PASSIVE IMMUNIZATION TO TREAT NICOTINE DEPENDENCE IN RATS

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Overview/Abstract

Cigarette smoking is the leading preventable cause of death in the United States, primarily due to nicotine addiction. Currently available medications are only partially effective in increasing smoking cessation, and additional therapies are needed. Immunotherapy is an alternative strategy for attenuating nicotine's addictive effects. Vaccination against nicotine alters nicotine pharmacokinetics and nicotine's behavioral effects in rats. In clinical trials, nicotine vaccines reduce smoking, but efficacy is limited by high variability and low mean serum nicotine-specific antibody (NicAb) levels.

Passive immunization is an alternative method of immunization, and can potentially circumvent the limitations of vaccination by allowing control over the dose and timing of NicAb administration. The overall goal of this thesis was to examine the use of passive immunization to treat tobacco addiction. The specific aims were to characterize the efficacy of passive immunization on nicotine pharmacokinetics and nicotine-induced behavior, explore the effects of combining vaccination with passive immunization, and examine a potential adverse effect of passive immunization, the precipitation of withdrawal.

Passive immunization with the nicotine-specific monoclonal antibody Nic311 reduced nicotine clearance and steady-state volume of distribution, and prolonged nicotine's elimination half-life, demonstrating the underlying mechanism of immunotherapy efficacy. Nic311 attenuated nicotine-induced locomotor sensitization, and combining Nic311 with vaccination resulted in greater reductions in brain nicotine levels and nicotine-induced locomotor sensitization compared to either immunotherapy alone, demonstrating the behavioral efficacy of Nic311 and suggesting a possible clinical role as a supplement for vaccination. Administration of Nic311 to nicotine-dependent rats substantially reduced brain nicotine levels but did not elicit a nicotine withdrawal syndrome. These findings demonstrate the benefits and safety of passive immunization with Nic311, and point to its potential in treating nicotine addiction.

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Abbreviations

ANOVA:	analysis of variance
Cl_t:	total clearance
CYP:	cytochrome P-450
ELISA:	enzyme-linked immunosorbent assay
HAMA:	human-anti-mouse antibody
i.v.:	intravenous
i.p.:	intraperitoneal
i.m.:	intramuscular
ICSS:	intracranial self-stimulation
IgG:	immunoglobulin G
IgM:	immunoglobulin M
kD:	kiloDaltons
K_d:	equilibrium dissociation constant
LMS:	locomotor sensitization
nAChR:	nicotinic acetylcholine receptor
Nic311:	nicotine-specific monoclonal antibody 311
Nic-IgG:	polyclonal nicotine-specific antiserum
NicAb:	nicotine-specific antibody
NICmAb:	nicotine-specific monoclonal antibody
NRT:	nicotine replacement therapy
rEPA:	recombinant *Pseudomonas aeruginosa* exoprotein A
s.c.:	subcutaneous
$V_{d,ss}$:	volume of distribution at steady-state

CHAPTER 1

Introduction

This chapter provides a summary of background material and introduces the goals of this thesis.

1. The problem: Cigarette Smoking

Cigarette smoking is the leading preventable cause of death in the United States; each year, over 400,000 people in the United States die due to disease and illness caused by smoking [1]. The causal role of smoking in cardiovascular disease, respiratory disease, and numerous cancers is well-established [2]. Many continue to smoke: in the United States, an estimated 19.8% of the population (about 45.3 million individuals) currently smoke cigarettes, 78% of whom are daily smokers [3]. The vast majority (> 80%) of individuals who smoke cigarettes wish to quit, but less than 5% of smokers quit smoking for more than 6 months each year [4]. Currently available pharmacologic treatments such as nicotine replacement therapy, varenicline, and bupropion, and behavioral counseling are able to increase the success rate of smoking cessation to ~30% [5], yet most smokers are unable to stop. New approaches are needed to help smokers quit smoking.

Tobacco smoking is an addiction, and induces dependence. Addiction is defined as a chronic neurobiological disease with genetic, cognitive, and environmental components, characterized by behaviors including loss of control over drug use, continued drug use in spite of harmful effects, and increased drug seeking behavior and craving in the absence of drug. Drug dependence in this thesis will be defined as a physiological condition that includes tolerance and is observed as a withdrawal syndrome when the drug is removed. Smoking fits the definition of addiction as described in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). Most individuals are unable to stop smoking, even though they are aware of the harmful consequences. When individuals stop

smoking, a nicotine withdrawal syndrome is seen, evidence of physical dependence. To develop improved treatments to help individuals quit smoking, it is important to discuss the factors that are responsible for tobacco dependence and addiction. A combination of pharmacologic, environmental, and behavioral factors contribute to tobacco addiction.

2. Pharmacological factors contributing to tobacco dependence

Tobacco smoke contains over 4000 chemicals (reviewed in [6]). This discussion will focus on those chemicals in tobacco smoke that have documented psychoactive effects and contribute to tobacco addiction.

2.1. Nicotine

Nicotine (Figure 1) is the primary chemical in tobacco that produces and maintains tobacco dependence and addiction [7]. To understand the role of nicotine in tobacco addiction, the following sections will discuss nicotine's neuronal mechanism of action.

2.1.1 Mechanism of action

During smoking, nicotine is rapidly absorbed from the lungs and quickly distributes into the brain, which is the target organ for nicotine addiction (see below for more detail on nicotine pharmacokinetics). In the brain, nicotine binds to and activates nicotinic acetylcholine receptors (nAChRs), which are ligand-gated ion channels located on presynaptic and postsynaptic nerve terminals. Upon nicotine binding, the pores of these channels open, resulting in a transient inward flow of positively charged sodium and

calcium ions across neuronal cell membranes that is followed by a period of receptor desensitization where nicotine remains bound but the ion channel is closed. If a sufficient quantity of receptor pores open simultaneously, the cellular membrane will be depolarized, facilitating neurotransmission.

2.1.2 nAChR structure and diversity

nAChRs are composed of 5 individual protein subunits that assemble together to form the functioning ion channel (Figure 2). There is a great deal of diversity in nAChR subtypes and compositions (reviewed in [8]). Nine α-subunit isoforms (α2-α10) and three ß-subunit isoforms (ß2-ß4) have been identified in the brain, and are highly conserved among mammalian species. nAChRs may be homomeric (e.g., composed of only α7 subunits) or heteromeric (composed of a combination of α2, α4, α5, α6, ß2, ß3, etc.) subunits. These different receptor compositions have different brain cellular and anatomical distributions and affinities for nicotine and other nicotinic receptor ligands (K_d = 2-4 nM for α4ß2* receptors, and K_d = 40 – 80 nM for lower affinity receptors) [9]. The α4ß2* receptors (* denoting any other subunit such as α5, α6, etc) play an important role in mediating the reinforcing effects of nicotine, although other receptors are also implicated [10].

2.1.3 nAChR localization in the brain

nAChRs (especially the high-affinity α4ß2* nAChRs) are located in structures that are part of the brain reward circuit (see below) [11]. This indicates that nicotine directly activates brain structures that are relevant in the development of addiction, and predicts

that nicotine produces rewarding effects. In addition, nAChRs are widely distributed throughout the brain, reflecting their roles in complex functions such as cognition, attention, learning, and memory [8, 12]. The action of nicotine in these areas may also contribute to nicotine addiction.

2.1.4 Overview of the brain reward circuit and addiction

The brain reward circuit is a pathway that mediates the effects of all addictive drugs, including nicotine. The brain reward circuit consists of inter-connected structures that include components of the mesocorticolimbic dopaminergic system. A common pathway for all drugs of abuse is the projection from the ventral tegmental area (VTA) in the midbrain to the nucleus accumbens and the prefrontal cortex, among other structures (Figure 3). The brain reward circuit is a key mediator of appetitive behavior [13] and is activated by naturally rewarding stimuli, including food and sex [14]. Drugs of abuse enhance the signaling of neurons located in the VTA that project to the nucleus accumbens via the medial forebrain bundle [15]. An intact reward circuit is necessary for drug self-administration by animals. Chemical and electrolytic lesion of the VTA, the medial forebrain bundle, or the accumbens prevents the self-administration of most drugs of abuse [16-18].

2.1.5 Nicotine's effects on the brain reward system: animal models

Nicotine binds to and activates nAChRs on neurons located in the VTA and nucleus accumbens. Application of nicotine to brain slices *in vitro* increases extracellular dopamine in the nucleus accumbens [19, 20], demonstrating nicotine's activity on the

brain reward circuit. Nicotine is self-administered by rodents, dogs, and monkeys [21-24], reflecting nicotine's reinforcing efficacy. Chronic administration of nicotine leads to dependence, seen as the onset of a well-defined withdrawal syndrome upon spontaneous cessation of chronic nicotine exposure or precipitated by the administration of a nAChR antagonist [25-27]. This withdrawal syndrome is alleviated by the further administration of nicotine [28]. When nicotine injections are paired with distinct environmental cues, rats and mice develop a conditioned place preference for the environment where nicotine was administered, demonstrating the reinforcing and rewarding effects of nicotine [29, 30]. Repeated nicotine dosing produces sensitization to nicotine-induced locomotor activity in rats [31], which is mediated by the same neural pathways underlying reward and reinforcement. Rats also learn to discriminate nicotine from saline [32], providing further evidence of nicotine's psychoactive and addictive properties. Transgenic mice that lack the ß2 nAChR subunit and have no functional α4ß2* nAChRs demonstrate a loss of nicotine-evoked dopamine release in the accumbens, and these mice do not self-administer nicotine or discriminate nicotine from saline, nor does nicotine evoke a conditioned place preference or locomotor activity (reviewed in [33]). These findings in animals predict the addictive potential of nicotine in humans, and demonstrate the fundamental role of nicotine in tobacco addiction.

2.1.6 Nicotine's effects on the brain reward system: Human data

The effects of nicotine in humans are consistent with the animal data, and point to the important role of nicotine in tobacco dependence. Studies indicate that individuals change their smoking patterns to titrate their nicotine intake (reviewed in [34]). Nicotine by

itself is self-administered by humans [35, 36], and brain imaging studies show that nicotine activates the brain reward pathway [37]. Chronic nicotine exposure also evokes dependence in humans. The abrupt cessation of nicotine exposure or administration of an nAChR antagonist to individuals chronically exposed to nicotine results in a nicotine withdrawal syndrome [38], a negative affective and cognitive state characterized by anhedonia, irritability, depressed mood, and inability to concentrate [39, 40]. Subsequent administration of nicotine alleviates this withdrawal syndrome. For example, nicotine replacement therapy reduces craving and withdrawal during smoking cessation [41]. These data together with the animal data indicate that nicotine is the primary addictive substance in tobacco, and that attenuating nicotine's effects in the brain is important for smoking cessation treatments.

2.2 *Non-nicotine psychoactive chemicals in Tobacco smoke*

Both nicotine and non-nicotine chemicals in tobacco smoke are important in continued smoking [42], and nicotine is not the *sole* psychoactive compound present in tobacco smoke. However, what is notable of other psychoactive components present in tobacco smoke is that they are secondary to nicotine: they enhance nicotine's effects and are not rewarding in the absence of nicotine. For example, acetaldehyde enhances nicotine-induced locomotor activity and increases the nicotine-induced expression of c-fos mRNA in rat forebrain regions [43]. Tobacco smoke also contains beta-carbolines, which are monoamine oxidase inhibitors [44-46]. Monoamine oxidase inhibitors increase the number of nicotine infusions taken during fixed-ratio nicotine self-administration

sessions in rats and also increase the break-point for nicotine during progressive-ratio self-administration testing [47, 48].

2.3 Current smoking cessation treatments

Pharmacologic treatments for smoking cessation can be grouped into two categories, based on their mechanism of action in blocking the effects of nicotine. Nicotine replacement therapy (NRT) and varenicline target nAChRs. The goal of NRT is to provide nicotine to prevent craving and withdrawal [49]. Varenicline is a partial agonist at nAChRs, including at the $\alpha4\beta2^*$ receptors, and provides some activity while also inhibiting the effects of nicotine [50]. Bupropion is an antidepressant that inhibits neuronal uptake of dopamine and norepinephrine, which alters nicotine's downstream effects [51]. All of these therapies enhance smoking quit-rates above that of placebo. Their efficacy is limited because they target receptors in the brain, which modulate endogenous processes in addition to mediating the effects of nicotine. One limitation is that this results in dose-limiting side-effects for pharmacotherapy. In addition, nicotine modulates the signaling of multiple neurotransmitters, which makes it difficult to find a single pharmacotherapy to counteract all of nicotine's effects. Immunotherapy (discussed in further detail below) is a different treatment strategy because it provides antibodies that selectively bind nicotine. Immunotherapy thus targets the drug rather than the brain, which circumvents these limitations.

3. Nonpharmacologic factors that contribute to tobacco addiction

Nonpharmacologic factors also play a role in tobacco addiction. Environmental cues such as the sight or smell of cigarette smoke, or the setting such as after a meal or in a bar produce drug craving and elicit drug seeking behavior [52]. Cognitive-behavioral counseling is an important component of smoking cessation treatment regimens [53], which indicates the role of these psychosocial aspects of tobacco dependence. These nonpharmacologic factors do not alone initiate smoking, but rather enhance nicotine's effects in the development and continuation of tobacco addiction.

4. Role of drug pharmacokinetics in addiction

The rewarding or reinforcing efficacy of drugs is greatly influenced by their pharmacokinetics. The dose, formulation, route of administration, rate of administration, rate of elimination, and the presence or absence of active metabolites can alter the psychoactive effects of nicotine [54] and other drugs of abuse such as cocaine [55, 56] and opiates [57]. This suggests that altering drug pharmacokinetics is a potential target for treating drug addiction.

4.1 Drug dose, rate and route of administration, and addiction: animal studies

The pharmacokinetic factors listed above affect the reinforcing efficacy of drugs because they alter the dose and rate of drug reaching the brain. For drug addiction, there appears to be an optimal range of drug doses that produce reward and reinforcement. Higher drug doses are aversive and lower drug doses do not sufficiently activate brain reward circuits.

Nicotine, cocaine, methamphetamine, and heroin self-administration studies show that drug doses that are either too high or too low result in reduced drug self-administration [58-61]. This demonstrates the importance of drug dose in producing reinforcement, and suggests that pharmacokinetic strategies that reduce the dose of drug that reaches the brain may serve as a mechanism to treat drug addiction.

Not only does drug dose play a role in a drug's addictive effects, but the rate at which a given dose distributes into the brain affects a drug's reinforcing efficacy. This can be demonstrated by changing either the rate or the route of drug administration. Changing the rate of drug administration results in different concentration-time profiles in serum, which affects the time-course of drug distribution to the brain. This alters the reinforcing efficacy of a drug; a dose infused over a longer period of time is less reinforcing compared to when the same dose is infused rapidly. In monkeys trained to self-administer i.v. cocaine, a longer cocaine infusion of the same dose (a 100 second infusion vs. a 5 second infusion) reduced the number of cocaine infusions taken by the animal, similar to the effect of lowering the cocaine dose from 800 μg/kg to 20 μg/kg [62], see also [63, 64]. In monkeys trained to self-administer i.v. nicotine, slowing the infusion rate from 5.2 to 0.3 μg/sec reduced self administration of nicotine to that of saline [65]. Similarly, a longer (slower) i.v. nicotine infusion (100 seconds vs. 5 seconds) reduced nicotine-induced locomotor sensitization in rats and reduced expression of the transcription factor c-fos in the striatum [66], indicating that slower nicotine infusions resulted in reduced neuronal activity and the attenuation of nicotine's psychoactive

effects. Thus, slowing the rate at which a drug reaches its target organ is another example of the importance of drug pharmacokinetics in mediating drug addiction.

Different routes of drug administration also produce different drug concentration – time profiles in serum and affect patterns of drug distribution. Intravenous administration results in immediate peak venous serum drug concentrations, while other extravascular routes of administration require drug absorption into serum, resulting in a delay before drug levels peak in serum and the need to administer higher doses to achieve similar peak serum levels [67]. For example, a 30-fold larger cocaine dose was required to elicit conditioned place preference in rats when administered i.p. compared to cocaine administered subcutaneously (s.c.) [68]. This illustrates the importance of different routes of administration in mediating a drug's reinforcing and addictive properties. In addition, this provides important information for model selection to study addiction and is discussed in further detail below.

4.2 Drug dose, rate and route of administration, and addiction: human studies

Studies in humans are consistent with animal data and also demonstrate the influence of drug pharmacokinetics on reinforcing efficacy, providing further support for pharmacokinetic intervention as a potential target for treating drug addiction. Human self-administration studies are consistent with animal studies indicating that there is an optimal range of drug doses which are reinforcing [61]. With regard to dosing rate, subjects preferred a single large dose of barbiturate rather than incremental administration of the same cumulative dose, and reported greater subjective effects after

the single dose [69]. Drug formulation and route of administration also modify a drug's reinforcing efficacy by altering the time-course of drug absorption. Smoked or intravenous cocaine produce higher serum and brain cocaine levels more rapidly than snorted cocaine because of more extensive uptake into blood through the lungs, resulting in higher abuse potential [55]. Cigarettes rapidly produce high serum and brain nicotine concentrations due to extensive absorption in the lungs and oral mucosa. Nicotine replacement products such as transdermal nicotine patches or nicotine gum/lozenges result in slower increases in serum nicotine levels, are not as reinforcing as cigarettes, and have little abuse potential [54]. Altering the pharmacokinetics of nicotine to attenuate the rapidity and extent to which nicotine distributes into the brain is a potential strategy to help smokers quit smoking.

4.3 Drug elimination

The above sections discuss how changing the way a drug enters the body (absorption, route of administration, distribution) can change a drug's reinforcing and addictive effects. Manipulating drug elimination is another pharmacokinetic mechanism by which a drug's addictive effects can be altered. Drug elimination results in the removal of drug from the body, which terminates drug action and can affect patterns of drug use. A drug's elimination rate is determined by its clearance, which also affects serum drug concentrations at steady-state. This discussion will be limited to nicotine elimination and how it relates to addiction, since this aspect of drug pharmacokinetics has been studied more for nicotine than other addictive drugs.

Nicotine is cleared mostly by hepatic metabolism by the enzyme CYP2A6 (see below for further discussion of nicotine pharmacokinetics). Studies of nicotine metabolism in smokers suggest that changing nicotine clearance has three potential consequences for smokers. First, changing nicotine clearance changes the amount smoked. Administration of the CYP2A6 inhibitor methoxsalen to smokers inhibits nicotine metabolism, which increases serum nicotine levels and decreases the number of cigarettes smoked [70]. Consistent with this finding, individuals with reduced CYP2A6 activity (due to single-nucleotide polymorphisms that result in attenuated/lost enzymatic activity) exhibit reduced nicotine clearance, a longer nicotine elimination half-life, and also smoke less compared to their wild-type counterparts [71]. Conversely, increasing nicotine renal clearance through urine acidification significantly increases the number of cigarettes smoked [72]. These findings demonstrate the importance of nicotine clearance on serum nicotine levels and that altering nicotine clearance alters smoking behavior.

Second, evidence suggests that changing nicotine clearance changes the onset of nicotine withdrawal. Individuals with higher nicotine clearance (more rapid nicotine metabolism) exhibit a more rapid onset of drug withdrawal: a recent study of adolescent smokers indicated that after 24 hours of abstinence, individuals who were rapid nicotine metabolizers exhibited significantly more withdrawal symptoms than individuals who were slow nicotine metabolizers [73]. This indicates that decreasing clearance may delay the onset of withdrawal, and is a potential strategy to reduce the negative reinforcement associated with smoking to avoid nicotine withdrawal.

Finally, reduced nicotine clearance may result in higher quit rates. Lerman et al. demonstrated an association between the rate of nicotine metabolism and smoking cessation during transdermal nicotine treatment, where individuals who were rapid metabolizers of nicotine were least likely to quit smoking compared to individuals who were normal and slow metabolizers [74]. In another study of nicotine metabolism, fewer individuals with reduced CYP2A6 activity were current smokers compared to individuals with wild-type CYP2A6 activity [71], which suggests that slower metabolizers of nicotine quit smoking more than rapid metabolizers. This suggests that reducing nicotine clearance and thereby prolonging nicotine's elimination half-life is a potential pharmacokinetic strategy to reduce nicotine intake and promote smoking cessation.

5. Nicotine pharmacokinetics

Since immunotherapy acts by altering nicotine pharmacokinetics (see below), it is important to describe the pharmacokinetics of nicotine in humans and rats.

5.1 Nicotine pharmacokinetics in humans

The pharmacokinetics of nicotine in humans have been extensively characterized (Table 1; reviewed in [75]). Nicotine inhaled during smoking sequentially enters the pulmonary venous circulation, the left heart, and arterial blood. This provides a bolus of high concentration nicotine in arterial blood that rapidly distributes to the brain. The lag-time between inhalation and the onset of nicotine entering the brain is 10 – 20 seconds, and nicotine levels in blood and brain peak at the end of smoking a cigarette. Brain nicotine

levels then fall as serum nicotine levels decline due to distribution to other tissues (steady-state volume of distribution = 2.2 – 3.3 L/kg) and hepatic metabolism (total clearance = 16 - 21 ml/min/kg = 0.9 - 1.2 L/hr/kg, 90% hepatic).

The greatest subjective effects of nicotine occur during smoking a cigarette. The brief interval between nicotine intake and perceived drug effect is a key characteristic of addictive drugs [76], and this allows smokers to titrate the levels of nicotine entering the brain by inhaling as frequently or deeply as desired. A pharmacokinetic intervention that would attenuate the early distribution of nicotine to the brain during smoking could reduce nicotine's subjective effects, and may be beneficial for improving smoking cessation.

The elimination half-life of nicotine in humans is ~2 hours. Nicotine is rapidly cleared from the body (clearance = 16 - 21 ml/min/kg), mostly by hepatic metabolism. Nicotine is metabolized primarily by the hepatic cytochrome P450 enzyme CYP2A6, which converts about 70-80% of the total nicotine dose to cotinine. Cotinine is a weak agonist at nAChRs [77], with 100-fold less potency than nicotine, and does not significantly contribute to tobacco addiction *in vivo*. The metabolism of nicotine to cotinine thus effectively inactivates nicotine. Another metabolite of nicotine that has been shown to be pharmacologically active is nornicotine [78, 79]. Nornicotine represents a very minor fraction of total nicotine metabolism (0.4 % in humans, 8% in rats) [80]. Although this metabolite has been shown to accumulate in the brain [80], concentrations are quite low, such that it does not significantly contribute to tobacco addiction. Thus the metabolism

of nicotine terminates nicotine effect, and active metabolites are not expected to confound immunotherapy against nicotine.

5.2 Nicotine pharmacokinetics in rats

The pharmacokinetics of nicotine in rats is quite similar to that of humans (Table 1, [81-83]). A single i.v. dose of nicotine exhibits a brief distribution phase, detectable if adequate serum sampling is performed early (during the first 15 minutes). In serum at physiological pH, nicotine is 69% ionized and 31% un-ionized, and >90% unbound to serum proteins. Nicotine distributes rapidly and extensively to the brain and other tissues, with a large volume of distribution at steady-state (2.5 L/kg).

The clearance of nicotine in rats is similar to that of humans in that nicotine is rapidly metabolized via hepatic enzymes, with an elimination half-life of ~1 hour. In rats, nicotine is metabolized via hepatic CYP1B1/2; the rat homologue of human CYP2A6 is inactive for nicotine metabolism [84, 85]. The mean nicotine clearance = 50 ml/min/kg (3 L/hr/kg), ~90% of which is by hepatic metabolism [81-83]. This clearance of nicotine in rats is higher than in humans, and since the volumes of distribution are similar between rat and human, this results in the more rapid elimination half-life in rats.

5.3 The rat as a model to study the effects of nicotine

For this study, the rat was chosen as a model to examine the effects of nicotine and how immunotherapy alters nicotine's pharmacokinetics and behavioral effects because rat and human pharmacokinetics are similar, as outlined above. The most significant differences

in nicotine pharmacokinetics between humans and rats are the shorter half-life and different metabolite profile in rats (in humans, 70 – 80% of nicotine is metabolized to cotinine, in rats, ~40% of nicotine is metabolized to cotinine [86]). However, the elimination half-life is more similar than different (1 hour vs. 2 hours), and the metabolite profile does not alter the efficacy of immunotherapy because the antibodies specifically bind nicotine and not nicotine metabolites (see below). In addition, the neuropharmacological effects of nicotine are similar in rats and humans (as reviewed above). The rat also serves as a good model because the behavioral pharmacology of nicotine in rats is well-established, which permits the assessment of how changes in nicotine pharmacokinetics due to immunotherapy alter nicotine's psychoactive and reinforcing properties.

5.3.2 Nicotine administration to rats

In studying the effects of nicotine in rats, there is no single route of nicotine administration that perfectly replicates smoking. Dosing intravenously via a jugular cannula is similar to smoking, in that nicotine rapidly enters the heart, circulates through pulmonary vessels, and then enters the arterial circulation, presenting a bolus of high concentration nicotine to the brain and other tissues. This route of nicotine administration is also used for nicotine self-administration in rats and other species [87], which demonstrates the behavioral relevance and efficacy of this route of dosing.

Most studies that have examined the effects of immunotherapy on nicotine pharmacokinetics and behavior in rats used intravenous dosing. For initial studies on

vaccination against nicotine, it was important to establish the effects of immunotherapy on the early distribution of nicotine to the brain, since the first few minutes of smoking produce the greatest subjective and rewarding effects. Vaccination against nicotine reduced the early distribution of nicotine to the brain after an i.v. nicotine dose [88, 89], and also reduced nicotine self-administration (where nicotine was also dosed i.v.) [90]. These studies demonstrated the efficacy of immunotherapy on nicotine pharmacokinetics and behavior when nicotine was administered intravenously.

In the behavioral studies described in this thesis, rats were administered acute nicotine doses via subcutaneous injection. A single s.c. injection of nicotine is rapidly absorbed and produces high serum (100 ng/ml, unpublished data) and brain nicotine levels (100 ng/kg, [91]) 3-10 minutes after administration in rats. This is similar to the peak in serum nicotine levels seen in smokers during smoking, where serum nicotine levels peak during the end of cigarette smoking (50 – 100 ng/ml, at about 5 - 9 minutes) [54, 92]. This similarity is important because it predicts that subcutaneous nicotine would have psychoactive effects, similar to smoking. This route of administration is commonly used to study the behavioral effects of nicotine in rats, and promotes nicotine-induced conditioned place preference [93], locomotor sensitization [94], and rats are able to discriminate s.c. nicotine from saline [32]. These data indicate that, insofar as an acute subcutaneous nicotine injection produces a rapid peak in serum nicotine levels and is psychoactive, it is a useful route of administration for studying nicotine's psychoactive effects. As such, it is also a relevant route of administration for studying the effects of immunotherapy on nicotine.

In this thesis, nicotine was also administered to rats by a subcutaneous infusion using an osmotic minipump. This method of nicotine delivery is not intended for studying nicotine's early effects, but rather this is a well-characterized and commonly used nicotine administration protocol used to induce nicotine dependence and study nicotine withdrawal [95]. Subcutaneous nicotine infusion is a good model to study withdrawal, because it provides fairly constant serum nicotine levels that approximate the serum nicotine levels seen in chronic smokers. Cessation of this infusion (via minipump removal) or administration of a nicotinic receptor antagonist results in a well-characterized withdrawal syndrome [25, 26, 95].

