleur » quand il est consommé en même temps qu'une cigarette). Ces sources distinctes de motivation pour la cigarette sont bien connues. La plupart des études précliniques axées sur la compréhension des mécanismes neurobiologiques de la dépendance à la nicotine considère qu'elles sont identiques chez l'ensemble des individus car elles sont observées en moyenne sur l'ensemble de la population testée. Néanmoins, il existe de nombreuses données psychologiques, génétiques et neurobiologiques, issues d'études cliniques et précliniques, qui indiquent désormais que le poids respectif de ces mécanismes psychopharmacologiques pourrait varier d'un fumeur à l'autre. Cette hétérogénéité pourrait contribuer à l'inégale efficacité de la varénicline, dont les cibles psychopharmacologiques sont encore mal connues, ainsi qu'à la faible validité prédictive des modèles précliniques, qui ne tiennent pas compte de cette possible hétérogénéité individuelle.

Dans ce travail de thèse, au moyen de l'auto-administration intraveineuse de nicotine chez le rat, nous avons exploré les variations individuelles dans la sensibilité aux effets renforçants primaires de la nicotine et aux effets de la nicotine sur la sensibilité aux effets renforçants de stimuli environnementaux associés. 59 rats ont été entraînés à l'auto-administration de nicotine, accompagnée de la présentation d'un bref stimulus visuel. Ce stimulus visuel, qui possède par lui-même de légères propriétés renforçantes primaires, agirait comme un stimulus associé à la nicotine, qui peut acquérir les propriétés de la nicotine par conditionnement pavlovien ou dont la nicotine peut augmenter les propriétés renforçantes primaires. Après l'acquisition de l'auto-administration, le rôle de la nicotine et des stimuli associés a été évalué lors de deux séances différentes. Lors d'une session ("cue-omission") l'auto-administration de nicotine, le stimulus visuel a été omis. Après un retour aux conditions de

base, les rats ont ensuite été soumis à une deuxième session ("nicotine-omission") au cours de laquelle la nicotine a été remplacée par une solution de solvant. Le stimulus visuel était, lui, toujours présent. Ces deux tests nous ont permis d'explorer indépendamment le rôle de la nicotine et de son stimulus visuel associé, dans la recherche de drogue.

Nous avons mis en évidence trois sous-populations d'individus. Chez les uns (groupe 1, 35% des individus) c'est le stimulus visuel associé à la nicotine qui apparaît comme le principal moteur du comportement d'auto-administration, alors que la nicotine en elle-même semble limiter leur comportement d'auto-administration. Ce groupe suggère que certains individus sont plus sensibles aux aspects négatifs de la nicotine, limitant leur auto-administration. Certaines études cliniques indiquent que certaines personnes semblent protégées de la dépendance au tabac, car elles sont plus sensibles aux propriétés aversives de la nicotine.

Les deux autres sous-populations (groupes 2 et 3) présentaient des interactions entre la nicotine et son stimulus associé: pour le groupe 2 (41% des individus) les rats étaient très sensibles aux effets renforçants primaires de la nicotine et le stimulus visuel était devenu un renforçateur conditionné capable de stimuler l'auto-administration même en l'absence de nicotine. Cette sous-population suggère que chez les individus particulièrement sensibles aux effets renforçants primaires de la nicotine, les stimuli environnementaux associés à la nicotine peuvent de façon complémentaire être des incitateurs de la consommation. Pour la troisième sous-population (24% des individus), les rats semblaient être principalement sensibles à la capacité de la nicotine à amplifier les effets renforçants primaires du stimulus visuel associé. Chez ces individus, l'auto-administration n'était possible qu'en présence du stimulus visuel et de la nicotine, suggérant que l'auto-administration était motivée par l'effet de la nicotine sur le stimulus visuel et pas principalement par la nicotine ou le stimulus visuel en soi.

Les phénotypes de ces sous-populations ont ensuite été caractérisés et validés par des marqueurs comportementaux mesurés avant toute consommation de nicotine (l'approche conditionnée pavlovienne), par des réponses comportementales provoquées par une réduction de la dose de nicotine

par injection, par des marqueurs du métabolisme de la nicotine et des marqueurs neurobiologiques des neurotransmissions cholinergique et dopaminergique dans des structures cérébrales clés. Cette caractérisation permettra aux futures études d'explorer la neurobiologie de ces différences individuelles dans la recherche de nicotine.

En parallèle, nous avons exploré les cibles psychopharmacologiques de la varénicline. Nous avons développé une nouvelle approche qui permet de révéler la facilitation par la nicotine des effets renforçants primaires d'un stimulus visuel associé. Cette approche implique la manipulation de la saillance du stimulus visuel associé à la nicotine, au moyen d'une interférence visuelle. Dans un premier temps, nous avons montré que la suppression de l'interférence visuelle augmente le comportement d'autoadministration et que cette augmentation résulte d'une amplification par la nicotine des effets renforçants primaires du stimulus visuel. Ensuite, nous avons montré qu'une exposition aiguë à la varénicline antagonise cet effet de la nicotine. La varénicline agit d'autant plus que l'individu est sensible à ces effets amplificateurs de la nicotine sur les effets renforçants du stimulus visuel. Ces résultats soulèvent des implications thérapeutiques: la varénicline pourrait être plus bénéfique pour le sevrage tabagique chez ceux qui sont particulièrement sensibles aux effets de la nicotine sur les stimuli environnementaux et notamment ceux possédant des propriétés renforçantes, mais moins pour ceux dont la consommation est motivée principalement par les effets renforçants primaires de la nicotine.

Ce travail de thèse met en évidence et valide des variations individuelles dans les mécanismes qui régissent le comportement de recherche de nicotine dans un modèle préclinique. Il offre pour perspective d'explorer les mécanismes neurobiologiques responsables de ces variations individuelles et l'impact à long terme de ces variations sur le développement de la dépendance à la nicotine, ainsi que de tester l'efficacité relative de la varénicline chez ces sous-populations.

Mots clés: nicotine, auto-administration, différences individuelles