6. Immunotherapy for addiction

The current pharmacotherapies used in smoking cessation treatment such as bupropion and varenicline bind to drug targets in the brain (bupropion binds a biogenic amine transporter [51], varenicline targets nAChR receptors [50]). These drug targets are in part responsible for mediating many endogenous functions in the brain. Drugs that bind to these targets (in addition to enhancing smoking cessation) also affect these endogenous processes. This limits the drug doses that can be used, because larger doses result in the onset of side effects due to alterations in the other endogenous processes. Immunization targets the drug rather than the brain. Thus, in theory, as much antibody could be given as needed, since the antibodies only bind nicotine. Immunotherapy thus provides an alternative way to inhibit the effects of nicotine without undesirable side effects caused

by interfering with endogenous receptors. This complementary mechanism of action could be used together with the other pharmacotherapies that affect neurotransmitter homeostasis to reduce nicotine's addictive effects.

6.1 History of immunotherapy in treating drug addiction

The efficacy of immunotherapy in modifying the effects of addictive drugs was first examined for heroin/morphine in the early 1970s. Berkowitz et al demonstrated that heroin/morphine vaccines were immunogenic, and elicited antibodies that were capable of binding heroin, retaining it in serum, and reducing brain drug levels [96]. Bonese et al in 1974 [97] demonstrated that immunization against morphine effectively reduced heroin self-administration by monkeys. A nicotine vaccine was also developed at the time to provide nicotine-specific antibodies to detect nicotine in blood and tissues using radioimmunoassay, but was not studied to treat smoking cessation [98]. Immunotherapy was not pursued as a treatment for opiate abuse because of the development of other addiction treatments that appeared more promising, such as methadone maintenance therapy for opiate addiction. However, recognition of the lack of effective long-term treatments for other addictions resulted in the re-evaluation of immunotherapy as a strategy for treating drug addiction. In the 1980s and 90s, initial reports of immunization as a treatment for phencyclidine [99, 100], cocaine [101, 102], and nicotine [103] suggested efficacy in attenuating drug effects, which has lead to more extensive study of immunotherapy for addiction. Currently, three nicotine vaccines and one cocaine vaccine are in clinical trials, and immunotherapies are under investigation for most major drugs of abuse (reviewed in [104]).

6.2 Immunization overview

Immunization against nicotine may be accomplished by either vaccination or passive immunization. Vaccination is the administration of an immunogen with an adjuvant (which together form the vaccine) directly to an individual or animal, which results in the stimulation of the organism's immune system to produce antibodies against the immunogen. Passive immunization is the administration of antibodies derived from exogenous sources. Both immunization strategies produce nicotine-specific antibodies (NicAbs) that bind nicotine in serum. Antibodies are large proteins (molecular weight ~150 kD), and do not distribute into the brain; they are mostly confined to blood and some extra-cellular fluid (Figure 4). When antibodies bind nicotine in serum, the free nicotine concentration is substantially reduced. Reducing the concentration of free nicotine in serum reduces the nicotine available for distribution into the brain or for metabolism, and results in three distinct pharmacokinetic consequences: 1) the acute distribution of nicotine into the brain is reduced; 2) during chronic nicotine exposure, the chronic accumulation of nicotine in the brain is slowed; and 3) nicotine clearance is reduced if the antibody has a longer elimination half-life than nicotine, prolonging nicotine's elimination half-life. Data from vaccination studies are presented below that demonstrate these effects. By altering nicotine pharmacokinetics, immunization can alter the psychoactive effects of nicotine and serve as a treatment in smoking cessation.

6.3 Immunization against nicotine: Vaccination

Most studies of immunization against nicotine have addressed vaccination, because vaccination is an inexpensive, presumably safe, long-lasting, and effective way to produce nicotine-specific antibodies. A number of studies in rats and humans have demonstrated the efficacy of vaccination (described below), establishing proof-of-principle for vaccination as a treatment for nicotine dependence.

6.3.1 Nicotine vaccine

Nicotine is a small molecule and is not itself immunogenic. Formation of a nicotine immunogen follows a standard protocol for rendering small molecules immunogenic via conjugation to a large carrier protein [105]. A nicotine vaccine is composed of a hapten, which is a modified nicotine molecule with a 'handle' to which a linker can be attached (Figure 1). The hapten is then covalently bound to a large carrier protein via this linker (e.g., succinic acid). Conjugation of hapten-linkers to carrier proteins yields approximately 4-12 moles of hapten per mole of carrier protein (unpublished data, [106]). The hapten-linker-carrier protein together form an immunogen (Figure 1), which when mixed with an adjuvant such as Alum (used in humans) or Freund's Adjuvant (used in rats) comprises the nicotine vaccine.

One nicotine immunogen that has been extensively characterized and is currently in clinical trials is made up of the hapten 3'-aminomethyl nicotine (3'-AmNic, Figure 1) conjugated to the carrier protein recombinant *Pseudomonas* exoprotein A (rEPA) [106]. A dosing schedule of one vaccine injection every 3 weeks in rats for 12 weeks (four total

injections) produces substantial serum titers (a measurement of serum antibody levels, determined as a function of antibody concentration and affinity) of nicotine-specific antibodies (NicAbs) [107]. In humans, vaccination using this immunogen (one injection every four weeks, 3 injections) also elicited serum NicAbs that have an approximate half-life of 2-3 months [108]. This immunogen has been used with Freund's adjuvant to study the effects of vaccination against nicotine in rats in our lab, and results of these studies are discussed below.

6.3.2 Vaccine-elicited NicAbs

Vaccination of rats generates NicAbs that have high affinity and specificity for nicotine (mean K_d = 38 nM = 3.8×10^{-8} M, no cross-reactivity to nicotine metabolites, acetylcholine, or other endogenous neurotransmitters) [106]. Mean serum NicAb levels elicited in rats after vaccination are ~200 μg/ml, (1.3×10^{-9} mol/ml of antibody) [107]. Assuming each antibody binds two nicotine molecules and that the volume of distribution of IgG in rats is 0.13 L/kg, this suggests a total body nicotine binding capacity of 3.5×10^{-7} mol/kg. An equimolar dose of nicotine is 0.056 mg/kg, which is a unit dose commonly used in nicotine self-administration and is approximately equivalent to the nicotine from 3 cigarettes/kg [61].

Compared to nAChRs, NicAb affinity for nicotine is less than that of the high-affinity nAChRs (α4β2* receptors) for nicotine (K_d = 2-4 nM), but on the lower end of the range of affinities for other nAChRs (K_d = 40-80 nM) [9]. The specific binding of nicotine to nAChRs in the brain (nAChR brain $B_{max} \approx 4 \times 10^{-13}$ mol nicotine, [109]) is much lower

than the total antibody binding capacity as calculated above (3.5 x 10^{-7} mol). Nicotine serum and brain levels are 4-5 orders of magnitude greater than the reported brain B_{max} values, indicating that most of the nicotine in the brain is due to nonspecific binding. The binding affinity of NicAbs for nicotine indicates that NicAbs can effectively compete with nAChRs for nicotine binding, but the greatest effect is most likely on nonspecific nicotine binding.

6.3.3 Nicotine vaccine: effects on nicotine pharmacokinetics

In vaccinated rats, NicAbs bind nicotine in serum and reduce the concentration of unbound (free) nicotine, which alters nicotine pharmacokinetics in three distinct ways. First, when a vaccinated rat is administered a single i.v. nicotine dose of 0.1 mg/kg (equivalent to the mg/kg nicotine dose delivered to a human from 6 cigarettes, and twice the antibody molar binding capacity in vaccinated rats), brain levels 1 minute after the nicotine injection are substantially and significantly reduced to 35% that of control vaccinated rats [89, 107]. Secondly, during chronic i.v. nicotine administration, where the total amount of nicotine administered over the course of a day is 1 mg/kg (equivalent to the mg/kg nicotine delivered to a human from 2 packs of cigarettes, 10 times more nicotine compared to the acute dose), the total accumulation of nicotine in the brain is largely unchanged. However the distribution of a single nicotine dose to the brain in this setting is significantly reduced, similar to the acute effect on a single nicotine dose [110, 111]. This suggests that during chronic nicotine exposure, vaccination slows rather than prevents the distribution of nicotine into the brain. This may be one mechanism by which immunotherapy attenuates the addictive and reinforcing effects of nicotine. Finally, in

rats vaccinated against nicotine, the elimination half-life of nicotine is increased from 1 hour to 6 hours [112], demonstrating that vaccination reduces nicotine clearance, and may be a potential mechanism by which immunotherapy reduces smoking. These data provide evidence for the efficacy of a nicotine vaccine in altering nicotine pharmacokinetics, and provide points of reference for the assessment of passive immunization against nicotine.

Immunotherapy efficacy is thought to be due to the presence of NicAbs in serum, which bind and sequester nicotine in serum and alter nicotine distribution into the brain because NicAbs are not able to distribute into the brain due to their size and exclusion by the blood-brain barrier. Even if NicAbs could distribute into the brain, immunotherapy against nicotine most likely still would be highly effective since nicotine bound to antibodies is pharmacologically inactive. The key factor is the substantial reduction in unbound nicotine, which results in the changes in nicotine pharmacokinetics as described above.

6.3.4 Nicotine vaccine: effects on nicotine-induced behavior

These effects of the nicotine vaccine on nicotine pharmacokinetics result in the attenuation of nicotine's psychoactive and reinforcing effects, as determined by reductions in nicotine-induced behavior. This has been demonstrated for a number of different nicotine vaccines. Vaccinating rats against nicotine reduces the acquisition, maintenance, and reinstatement of nicotine self-administration [90, 113]. The strength of these behavioral assays are that the doses of nicotine self-administered by rats are

clinically relevant, and (on a mg/kg basis) in the range of nicotine doses that smokers obtain by smoking (~1 mg/kg/day, or about 2 packs/kg). In addition, they involve a chronic nicotine dosing regimen, mimicking smokers under a chronic nicotine exposure. Among other nicotine-induced behaviors, vaccination against nicotine attenuates nicotine-induced locomotor activity and blocks nicotine discrimination [91, 106, 114]. These behavioral assays use acute nicotine doses that elicit serum nicotine levels in the range of smokers (10 – 50 ng/ml). These data from animal studies demonstrate the efficacy of vaccination against nicotine administered either s.c. (locomotor activity, discrimination) or i.v. (nicotine self-administration, discrimination) in altering nicotine's pharmacokinetics and reinforcing properties.

6.3.5 Nicotine Vaccine clinical trials

Based on the data from the preclinical studies, three different nicotine vaccines are in clinical trials [108, 115, 116]. Preliminary data are consistent across all three vaccines. They elicit nicotine-specific antibodies that bind nicotine and significantly increase smoking cessation rates to >50% among individuals with the highest third of NicAb titers, compared to 30% for placebo and low or medium NicAb titers [116, 117]. No serious adverse effects have been reported, providing support for the safety of the nicotine vaccines, and the presence of nicotine did not compromise immunogenicity in smokers [108]. These clinical results confirm that immunotherapy against nicotine binds nicotine in serum and attenuates nicotine's addictive properties, and is a potential treatment for nicotine addiction. An interesting finding from clinical trials is that the mean serum NicAb levels elicited by vaccination are about one-tenth that of rats

(vaccine-generated mean NicAb levels: 25 μg/ml clinically, ~200 μg/ml in rats) [108]. This suggests that efficacy may be achieved with lower NicAb levels than the high NicAb levels observed in rats. Since only individuals with the highest third of NicAb levels showed efficacy and enhanced smoking cessation rates, this illustrates the necessity of consistently eliciting high NicAb levels to provide maximal efficacy of immunotherapy.

6.3.6 Vaccine limitations

The animal and clinical data provide evidence for the efficacy of vaccination to treat nicotine addiction. However, vaccine efficacy is limited due to three main factors. First, in a given human population, vaccines produce highly variable nicotine-specific antibody levels in serum, and mean NicAb levels have been low (especially in humans, where NicAb levels are one-tenth that of rats). This is most likely because the human vaccine contains alum instead of Freund's Adjuvant in the nicotine vaccine administered to rats, and the human vaccine is administered i.m. vs. i.p. [108, 118]. Secondly, there is a practical upper limit of serum antibody levels generated by vaccination. The concentration of total serum IgG is 10 mg/ml in humans, and a maximum of only 1-2% of this total concentration (0.1 mg/ml) can be specific for a given antigen, demonstrating that there is some limit to the amount of antigen-specific antibody that can be produced [119]. This is because the immune system is also dealing with all other foreign antigens and challenges presented to it, and may present the upper limit of efficacy achievable by vaccination. Finally, there is also a considerable delay before effective antibody serum titers are elicited, which delays the efficacy of immunotherapy in reducing nicotine

distribution into the brain. There is a need to improve the efficacy of immunotherapy such that all individuals would promptly develop high and consistent serum NicAbs levels to provide maximum efficacy of immunotherapy.

6.4 Passive Immunization as an alternative to Vaccination

6.4.1 Advantages of Passive Immunization

Passive immunization is the administration of antibodies produced exogenously. Passive immunization has potential advantages over vaccination for the treatment of nicotine addiction. Passive immunization allows control of the dose of antibody administered, which provides two opportunities for enhancing immunotherapy. First, this enables the use and study of nicotine-specific antibody serum levels not reachable by vaccination, which may lead to a greater reduction of nicotine distribution to the brain and nicotine-related behavior compared to vaccination. Second, this allows the selection and administration of an antibody dose that is consistent across a given population, reducing the variability in NicAb serum levels seen in vaccination. Passive immunization also results immediately in substantial NicAb serum levels, avoiding the delay in generating NicAb levels that is inherent to vaccination.

Passive immunization also has advantages over vaccination as an experimental tool, to study characteristics of immunotherapy. Passive immunization allows the characterization of dose-response relationships, which is difficult to perform in vaccination studies. The use of a monoclonal antibody may also permit investigator selection of antibody affinity and the study and selection of an optimal affinity.

6.4.2 Passive Immunization: other drugs

Passive immunization has been studied in animal models of cocaine [101, 120, 121], methamphetamine [122-124], and phencyclidine [125, 126] dependence, demonstrating the potential utility of passive immunization. For example, passive immunization (using antibody doses of 4 and 12 mg/kg) against cocaine reduced cocaine levels in the brain and attenuated cocaine self-administration [120]. In addition, the higher dose resulted in greater serum cocaine-specific antibody levels than a cocaine vaccine, and reduced cocaine self-administration to a greater extent than the lower cocaine monoclonal antibody dose [120]. A monoclonal antibody against phencyclidine (PCP) reduced brain PCP levels in an antibody-dose dependent manner (antibody doses from 5 – 1000 mg/kg; antibody:drug molar ratios from 0.003 up to 1) [126]. Higher doses of a monoclonal antibody against methamphetamine (175, 300, 525 mg/kg; antibody:drug molar ratios of 0.32, 0.56, 1) resulted in greater reductions in methamphetamine induced-locomotor activity [127]. These studies demonstrate the feasibility of passive immunization to treat drug addiction, and indicate that increasing doses provide greater effects on drug distribution to the brain and drug-induced behavior. In addition, the antibody doses used across these studies suggest that the doses of antibody used in these studies for passive immunization against nicotine are realistic and practical, because the drug:antibody molar ratios are comparable to those reported for passive immunization against cocaine, methamphetamine, and PCP.

6.4.1 Efficacy of Passive Immunization against Nicotine:

Passive immunization against nicotine has been less well-studied compared to vaccination. Passive immunization with polyclonal anti-nicotine NIC-IgG reduced the distribution of nicotine to the brain in a dose-dependent manner, prevented nicotine from relieving nicotine withdrawal, and blocked nicotine-induced locomotor activity [114, 128]. In addition, one monoclonal antibody against nicotine reduced brain nicotine levels 3 minutes after a nicotine dose and dose-dependently attenuated nicotine-induced locomotor activity [91]. The use of a monoclonal antibody may have important advantages over polyclonal antibodies, such as control over affinity and uniformity of antibodies. In addition, the clinical use of polyclonal antisera has resulted in allergic reactions.

6.4.2 Limitations of Passive immunization

Passive immunization has one significant limitation and two other limitations that are more easily addressed. The use of passive immunization is largely limited by its cost. It is expensive to produce and purify monoclonal antibodies in large quantities. For example, custom production of antibody on a small scale for experimental use is about $1-3/mg. Clinically, the costs associated with monoclonal antibodies are high. For example, the cost for one year of treatment of colorectal cancer with the monoclonal antibody bevacizumab is $52,800 [129, 130]. As biotechnology develops, new methods may be developed to produce large quantities of monoclonal antibodies and reduce expenses [131]. In addition, cost does not necessarily preclude clinical use; passive

immunization is used clinically in a number of cancer chemotherapies and inflammatory diseases [132].

Passive immunization is mostly safe, but clinical reports of passive immunization indicate that murine-derived antibodies are often recognized as foreign proteins and attacked by the host immune system in a Human Anti-Mouse Antibody (HAMA) response and efficacy is lost [132, 133]. During a HAMA response, the host immune system produces antibodies that bind to the therapeutic antibodies, which are targeted for antigenic processing and subsequent degradation. This process rapidly lowers serum concentrations of the therapeutic antibodies, curtails efficacy, and can result in allergic reactions. This was a more significant issue when monoclonal antibodies were derived from murine sources. Methods have been developed to reduce this problem. For example, the humanization of monoclonal antibodies (replacing amino acid and sugar residues from mice or other animals with human motifs) reduces the likelihood of a HAMA response [134, 135].

Another limitation is that passive immunization requires more frequent dosing to maintain serum antibody levels compared to vaccination. The elimination half-life of IgG in humans is 21 days, which necessitates dosing every 3 weeks to maintain effective serum antibody levels [136]. Vaccination could be limited to much more infrequent dosing. In one nicotine vaccine clinical trial, after the initial vaccination protocol to produce antibody levels, vaccine-elicited NicAbs fell by half in about 2-3 months, but immediately increased upon booster vaccination [108]. Another nicotine vaccine clinical

trial reported a NicAb elimination half-life of 90 days [116]. This suggests that after an initial vaccination protocol, additional vaccinations could be limited to every 2-3 months to maintain serum NicAb levels.

6.5 Nic311

6.5.1 Background

The monoclonal antibody used in this thesis (Nic311) is an immunoglobulin G (IgG) 1_{κ} derived from mice immunized with 3'-AmNic-rEPA (Figure 1). Nic311 has high affinity for nicotine, with a K_d for nicotine = 60 nM (similar to that seen in vaccine-generated antibodies). Nic311 is highly specific for binding nicotine, having < 1% cross-reactivity with the nicotine metabolites cotinine, nornicotine, and nicotine-n-oxide and acetylcholine or other endogenous neurotransmitters (cross-reactivity of 1.9% with anatabine, another alkaloid with structure similar to that of nicotine in tobacco plants – see chapter 3). Initial data showed a dose-dependent relationship between Nic311 and the distribution of a single i.v. nicotine dose to rat brain (Chapter 3, [137]). This study demonstrated that higher doses of Nic311 resulted in greater reductions in brain nicotine levels (Figure 5). In addition, larger doses of Nic311 produced higher nicotine-specific antibody concentrations compared to those generated by vaccination, and increased nicotine binding in serum. Serum Nic311 levels higher than the best mean NicAb serum level achieved by vaccination reduced brain nicotine levels to a greater extent than the best mean effect of vaccination, demonstrating that passive immunization is able to surpass the upper limit of efficacy of vaccination.

6.5.2 Nic311 selection

Nic311 was selected from a panel of monoclonal antibodies against nicotine because of its high affinity for nicotine compared to the other monoclonal antibodies. Characterization of the effects of antibody affinity demonstrated that for an acute dose of nicotine, the reduction in brain nicotine levels was greater with higher-affinity antibodies. For example, a 10 mg/kg dose of a high-affinity polyclonal antiserum (Nic-IgG, K_d for nicotine = 1.6 nM) reduced acute brain nicotine levels to the same extent as an 80 mg/kg dose of Nic311 (K_d for nicotine = 60 nM) (Figure 5). However, in the setting of chronic nicotine dosing, Nic311 was as effective as Nic-IgG in reducing the distribution of an acute nicotine dose to the brain (Figure 6, [138]). This suggests that although antibody affinity is important, antibody concentration appears to be the determining factor for reducing brain nicotine levels in the setting of chronic nicotine exposure (a more clinically-relevant nicotine dosing condition). In addition, these data indicate that the limitation of lower affinity for nicotine can be overcome by using higher antibody doses.

An additional study examined the effect of passive immunization with Nic311 on acute and chronic brain nicotine levels [138]. Rats were administered an i.v. nicotine bolus of 0.03 mg/kg every 14 minutes for 16 hours, resulting in a total dose of 2 mg/kg/day (~ 4 packs of cigarettes). At the end of the first day, rats were administered Nic311 10, 30, 80, and 240 mg/kg, and the nicotine dosing protocol continued for another day. The final nicotine dose of the multiple infusions was radiolabeled with tritiated nicotine, which made it possible to distinguish the distribution of the final dose compared to the chronically accumulated nicotine. Nic311 did not alter the chronic accumulation of

nicotine into the brain, but acute brain nicotine levels of the radiolabeled nicotine were dose-dependently reduced (Figure 6), even in the presence of chronic nicotine. Nic311 doses that produced serum NicAb levels greater than those achieved by vaccination were more effective in reducing the acute distribution of nicotine to the brain, yet chronic brain nicotine levels were unaltered at all doses of Nic311. This indicated that in the setting of chronic nicotine exposure, Nic311 slows the distribution of nicotine into the brain, but the chronic accumulation of nicotine in the brain is not significantly reduced.

This effect of passive immunization is similar to the results from vaccine studies, which demonstrated that brain nicotine levels from an acute nicotine dose were significantly reduced during chronic nicotine exposure. These findings indicate that passive immunization and vaccination both alter nicotine brain distribution in a similar fashion, and support the observation that immunotherapy slows the distribution of nicotine to the brain during chronic nicotine exposure. This is important because by slowing the distribution of nicotine in to the brain, the rewarding effects of nicotine are attenuated, which may serve as a mechanism by which immunotherapy affects nicotine addiction.

7. Thesis goals:

7.1 Summary

The goal of this thesis was to characterize the use of passive immunization against nicotine as a potential treatment for tobacco addiction. Studies of immunotherapy against nicotine demonstrate that nicotine vaccines alter nicotine pharmacokinetics, which

diminishes nicotine's addictive effects in rats. The feasibility of this strategy as a potential treatment for nicotine addiction has been established, and three nicotine vaccines are in clinical trials. In clinical trials, findings confirmed the preclinical results and nicotine vaccines demonstrated efficacy as determined by higher quit-rates compared to placebo, but only among individuals with the highest third of NicAb titers. These data indicate that two-thirds of vaccinated individuals have serum NicAb levels that are low and ineffective in attenuating nicotine's addictive effects. Efficacy of the nicotine vaccines in clinical trials is limited by high variability in serum NicAb levels and low mean serum NicAb levels. In addition, there is an apparent upper limit to the maximum NicAb levels achievable after vaccination, and there is a delay before effective serum NicAb levels are produced.

Passive immunization has great potential to improve the efficacy of immunotherapy to treat nicotine addiction, and provides a solution for the limitations of vaccination. Previously, passive immunization against nicotine was studied and used as an experimental tool for the convenience of immediately effective serum NicAb levels and the ability to study dose-effect relationships. These experimental conveniences exemplify a major advantage of passive immunization that could provide benefit clinically: Passive immunization provides the investigator or clinician control of antibody dose. This enables the administration of as high doses of NicAbs as needed, avoiding low mean serum NicAb levels and the apparent upper limit of vaccine-generated NicAb levels. A population of individuals can be administered the same dose, eliminating variability in NicAb serum levels. The timing of the presence of effective

serum NicAb levels is also controllable, since the antibodies are immediately effective upon infusion.

7.2 Gaps in the literature

This thesis examines the use of passive immunization to treat nicotine addiction. Passive immunization has many potential advantages, and also a potential disadvantage. Three distinct gaps in the literature regarding potential advantages and disadvantages of passive immunization as an immunotherapy to treat nicotine addiction are described below.

First, the effects of passive immunization on nicotine-induced behavior have not been studied extensively. Preliminary reports demonstrate that passive immunization against nicotine dose-dependently reduces the acute distribution of nicotine to the brain, and that larger doses can surpass the reductions seen after vaccination. However, it is important to demonstrate that these pharmacokinetic effects result in behavioral efficacy to fully validate the use of passive immunization as a therapy to treat nicotine addiction. This thesis provides data to address this question.

Second, the clinical role of passive immunization against nicotine in clinical use has not been fully explored. The limiting factor for using passive immunization clinically to treat tobacco addiction is its cost, and the administration of large doses of NicAbs as a stand-alone therapy is unrealistic. However, it may be possible to now use passive immunization in ways that do not require large antibody doses but still take advantage of the benefits of passive immunization, such as in combination with vaccination or even

other medications. This thesis begins to explore potential clinical uses of passive immunization.

Thirdly, no studies have addressed a potential adverse effect specific for passive immunization; the precipitation of nicotine withdrawal. Passively administered antibodies are immediately effective and alter the distribution of nicotine to the brain. To an individual who is a current smoker, the administration of a large dose of NicAbs may result in a very large and rapid change in nicotine pharmacokinetics and distribution such that a substantial redistribution of nicotine out of the brain would occur and precipitate a nicotine withdrawal syndrome. This has not been a concern with vaccination against nicotine, since antibody levels in serum increase gradually, and the effects on nicotine pharmacokinetics would be gradually apparent. The effects of passive immunization on nicotine brain levels have not been studied in this manner, and no studies have determined if passive immunization triggers nicotine withdrawal. This is a third gap in the literature that this thesis begins to address.

7.3 Thesis Goals

The five specific aims of this thesis provide information to understand how passive immunization against nicotine may serve as a treatment for tobacco addiction. The first goal was to characterize the pharmacokinetics of an acute nicotine dose in the presence of Nic311, to determine how passive immunization alters nicotine elimination, a mechanism by which immunization alters smoking behavior. Second, the pharmacokinetics of Nic311 in the presence and absence of chronic nicotine exposure were assessed to

provide information for dosing of Nic311 and determine if nicotine binding alters Nic311 elimination, which could change dosing of Nic311 in the presence of nicotine. Third, the effects of passive immunization on nicotine-induced behavior were studied to provide a more complete assessment of the efficacy and utility of passive immunization against nicotine. The fourth goal was to explore a potential clinical use of passive immunization, as a supplement to vaccination in a combination immunotherapy. The fifth goal of this thesis was to study a potential adverse effect specific for passive immunization, the precipitation of nicotine withdrawal. These five goals are addressed in the specific aims of this thesis as outlined in chapter 2.

CHAPTER 1 FIGURES AND TABLES

Table 1. Nicotine pharmacokinetic parameter estimates in rats and humans.

Cl_t, total body clearance; *ƒ*-hepatic, hepatic fraction of total clearance; *ƒ*-renal, renal fraction of total clearance; $V_{d,ss}$, Volume of distribution at steady-state; $t_{½}\beta$, elimination half-life; *ƒu*, fraction unbound in serum. Values in parentheses represent range. Data from [75, 82, 83].

Nicotine Pharmacokinetic Parameters		
	Rat	**Human**
Cl_t (L/hr/kg)	3 ± 0.3	1.3 (1.1 – 1.5)
(ml/min/kg)	50.3	18.6 (16 – 21)
ƒ-hepatic:	0.91	0.85
ƒ-renal:	0.09	0.15
$V_{d,ss}$ (L/kg)	2.8 ± 0.7	2.75 (2.2 – 3.3)
$t_{½}\beta$ (hr)	0.9 ± 0.1	2.1 (1.7 – 2.5
% bound in plasma	9.5 ± 0.5	< 5
ƒu	0.91	> 0.95

Figure 1. Nicotine and the nicotine immunogen 3`AmNic-rEPA

(Left) Nicotine is a weak base. It is comprised of a pyridine and a pyrrolidine ring, with a molecular weight of 162 Daltons, and a pKa of 8.0.

(Right) A nicotine immunogen (3`AmNic-rEPA) is composed of a hapten (3`AmNic) conjugated to a carrier protein (rEPA, recombinant *Pseudomonas* exoprotein A) via a succinic acid linker.

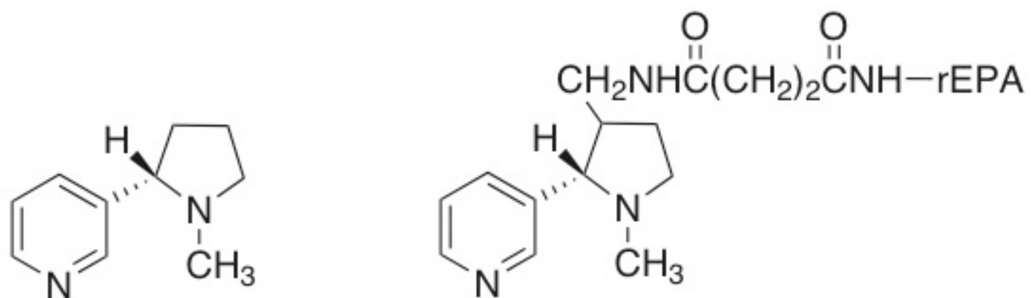


Figure 2. The nicotinic acetylcholine receptor

The nicotinic acetylcholine receptor (nAChR) is made up of five distinct subunits (each with four transmembrane domains) that together form an ion channel. These ligand-gated ion channels are found throughout the central nervous system. Neuronal nAChRs may be homomeric or heteromeric. (Adapted from [139]).

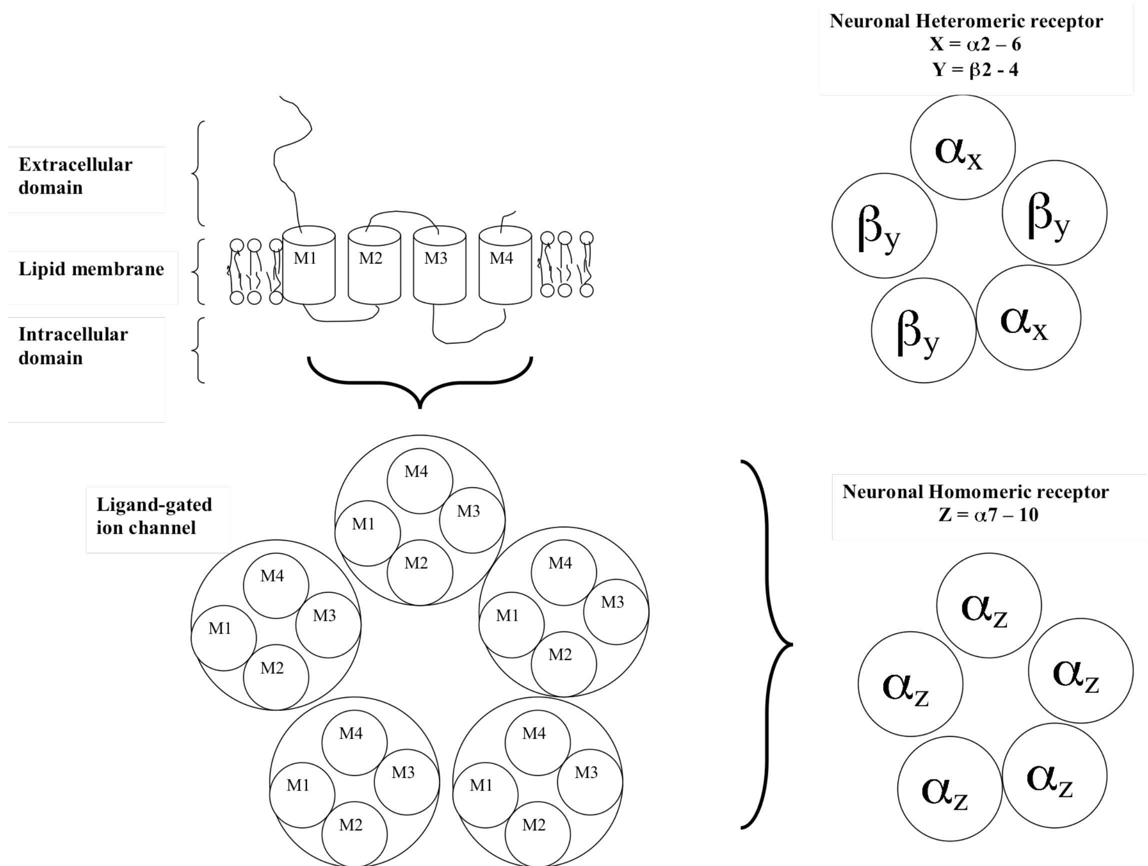
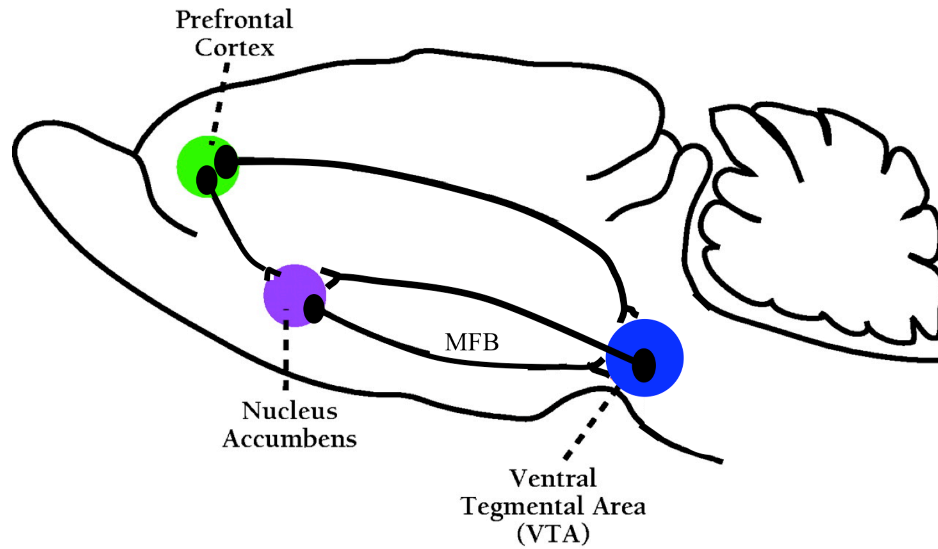


Figure 3. The Brain Reward circuit in rat and human brain.

The key components of the Brain Reward Circuit are analogous regions in the rat (top) and human (bottom) brain, and both are activated by natural rewards and drugs of abuse. VTA, ventral tegmental area.



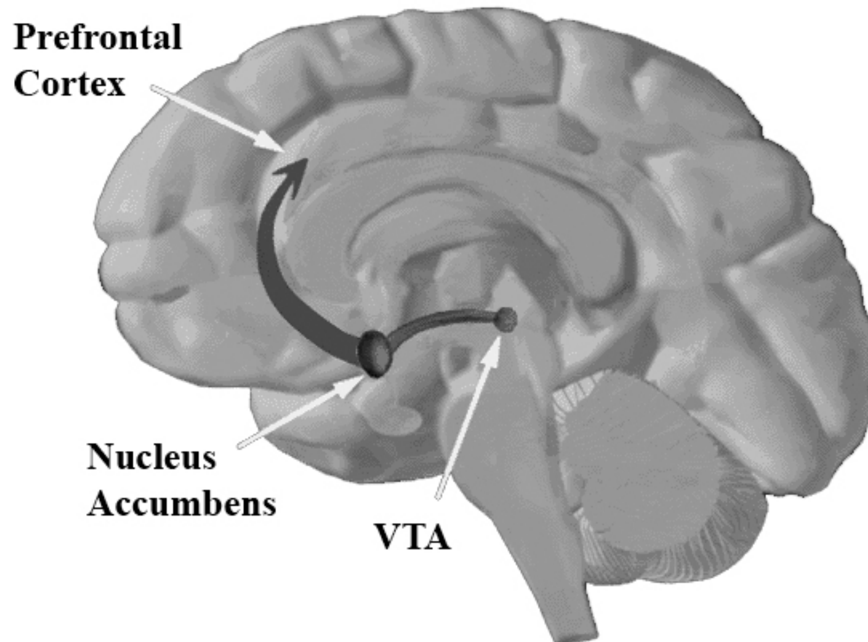


Figure 4. The mechanism of action of immunotherapy against nicotine.

Nicotine rapidly distributes into the brain, but antibodies are large molecules and are excluded from the brain by the blood-brain barrier. When a vaccinated individual smokes, antibodies bind nicotine and alter nicotine distribution into the brain.

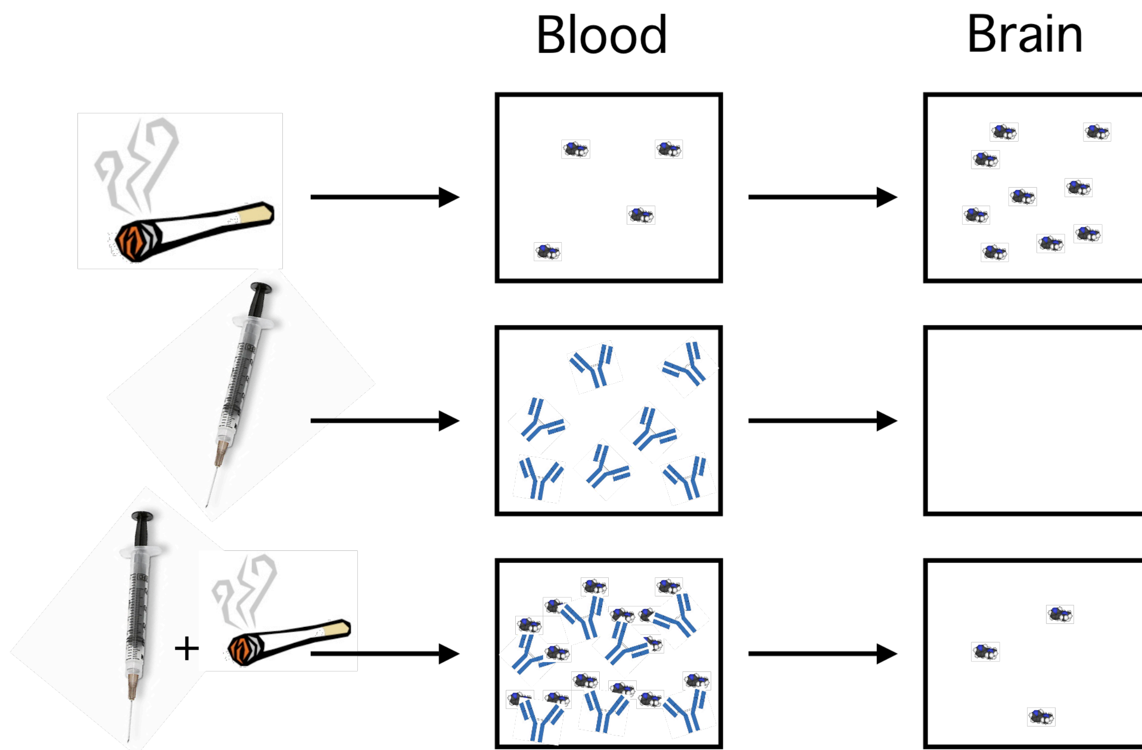


Figure 5. Nic311 dose-response for serum and brain nicotine levels (single-dose) This figure is modified and adapted from [137]. Serum (top) and brain (bottom) nicotine concentrations were measured 3 minutes after administration of nicotine 0.3 mg/kg. Data points for each of 2 rats at each Nic311 dose are shown due to limited supply of antibody. Nic-IgG is a polyclonal antiserum with higher affinity for nicotine than Nic311 (Nic-IgG Kd = 1.6 nM, Nic311 Kd = 60 nM), which is as effective as Nic311 80 mg/kg in this protocol. Red dashed line indicates maximal mean effect observed after vaccination, which is surpassed by higher doses of Nic311.

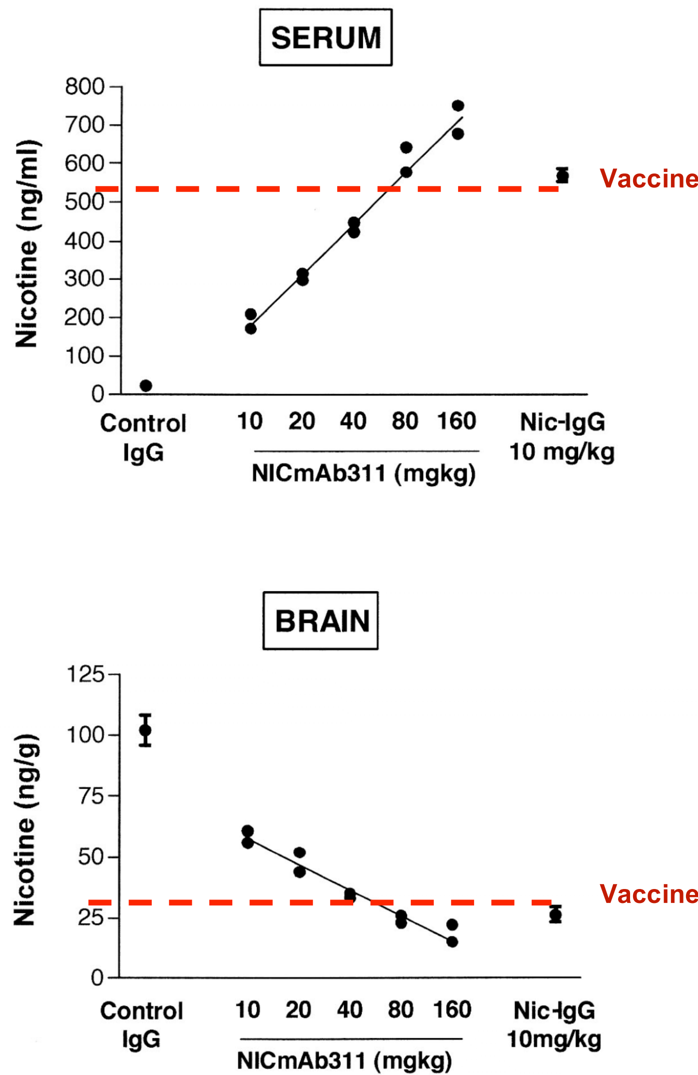
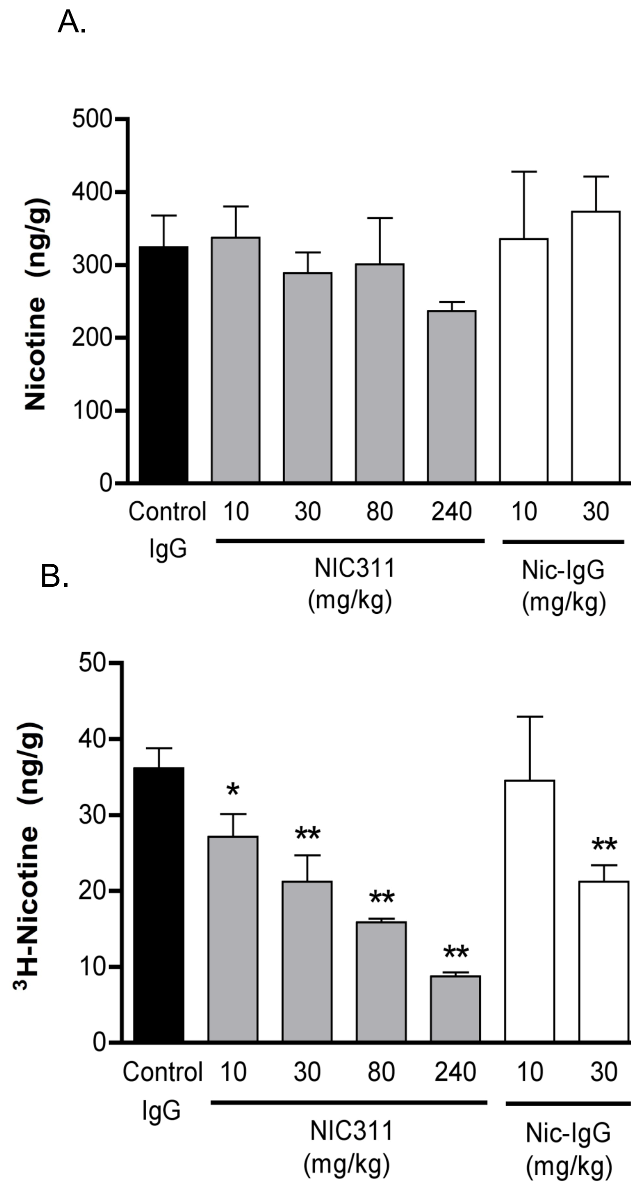


Figure 6. Nic311 dose-response on brain nicotine levels during chronic dosing

(Top) Total (chronic accumulation) brain nicotine levels after 0.03 mg/kg/16 hours, in the presence of increasing doses of Nic311 or Nic-IgG. No significant difference was found between groups.

(Bottom) Acute brain nicotine levels 5 minutes after the final ^{3}H-nicotine dose of 0.03 mg/kg. Nic311 and Nic-IgG dose-dependently reduced acute brain nicotine levels (*, **; $p < 0.05$, $p < 0.01$ compared to control).



CHAPTER 2

Specific Aims and Introduction to Chapters 3, 4, 5

This chapter provides an overview of the Specific Aims of this thesis and the hypotheses posed that address these Aims. In addition, chapters 3, 4, and 5 are introduced, including discussion on how they address the Aims of this thesis.

1. Overall goal

The overall goal of this thesis was to study the use of passive immunization against nicotine as a treatment for nicotine addiction. The specific goals were to examine the efficacy and safety of passive immunization to treat nicotine dependence in rats, and begin exploring the role of passive immunization as a treatment for tobacco addiction. Studies were conducted to characterize the effects of passive immunization with the nicotine-specific monoclonal antibody Nic311 on nicotine distribution to the brain, nicotine-induced locomotor sensitization, and the precipitation of nicotine withdrawal in rats. These pharmacokinetic and behavioral studies provide insight into the circumstances where passive immunization has advantages over vaccination in treating nicotine dependence, and the clinical utility of passive immunization to treat drug addiction.

2. Specific Aims and Hypotheses

Specific aim #1 was to determine how passive immunization with the nicotine-specific monoclonal antibody Nic311 altered the pharmacokinetics of an acute nicotine dose. Since the subjective effects of smoking occur during the first few minutes of cigarette smoking, it was important to characterize how passive immunization altered the pharmacokinetics of an acute dose of nicotine. The hypothesis tested was that <u>Passive immunization with Nic311 significantly reduces the clearance and steady-state volume of distribution and prolongs the elimination half-life of nicotine in rats, compared to that of controls.</u>

Specific aim #2 was to characterize the pharmacokinetics of Nic311 in the presence and absence of chronic nicotine exposure. Characterizing the pharmacokinetics of Nic311 was necessary to guide antibody dosing in other experiments. This study also determined if the presence of nicotine altered the pharmacokinetic parameter estimates of Nic311, because Nic311 would be administered to animals receiving either nicotine or saline. If Nic311 pharmacokinetics are altered in the presence of nicotine, this would complicate dosing of antibody and could compromise the efficacy of passive immunization. The hypothesis tested was that Nic311 pharmacokinetic parameters are similar to those of previously reported values of mouse IgG in rat, and that the presence of nicotine does not significantly alter Nic311 pharmacokinetics.

Specific aim #3 was to examine the effects of passive immunization with Nic311 on nicotine-induced behavior. The effects of Nic311 on brain nicotine levels were previously established, and it was important to determine if these pharmacokinetic effects result in a behavioral effect. Nicotine-induced locomotor sensitization (LMS) is a well-characterized behavioral model of nicotine's psychoactive effects and was used to study the behavioral effects of passive immunization with Nic311. The hypothesis tested was that when Nic311 is dosed to produce serum NicAb levels equivalent to those generated by vaccination, brain nicotine levels and nicotine-induced locomotor sensitization are attenuated as much as by vaccination. That is, equivalent serum NicAb levels result in equivalent reductions in brain nicotine levels and nicotine-induced LMS.

Specific aim #4 was to explore the potential clinical use of passive immunization with Nic311. Currently, passive immunization is expensive [130], and is not likely to be used broadly. Combining vaccination with intermediate doses of passive immunization is one way to take advantage of the benefits of passive immunization. The hypothesis tested was that combining Nic311 with vaccination reduces brain nicotine levels and nicotine-induced locomotor sensitization to a greater extent compared to vaccination alone.

Specific aim #5 was to determine if Nic311 precipitates nicotine abstinence syndrome (nicotine withdrawal). Nicotine withdrawal can be triggered by the administration of a nicotinic antagonist or cessation of nicotine, and produces characteristic changes in brain reward thresholds in nicotine dependent rats [25, 141]. A concern of passive immunization was that an acute dose of Nic311 to a nicotine-dependent individual currently exposed to nicotine could substantially reduce brain nicotine levels and precipitate withdrawal, which would be an important adverse effect of passive immunization. This precipitation of withdrawal after administration of Nic311 was compared to that precipitated by the nicotine antagonist mecamylamine. Mecamylamine is a nonspecific antagonist at nAChRs that reduces nicotine signaling, and elicits a robust withdrawal signal when administered to nicotine-dependent animals that are currently exposed to nicotine. Passive immunization does not directly block nicotine signaling at nAChRs but rather reduces brain nicotine levels. Reducing brain nicotine levels may indirectly cause a reduction in nicotine bound to nAChRs and reduce nicotine signaling, but it is not expected to remove all nicotine bound to nAChRs. For

this reason, the withdrawal precipitated by Nic311 may be more modest compared to that of mecamylamine. The hypothesis tested here was that Nic311 precipitates less severe nicotine withdrawal compared to that induced by the nicotine antagonist mecamylamine.

3. Summary of how chapters 3, 4, and 5 address these Aims.

Chapter 3 [137] includes data that address Aim 1. The pharmacokinetics of an acute nicotine dose were assessed by collecting serial blood samples after an acute i.v. dose of nicotine, in the presence of Nic311 or Control IgG. These data were included with a more comprehensive assessment of Nic311 and published in the manuscript included as chapter 3. The sections relevant to Aim 1 have been excerpted and precede chapter 3 to indicate the specific experiment that is part of this thesis.

Chapter 4 [142] addresses Aims 2, 3, and 4. In Chapter 4 the pharmacokinetics of Nic311 were assessed in the presence and absence of nicotine to address Aim 2. Two groups of rats (one exposed to nicotine, the other not) were administered Nic311 30 mg/kg and serial blood samples were collected through 56 days to determine the pharmacokinetic parameters of Nic311. These data guided the dosing of Nic311 in the locomotor sensitization study.

Chapter 4 also includes data that address Aims 3 and 4 and demonstrates the effects of immunotherapy on nicotine-induced locomotor sensitization (LMS). Nicotine-induced LMS was used for this study since LMS is a robust and well-characterized model of nicotine's psychoactive effects and is mediated by brain structures that overlap the brain

reward pathway. The nicotine dose used to induce LMS produces serum nicotine concentrations similar to that of smokers and those observed in rats during nicotine self-administration [143], and also produces conditioned place preference [93]. In addition, nicotine-induced LMS has been shown to be inhibited by lobeline [144], indicating that this behavioral assay is responsive to therapies. This study examined the effects of passive immunization using Nic311 alone, vaccination alone, and the combination of vaccination and passive immunization with Nic311 on a well-characterized LMS protocol. Brain nicotine levels and nicotine-specific antibody (NicAb) concentrations were measured in addition to locomotor activity to examine relationships between NicAbs, brain nicotine levels, and LMS.

Chapter 5 [145] addresses Aim 5. This study assessed the ability of Nic311 to precipitate withdrawal. Withdrawal was measured using intracranial self-stimulation (ICSS) and somatic withdrawal signs. ICSS is a quantitative and central measure of anhedonia, and has been widely used in studying an animal's affective state such as in withdrawal or depression [146]. Here, ICSS was used to provide an objective and accurate measurement of withdrawal precipitated by passive immunization with Nic311 and compare it to that precipitated by mecamylamine, a noncompetitive antagonist at nicotinic acetylcholine receptors [27]. Brain nicotine levels were measured in a parallel experiment to provide insight into the pharmacokinetic aspects of withdrawal.

CHAPTER 3

Monoclonal nicotine-specific antibodies reduce nicotine distribution to brain in rats: Dose- and affinity-response relationships

D.E. Keyler, S.A. Roiko, E. Benlhabib, M.G. LeSage, J.V. St.Peter, S. Stewart, S. Fuller, C.T. Le, P.R Pentel

This manuscript was published in the journal *Drug Metabolism and Disposition* (Keyler, D.E., Roiko, S.A., et al. Drug Metab Dispos, 2005 **33**(7):1056-61) and has been adapted for this dissertation. Sections relevant to this thesis are excerpted in a preface, which immediately follows this title page.

Abbreviations: NICmAb - monoclonal anti-nicotine antibodies; Nic-IgG - polyclonal rabbit anti-nicotine antiserum; rEPA - recombinant *Pseudomonas* exoprotein A

ABSTRACT

Vaccination against nicotine is being studied as a potential treatment for nicotine dependence. Some of the limitations of vaccination, such as variability in antibody titer and affinity, might be overcome by instead using passive immunization with nicotine-specific monoclonal antibodies. The effects of antibodies on nicotine distribution to brain were studied using nicotine-specific monoclonal antibodies (NICmAbs) with K_d values ranging from 60 to 250 nM and a high affinity polyclonal rabbit antiserum (K_d = 1.6 nM). Pretreatment with NICmAbs substantially increased the binding of nicotine in serum following a single nicotine dose, reduced the unbound nicotine concentration in serum, and reduced the distribution of nicotine to brain. Efficacy was directly related to antibody affinity for nicotine. Efficacy of the highest affinity NICmAb, Nic311, was dose-related, with the highest dose reducing nicotine distribution to brain by 78%. Nic311 decreased nicotine clearance by 90% and prolonged the terminal half-life of nicotine by 120%. At equivalent doses, Nic311 was less effective than the higher affinity rabbit antiserum, but comparable efficacy could be achieved by increasing the Nic311 dose. These data suggest that passive immunization with nicotine-specific monoclonal antibodies substantially alters nicotine pharmacokinetics in a manner similar to that previously reported for vaccination against nicotine. Antibody efficacy is a function of both dose and affinity for nicotine.

1. Preface to Chapter 3: Effects of Nic311 on nicotine pharmacokinetics

The following manuscript includes an experiment to address Aim 1. Since this was part of the initial characterization of Nic311 and contained within a larger manuscript, the sections relevant to my work are excerpted here.

1.1 Introduction

This experiment was conducted to determine how passive immunization with Nic311 altered the pharmacokinetic parameters of an acute nicotine dose. Previous work demonstrated that vaccination reduced the clearance and volume of distribution of an acute nicotine dose, resulting in a prolonged elimination half-life. It was important to assess the effects of passive immunization on nicotine pharmacokinetic parameters after an acute nicotine dose, since the subjective effects of smoking occur during the first few minutes of smoking. These data address the question, how is the pharmacokinetics of an acute nicotine dose altered in the presence of passively administered antibodies?

The experimental methods and findings are briefly described below.

1.2 Methods:

Groups of six rats were pretreated with 27 mg/kg of either Nic311 or Control-IgG (a polyclonal human IgG Sandoglobulin, Sandoz, Vienna, Austria). The 27 mg/kg dose was chosen to approximate the nicotine-specific antibody content of a rat vaccinated with the same immunogen [106, 107]. This calculation was based upon a typical nicotine-specific

serum antibody concentration of 0.2 mg/ml in previous studies of rats vaccinated with this immunogen [106, 107], and a steady state volume of distribution of 0.125 L/kg for mouse IgG administered to rats [147]. Antibody was administered i.p. in 0.5 ml PBS. Twenty-four hours later, rats were anesthetized and jugular and femoral venous catheters placed. Nicotine 0.1 mg/kg was administered via the jugular catheter over 10 sec. This nicotine dose (the equivalent of ~ 10 cigarettes) was used to ensure that blood nicotine levels would be above the lower limit of quantitation (2 ng/ml) 4 hours after administration. Blood samples of increasing volume (0.5 to 1.5 ml) were collected from the femoral catheter at intervals of up to 4h for controls and 20 h for rats treated with Nic311.

Nic311 was administered i.p. in this experiment, rather than i.v. as in the other experiments, as a convenience. Data included in this manuscript demonstrated that serum Nic311 concentrations were comparable 24 hours after either i.p. or i.v. dosing, and serum Nic311 concentrations were measured throughout this experiment.

Noncompartmental pharmacokinetic parameters were estimated from individual concentration-time data using WinNonlin version 4.1 (Pharsight, Mountain View, CA). C_{max} was the highest measured value. The terminal elimination rate constant (*k*) was estimated by iterative least squares regression using the terminal 5 to 7 serum concentrations. Half-life was determined from the ratio Ln2/*k*. The area under the concentration-time curve (AUC) from time t_0 to last observed concentration (C_{last}) was estimated using the log-linear trapezoidal rule. The terminal area from C_{last} to infinity

was estimated as the ratio C_{last}/k. The AUC from time 0 to infinity was the sum of these area estimates. Total clearance (Cl_t) was estimated as the dose/AUC, and the steady state volume of distribution as the product of clearance and area under the moment curve divided by AUC.

1.3 Results

Pretreatment with Nic311 decreased nicotine $V_{d,ss}$ by 82% ($p < 0.01$), decreased Cl by 90% ($p < 0.01$), and prolonged the terminal half-life by 120% ($p < 0.001$ compared to controls; Figure 4). Serum Nic311 concentrations were stable throughout the study period with values of 120±33 μg/ml at 30 min, 100±10 at 6 h, and 110±30 μg/ml at 24 h after nicotine dosing. Nicotine pharmacokinetic parameter estimates are shown in Table 3.

1.4 Discussion

The effects of Nic311 on nicotine pharmacokinetic parameters were quite similar to those reported previously after vaccination of rats against nicotine [112]. Nic311 markedly reduced nicotine clearance, decreased $V_{d,ss}$ and increased the terminal half-life. The slowing of nicotine elimination by nicotine-specific antibody presents the possibility that antibody could become saturated with nicotine after repeated or chronic dosing, and become less effective in binding subsequently administered nicotine. However, any such saturation occurring after vaccination of rats did not appear to prevent vaccination from attenuating the effects of nicotine even under chronic dosing conditions which simulate cigarette smoking [110].

It is possible that the reduced clearance and longer half-life of nicotine produced by Nic311 could be beneficial. Population data suggest that smokers who have slower nicotine metabolism smoke less, perhaps because the effects of each cigarette last longer [148, 149]. This represents one possible mechanism by which immunization could facilitate smoking reduction.

2. INTRODUCTION

Immunization has been studied as a potential treatment strategy for a variety of drug addictions, including heroin [150], cocaine [151, 152], phencyclidine [126], methamphetamine [124], and nicotine [91, 106, 113, 153, 154]. Vaccines consisting of the drug linked to a foreign carrier protein elicit the production of drug-specific antibodies that bind drug in serum and extracellular fluid, reduce the unbound drug concentration, and reduce drug distribution to brain. Immunization has been shown to block or attenuate a variety of drug-induced behaviors in rats that are relevant to addiction, including locomotor activation, drug discrimination, and drug self-administration (reviewed in [155, 156]). These data suggest that immunization may have potential for the prevention or treatment of drug addiction. Clinical trials of vaccines for cocaine and nicotine addiction have been initiated (personal communication, D. Hatsukami, and [157]).

Two immunization strategies have been studied; active immunization, in which the experimental animal is vaccinated to elicit an immune response, and passive immunization in which the experimental animal is administered exogenously produced drug-specific antibody [156]. Clinical interest has focused on vaccination because of its safety, prolonged effect, convenience (obviating the need for daily medication), and low cost. Passive immunization has been used primarily as an experimental expedient because of its immediate onset of effect compared to the 1 to 2 months required to develop a satisfactory antibody response to vaccination, and because it allows control of

the antibody dose [158]. These same experimental advantages of passive immunization over vaccination could prove clinically useful as well. Rapid onset of effect could be advantageous for initiating treatment of patients in a timely manner, while they are motivated. Control of antibody dose could be important because individual responses to vaccination are variable [159]. Some patients may not achieve satisfactory antibody concentrations after vaccination, or others may require higher antibody concentrations than are achievable with vaccination. In addition, if a monoclonal antibody is used for passive immunization, an antibody with defined affinity and specificity can be selected for administration.

Nicotine addiction provides a useful model for studying passive immunization because both the pharmacokinetic and behavioral effects of vaccination have been well studied in rats. Vaccination reduces nicotine distribution to the brain under a variety of clinically relevant acute and chronic nicotine dosing conditions [106, 110, 160], reduces nicotine-induced dopamine release from the nucleus accumbens [161], and attenuates the reinstatement of nicotine self-administration [90, 113]. Passive immunization of rats with a nicotine-specific polyclonal rabbit antiserum, although less well studied, has similar effects on nicotine distribution to brain and attenuates nicotine discrimination [114] and the relief of nicotine abstinence by nicotine [128]. In a recent study, passive immunization with a monoclonal antibody reduced nicotine-induced locomotor activation in a dose-related manner [91]. Although these data support the potential clinical use of passive immunization, the specific antibody characteristics which would provide optimal efficacy are unclear. Efforts with existing vaccines have focused on producing very high

affinity antibodies to maximize their ability to bind drug. However, the relationship of antibody affinity to efficacy is not established, and it is possible that antibodies with more modest affinities for nicotine would suffice. This is an important practical consideration because obtaining very high-affinity monoclonal antibodies to some antigens can be difficult. In addition, it is also possible that a very high affinity antibody could result in greater antibody saturation with drug when large or repeated drug doses are administered, and thus compromise efficacy [107, 112].

In the current study, a variety of monoclonal nicotine-specific antibodies (NICmAbs) were evaluated to determine the relationship of antibody affinity to pharmacokinetic efficacy. Because it proved difficult to produce very high-affinity mAbs, a polyclonal nicotine-specific rabbit antiserum with higher affinity for nicotine was also studied. The most effective of the NICmAbs was further studied to determine the dose-response relationship for reducing nicotine distribution to brain and the effects of immunization on nicotine pharmacokinetic parameters.

3. METHODS

3.1 Drugs and reagents.

(-)-Nicotine bitartrate and goat anti-IgG-peroxidase conjugate were obtained from Sigma-Aldrich (St. Louis, MO). Internal standards for nicotine assay were a gift from Dr. Peyton Jacob. All nicotine doses and measured concentrations are expressed as the weight of the base.

3.2 Production of rabbit nicotine-specific IgG (Nic-IgG).

New Zealand white rabbits were immunized with *trans*-3'-aminomethyl nicotine conjugated to recombinant *Pseudomonas aeruginosa* exoprotein A in Freund's adjuvant as described previously [106]. Immune rabbit serum was purified on a Protein G Sepharose 4 Fast Flow column (Amersham Biosciences, Inc., Piscataway, NJ) equilibrated with PBS, eluted with 0.1 M glycine buffer at pH 2.7, and neutralized with 1 M Tris buffer at pH 9. The IgG was diafiltered, concentrated and brought to a concentration of 50 mg/ml total IgG in PBS. The nicotine-specific IgG content of this fraction determined by ELISA was 5%. All doses of Nic-IgG administered to rats are expressed as mg of nicotine-specific IgG. This antiserum has been previously characterized as having low cross-reactivity with nicotine metabolites or acetylcholine [106].

3.3 Production of nicotine-specific monoclonal antibodies (NICmAb).

Mice were immunized with *trans*-3'-aminomethyl nicotine conjugated to recombinant *P. aeruginosa* exoprotein A adsorbed to alum, and hybridomas were prepared as previously described [119]. The fused cells were resuspended into a selection medium and seeded into 96-well tissue culture plates that contained a macrophage feeder layer [24]. Supernatants of growing cultures were screened for nicotine-specific mAb secretors on a polyglutamate-hapten conjugate used as antigen in ELISA assays [162]. ELISA positives were re-screened on the nicotine metabolite cotinine to eliminate cotinine cross-reactive MAb secretors. Ascites produced by injecting established clones into mice were purified on a protein A column and further characterized. Affinity for nicotine was measured by radioimmunoassay [163].

3.4 Control antibodies.

Two control immunoglobulins were used in these experiments. The first experiment used control-mAb, a monoclonal IgG1 kappa directed at the unrelated hapten desipramine. Because the availability of this monoclonal antibody was limited, polyclonal human IgG (Control-IgG; Sandoglobulin, Sandoz, Vienna, Austria) was used for subsequent experiments [106]. Serum and brain nicotine concentrations are comparable in rats treated with either of these control antibodies and then administered nicotine (see control groups, Figures 1 and 2).

3.5 Characterization of antibody.

Antibody affinity for nicotine was measured by radioimmunoassay [163]. Nicotine-specific antibody concentrations in the serum of rats treated with NICmAbs were measured by ELISA calibrated using purified NICmAb [111]. Nicotine-specific antibody concentration in rabbit antiserum was calculated from the binding capacity measured by radioimmunoassay [163]. Antibody specificity was measured by competitive ELISA.

3.6 Nicotine assay.

Concentrations of nicotine and cotinine in serum or brain were measured by gas chromatography with nitrogen-phosphorus detection [103, 164]. Brain nicotine concentrations were corrected for brain blood content [112].

3.7 Effects of NICmAbs and Nic-IgG on nicotine distribution.

The purpose of this experiment was to compare the effects of antibodies with a range of affinities for nicotine (three NICmAbs and Nic-IgG) on nicotine distribution. Male Holtzman rats weighing 320 to 410 g and housed in pairs on a 12-h light dark cycle were used for all experiments. Five groups of 5-6 rats were anesthetized, and femoral and jugular venous catheters placed. Antibody pretreatment was administered via the femoral catheter. Controls received 10 mg/kg control-mAb, and the four antibody groups received 10 mg/kg of Nic079 (K_d = 250 nM), Nic810 (K_d = 70 nM), Nic311 (K_d = 60 nM) or Nic-IgG (K_d = 1.6 nM) in 1 ml PBS. Thirty minutes later, rats received 0.03 mg/kg nicotine over 10 sec via the jugular catheter. Three minutes after nicotine dosing, rats

were decapitated, and blood and brain collected. Serum was separated for drug, antibody and protein binding assay.

3.8 Dose-response for effects of Nic311 on nicotine distribution.

The purpose of this experiment was to study the relationship between NICmAb dose and nicotine distribution. Nic311 was chosen to study because it had the lowest K_d and the largest pharmacokinetic effect of the NICmAbs evaluated previously. Because the availability of Nic311 was limited, antibody administration was restricted to 2 rats per dose. Rats in this experiment were prepared and studied as described immediately above, receiving Nic311 at doses of 10 to160 mg/kg i.v., followed in 30 minutes by nicotine 0.03 mg/kg and decapitation and sampling 3 minutes after the nicotine dose.

3.9 Nicotine pharmacokinetic parameters.

Groups of six rats were pretreated with 27 mg/kg of either Nic311 or control-IgG. The 27 mg/kg dose was chosen to approximate the nicotine-specific antibody content of a rat vaccinated with the same immunogen [106, 107]. This calculation was based upon a typical nicotine-specific serum antibody concentration of 0.2 mg/ml in previous studies of rats vaccinated with this immunogen [106, 107], and a steady state volume of distribution of 0.125 L/kg for mouse IgG administered to rats [147]. Antibody was administered i.p. in 0.5 ml PBS. Twenty-four hours later, rats were anesthetized and jugular and femoral venous catheters placed. Nicotine 0.1 mg/kg was administered via the jugular catheter over 10 sec. Blood samples of increasing volume (0.5 - 1.5 ml) were

collected from the femoral catheter at intervals of up to 4h for controls and 20 h for rats treated with Nic311.

Nic311 was administered i.p. in this experiment, rather than i.v. as in the other experiments in this study, as a convenience. To confirm that absorption of Nic311 via the i.p. route was satisfactory, six rats were anesthetized, and jugular and femoral venous catheters placed. Nic311 10 mg/kg was administered to one group i.v. and to the other group i.p. Blood was sampled at 0.5, 1, 6 and 24 h for measurement of serum nicotine-specific antibody concentrations.

3.10 Protein binding.

Protein binding was measured by equilibrium dialysis of 0.9 ml serum against PBS for 4h at 37°C using Spectrapor 2 membranes (Spectrapor Labs, Ranco Dominguez, CA) as described previously [165].

3.11 Estimation of nicotine pharmacokinetic parameters.

Noncompartmental pharmacokinetic parameters were estimated from individual concentration-time data using WinNonlin version 4.1 (Pharsight, Mountain View, CA). C_{max} was the highest measured value. The terminal elimination rate constant (k) was estimated by iterative least squares regression using the terminal five to seven serum concentrations. Half-life was determined from the ratio Ln2/k. The area under the concentration time curve (AUC) from time 0 to last observed concentration (C_{last}) was estimated using the log-linear trapezoidal rule. The terminal area from C_{last} to infinity

was estimated as the ratio C_{last}/k. The AUC from time 0 to infinity was the sum of these area estimates. Total clearance (Cl_t) was estimated as the dose/AUC, and the steady state volume of distribution as the product of clearance and area under the moment curve divided by AUC.

3.12 Statistical methods.

Correlations between antibody Kd or antibody dose and various parameters (serum or brain nicotine concentration) were analyzed by linear regression, as were correlations between the unbound serum nicotine concentration and brain nicotine concentration. Differences between individual antibody-treated groups and controls were analyzed by one-way analysis of variance with Dunnett's post-hoc contrast if the overall test was significant at $p < 0.05$. Pharmacokinetic parameters were compared using two-sided t tests.

4. RESULTS

4.1 Antibody characteristics.

Antibody affinities for nicotine were Nic079 (K_d = 250 nM), Nic810 (K_d = 70 nM), Nic311 (K_d = 60 nM) and Nic-IgG (K_d = 1.6 nM) . These antibodies provided a range of affinities varying by more than 2 orders of magnitude. Nic311 had the highest affinity of the available mAbs and was therefore focused upon in this study. Both Nic311 and Nic-IgG were highly specific for nicotine. Nic311 cross reactivity was <1% for the nicotine metabolites (-)-cotinine, (±)-nicotine-N-oxide and (±)-nornicotine, and <1% for the endogenous nicotinic receptor ligand acetylcholine. Nic-IgG cross reactivity was (-)-cotinine 1.9%, (±)-nicotine-N-oxide <1%, (±)-nornicotine 1.5% , and acetylcholine <1%.

4.2 Effects of antibody affinity on nicotine distribution.

All antibodies tested, except Nic079, significantly increased nicotine retention in serum and all antibodies including Nic079 reduced nicotine distribution to brain compared to controls (Figure 1). The magnitude of these effects was directly related to antibody affinity for nicotine ($r = 0.67$, $p < 0.001$); Nic-IgG (K_d = 1.6nM) had the greatest effect, and Nic311 (K_d = 60 nM) had the greatest effect among mAbs. Protein binding showed similar affinity-related effects (Table 1) with the Nic-IgG group having the highest protein binding of nicotine ($r = 0.90$, $p < 0.001$) and the lowest unbound serum nicotine concentration ($r = 0.68$, $p < 0.001$). There was a close correlation between the unbound nicotine concentration in serum ($r = 0.99$, $P < 0.01$) and the brain nicotine concentration ($r = 0.99$, $p < 0.001$, Figure 2, top).

Serum antibody concentrations were comparable 30 min after administration of 10 mg/kg of the various NICmAbs or Nic-IgG (230±30, 230±30, 230±20, and 247±31 μg/ml) for Nic079, Nic810, Nic311 and Nic-IgG respectively (p = NS).

4.3 Effects of Nic311 dose on nicotine distribution.

There was a strong correlation between the log Nic311 dose and nicotine retention in serum ($r = 0.99$, $p<0.001$) and nicotine distribution to brain ($r = 0.99$, $p <0.01$) (Figure 3). The highest NICmAb dose of 160 mg/kg reduced brain nicotine concentration by 78% compared to Control-IgG. The reduction in brain nicotine concentration produced by the 80 mg/kg Nic311 dose (71%) was similar to that of 10 mg/kg Nic-IgG determined in the previous experiment (69%). Serum protein binding of nicotine increased, and the unbound nicotine concentration decreased, with increasing Nic311 dose (Table 2, $p <0.001$). There was a close correlation between the unbound nicotine concentration in serum and the brain nicotine concentration ($r = 0.99$, $p <0.001$) Figure 2, bottom). Serum Nic311 concentrations (mean of 2 values) measured 30 min after the antibody dose were dose-related; 220, 370, 930, 1,570 and 4,230 μg/ml with increasing Nic311 dose.

4.4 Nicotine pharmacokinetics (Figure 4).

Pretreatment with 27 mg/kg Nic311 decreased nicotine $V_{d,ss}$ by 82% ($p <0.01$), decreased Cl by 90% ($p <0.01$), and prolonged the terminal half-life by 120% ($p <0.001$ compared to controls). Serum Nic311 concentrations were stable throughout the study period with

values of 120±33 μg/ml at 30 min 100±10 at 6 h, and 110±030 μg/ml at 24 h after nicotine dosing.

4.5 Route of Nic311 dosing.

Serum antibody concentrations at 0.5, 1, 6, 24 and 48 h after antibody dosing (Nic311 10 mg/kg) were 195±23, 188±14, 133±23, and 70±10 μg/ml (i.v.) and 1±1, 10±7, 79±36, and 70±20 μg/ml (i.p.). Thus serum antibody concentrations were comparable at 24 h, validating the use of the i.p. route for antibody administration in the preceding experiment which examined nicotine pharmacokinetic parameters starting 24 h after i.p. administration of Nic311.

5. DISCUSSION

These data show a clear relationship between monoclonal antibody affinity, dose, and efficacy for altering the distribution of a single dose of nicotine in the rat. Polyclonal nicotine-specific antiserum (Nic-IgG, K_d = 1.6 nM) was most effective, but a comparable effect was obtained with Nic311 (K_d = 60 nM) when administered at an 8-fold higher dose. Nic311 also markedly reduced nicotine clearance and prolonged its terminal half-life. These effects of passive immunization are strikingly similar to those reported previously after vaccination of rats against nicotine [106, 107]. The data suggest that passive immunization with monoclonal antibodies could provide an alternative strategy to vaccination, and offer preliminary information regarding the K_d and antibody dose required.

The NICmAbs used in this study represented a modest range of K_d values for nicotine (60 to 250 nM). Because identification of higher affinity monoclonal antibodies has not been successful, polyclonal Nic-IgG was used to extend the range of Kd values studied. It is possible that differences between the effects of Nic-IgG and the various NICmAbs were due to antibody characteristics other than K_d. We are aware of no data regarding the comparative pharmacokinetics of rabbit and mouse IgG in the rat. However, the measured serum nicotine-specific antibody concentrations in this study after either 3 mg of Nic-IgG or the various NICmAbs were similar. It is therefore likely that differences in

efficacy between NIC-IgG and NICmAbs in the current study were predominantly due to their differences in K_d for nicotine.

In the range of K_d values evaluated, lower K_d was associated with greater efficacy for retaining nicotine in serum, increasing protein binding of nicotine in serum, reducing unbound nicotine concentration in serum, and reducing nicotine distribution to brain. These data suggest that a monoclonal antibody with a lower K_d than that of Nic311 would be even more effective than this monoclonal antibody. Nevertheless, several considerations suggest that Nic311 is sufficiently effective to be of interest as a means of reducing nicotine effects. First, the K_d of 60 nM is only slightly higher than that of antibodies elicited by vaccination of rats (range 19 to 40 nM) with this same immunogen [106, 110, 166], and vaccination has been shown to attenuate many nicotine-related effects in rats that are relevant to nicotine addiction [90, 113]. In addition, the monoclonal nicotine-specific antibody studied by Carrera et al (2004), with an even higher K_d of 200 nM, produced a substantial reduction of the locomotor activating effect of a single nicotine dose [91]. Second, the magnitude of Nic311 effect is a function of dose. The 40 mg/kg dose of Nic311, which is within the range of antibody content of a vaccinated rat, produced a 20-fold increase in serum nicotine concentration and a 60% decrease in brain concentration compared with controls, values essentially identical to those reported after vaccination using the same protocol [106]. Higher Nic311 doses produced greater effects, and at the 80 mg/kg dose these effects were comparable to those of 3 mg Nic-IgG. Thus the lesser efficacy of Nic311 compared with Nic-IgG could be compensated with a suitable increase in Nic311 dose.

The feasibility of using passive immunization as a clinical therapy rests in part on the dose of antibody required. Even the highest NICmAb dose used in this study is well within the range of antibody doses used in other settings. Doses of methamphetamine- or phencyclidine-specific IgG of up to 1 g/kg [124, 125], and desipramine-specific IgG doses of up to 5.2 g/kg [167] have been administered to rats without adverse effect. Nonspecific IgG is administered to humans in doses of up to 2 g/kg for treatment of immunologic disorders [168], and is well tolerated. Even the highest NICmAb dose of 160 mg/kg used in this study is well within this range. The cost of such high antibody doses is an important consideration, but it is not clear that such high doses are required for efficacy. As noted above, the 40 mg/kg dose used in this study is similar to the estimated amount of antibody that is produced by vaccination and that is effective in reducing nicotine effects in rats, and is also similar to the 50 mg/kg monoclonal nicotine-specific antibody dose recently reported to attenuate nicotine-induced locomotor activation in the rat [91]. Data are not yet available from ongoing clinical trials of vaccination to estimate effective serum antibody concentrations in humans.

The binding data confirm that NICmAb effects and mechanism of action are similar to those of vaccination against nicotine [156]. Antibodies are largely excluded from the brain by the blood brain barrier owing to their large size, so that only unbound nicotine can distribute to brain [107]. The highly significant correlations between unbound nicotine concentration in serum and brain nicotine concentration found in this study

strongly support the hypothesis that immunization reduces nicotine distribution to brain by reducing the concentration of the unbound nicotine in serum.

This study examined nicotine distribution at 3 minutes after the nicotine dose. This time point is of interest because the rewarding effects of a cigarette are maximal in the first few minutes after a cigarette, and it is presumably these early behavioral effects that immunization will need to attenuate if it is to be of benefit in treating nicotine addiction [169]. Previous studies with vaccination of rats against nicotine have shown similar effects on nicotine distribution as early as 30 sec after the nicotine dose [29] and persisting to at least 25 minutes [22]. Although such studies need to be repeated with passive immunization, the close parallels between effects of vaccination reported previously and passive immunization found in the current study argue that passive immunization will be similarly effective.

The effects of Nic311 on nicotine pharmacokinetic parameters were also very similar to those reported previously after vaccination of rats against nicotine [112]. Nic311 markedly reduced nicotine clearance, decreased $V_{d,ss}$ and increased the terminal half-life. The increase in terminal half-life (from 1.1 h in controls to 2.5 h in rats pretreated with Nic311) contrasts with the much greater prolongation of phencylidine's terminal half-life to over 15 days in rats treated with phencyclidine-specific monoclonal antibody [170]. Although the reasons for this difference between drugs are not entirely clear, there were several substantial differences between the nicotine and phencyclidine protocols. The phencyclidine antibodies had a higher affinity for drug (K_d = 1.8 nM, [170]), and were

administered at a much higher dose (1 g/kg v. 27 mg/kg in the current study), both of which could have led to enhanced drug binding and a more profound effect on drug elimination. In addition, phencyclidine antibodies were administered at steady state produced by a continuous phencyclidine infusion rather than the single nicotine dose used in the current study.

The slowing of nicotine elimination by a nicotine-specific antibody presents the possibility that the antibody could become saturated with nicotine after repeated or chronic dosing, and become less effective in binding subsequently administered nicotine. However, any such saturation occurring after vaccination of rats does not seem to prevent vaccination from attenuating the effects of nicotine even under chronic dosing conditions which simulate cigarette smoking [110]. Since Nic311 has a similar affinity for nicotine as does immune serum in vaccinated rats, a similar preservation of efficacy with chronic nicotine dosing would be expected with passive immunization, but this remains to be studied. It is also possible that the slower clearance and longer half-life of nicotine produced by Nic311 could be beneficial. Population data suggest that smokers who have slower nicotine metabolism smoke less, perhaps because the effects of each cigarette last longer [148, 149]. If so, it is possible that immunization could be used to facilitate smoking reduction. Further studies under chronic nicotine dosing conditions will be needed to evaluate this possibility and comment further upon the optimal K_d of antibodies used to modify the pharmacokinetic or behavioral effects of chronic nicotine.

ACKNOWLEDGMENTS

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FIGURES AND TABLES

Table 1. Effects of antibody affinity on nicotine binding in serum

Effects of monoclonal nicotine-specific antibodies and Nic-IgG on nicotine protein binding in serum (mean±SD). The corresponding total serum and brain nicotine concentrations are shown in Figure 1. There was a significant relationship between antibody Kd and both the % nicotine bound (r = 0.90, p <0.001) P and the unbound nicotine concentration in serum (r = 0.68, p <0.001). ANOVA showed that all antibody groups differed from the control group.

Treatments (n = 6)	**Kd (nM)**	**Nicotine % Bound**	**Unbound Nicotine ng/ml**
Control-mAb	---	7±7	21±4
Nic079	250	64±7 **	16±3 *
Nic810	70	81±5 **	15±4 **
Nic311	60	95±1 **	10±1 **
Nic-IgG	1.6	99±0.1 **	3±1 **

* p<0.01, ** p < 0.01 ** compared to control group

Table 2. Nic311 dose-response on nicotine binding in serum

Effects of Nic311 dose on nicotine binding in serum. Values are the mean±SD except for unbound nicotine concentrations in the NICmAb groups, which show only the means because there were just 2 animals per group. Values for Nic-IgG, taken from the experiment illustrated in Table 1, are shown for comparison. Corresponding total serum and brain nicotine concentrations are shown in Figure 3. There was a significant correlation between Nic311 dose and both the % nicotine bound ($r = .93$, $p < 0.01$) and the unbound nicotine concentration in serum ($r = .98$, $p < 0.05$).

Treatment	Dose (mg/kg)	Nicotine % Bound	Unbound Nicotine ng/ml
Control-IgG	160	32±8	18±2
Nic311	10	95.0	10.1
	20	97.3	8.5
	40	98.9	5.0
	80	99.5	3.1
	160	99.7	2.2
Nic-IgG	10	99.5±0.1	2.7±0.7

Table 3. Nicotine pharmacokinetic parameter estimates

Noncompartmental pharmacokinetic parameter estimates for nicotine in rats pretreated with Nic311 or Control-IgG. Rats received a single i.v. bolus dose of nicotine 1.0 mg/kg. Nic311 substantially reduced the $V_{d,ss}$, decreased Cl, and increased the terminal half-life.

Treatment	AUC h·ng/ml	$V_{d,ss}$ L/kg	Cl_t L/h·kg	$T_{1/2}$ hours
Control-IgG	48 ± 21	3.48 ± 1.24	2.15 ± 0.60	1.1 ± 0.2
Nic311	473. ± 120**	0.64 ± 0.10**	0.22 ± 0.05 **	2.5 ± 0.3 ***

**p<0.01, ***p < 0.001 compared to controls

Figure 1. Serum and Brain nicotine concentrations after NICmAb treatment

Serum and brain nicotine concentrations after a single nicotine dose in rats pretreated with 10 mg/kg Control-mAb, monoclonal NICmAbs or Nic-IgG. Nicotine 0.03 mg/kg was administered 30 minutes after the pretreatment and serum and brain collected 3 min after the nicotine dose. Pretreatment with each of the NICmAbs or with Nic-IgG increased nicotine retention in serum and reduced nicotine distribution to brain. Decreasing Kd (values above bars) was associated with increasing nicotine concentration in serum ($r = 0.67$, $p < 0.001$) and decreasing nicotine concentration in brain ($r = 0.69$, $p < 0.001$). ** $p < 0.01$ compared to Control-mAb.

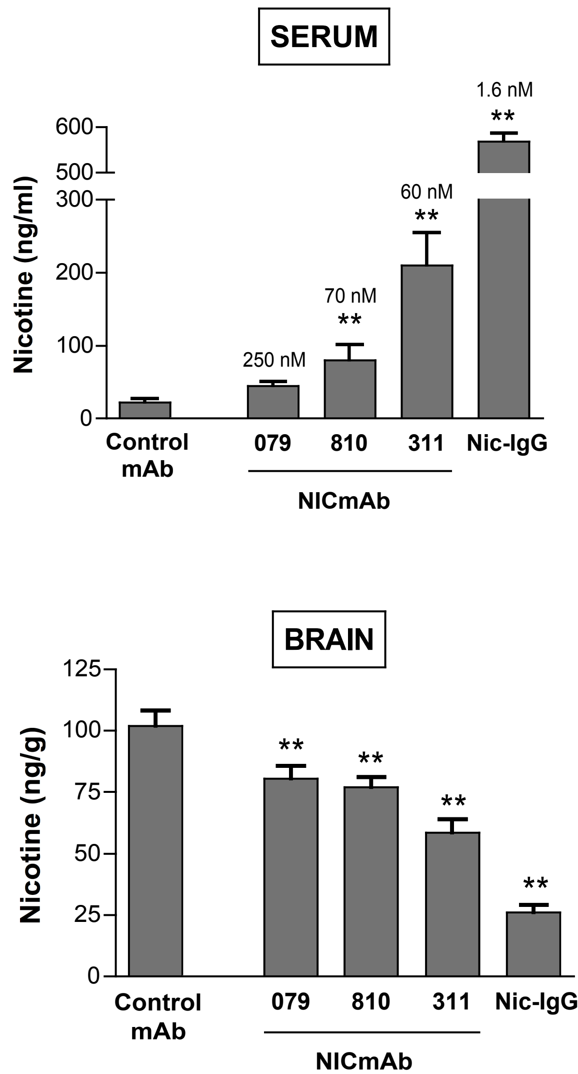
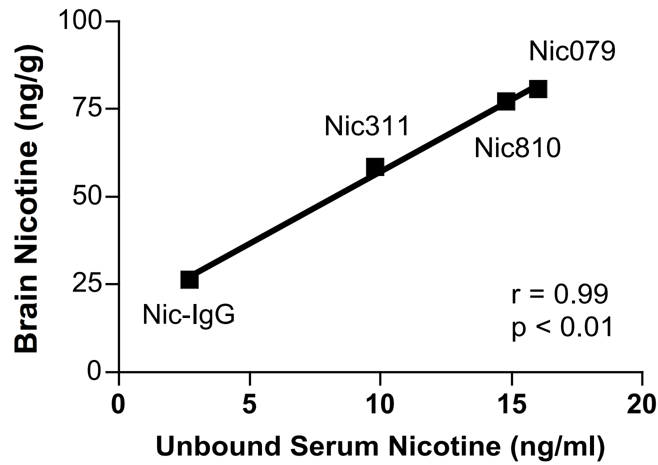


Figure 2. Correlations between unbound serum nicotine and brain nicotine

Correlations between the serum unbound nicotine concentration and brain nicotine concentration. Top panel shows data from the four different antibodies, all administered at a dose of 10 mg/kg. Bottom panel shows dose-response data for Nic311.



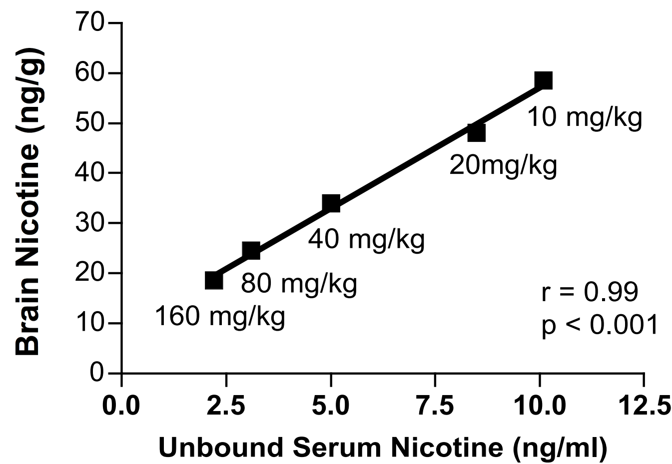


Figure 3. Nic311 dose-response for nicotine distribution to the brain

Dose-response relationship for nicotine distribution after treatment with Nic311 compared to Control-mAb. Increasing Nic311 dose was associated with increasing serum nicotine concentration measured 3 minutes after a nicotine dose ($r = 0.99$, $p < 0.001$) and decreasing brain nicotine concentration ($r = 0.99$, $p < 0.01$). Values for rats pretreated with Nic-IgG, taken from the experiment described in Figure 1, are shown for comparison.

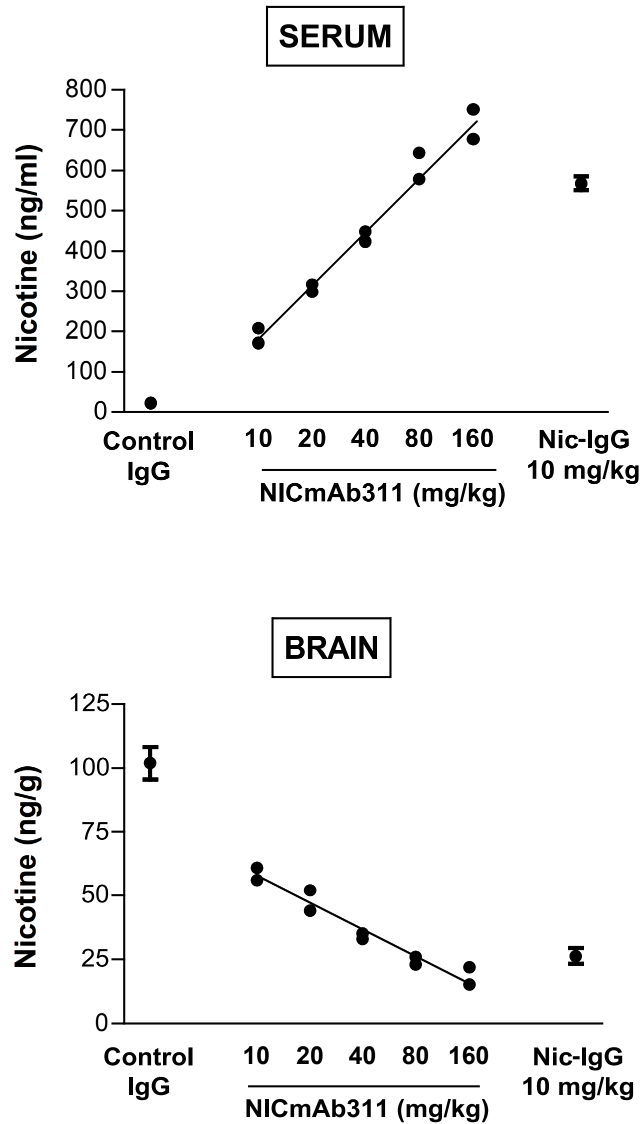
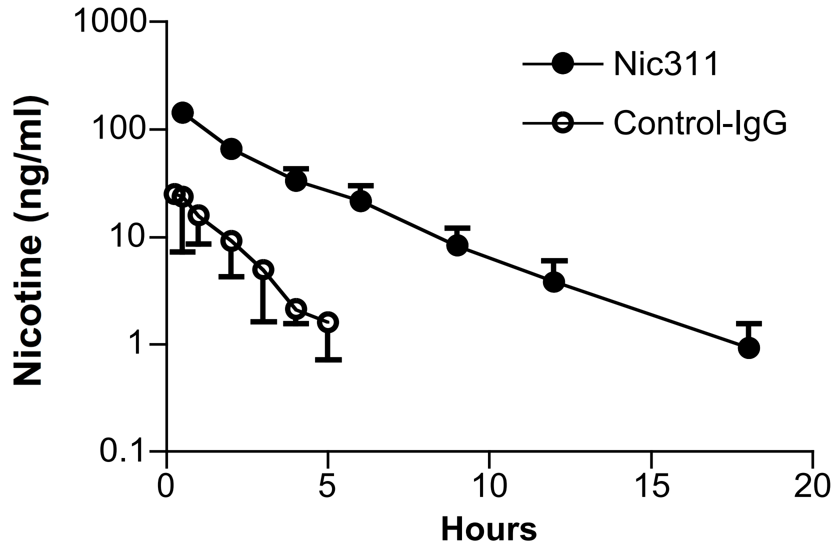


Figure 4. Nicotine concentrations vs. time in the presence and absence of Nic311

Serum nicotine concentrations (mean±SD) after a single dose of nicotine 0.1 mg/kg in rats pretreated with Nic311 27mg/kg or control-IgG. Pharmacokinetic parameter estimates are shown in Table 3.



CHAPTER 4

Combined active and passive immunization enhances the efficacy of immunotherapy against nicotine in rats.

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ABSTRACT

Vaccination against nicotine reduces the behavioral effects of nicotine in rats and is under clinical evaluation as a treatment for tobacco addiction. Efficacy is limited by the need for high serum nicotine-specific antibody (NicAb) levels, and currently available nicotine vaccines do not uniformly generate the required NicAb levels. Passive immunization with a nicotine-specific monoclonal antibody (Nic311) has also shown efficacy in rats. The principal aim of this study was to determine whether the combined use of vaccination and passive immunization would produce greater effects than vaccination alone on nicotine pharmacokinetics and locomotor sensitization (LMS) to nicotine. Rats were treated with vaccination alone, Nic311 alone, both or neither and were then administered 10 daily injections of nicotine 0.3 mg/kg subcutaneously. Treatment with Nic311 or vaccination alone increased the binding of nicotine in serum, reduced the unbound serum nicotine concentration and nicotine distribution to brain, and attenuated the development of LMS. Combined use of vaccination and passive immunization produced higher total serum NicAb levels, greater changes in nicotine pharmacokinetics and a greater attenuation of LMS than either treatment alone. The total serum NicAb concentration was significantly correlated with brain nicotine levels and locomotor activity. These data indicate that providing higher serum NicAb concentrations improves the efficacy of immunotherapy against nicotine, and that supplementing vaccination with passive immunization is a potential strategy to accomplish this.

1. INTRODUCTION

Cigarette smoking is the leading preventable cause of death in the United States [1]. Nicotine is the principle addictive component of tobacco smoke [171], and the rapid absorption and distribution of nicotine from cigarette smoke into the brain produces reinforcing effects that help initiate and maintain tobacco dependence [54]. Interventions that suppress or slow the distribution of nicotine to brain may be useful to treat tobacco dependence.

Immunization against nicotine is being studied as a means of altering the pharmacokinetics of nicotine, and has substantial effects in animal models of nicotine addiction. Immunization of rats generates nicotine-specific antibodies (NicAbs) that bind nicotine in serum, reduce the unbound concentration of nicotine, and reduce nicotine distribution to brain [88, 89, 106, 107, 117, 172]. Vaccination against nicotine attenuates a wide range of nicotine's behavioral effects relevant to addiction including the acquisition, maintenance, and reinstatement of nicotine self-administration [90, 113]. Three nicotine vaccines are currently in clinical trials, and preliminary data indicate efficacy for enhancing smoking cessation rates [108, 173] (personal communication, C Bunce, A Fattom).

Although this approach appears to be promising, a major limitation of vaccination against nicotine as a strategy for treating tobacco addiction is the need to reliably generate high serum NicAb levels. In rats, the effects of immunization on nicotine pharmacokinetics

are strongly correlated with the serum NicAb levels achieved; higher serum NicAb concentrations are associated with greater binding of nicotine in serum and reduced distribution of nicotine to brain [88, 89, 137]. Enhanced smoking cessation rates have been reported in clinical trials of nicotine vaccines, but they are confined to those subjects with the highest serum antibody concentrations [108, 173]; (personal communication, C Bunce, A Fattom). Two limitations of vaccination are apparent from clinical trials; the mean serum NicAb concentrations are lower than those reported in rats or mice [106, 174], and there is substantial variability (up to a 30-fold range) in those concentrations [108, 173]. Developing strategies to reliably produce high serum antibody concentrations in response to nicotine vaccines is critical to maximizing their efficacy.

Immunization against nicotine can be accomplished via either active immunization (vaccination) or passive immunization (administration of pre-formed antibodies). Initial efforts to develop immunotherapies against nicotine have used vaccination because of its long-lasting effects, reasonable cost, and the excellent safety record of vaccines in general. Passive immunization with nicotine-specific monoclonal antibodies has also been studied in rats and produces effects on nicotine pharmacokinetics that are similar to those of vaccination [91, 106, 137, 138]. Effects of passive immunization on nicotine-related behaviors are less well studied, but attenuation of nicotine-induced locomotor activity has been reported [91, 106]. Passive immunization has potential advantages over vaccination in that higher doses of antibody can be administered than can be elicited by vaccination, the antibody dose can be controlled so that individual variability in serum antibody levels might be minimized, and the onset of effect is immediate. For these

reasons, passive immunization is of interest as a possible therapeutic alternative to vaccination and is also being studied for the treatment of methamphetamine, phencyclidine, and cocaine addictions [121, 175, 176]. The main disadvantage of passive immunization is that it is considerably more expensive than vaccination. Nevertheless, passive immunization with various monoclonal antibodies, in some cases at cumulative doses comparable to those used in the current study, is now widely used to treat cancer, inflammatory diseases, and other disorders [132].

In the current study, the effects of vaccination, passive immunization or a combination of the two were studied with regard to their effects on serum NicAb levels, nicotine pharmacokinetics, and locomotor sensitization (LMS) to nicotine. The purposes of this study were to 1) compare the effects of vaccination with those of passive immunization, 2) assess whether providing serum NicAb levels higher than those achieved by vaccination alone would further improve efficacy, and 3) evaluate the feasibility of using the combination of vaccination and passive immunization as a general strategy for enhancing the efficacy of immunotherapy for nicotine addiction. The pharmacokinetics of the nicotine-specific monoclonal antibody Nic311 was also characterized to guide Nic311 dosing.

2. METHODS

2.1 General Methods

2.1.1 Animals:

Male Holtzman Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 275-300 g were housed individually in temperature and humidity controlled colony rooms with unlimited access to water under a 12-h light/dark cycle (lights off at 10:00 pm). Rats were food-restricted to 18 g/day rat chow to minimize weight gain and chronic catheter displacement throughout the chronic studies. All protocols were approved by the Minneapolis Medical Research Foundation Animal Care and Use Committee and were in accordance with NIH guidelines.

2.1.2 Reagents:

Nicotine bitartrate (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile heparinized saline (30 units/ml). The pH of the solution was adjusted to 7.4 with dilute NaOH. All nicotine doses and concentrations are expressed as that of the base.

The nicotine immunogen used in this study is a well-characterized conjugate consisting of the hapten 3′-aminomethyl nicotine (3′-AmNic) linked to the carrier protein recombinant *Pseudomonas* exoprotein A (rEPA) [106]. This immunogen (3′-AmNic-rEPA) generates antibodies in rats that have a high affinity for nicotine (K_d = 19-40 nM) and < 1 % cross-reactivity with acetylcholine, the major nicotine metabolites cotinine and nicotine-N-oxide, and a variety of other neurotransmitters or medications [106]. Control

immunogen was the unconjugated rEPA alone. Vaccine doses consisted of 25 µg of immunogen in complete Freund's adjuvant for the first dose and in incomplete Freund's adjuvant for subsequent booster doses. The nicotine-specific monoclonal antibody used in this study (Nic311) is an immunoglobulin G (IgG) 1_κ isoform derived from mice immunized with 3′-AmNic-rEPA with a K_d for nicotine of 60 nM. Nic311 was purified by protein G chromatography to ≥ 95% of total protein content with endotoxin levels of < 0.2 EU/mg. Nic311 was diluted in phosphate-buffered saline to a concentration of 10mg/ml for administering doses of 30 mg/kg in the pharmacokinetic experiment and 30 mg/ml for doses of 80mg/kg in the locomotor experiment. Control IgG was human polyclonal IgG (Sandoglobulin®) which does not bind nicotine or alter nicotine pharmacokinetics or behavior in rats [137]. Nic311 has < 1% cross-reactivity with nicotine metabolites or acetylcholine [138].

2.1.3 Nicotine and nicotine-specific antibody (NicAb) assays.

Serum and brain nicotine levels were measured by gas chromatography with nitrogen-phosphorous detection [164]. Brain nicotine concentrations were corrected for brain blood content [88].

Nicotine-specific antibody concentrations generated by vaccine were measured by ELISA using 3′-AmNic-polyglutamate as the coating antigen and goat anti-rat IgG-horse radish peroxidase (HRP) as detecting antibody. The same coating antigen was used to measure Nic311 concentrations, and the detecting antibody was goat anti-mouse-IgG-HRP. Because Nic311 was administered to one group of rats that had already been

vaccinated, it was necessary to determine cross reactivity of the antibodies generated by vaccination in the ELISA used to quantitate Nic311 levels. This was accomplished by assaying serum samples using both the rat and mouse quantitative ELISAs. All reported Nic311 levels were corrected for this cross reactivity. In contrast, serum nicotine-specific antibody levels generated by vaccination were measured prior to Nic311 administration, so no correction of these values was required.

2.1.4 ELISA to detect antibodies against Nic311

The purpose of this assay was to detect the potential presence of rat anti-mouse antibodies in the Nic311 pharmacokinetic parameters experiment. Plates were coated with 1 ng/well of Nic311. Serum samples from days 8-35 were added to wells, serially diluted, and incubated overnight at 4°C. Detecting antibody was goat anti-rat IgG or horse anti-rat IgM conjugated to HRP.

2.1.5 Protein Binding

Equilibrium dialysis of serum was performed in 1.0 ml Teflon cells with Spectrapor 2 membranes (Spectrapor Labs, Rancho Dominguez, CA) as described previously [177]. The fraction unbound was the ratio of the nicotine concentrations in dialysate and serum. Unbound nicotine concentration was calculated as the product of the total serum nicotine concentration prior to dialysis and the fraction unbound.

2.2 Nic311 Pharmacokinetic Parameter Estimates

Two groups of 6-7 rats were anesthetized with intramuscular droperidol 2.0 mg/kg and

fentanyl 0.04 mg/kg and cannulae were inserted into the jugular and femoral veins, externalized between the scapulae, and attached to a guide cannula mounted in a harness assembly (Instech, Plymouth Meeting, PA). A stainless steel spring tether attached to the guide cannula allowed connection to a fluid swivel for antibody administration and sample collection. The femoral cannula was used to administer Nic311 and the jugular cannula was used for sampling.

Rats were administered a single dose of Nic311 (30 mg/kg) i.v. in a volume of 1 ml via the femoral cannula. One group of rats was implanted with a subcutaneous osmotic minipump (Alzet 2ML4, Durect Corp) delivering nicotine 3.2 mg/kg day to determine Nic311 pharmacokinetics in the presence of nicotine, since nicotine would be present in the LMS protocol (see below). The minipump was replaced on day 28. Blood samples (0.5 ml) were collected from the jugular cannula at intervals for 56 days (> 5 terminal half-lives) and serum was stored at -20°C until analyzed for nicotine content as described above. Pharmacokinetic parameters were estimated by two-compartment analysis using WinNonlin 4.0 (Pharsight Corp).

<u>2.3 Locomotor sensitization to nicotine</u>

2.3.1 Protocol:

Five groups of rats (n = 9-15/group) were immunized with 3′-AmNic-rEPA or control vaccine on days 1, 21, 42, and 59 (see timeline, Figure 1). On days 62-64 a chronic jugular cannula was implanted and a serum sample was taken to measure vaccine-generated nicotine-specific antibodies. On Day 69, rats began the nicotine sensitization

protocol of 10 daily doses of saline or nicotine 0.3 mg/kg s.c. Rats were not exposed to the testing chambers before beginning the sensitization protocol. Locomotor activity (horizontal distance traveled) was measured for 30 minutes immediately following each nicotine dose. One hour prior to the first and the 7th session rats were administered Nic311 or Control IgG via the jugular cannula. The Nic311 doses of 80 mg/kg on day 1 and 40 mg/kg on day 7 were based on the terminal half-life of Nic311 in the presence of nicotine as determined above (7.6 days, Table 2) and intended to maintain serum Nic311 concentrations equal to those expected from vaccination (about 200 µg/ml) throughout the locomotor sensitization period. Group treatments are shown in Table 1. Immediately after the final (10th) locomotor session (40 minutes after the nicotine dose), all animals were administered a terminal dose of pentobarbital (50 mg/kg) to confirm catheter patency, immediately sacrificed, and brain and serum samples were collected for analysis. Data from animals with catheters that had failed (n = 3) were not used.

2.3.2 Locomotor Apparatus:

Locomotor monitoring sessions were conducted in open field activity chambers (Med Associates, Inc., St. Albans, VT) measuring 43 x 43 cm. Each chamber had two 16-beam photocell arrays placed 2.5 cm and one array 8 cm above the chamber floor to monitor horizontal and vertical activity. Chambers were placed inside sound-attenuating cubicles that had exhaust fans providing masking noise and ambient lighting. A computer with open-field activity software (Med Associates, Inc., St. Albans, VT) was used for operating the apparatus and recording data.

2.4 Statistical analyses

Nic311 pharmacokinetic parameters in the presence and absence of nicotine were compared by Wilcoxon's rank sum test. This nonparametric test was used because of small group sizes. Serum NicAb concentrations, serum and brain nicotine levels, free nicotine levels, and % bound were analyzed by Kruskal-Wallis tests with the Wilcoxon rank sum test for multiple comparisons and Bonferroni post-tests. This nonparametric test was used because of unequal group variances.

Locomotor activity was measured as total horizontal distance traveled over the 30-minute session. Locomotor activity analysis consisted of an overall 2-way ANOVA with group as a between-subjects factor and day as a within-subjects factor. Subsequent one-way ANOVAs were used to analyze within-group distance traveled across days, with Dunnett's post-test to compare day 1 to the subsequent days. In addition, a one-way ANOVA was used to examine locomotor activity between groups on day 1.

Because the nicotine group exhibited significant sensitization on days 7-10 compared to day 1, data from these days were used to analyze treatment effects on locomotor activity between groups. Distance traveled across days 7-10 was analyzed using two-factor ANOVA with group as a between-subjects factor and day as a within-subjects factor, followed by Bonferroni's post-test to compare marginal means between groups. Since most locomotor activity occurs during the beginning of the locomotor activity sessions, a second planned comparison was analysis of within session data on days 1 and 10. Within-session data were separated into 5-minute blocks, and day 1 and day 10 were

analyzed separately using two-factor ANOVA with group as a between-subjects factor and time as a within-subjects factor, followed by Bonferroni's post-test to compare mean activity scores between groups at each 5-minute bin.

The relationships between total serum NicAb concentrations and free serum nicotine levels, brain nicotine levels, and mean locomotor activity across days 7-10 were analyzed using linear regression analysis. Relationships between free serum nicotine and brain nicotine levels, and brain nicotine levels and locomotor activity were similarly analyzed.

Inspection of the serum NicAb concentration vs. locomotor activity data (Figure 7, bottom) suggested that a serum NicAb concentration of ≥ 300 μg/ml was associated with maximal efficacy in the attenuation of locomotor activity (i.e., activity comparable to that of saline controls). To examine this further, a t-test was used to compare mean locomotor activity across days 7-10 for rats with total serum NicAb concentrations < 300 μg/ml (n = 35) vs. ≥ 300 μg/ml (n = 14). The proportion of animals in these subgroups exhibiting mean locomotor activity > 2000 cm across days 7-10 were also compared using Chi-square analysis. This cutoff (2000 cm) was used in the Chi-square analysis because it approximated the mean day 1 activity level.

3. RESULTS

3.1 General:

Food intake and weight gain did not differ among groups in the locomotor sensitization experiment [mean ± SD weight gain: control (nicotine + saline) 103±19 g, vaccine 111±21 g, Nic311 94±18 g, combined 110±24 g]. No adverse effects of antibody treatment were observed in either the Nic311 pharmacokinetic experiment or the locomotor sensitization experiment.

3.2 Nic311 Pharmacokinetic Parameter Estimates

Serum Nic311 levels in the presence or absence of nicotine are shown in Figure 2, and pharmacokinetic parameter estimates are shown in Table 2. The Nic311 steady-state volume of distribution, clearance, and α (distribution) half-life were significantly lower in the presence of nicotine compared to its absence, but the elimination half-life was not statistically different. Three rats (depicted individually in Figure 2) were excluded from the pharmacokinetic parameter estimation (1 rat in the presence of nicotine and 2 rats in the absence of nicotine) because their serum Nic311 levels declined considerably more rapidly after day 11 and by day 17 were > 3 SD below the means of their respective groups. Because rat anti-mouse antibodies were a possible cause of the more rapid decline of Nic311 levels in these outliers, ELISAs were performed on all samples collected between days 8 and 35. No anti-mouse IgG or IgM titers above background were detected in any of the rats, including the outliers.

3.3 Locomotor Sensitization

3.3.1 Antibody and Nicotine Levels:

Cross-reactivity of serum antibodies generated by vaccination in the ELISA used to quantitate Nic311 levels was 3.2%. All reported Nic311 levels measured in rats previously vaccinated with 3′-AmNic-rEPA were corrected for this cross-reactivity. Serum NicAb levels measured after the 10th nicotine dose were comparable in the groups receiving vaccination or Nic311 alone (Table 3). Total serum NicAb levels in the combined immunotherapy group were approximately double that of the two monotherapy groups (p < 0.001). NicAb concentrations generated by vaccination did not differ between the vaccine alone group and the combined immunotherapy group, indicating that Nic311 treatment had no effect on vaccine-elicited antibody levels. One rat receiving combined immunotherapy had an unexpectedly low serum Nic311 level reminiscent of the outliers in the pharmacokinetic experiment. The exclusion of data from this rat did not alter the results of statistical analyses and all analyses presented include data from this rat. There was a trend toward less variability in serum NicAb levels in the combined immunotherapy group, with coefficients of variation for serum NicAb levels of 63%, 46%, and 35% in the vaccine alone, Nic311 alone, and combined immunotherapy groups respectively.

Serum nicotine levels measured 40 minutes after the 10th (final) nicotine dose of the LMS protocol were significantly higher after vaccination (p < 0.01) or Nic311 alone (p < 0.05) compared to the nicotine control group (Figure 3 top). Serum nicotine levels were higher after combined immunotherapy than after either vaccination or Nic311 alone (p < 0.01).

Vaccination or Nic311 alone did not significantly reduce brain nicotine concentrations compared to controls, but brain nicotine concentrations in the combined immunotherapy group were significantly lower than in all other groups ($p < 0.01$, Figure 3 bottom).

3.3.2 Protein Binding:

The percent of nicotine bound in serum was increased and the free nicotine concentration was reduced by all antibody treatments compared to controls (Table 4), and to a significantly greater extent ($p < 0.01$) by combined immunotherapy than by either vaccination or Nic311 alone. For all groups together, there was a strong correlation between the free serum nicotine concentration and the brain nicotine concentration ($r^2 = 0.74$, $p < 0.001$; Figure 4).

3.3.3 Locomotor Activity:

Figure 5 shows locomotor activity from each 30 min session throughout testing. Two-way ANOVA indicated a significant effect of group (F = 4.35, $p = 0.004$) and day (F = 2.45, $p < 0.01$), as well as a significant interaction between group and day (F = 2.82, $p < 0.0001$). A one-way ANOVA of overall locomotor activity on Day 1 indicated no differences between groups (F = 0.90, $p = 0.47$; Figure 5).

Analysis of locomotor activity in the nicotine control group (no immunotherapy) across days indicated an overall effect of day (F = 2.46, $p = 0.015$). Locomotor activity on days 7-10 was significantly greater than on day 1 ($p < 0.05$), indicating sensitization to nicotine (mean ± SD increase 49±77%; Figure 5). In contrast, locomotor activity in the saline control group declined significantly throughout testing (F = 18.31, $p < 0.0001$),

reflecting habituation to the experimental setting [178]. Locomotor activity across days did not change in the vaccine alone (F = 0.89, $p = 0.53$) or Nic311 alone groups (F = 1.49, $p = 0.16$); that is, these groups did not exhibit sensitization. The combined immunotherapy group also failed to show sensitization and in addition, locomotor activity declined significantly across sessions in a manner similar to the saline control group (F = 2.804, $p = 0.005$).

Locomotor activity was compared among groups on days 7-10 (days that the nicotine control group exhibited sensitization). There was a significant effect of group (F = 6.28, $p = 0.0003$) and also a significant effect of day (F = 3.39, $p = 0.019$), but no significant interaction between group and day (F = 0.57, $p = 0.87$). Thus overall group data across days 7-10 was used for post-hoc comparisons. The Nic311, combined, and saline control groups had significantly lower activity levels than the nicotine control group (Figure 5; $p < 0.05$ for Nic311; $p < 0.01$ for saline control or combined); and there was a non-significant trend toward lower locomotor activity in the vaccine alone group ($p = 0.07$). The combined immunotherapy group and the saline control group had significantly lower locomotor activity compared to the vaccine alone group ($p < 0.05$).

Since the majority of activity occurs early in the locomotor sessions, data during sessions 1 and 10 (in 5 minute bins) were analyzed to further clarify treatment effects (a planned comparison). Within-session analysis of locomotor activity during session 1 demonstrated a significant effect of time (F = 125.19, $p < 0.0001$) but no significant effect of group (F = 0.90, $p = 0.47$) and no interaction (F = 1.02, $p = 0.44$; Figure 6 top).

Analysis of locomotor activity during session 10 showed a significant effect of time (F = 100.76, $p < 0.0001$) and group (F = 8.27, $p < 0.0001$), and also a significant interaction (F = 3.07, $p < 0.0001$; Figure 6 bottom). The nicotine control group had significantly increased locomotor activity compared to all other groups during the initial 10 minutes of the session ($p < 0.001$ at 5 minutes, $p < 0.05$ at 10 minutes). The combined immunotherapy group had significantly reduced locomotor activity compared to the vaccine alone group ($p < 0.01$) and the Nic311 alone group ($p < 0.05$) during the first 5 minutes of the session.

3.3.4 Correlations

For all groups considered together, higher serum NicAb levels were associated with larger effects on nicotine pharmacokinetics by several measures. There was a significant negative correlation between the total serum NicAb concentration and free serum nicotine concentration ($r^2 = 0.36$, $p < 0.001$), and between the total serum NicAb concentration and the brain nicotine concentration ($r^2 = 0.43$, $p < 0.001$; Figure 7 top and middle). Higher NicAb levels were also significantly associated with greater behavioral efficacy (reduced locomotor activity) on days 7-10, although the relationship was weak ($r^2 = 0.11$, $p = 0.047$; Figure 7 bottom). In sum, rats with the highest total serum antibody concentrations had the highest total serum nicotine levels, the lowest free serum nicotine levels, the lowest brain nicotine levels, and the lowest locomotor activity scores.

Rats with total serum NicAb concentrations ≥ 300 µg/ml (regardless of the source of the antibody) had significantly lower activity scores compared to rats with serum NicAb

concentrations < 300 μg/ml (1310±450cm vs. 2080±800 cm, mean ± SD, $p < 0.01$). In addition, there was a lower proportion of rats with activity scores > 2000 cm among rats with total serum NicAb concentrations ≥ 300 μg/ml compared to rats with total serum NicAb concentrations < 300 μg/ml (0/14 vs. 19/35, $p < 0.001$). The mean activity score of rats with total serum NicAb levels of ≥ 300 μg/ml did not differ from that of saline controls (1310±450 cm vs. 1035±411 cm, $p = 0.13$).

4. DISCUSSION

The main findings of this study were that 1) both vaccination alone and Nic311 alone attenuated the development of LMS to nicotine, 2) vaccination and Nic311 were equally effective in altering nicotine pharmacokinetics and attenuating LMS to nicotine when dosed to produce equivalent serum NicAb concentrations, and 3) combined immunotherapy produced higher serum NicAb concentrations, greater effects on nicotine pharmacokinetics, and greater effects on LMS than either vaccination or Nic311 alone. These data indicate that interventions which produce higher serum NicAb concentrations than vaccination alone can enhance the efficacy of immunotherapy for nicotine addiction, and that the combination of vaccination and passive immunization is a feasible strategy for augmenting serum NicAb concentrations.

The effects of vaccination and of passive immunization on nicotine pharmacokinetics in rats are qualitatively similar [88, 91, 111, 137, 138] but have not previously been directly compared. A comparison of vaccination using 3′-AmNic-rEPA and passive immunization using Nic311 was feasible because the affinity of NicAbs elicited by vaccination is similar to that of Nic311, and the dosing regimen of Nic311 used in the LMS experiment produced serum NicAb levels equal to those generated by vaccination. The effects of these treatments on nicotine protein binding in serum and nicotine distribution to brain were comparable. Vaccination alone and Nic311 alone also both attenuated LMS to nicotine to comparable extents. Previous studies have reported attenuation of *acute* nicotine-induced locomotor activity (as distinct from sensitization)

following passive immunization with a polyclonal anti-nicotine antiserum [106], and vaccination or passive immunization with a monoclonal antibody [91]. The current study extends these data to LMS, a behavior mediated by pathways which overlap substantially with those mediating drug reinforcement and which is relevant to addiction treatment [94]. The data show that both vaccination alone and passive immunization alone are effective in attenuating LMS to nicotine, and suggest that the pharmacokinetic and behavioral effects of immunotherapy depend on the total antibody concentration rather than their source (endogenously produced following vaccination or passively administered).

Combination immunotherapy (vaccination + Nic311) generated total serum NicAb levels approximately twice that of either single immunotherapy and was more effective than the single immunotherapies in reducing LMS to nicotine. While all immunotherapies reduced LMS compared to the nicotine control group on days 7-10 of testing, only the combined treatment group (and the saline-control group which received no nicotine) showed a *decrease* in activity over the course of the protocol. A decrease in locomotor activity in controls has been reported by others and is attributed to habituation to the testing procedure [178]. The effects of combination immunotherapy on LMS as measured by total activity during 30 minutes of testing were greater than those of the vaccine group. Combination immunotherapy by this measure was not more effective than Nic311 alone. However, the attenuation of LMS by combination immunotherapy during the first 5 minutes of the session, during which most activity occurred, was greater than that of either vaccination or Nic311 alone. In support of the LMS data, combination

immunotherapy produced greater effects on nicotine pharmacokinetics than the individual treatments alone. In addition, there were significant correlations between the total serum NicAb concentration (for all groups analyzed together) and the free nicotine concentration in serum, the brain nicotine concentration, and LMS to nicotine. Taken together, these data provide strong support for the hypothesis that increasing NicAb concentrations above those that are achievable with vaccination alone can enhance the efficacy of immunotherapy.

One prior study of immunization against nicotine in rats found no relationship between serum NicAb concentrations generated by vaccination and reductions in nicotine-self administration. However, group sizes were small and may not have been adequately powered to detect this relationship [90]. Another study found that the attenuation of nicotine-induced locomotor activity by a nicotine-specific monoclonal antibody was related to antibody dose [91]. Studies of immunotherapies for other addictive drugs have reported similar relationships between antibody doses or serum concentrations and methamphetamine-induced locomotor activity, heart-rate, blood pressure and self-administration [124, 127], phencyclidine-induced locomotor activity [100], and cocaine seeking and self-administration [120, 151, 179]. Thus, most animal studies are consistent in indicating that higher serum antibody concentrations result in a greater attenuation of drug-induced behavior across a variety of drugs and immunotherapies. In two Phase II clinical trials of nicotine vaccines, increased smoking cessation rates were observed only in the one third of subjects who had the highest serum NicAb titers [108, 173]; (personal communication, C Bunce, A Fattom). These data support the need to achieve uniformly

high serum antibody concentrations to maximize the efficacy of immunotherapy to treating drug addiction.

A previous report examined the combination of vaccination and passive immunization with a monoclonal antibody against cocaine in rats and noted that the combination was no more effective than either treatment alone in attenuating cocaine self-administration [179]. However, the study was not specifically designed to compare combination immunotherapy with vaccination or passive immunization alone, and serum antibody levels from vaccination may have been low at the time of comparison.

A limitation of this study is that the locomotor sensitization experiment did not include an untreated control group to control for effects of nonspecific antibodies: the saline group received both control vaccine and control IgG. Nonetheless, the present study provided appropriate control conditions for detecting effects of nicotine-specific antibodies. It is unlikely that nonspecific effects of either vaccination or Nic311 on locomotor activity influenced the current findings since antibodies generated from vaccination or Nic311 are highly specific for nicotine and do not bind endogenous neurotransmitters or the major nicotine metabolites [106, 138]. Immunization against nicotine also produces effects that are behaviorally specific, as vaccination or passive immunization with a nicotine-specific antiserum have no effect on cocaine self-administration, cocaine-induced locomotor activity, or operant food-maintained behavior [180].

Although this study was not designed to establish a threshold NicAb level for efficacy, all but one of the rats in the combined immunotherapy group had serum NicAb levels of ≥ 300 μg/ml. Rats with serum NicAb levels above this arbitrary value exhibited levels of locomotor activity significantly lower than those with NicAb concentrations < 300 μg/ml and similar to that of the saline controls. Rats with NicAb levels ≥ 300 μg/ml also had a significantly lower proportion of animals with activity scores > 2000 cm (the approximate mean day 1 baseline) during days 7-10 than those with lower NicAb levels (0/12 vs. 19/35). These findings suggest that a serum NicAb concentration of ≥ 300 μg/ml provided reliable protection against nicotine's psychoactive effects in this protocol, and that a target concentration strategy might be feasible as a rational clinical approach to immunotherapy. A threshold concentration of 300 μg/ml as discussed above is 10 times the mean serum NicAb levels reported in clinical trials and is not intended as a specific target for human treatment; the LMS protocol differs in many ways from smoking and uses nicotine doses more than 10 times higher than the nicotine dose absorbed from a cigarette [171]. Nonetheless, these data provide a basis for further exploration of a target antibody concentration strategy.

A target antibody concentration strategy for treating nicotine addiction would be challenging to implement clinically using currently available nicotine vaccines owing to the large variability in serum NicAb concentrations they produce. Passive immunization alone is an alternative strategy and has been effective in blocking the effects of nicotine [91, 128, 137], phencyclidine [125, 170, 181], methamphetamine [124, 127, 175], and cocaine [120, 121] in rats but the required doses may be quite high. The current study

suggests that the combination of vaccination and passive immunization may offer some advantages. While this study used a fixed Nic311 dose, this could be adjusted so that vaccinated rats receive only as much Nic311 as is needed to achieve a target serum NicAb concentration. The advantage of using combination immunotherapy rather than Nic311 alone lies in exploiting the ability of passive immunization to rapidly produce uniformly effective serum NicAb concentrations while minimizing the dose, the cost and the number of subjects requiring supplemental Nic311 after vaccination.

The use of combined immunotherapy could also serve to compensate for the substantial individual variability in serum NicAb levels produced by vaccination. The coefficient of variation of the serum NicAb concentrations for the combined immunotherapy group was lower than for either of the single treatments. This suggests that the factors leading to variability in levels in the vaccine alone and Nic311 alone groups (presumably immunologic v. pharmacokinetic) are independent so that combining these approaches reduces overall variability.

While the purpose of characterizing Nic311 pharmacokinetics in the current study was simply to guide the selection of an appropriate Nic311 dosing regimen in the LMS experiment, several interesting observations were made. The terminal half-life of Nic311 was similar to previously reported values for mouse IgG in rat [147] and was not affected by the presence or absence of nicotine, but the volume of distribution, clearance, and alpha distribution half-life of Nic311 were modestly reduced in the presence of nicotine. However, group sizes were small and this finding requires confirmation. An additional

finding in the pharmacokinetic parameters experiment was the very rapid decline of serum Nic311 levels in several rats compared to the group mean. Rapid NicAb elimination did not appear to be due to the presence of nicotine, since two of these rats received concurrent nicotine but one did not, nor did it appear to be due to the presence of rat anti-mouse antibodies. One rat receiving combination immunotherapy in the LMS experiment also showed markedly reduced Nic311 serum levels compared to the group mean, perhaps reflecting the same process. While excluding this rat's data did not change the outcome of any of the statistical analyses in the LMS experiment, this observation nonetheless illustrates the value of confirming serum NicAb levels after the administration of heterologous monoclonal antibodies, for protocols lasting more than a few days.

In summary, combining vaccination with passive immunization improved the efficacy of immunotherapy for altering nicotine pharmacokinetics and attenuating nicotine-induced LMS. These data provide a basis for further study of combination immunotherapy as a means of more reliably obtaining uniformly high serum NicAb concentrations.

ACKNOWLEDGEMENTS

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FIGURES AND TABLES

TABLES

Table 1. Treatment groups

Locomotor activity groups and treatments. Vaccinated rats received their final vaccine dose 10 days prior to the LMS protocol. Nic311 was administered as 80 mg/kg i.v. one hour prior to LMS on day 1 and 40 mg/kg i.v. one hour prior to LMS on day 7 .

Group	Nicotine	Vaccine Immunogen	Passive Immunization
Nicotine control	+	rEPA	Control-IgG
Vaccine	+	3′-AmNic-rEPA	Control-IgG
Nic311	+	rEPA	Nic311
Combined	+	3′-AmNic-rEPA	Nic311
Saline control	-	rEPA	Control-IgG

Table 2. Nic311 pharmacokinetic parameter estimates

Nic311 pharmacokinetic parameters with and without concurrent nicotine infusion (3.2 mg/kg/day).

Group (n = 5/group)	Cl (ml/hr/kg)	$V_{d,ss}$ (ml/kg)	α $t_{1/2}$ (hours)	β $t_{1/2}$ (days)	AUC (mg-hr/ml)
Nic311	0.65 ± 0.04	163 ± 21	7.7 ± 1.6	8.0 ± 1.0	46.3 ± 3
Nic311 + Nicotine	0.50 ± 0.06	122 ± 12	5.6 ± 0.9	7.6 ± 1.0	61.4 ± 8
p value:	*0.012*	*0.022*	*0.022*	*0.676*	*0.012*

Table 3. Antibody concentrations among locomotor sensitization groups

Mean ± SD antibody concentrations among groups.

	Vaccine NicAb (µg/ml)	Nic311 (µg/ml)	Total NicAb (µg/ml)
Vaccine	166 ± 105	-	166 ± 105
Nic311	-	172 ± 79	172 ± 79
Combined	224 ± 112	203 ± 97	420 ± 148***

*** Significantly higher than either Vaccine or Nic311 alone ($p < 0.001$).

Table 4. Nicotine binding in serum among the locomotor sensitization groups

Nicotine serum protein binding (mean ± SD) in serum obtained 40 minutes after the 10th nicotine dose in the LMS experiment.

	Total serum nicotine (ng/ml)	Nicotine % bound	Unbound nicotine (ng/ml)
Nicotine control	66 ± 11	8 ± 5	60 ± 10
Vaccine	452 ± 246 **	84 ± 14 ***	47 ± 12 **
Nic311	359 ± 148 *	86 ± 5 ***	44 ± 5 **
Combined	1008 ± 448 ##	95 ± 4 ##	32 ± 8 #

*, **, *** $p < 0.05, 0.01, 0.001$ compared to nicotine control.

#, ## $p < 0.05, 0.01$ compared to all other groups.

FIGURES

Figure 1. Locomotor sensitization experiment timeline

Timeline for the locomotor sensitization experiment. Locomotor activity was measured following each daily dose of nicotine 0.3 mg/kg or saline s.c.

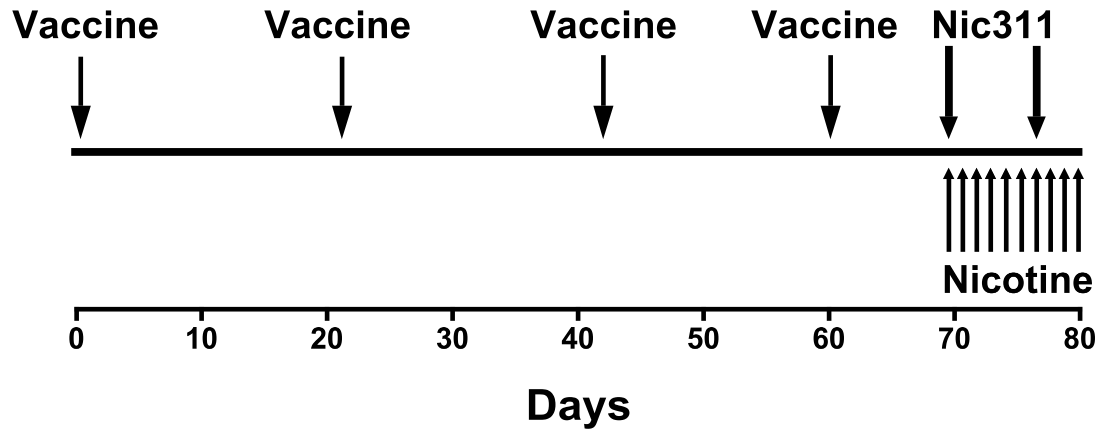


Figure 2. Nic311 concentration vs. time in the presence or absence of nicotine

Nic311 serum levels (mean ± SD) in the presence (-●-) and absence (—○—) of a nicotine infusion of 3.2 mg/kg/day. Inset shows levels over the initial 84 hours. Individual outliers (-△-) were not included in calculation of the mean values. Pharmacokinetic parameter estimates are shown in Table 2.

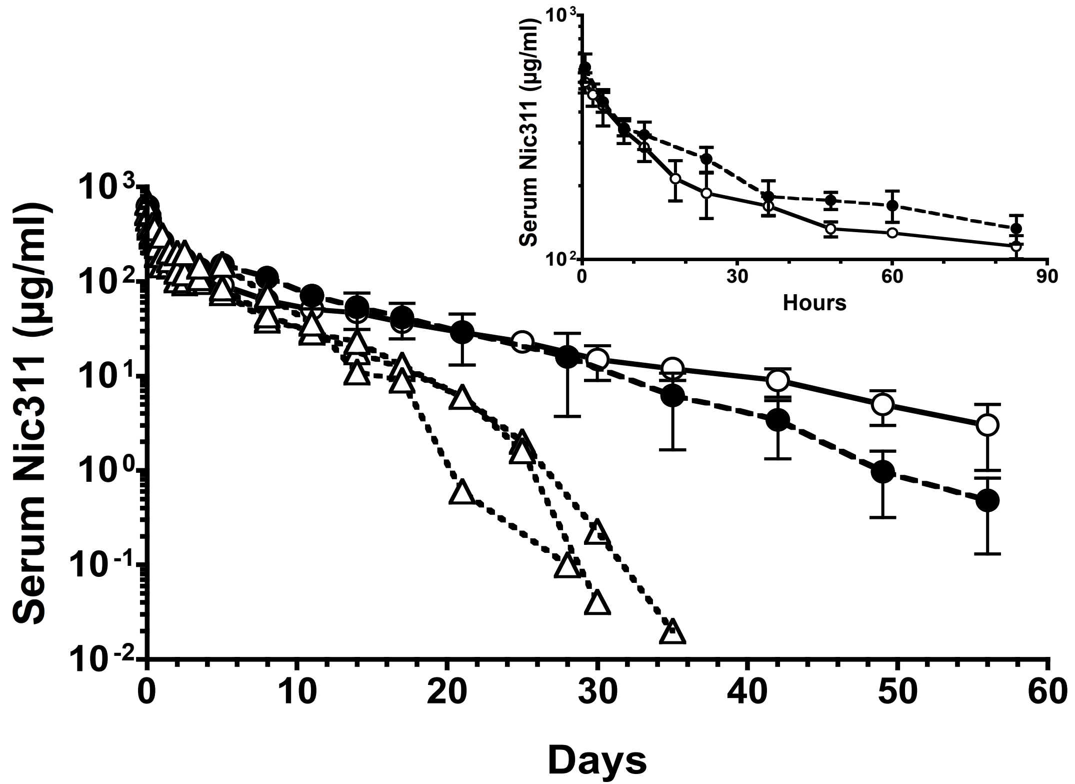


Figure 3. Serum and brain nicotine levels in the locomotor sensitization experiment. Ten daily s.c. doses of nicotine 0.3 mg/kg or saline were administered, and nicotine levels (mean ± SD) were obtained 40 minutes after the final dose. Serum nicotine levels were significantly increased and brain nicotine levels were significantly reduced by combined immunotherapy compared to all other treatments. *, ** $p < 0.05$, $p < 0.01$ compared to nicotine control; # $p < 0.01$ compared to all other groups.

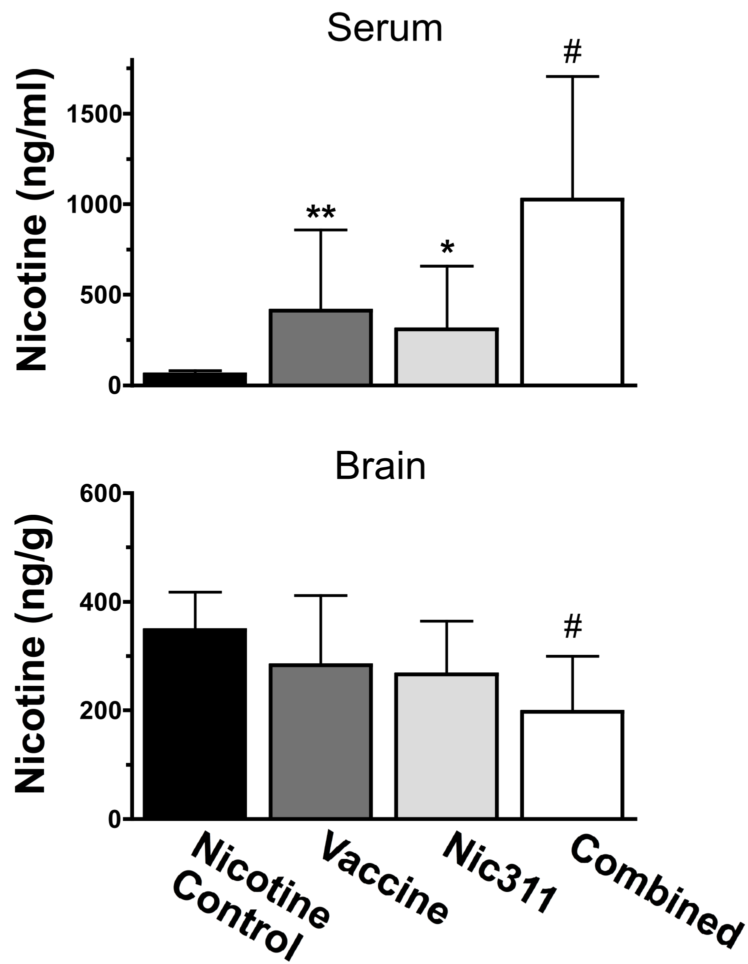


Figure 4. Unbound serum nicotine vs. brain nicotine in the locomotor sensitization experiment.

Relationship between free nicotine concentration in serum and the brain nicotine concentrations for rats in the locomotor sensitization protocol.

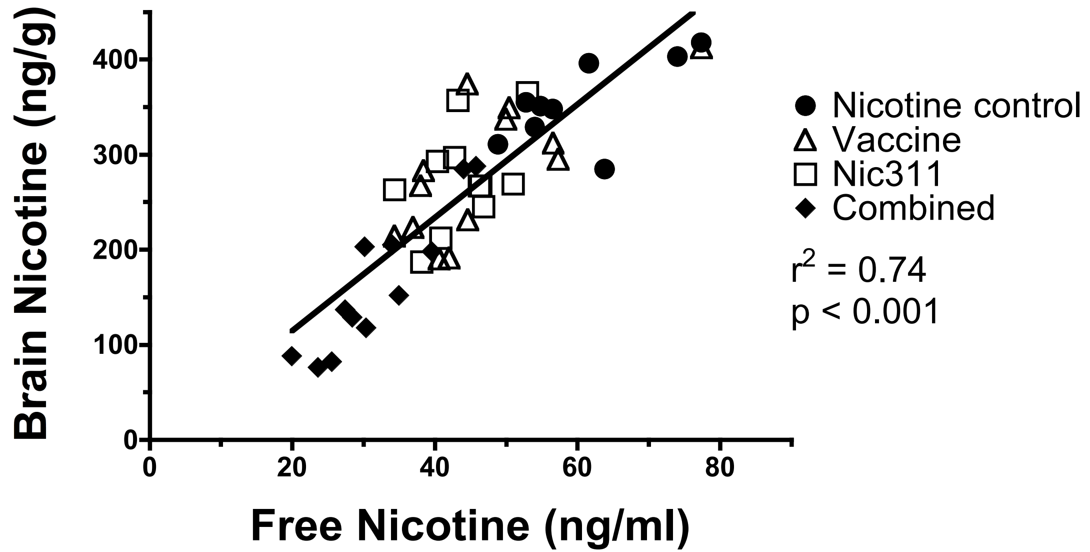


Figure 5. Effects of treatments on locomotor sensitization.

Total horizontal distance traveled (mean ± SE) was assessed for 30 minutes immediately after each daily dose of nicotine 0.3 mg/kg or saline s.c. Only the nicotine group exhibited a progressive increase in locomotor activity over the course of the experiment (sensitization). The combined immunotherapy group and the saline group showed reduced locomotor activity over the course of testing (* $p < 0.05$ day 10 compared to day 1), and also had significantly reduced locomotor activity compared to the vaccine alone group on days 7-10 (# $p < 0.05$).

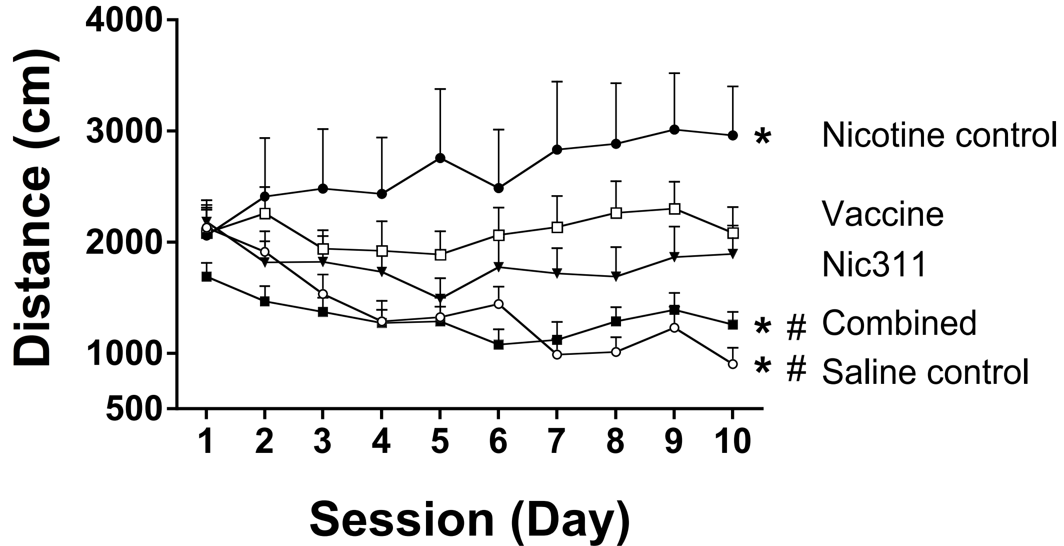


Figure 6. Locomotor activity during sessions 1 and 10

(Top) Locomotor activity (mean ± SE) in 5-minute blocks during the first session of the locomotor sensitization experiment. Locomotor activity did not differ among groups at any time. (Bottom) Locomotor activity during the 10th (final) session. The nicotine control group showed significantly greater activity at 5 minutes ($p < 0.001$) and 10 minutes ($p < 0.05$) compared to all other groups. The combined immunotherapy group had significantly reduced activity compared to rats treated with vaccine alone ($p < 0.01$) or Nic311 alone ($p < 0.05$) during the first 5 minutes of the session.

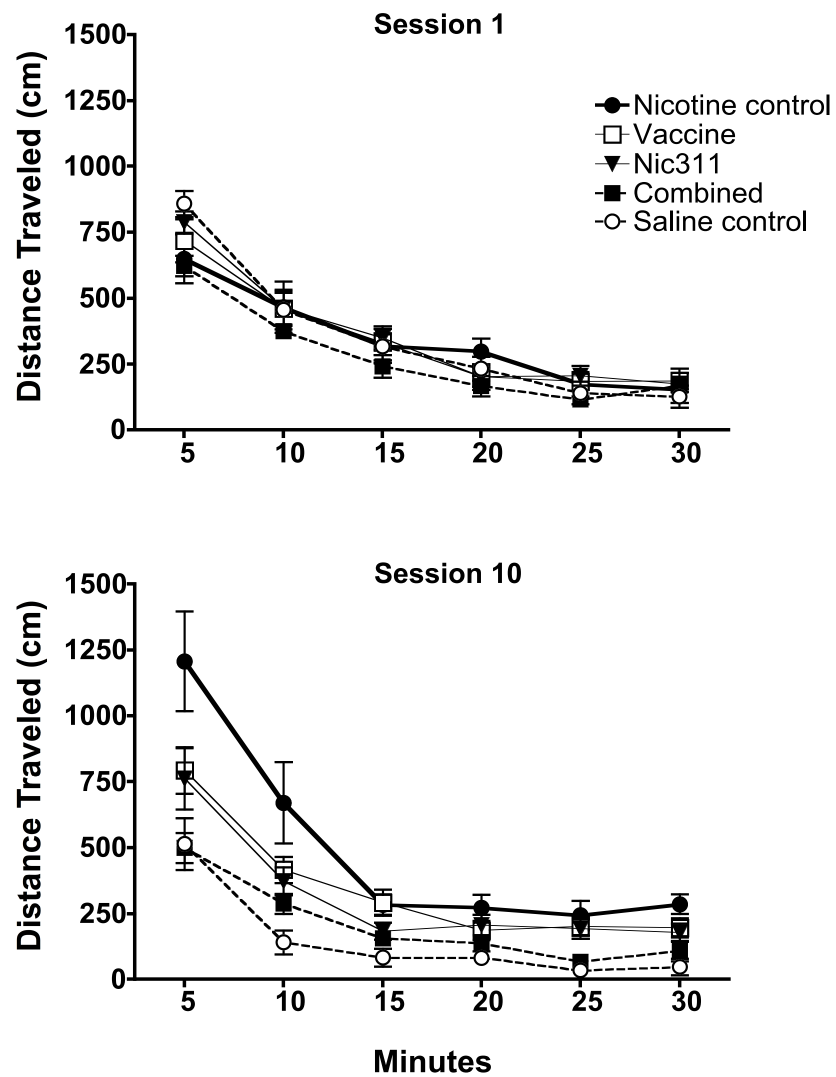
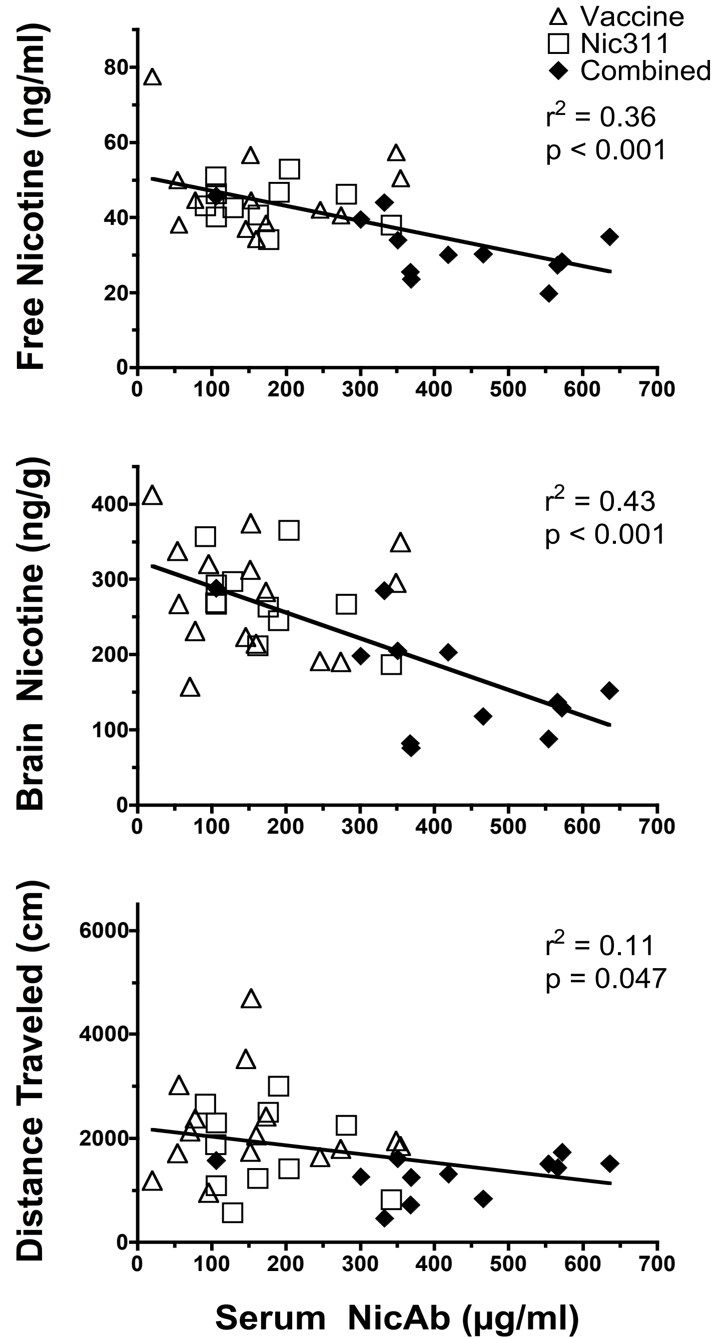


Figure 7. Total serum NicAb vs. free serum nicotine, brain nicotine, and behavior
Relationship of total serum NicAb concentration to the free serum nicotine concentration (top), brain nicotine concentration (middle), and the mean total distance traveled on days 7-10 (bottom).



CHAPTER 5

Passive immunization with a nicotine-specific monoclonal antibody decreases brain nicotine levels but does not precipitate withdrawal in nicotine-dependent rats

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ABSTRACT

Vaccination against nicotine is under investigation as a treatment for tobacco dependence. Passive immunization with nicotine-specific antibodies represents a complementary strategy to vaccination. A potential adverse effect of passive immunization in nicotine-dependent individuals is that it may lead to a rapid reduction in brain nicotine levels and trigger withdrawal. The goal of this study was to determine if passive immunization with the nicotine-specific monoclonal antibody Nic311 precipitated withdrawal in nicotine-dependent rats as measured by increases in brain reward thresholds and somatic signs. Another cohort of rats was used to measure brain nicotine levels after Nic311 administration. Nic311 30, 80 or 240 mg/kg reduced brain nicotine concentrations by 45, 83 or 92% compared to controls. None of these Nic311 doses precipitated withdrawal measured at intervals up to 72 hours following antibody administration. Administration of the nicotinic antagonist mecamylamine precipitated a robust nicotine withdrawal syndrome. A substantial, but not complete, acute reduction in brain nicotine levels following passive immunization was not sufficient to precipitate nicotine withdrawal in nicotine-dependent rats. The Nic311 doses used have been shown to attenuate the behavioral effects of nicotine, suggesting that the use of passive immunization to treat nicotine addiction is not likely to precipitate withdrawal.

1. INTRODUCTION

Nicotine is the primary addictive component in tobacco [171]. The pharmacokinetics of nicotine, including the extent and rate of nicotine entry into the brain and the rate of nicotine elimination, are key determinants of its rewarding and reinforcing effects [54, 75, 92]. Interventions that alter nicotine pharmacokinetics may be useful for attenuating the addictive effects of nicotine and aiding in smoking cessation.

Vaccination against nicotine elicits the production of nicotine-specific antibodies that bind nicotine in serum, reduce or slow its distribution to brain, and slow its elimination [180]. Vaccination of rats against nicotine attenuates a variety of nicotine-related behaviors including the acquisition, maintenance, and reinstatement of nicotine self-administration [90, 172]. Three nicotine vaccines are in clinical trials, and initial reports indicate efficacy in enhancing smoking cessation rates. However, efficacy is limited because it is strongly correlated with the serum nicotine-specific antibody concentrations or titers achieved, which are both modest and characterized by high individual variability [108, 116]. The development of alternative strategies to reliably produce sufficiently high antibody levels is critical to maximize the efficacy of immunotherapy against nicotine.

Passive immunization against nicotine (the administration of exogenously derived nicotine-specific antibodies, or NicAb) has been studied in animals as a complementary strategy to vaccination. The effects of passive immunization in rats are quite similar to

those of vaccination, such as lower brain nicotine concentrations and attenuation of nicotine-induced locomotor sensitization [91, 137, 142]. Passive immunization has a number of potential advantages over vaccination. First, passive immunization provides control over the antibody dose and resulting serum antibody concentration, and suitable doses can produce higher mean serum antibody levels than vaccination. Second, the large individual variability in immune response and resulting serum antibody levels seen with vaccination is circumvented. Third, the effect of passive immunization is immediate as compared to the 1–3 months needed for vaccination to elicit a maximal antibody response. Passive immunization is also being studied as a potential treatment for phencyclidine [170], cocaine [121], and methamphetamine addiction or toxicity [182]. The principal disadvantage of passive immunization with nicotine-specific monoclonal antibodies as a therapeutic strategy for tobacco addiction is its cost [130, 183].

A potential adverse effect of both vaccination and passive immunization against nicotine is that, by reducing nicotine levels in brain, they could precipitate a withdrawal syndrome if administered to current smokers. This is unlikely to be a problem with vaccination because NicAb levels increase gradually over 1–3 months [184]. Passive immunization differs from vaccination in that therapeutic levels of NicAb are achieved immediately, which could more abruptly reduce brain nicotine concentrations.

The goal of this study was to determine whether the acute administration of the monoclonal nicotine-specific antibody Nic311 precipitates a nicotine abstinence syndrome in nicotine dependent rats as assessed by increases in brain reward thresholds

(a measure of anhedonia associated with withdrawal) or somatic signs, two well-established measures of nicotine withdrawal [25, 26]. In a parallel experiment, brain nicotine concentrations were measured to quantitate the extent of reduction in brain nicotine concentrations after Nic311 administration.

2. MATERIALS AND METHODS

2.1. Animals

Male Holtzman Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 300-325 g at the time of surgery were housed individually in temperature and humidity controlled colony rooms with unlimited access to water under a reversed 12-h light/dark cycle (lights off at 10:00 am). Rats were food-restricted to 18 g/day rat chow to minimize weight gain and limit chronic in-dwelling catheter migration throughout the chronic studies. All protocols were approved by the Minneapolis Medical Research Foundation Animal Care and Use Committee and were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Reagents

Nicotine bitartrate or mecamylamine (Sigma Chemical Co., St. Louis, MO) were dissolved in sterile saline. The pH of the nicotine solution was adjusted to 7.4 with dilute NaOH. All nicotine doses and concentrations are expressed as that of the base. The nicotine-specific monoclonal antibody Nic311 is an $IgG1_{\kappa}$ derived from mice immunized with the immunogen 3′-aminomethylnicotine conjugated to recombinant Pseudomonas

exoprotein A, with a K_d for nicotine of 60 nM and < 1% cross-reactivity with nicotine metabolites or a variety of neurotransmitters including acetylcholine [138]. Nic311 was purified by protein G chromatography to ≥ 95% of total protein content with endotoxin levels of < 0.2 EU/mg. Nic311 was diluted in phosphate-buffered saline to a concentration of 10 mg/ml for administration of the 30 mg/kg dose, 30 mg/ml for the 80 mg/kg dose, and 50 mg/ml for the 240 mg/kg dose. The Nic311 dose of 80 mg/kg was selected because this is a likely clinical dose and produces serum NicAb levels similar to vaccination [142]. In addition, Nic311 80 mg/kg is equimolar to the steady-state rat body burden of nicotine (during a nicotine infusion of 3.2 mg/kg/day), and the Nic311 doses of 30 and 240 mg/kg bracket this dose (0.4 and 3.5 x nicotine total body burden, respectively). Control IgG was human polyclonal IgG (Sandoglobulin®; Sandoz, Vienna, Austria) that does not bind nicotine or alter nicotine pharmacokinetics or behavior in rats [137].

<u>*2.3. Effects of Nic311 on serum and brain nicotine levels*</u>

2.3.1. Protocol

Animals were anesthetized with isoflurane and an Alzet 2ML2 osmotic minipump was implanted (Durect Cupertino, CA) to deliver nicotine 3.2 mg/kg/day [185]. After two days of nicotine exposure (by which time nicotine concentrations had reached steady-state), rats were anesthetized with intramuscular (i.m.) droperidol 2.0 mg/kg and fentanyl 0.04 mg/kg. A temporary femoral cannula was implanted to deliver Nic311 or Control IgG i.v. and then removed. To characterize the effects of Nic311 over the time period corresponding to reward threshold measurement (see below), brain and blood samples

were collected 15 or 60 minutes after infusion of Nic311 80 mg/kg (n = 4/time-point) or Control IgG (n = 6/time point). To characterize the dose-response relationship, brain and blood samples were collected 60 minutes after infusion of Nic311 at doses of 30, 80, or 240 mg/kg (n = 4/dose). This time-point was used because the greatest effect on brain nicotine concentrations was observed 60 minutes following infusion of Nic311. Group size was smaller for rats administered Nic311 than for controls due to the limited availability and expense of producing Nic311.

2.3.2. Nicotine assay

Serum and brain nicotine levels were measured by gas chromatography with nitrogen-phosphorous detection [164]. Brain nicotine concentrations were corrected for brain blood content [88].

2.3.3. Pharmacokinetic calculations

The total body burden of nicotine during the nicotine infusion at the time treatments were administered was calculated as the product of the steady state concentration of nicotine (52 ± 13 ng/ml as measured on day 2 of the nicotine infusion in the control IgG group) and the steady-state volume of distribution of nicotine as previously reported in this strain of rat (3.5 L/kg) [137]. The molar dose of Nic311 was calculated based on a molecular weight of 150 000 kD for Nic311 and 2 nicotine-binding sites per molecule of Nic311. The amount of nicotine in serum after administration of treatments was calculated as the serum nicotine concentration x serum volume, where serum volume is

the product of the blood volume (64 ml/kg) and (1 - the estimated hematocrit 0.45) = 28.8 ml/kg.

2.4. Withdrawal assessment

2.4.1. Equipment

Intracranial self-stimulation (ICSS) training and testing occurred in operant conditioning chambers (29 cm x 26 cm x 33 cm high) (Med Associates, St. Albans, VT) placed inside sound-attenuated cubicles. A 5-cm wide metal wheel manipulandum was fixed to the front wall. Brain stimulation was administered with constant-current stimulators (Model #PHM-152, Med-Associates). Rats were connected to the stimulation circuit through bipolar leads (Plastics One, Roanoke, VA) attached to gold-contact swivel commutators (Plastics One). MED-PC IV software was used to control stimulation parameters and for data collection.

2.4.2. Stereotaxic surgery

Animals were anesthetized with i.m. ketamine (75 mg/kg) and xylazine (7.5 mg/kg) and implanted with a bipolar stainless steel electrode (Model MS303/2: Plastics One) in the medial forebrain bundle at the level of the lateral hypothalamus [AP -0.5 and ML ± 1.7 mm from bregma, DV -8.3 mm from dura with the incisor bar set 5 mm above the inter-aural line [186]]. The side of the brain in which the electrode was placed was alternated across subjects. Animals were allowed to recover for at least one week prior to ICSS training. During the first two days of recovery all animals received injections of the antibiotic enrofloxacin, 1.1 mg i.m.

2.4.3. Intracranial self-stimulation (ICSS) procedure

Rats were trained on a modified version of the Kornetsky and Esposito [187] discrete-trial current-threshold procedure as described previously [see 188]. Each trial was initiated with presentation of a non-contingent stimulus (0.1 ms cathodal squarewave pulses at a frequency of 100 Hz for 500 ms) followed by a 7.5-sec window during which a positive response on the wheel manipulandum produced a second, contingent stimulation identical to the first. Lack of responding in the 7.5-second time window was considered a negative response. Each positive or negative response was followed by a variable inter-trial interval averaging 10 sec (range = 7.5 to 12.5 sec), during which time additional responses delayed onset of the subsequent trial by 12.5 sec. Stimulus intensities were presented in four alternating descending and ascending series (step size = 5 μA), with five trials presented at each current intensity step. The current threshold for each series was defined as the midpoint between two consecutive current intensity steps that yielded three or more positive responses and two consecutive current intensity steps that yielded three or more negative responses. The overall threshold for the approximately 45 min session was defined as the mean of the current thresholds from the four alternating series. To assess performance effects (e.g., motor disruption), response latencies (time between onset of the non-contingent stimulus and a positive response) were averaged across all trials in which a positive response was made [188].

2.4.4. Assessment of somatic signs

Rats were habituated to clear plastic circular chambers for 10 minutes on each of 2 days prior to withdrawal testing. An experimentally blind, trained observer recorded behavior for 10 minutes as described by [26]. Behavioral categories included gasps/abdominal writhes, teeth chatter/chews, shakes/tremors, ptosis, and other miscellaneous less frequent signs (e.g., scratches, yawns). Multiple successive counts of any sign required a distinct pause between signs. If continuously present, ptosis was only counted once/minute and teeth chattering only once every 15 seconds.

2.4.5 Protocol

Rats were implanted with intra-cranial electrodes and trained for ICSS as described above for at least 15 sessions and until thresholds were stable (i.e., no more than 10% variation over a 5-day period). A chronic in-dwelling jugular cannula was then implanted for antibody delivery as described previously [185]. At least 7 days later, rats resumed ICSS threshold assessment, and after ICSS thresholds again stabilized, osmotic minipumps (2ML4) were implanted to deliver a continuous s.c. nicotine infusion of 3.2 mg/kg/day. This infusion rate has been shown to reliably induce nicotine dependence as measured using elevated ICSS thresholds and somatic signs following pump removal (spontaneous withdrawal) or administration of a nicotinic antagonist (precipitated withdrawal) [25]. Rats continued to be tested for ICSS during nicotine exposure. After 1–3 weeks of nicotine exposure, rats were assessed for precipitated withdrawal. Duration of nicotine exposure depended on stability of thresholds and was counter-balanced between

experimental groups, so that each group had the same average duration of nicotine exposure prior to assessment of precipitated withdrawal.

During assessment of precipitated withdrawal, rats were exposed to one of the following treatments: Control IgG 80 mg/kg i.v. + mecamylamine 1.5 mg/kg s.c. (positive control, n = 6); Control IgG 80 mg/kg i.v. + saline s.c. (negative control, n = 6); Nic311 80 mg/kg i.v. + saline s.c. (n = 6); Nic311 240 mg/kg i.v. + saline s.c. (n = 4). Mecamylamine or saline was administered s.c. immediately after antibody administration. It is well-established that this dose of mecamylamine precipitates robust increases in ICSS thresholds and somatic signs when administered to dependent rats while having no effect on these measures in drug-naïve rats [141, 189-191]. Therefore, the effects of Control IgG+mecamylamine in rats chronically infused with saline were not examined. Group size for rats administered Nic311 240 mg/kg was 4 rather than 6 due to its limited availability and expense.

ICSS thresholds were assessed 0.25, 3, 24, 48 and 72 hours after antibody administration. Somatic signs were assessed following the first three ICSS sessions (approximately 1, 4, and 25 hours after infusion).

<u>*2.5. Statistical analyses*</u>

2.5.1. Effects of Nic311 on serum and brain nicotine concentrations

Mean serum and brain nicotine concentrations following administration of Nic311 (80 mg/kg) or Control IgG were analyzed by two-factor ANOVA with time and treatment as

factors, followed by Bonferroni's post test to compare groups at each time point. Mean serum and brain nicotine concentrations and serum concentrations of the nicotine metabolite cotinine at 60 minutes following administration of Nic311 30, 80, or 240 mg/kg or Control IgG were analyzed by one-way ANOVA followed by Bonferroni's post-test to compare groups where appropriate.

2.5.2. Withdrawal assessment

Baseline ICSS thresholds were defined as the mean thresholds of the last 5 sessions during nicotine exposure prior to assessment of withdrawal. ICSS thresholds prior to osmotic pump implantation were compared to baseline as described above via t-test to confirm that ICSS thresholds were not altered by nicotine and the defined baseline was appropriate. Baseline thresholds were compared between groups using a one-way ANOVA. To compare differences between groups for the first 24 hours after antibody treatment, ICSS thresholds (expressed as % of baseline) were analyzed by repeated-measures two-factor ANOVA with treatment and session as factors followed by Bonferroni's post-test for between group comparisons at each test session. ICSS thresholds across sessions for each group were also compared to baseline using a one-way ANOVA followed by Dunnett's post hoc tests. Further, since the pharmacokinetic data indicated a more substantial reduction in brain nicotine concentrations at 60 minutes compared to 15 minutes, it was possible that averaging across the entire 45 min ICSS session would obscure drug effects. To address this possibility, within-session thresholds of the first ICSS session (15–60 minutes after drug administration) were assessed by t-test to compare the mean threshold from the first two ascending and descending current

series (obtained approximately from 0 to 25 minutes of the session) with the mean thresholds from the last two ascending and descending current series (obtained approximately from 25–45 minutes of the session) in each group. Response latencies (before and up to 24 hr after drug infusion) were also analyzed by two-factor ANOVA to check for nonspecific effects. To assess differences in somatic signs between groups, a repeated-measures two-factor ANOVA was used with treatment and session as factors followed by Bonferroni's post-test.

3. RESULTS

3.1. Effects of Nic311 on serum and brain nicotine concentrations

3.1.1. Time course

There was a significant effect of treatment ($F(1, 12) = 182.2$, $p < 0.0001$) and time ($F(1, 12) = 18.4$, $p = 0.001$) on serum nicotine concentration and a significant interaction ($F(1, 12) = 17.4$, $p = 0.001$). As shown in Figure 1, Nic311 80 mg/kg significantly increased serum concentrations at both 15 and 60 minutes following antibody infusion (Figure 1, $p < 0.001$). There was a significant effect of treatment ($F(1, 16) = 33.6$, $p < 0.001$) and time ($F(1, 16) = 12.94$, $p < 0.001$) on brain nicotine concentrations, but no significant interaction. Nic311 80 mg/kg significantly reduced brain nicotine levels compared to Control IgG at 15 minutes (61% that of controls, $p < 0.05$) and 1 hour (17% that of controls, $p < 0.001$) post-infusion.

3.1.2. Dose response

There was a significant relationship between Nic311 dose and serum nicotine concentrations 60 minutes post-infusion ($F(3, 17) = 63.6$, $p < 0.0001$). As shown in Figure 2, Nic311 30 and 80 mg/kg resulted in significantly higher nicotine serum concentrations compared to Control IgG ($p < 0.01$), and Nic311 240 mg/kg resulted in significantly higher nicotine serum concentrations than all other groups ($p < 0.0001$). Serum cotinine levels were not significantly different among groups 60 minutes following antibody infusion (control 546 ± 159 ng/ml, Nic311 30 mg/kg 458 ± 134 ng/ml, Nic311 80 mg/kg 451 ± 79 ng/ml, Nic311 240 mg/kg 749 ± 301 ng/ml, $p = 0.12$). There was a significant relationship between Nic311 dose and brain nicotine concentrations ($F(3, 20) = 15.0$, $p < 0.0001$). The Nic311 doses of 30, 80, and 240 mg/kg reduced brain nicotine concentrations to 55%, 17%, and 8% of controls ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively).

3.1.3. Pharmacokinetic calculations

The estimated amount of nicotine in the body at the time treatments were administered was 0.182 mg/kg = 1.12 µmol/kg. The molar doses of Nic311 were 0.4 µmol/kg (for the Nic311 30 mg/kg dose), 1.07 µmol/kg (Nic311 80 mg/kg) and 3.20 µmol/kg (Nic311 240 mg/kg). The molar ratio of Nic311 to amount of nicotine in the body were 0.34 (Nic311 30 mg/kg), 0.96 (Nic311 80 mg/kg) and 2.86 (Nic311 240 mg/kg). The total amount of nicotine in serum after treatment was 0.001 mg/kg (Control), 0.029 mg/kg (Nic311 30 mg/kg), 0.033 mg/kg (Nic311 80 mg/kg) and 0.130 mg/kg (Nic311 240 mg/kg). These amounts of nicotine in serum represented 0.8%, 16%, 18% and 71% of the total estimated

amount of nicotine in the body at the time of treatment for the 0 (control) 30, 80 and 240 mg/kg Nic311 doses.

<u>3.2. Withdrawal assessment</u>

3.2.1. Baseline measures

Baseline thresholds during nicotine exposure did not differ from thresholds during the last 5 sessions prior to nicotine pump implantation, indicating that nicotine did not alter ICSS thresholds (data not shown). Baseline thresholds prior to precipitated withdrawal did not differ among groups (Table 1).

3.2.2. ICSS Thresholds

Mecamylamine significantly increased ICSS thresholds whereas Nic311 did not (Figure 3, top). There was a significant overall effect of treatment ($F(3, 54) = 4.97, p = 0.011$) on ICSS thresholds but no significant effect of time and no significant interaction. ICSS thresholds in rats administered Nic311 80 mg/kg + saline did not differ from those administered Control IgG + saline at any time. Nic311 240 mg/kg + saline resulted in a slight increase in ICSS thresholds (mean 110%, range 109–112%) compared to baseline but this was not significantly different from Control IgG+saline ($p = 0.3$). Control IgG + mecamylamine resulted in significantly higher ICSS thresholds compared to Control IgG + saline or Nic311 80 mg/kg + saline ($p < 0.01$) 15 minutes after administration but did not differ from Nic311 240 mg/kg + saline at any time. Drug and antibody administration did not alter response latencies for up to 24 hr post-infusion (Table 1).

Analysis of within-group data indicated that only Control IgG + mecamylamine at 15 min after drug administration significantly elevated thresholds compared to baseline ($F(3, 20) = 4.696$, $p = 0.012$). Analysis of within-session data from the 15 min test showed stability of ICSS thresholds within sessions: there were no differences between the mean thresholds from the first two ascending and descending current series (obtained from approximately 0 to 25 minutes of the session) compared to the mean threshold of the last two ascending and descending current series (obtained from approximately 25–45 minutes of the session) for all groups. These data indicate that any potential effects of Nic311 on ICSS thresholds late in the session (i.e., when brain nicotine levels were lowest, see Figure 1) were not obscured by collapsing the data across all four current series.

3.2.3. Somatic signs

Mecamylamine significantly increased somatic signs of withdrawal compared to Nic311 80 mg/kg or Control IgG (Figure 3, bottom). There was a significant overall effect of treatment ($F(3, 36) = 4.12$, $p = 0.022$) and time ($F(2, 36) = 4.89$, $p = 0.013$) on somatic withdrawal signs, and a significant interaction ($F(6, 36) = 3.42$, $p = 0.009$). Control IgG + mecamylamine significantly increased somatic withdrawal signs 1 hour after injection compared to Control IgG + saline or Nic311 80 mg/kg+saline ($p < 0.001$) but not compared to Nic311 240 mg/kg + saline. Somatic withdrawal signs 1 hr after Nic311 240 mg/kg + saline were slightly elevated but were not significantly different from Control IgG + saline or Nic311 80 mg/kg + saline. There were no differences in somatic withdrawal signs among groups at the remaining time points.

4. DISCUSSION

Previous studies have shown that Nic311 reduces or slows nicotine distribution into brain if administered prior to nicotine administration [137, 138]. The current study found that Nic311 also rapidly redistributed nicotine out of the brain when administered during an ongoing nicotine infusion. The 80 mg/kg dose of Nic311, which was approximately equimolar to the body burden of nicotine, reduced the brain nicotine concentration by 83±7%. The 240 mg/kg dose, a 3-fold molar excess of Nic311 compared to the nicotine body burden, reduced the brain nicotine concentration by 92±8%. These large reductions are consistent with previous studies of the effects of passive immunization on brain concentrations of PCP [170], methamphetamine [182], and desipramine [192] at similar antibody:drug molar ratios. The corresponding serum nicotine concentrations were greatly increased after Nic311 administration. These very high serum nicotine levels are attributable to the high binding capacity of the administered Nic311 and to sampling shortly after Nic311 dosing when a large fraction of the administered antibody would have been present in serum [137, 142]. Comparably high serum nicotine concentrations were reported previously when the same Nic311 dose was administered prior to nicotine dosing. The high serum nicotine concentrations resulting from immunization are not toxic because most of the nicotine in serum is bound to antibody and is not pharmacologically active [137].

This study is the first to evaluate the potential for passive immunization to precipitate nicotine withdrawal, but vaccination has been previously studied in this regard. Vaccination of nicotine-dependent rats with a nicotine conjugate vaccine during

continuous nicotine infusion did not increase ICSS thresholds or somatic withdrawal signs [184]. In addition, a Phase I study of a nicotine vaccine did not report any signs or symptoms of nicotine withdrawal in smokers vaccinated against nicotine [108]. This is most likely attributable to vaccination producing a very gradual increase in serum NicAb concentration over weeks to months so that any resulting changes in brain nicotine concentration also occur gradually. The much more rapid increase in serum NicAb concentration produced by passive immunization would be expected to have a greater potential for producing withdrawal.

In the current study the rapid and substantial redistribution of nicotine out of brain produced by Nic311 did not precipitate nicotine withdrawal whereas the nicotinic acetylcholine receptor (nAChR) antagonist mecamylamine produced robust withdrawal as measured by both brain reward thresholds and somatic signs. The Nic311 dose of 80 mg/kg was studied because it produces substantial effects on nicotine pharmacokinetics and attenuates nicotine-induced locomotor sensitization in rats [142]. Nic311 80 mg/kg also produces serum NicAb concentrations comparable to those elicited by vaccination of rats against nicotine and higher than those produced by vaccination in clinical trials [108]. This Nic311 dose is therefore in the range that might be considered for clinical use. Nic311 80 mg/kg produced no measurable withdrawal, and 240 mg/kg produced only a nonsignificant trend toward increases in ICSS thresholds and somatic signs. It is possible that the small effect of the highest dose would be significant if evaluated using larger groups, but the magnitude of change in ICSS thresholds and somatic signs was quite small compared to that of mecamylamine. These findings imply that the

therapeutically desirable effects of Nic311 in rats as modeled by attenuation of locomotor sensitization [142] occur at Nic311 doses that do not precipitate withdrawal. Similarly, vaccination against nicotine blocked the ICSS threshold-reducing (i.e., rewarding) effects of an acute nicotine injection but did not precipitate withdrawal in nicotine-dependent rats [184]. These findings suggest that either active or passive immunization against nicotine should effectively attenuate nicotine's acute effects in current smokers without precipitating a withdrawal syndrome.

The rate and extent by which nicotine must be removed from the brain to precipitate withdrawal is not well established. Previously, the only means of initiating nicotine withdrawal in nicotine dependent animals were to abruptly discontinue chronic nicotine dosing or to administer an nAChR antagonist. Withdrawal following antagonist treatment is rapid and measurable within minutes. Only limited data are available on the early time course of spontaneous nicotine withdrawal following termination of a nicotine infusion. One study reported that the removal of a nicotine osmotic minipump resulted in the onset of withdrawal in rats 2.5–4 hours and peak withdrawal severity at 6–8 hours, as measured by ICSS and somatic signs [25]. Although serum and brain nicotine concentrations were not measured in this study, the elimination half-life for nicotine in serum or brain in rats is ~1 hour [80, 137]. As an approximation, this suggests that withdrawal after nicotine pump removal was first detected when 82– 94% of nicotine had been eliminated (2.5–4 nicotine half-lives), and peak severity occurred when >99% had been eliminated. Similarly, in humans the onset of tobacco withdrawal has been reported 3–6 hours after cessation of smoking (an estimated 1.5–3 nicotine elimination half-lives

in humans) and peak withdrawal at 10–12 hours (5–6 half-lives) [40, 193]. Based on these estimates, it is quite surprising that the rapid removal of 83±7% or 92±8% of nicotine from brain after the 80 or 240 mg/kg doses of Nic311, respectively, did not precipitate nicotine withdrawal.

The reasons for this unexpected absence of withdrawal after Nic311 treatment are unclear. Only one nicotine infusion rate was studied to induce dependence, but this nicotine infusion rate is used widely by others and results in robust withdrawal in a variety of strains of rats when terminated [25, 26]. It is possible that the development of withdrawal requires a delay between the offset of nicotine signaling and the manifestation of behavioral signs. However, consistent with previous reports, the administration of mecamylamine produced robust withdrawal within 15 minutes of its administration [141, 191]. Also possible is that Nic311 predominantly removed nicotine from the brain that was not specifically bound to nAChRs. The rapid removal of nicotine from brain by Nic311 may not have allowed nicotine redistribution within the brain to reach equilibrium over the period of withdrawal testing, so that exposure of nAChRs to nicotine was greater than predicted from the whole brain level. This could have resulted in a more gradual reduction in nAChR signaling compared to mecamylamine and a delay in the expression of withdrawal. However ICSS thresholds and somatic signs were observed repeatedly for 25 hours after administration of antibody, and ICSS thresholds were also measured at intervals up to 72 hours, with no trend toward increasing values. An additional difference between termination of a nicotine infusion and the administration of Nic311 as potential precipitants of withdrawal is that, in the current protocol, nicotine

infusion continued after Nic311 was administered to simulate continued smoking. This could have mitigated the ability of Nic311 to precipitate withdrawal at the later time points.

One potential use for passive immunization is in combination with vaccination, to supplement vaccination and ensure that desired antibody levels are achieved, or to provide a more rapid onset of effect. In support of this possibility, combining vaccination with passive immunization results in lower brain nicotine levels in rats and a greater attenuation of nicotine-induced locomotor sensitization than either treatment alone [142]. If passive immunization is used concurrently with vaccination, monoclonal antibody might be administered to patients who are still smoking. This study suggests that the precipitation of withdrawal by passive immunization under these circumstances is unlikely.

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FIGURES AND TABLES

Table 1. Group baseline thresholds and response latencies

Baseline thresholds and response latencies, and response latencies during the first 24 hr post-antibody infusion, for the four experimental groups.

Group	Baseline Threshold (μA)	Baseline Latency (sec)	Latency (sec) after treatment		
			15 min	3 hour	24 hour
Control IgG + Saline	121 ± 14	3.4 ± 0.2	3.4 ± 0.1	3.5 ± 0.2	3.5 ± 0.2
Control IgG + Mecamylamine	110 ± 16	2.7 ± 0.2	2.7 ± 0.7	3.3 ± 0.4	2.8 ± 0.3
Nic311 80 mg/kg + Saline	113 ± 15	2.7 ± 0.2	2.8 ± 0.2	2.6 ± 0.2	2.8 ± 0.2
Nic311 240 mg/kg + Saline	95 ± 12	2.5 ± 0.4	2.6 ± 0.3	2.5 ± 0.5	2.5 ± 0.2

Figure 1. Nicotine serum and brain 15 and 60 minutes after Nic311 80 mg/kg

Serum and brain nicotine concentrations (mean ± SD) 15 and 60 min after infusion of Nic311 80 mg/kg. *, ***; $p < 0.05$, 0.001 compared to control at each time point; (%) indicates brain nicotine levels as percent of control.

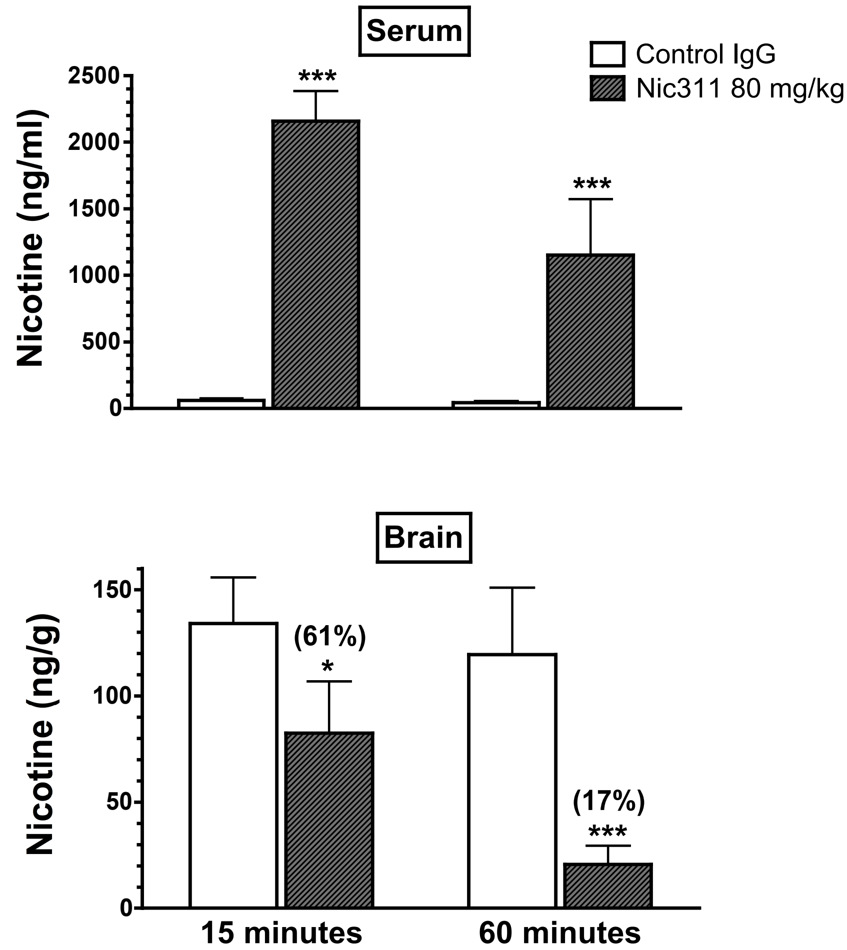


Figure 2. Dose-response of Nic311 vs. serum and brain nicotine levels at 60 minutes
Serum and brain nicotine concentrations (mean ± SD) measured 60 minutes post-antibody infusion. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to Control; ###, $p < 0.001$ compared to all other groups; (%) indicates brain nicotine levels as percent of control.

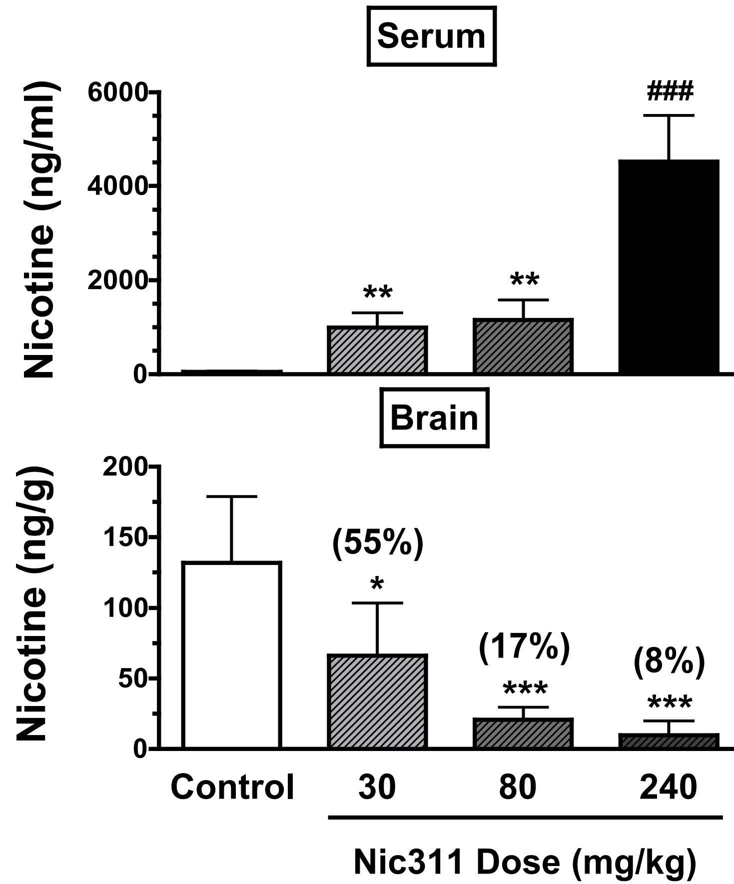
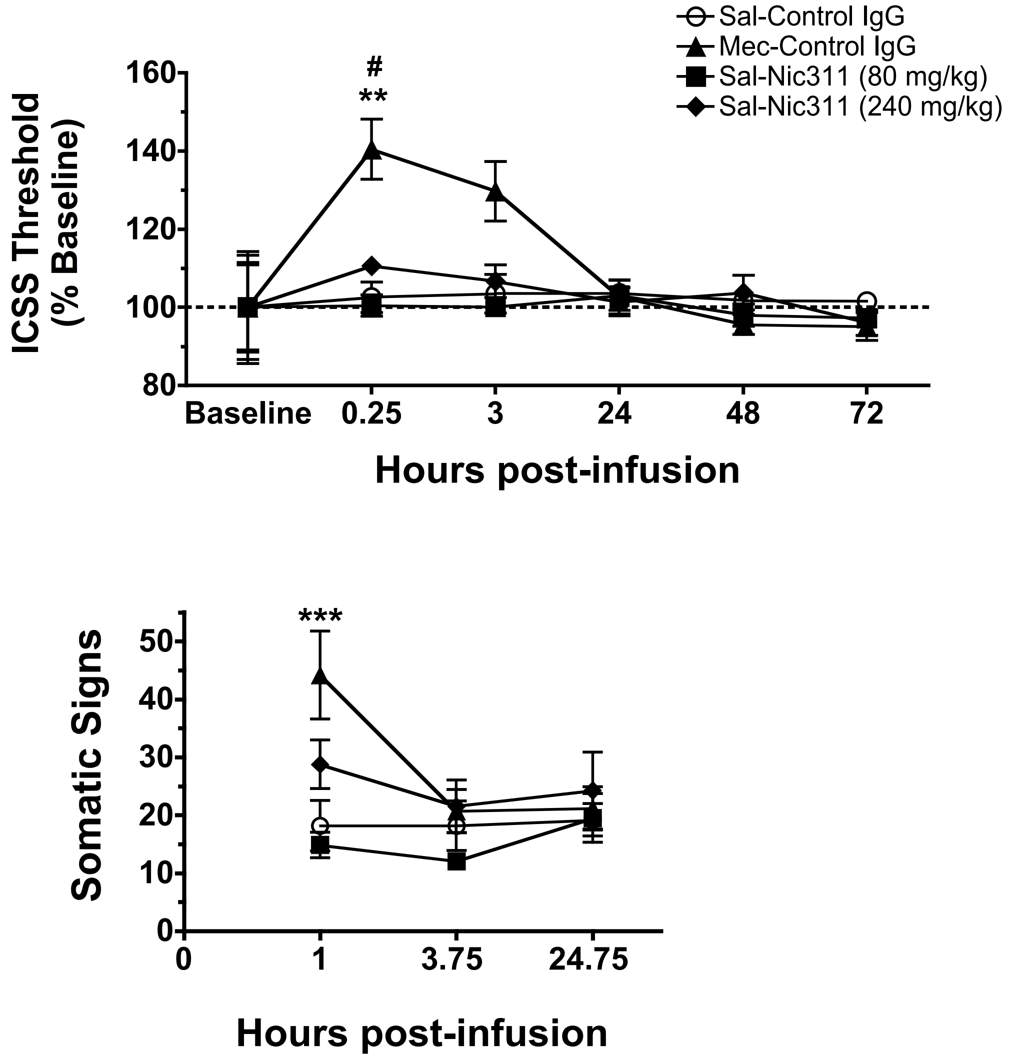


Figure 3. ICSS thresholds and Somatic signs after treatment

(Top) ICSS thresholds (as percent of baseline, mean ± SE) following administration of either i.v. Control IgG or Nic311, and s.c. mecamylamine or saline. **, $p < 0.01$ compared to Saline and Nic311 80mg/kg at 0.25 hours; # $p < 0.05$ compared to baseline. (Bottom) Somatic withdrawal signs observed during the first 25 hr post-infusion . ***, $p < 0.001$ compared to Control IgG + saline and Nic311 80 mg/kg + saline.



CHAPTER 6

Concluding Comments

This chapter provides concluding comments for this dissertation and discusses passive immunization to treat nicotine addiction.

1. Introduction

Most studies of immunotherapy for nicotine addiction have focused on nicotine vaccines, because vaccination against nicotine is relatively simple (using standard protocols for creating hapten-linker-immunogens), inexpensive, presumably safe, and long lasting. Data from preclinical and clinical studies using vaccination against nicotine demonstrated efficacy for attenuating nicotine's addictive effects and increasing smoking cessation. However, the efficacy of the nicotine vaccines are limited because of low mean serum NicAb levels that are highly variable, and an apparent upper limit to the amount of NicAb produced. In addition, there is a delay before the presence of substantial NicAb levels in serum.

Previously, passive immunization was used mostly as an experimental convenience, because it provided control over the dose and timing of serum antibody concentrations. These experimental advantages of passive immunization also provide significant potential advantages for clinical use. Passive immunization can circumvent the limitations of vaccination because the control of antibody dose allows the administration of antibody doses as high as deemed necessary, and each individual can be administered the same dose of antibodies to minimize variability in serum NicAb levels. There is no delay in efficacy because the administered antibodies are immediately effective upon administration.

The overall goal of this thesis was to examine the use of passive immunization as a therapy to treat nicotine dependence. Specific questions regarding the effects of passive immunization on nicotine pharmacokinetics and behavior were addressed in the five specific aims of this thesis: how passive immunization affects the pharmacokinetics of nicotine in the presence of Nic311, the pharmacokinetics of Nic311, the behavioral efficacy of passive immunization against nicotine and a potential clinical role for passive immunization, and the potential for precipitating nicotine withdrawal.

2. Implications of findings

2.1 Aim 1:

Passive immunization reduced the clearance and volume of distribution of an acute nicotine dose, which prolonged the elimination half-life of nicotine. Since reducing nicotine clearance can reduce smoking and may potentially increase quit-rates, this points to one mechanism by which passive immunization may reduce nicotine's addictive effects.

2.2 Aim 2:

Nic311 clearance and volume of distribution were significantly reduced in the presence of nicotine. This is the first report of which we are aware that demonstrates altered antibody pharmacokinetics in the presence of a small molecule antigen. The underlying mechanism for this is unknown. In this experiment, the elimination half-life of Nic311 in the presence of nicotine was not altered. Three rats exhibited a substantial decline in

serum Nic311 levels, and the mechanism for this is also unknown. These findings indicate that in clinical use in the setting of chronic nicotine exposure, nicotine most likely will not result in greater dosing frequency of passive immunization, and demonstrate the necessity of confirming antibody serum levels.

2.3 Aim 3 & 4:

Findings of the studies described here support the potential clinical benefits of passive immunization against nicotine. The efficacy of vaccination and passive immunization in attenuating nicotine-induced locomotor sensitization provide further evidence that immunotherapy attenuates nicotine's behavioral effects. This reduction in nicotine's behavioral effects is due to antibodies binding nicotine in serum and reducing the distribution of an acute nicotine dose to the brain. Results of this study suggested that antibodies from either passive immunization or vaccination are equally efficacious, and the quantity of antibodies appears to be more important than the source, to the point that combining vaccination and passive immunization resulted in significantly greater effects on brain nicotine levels and nicotine-induced locomotor sensitization. This points toward possible uses of passive immunization in clinical settings that are discussed below.

2.4 Aim 5:

Passive immunization did not precipitate nicotine withdrawal in the model of nicotine dependence used here, suggesting that withdrawal may not be a potential adverse effect if passive immunization was used clinically with nicotine-dependent individuals who were active smokers. This study also demonstrated a dissociation between brain nicotine

levels and the onset of withdrawal – at the time-points when brain nicotine levels were substantially reduced, no evidence of nicotine withdrawal was detected. No studies to date have fully addressed how changing the rate and extent of reducing brain nicotine levels affects the onset of nicotine withdrawal. This indicates that the relationship between nicotine elimination kinetics and withdrawal is complex.

3. Potential Clinical uses of Passive Immunization in treating nicotine dependence

The general goal of this thesis was to study the potential role of passive immunization as a treatment for tobacco addiction. Passive immunization could be used alone or in combination with vaccination or other current pharmacotherapies as a smoking cessation therapy. This section will speculate on the potential ways passive immunization could be used in clinical settings.

3.1 Passive immunization as an alternative to vaccination

Passive immunization could be used as a stand-alone therapy, but this is currently problematic because of the high cost associated with the production, purification, and administration of antibodies. New processes and technologies are under development to produce and purify antibodies for passive immunotherapies, and the use of passive immunization to treat smoking cessation may someday have a more reasonable price. For this reason, it is important and pertinent to demonstrate the efficacy of passive immunization alone, in addition to considering possible clinical uses.

3.2 Passive immunization in combination with vaccination: a target concentration strategy

Passive immunization might also serve as a potential strategy to enhance the efficacy of vaccination by combining vaccination with passive immunization. One potential role of passive immunization is to supplement those individuals who respond poorly to vaccination, providing an additive effect, since the source of NicAbs did not seem to alter efficacy. Measuring serum NicAb levels after vaccination could identify those individuals who have medium or low serum NicAb levels, and passive immunization could supplement the vaccine-generated NicAbs to provide effective serum NicAb levels.

Data in this thesis suggest there may be a target NicAb concentration above which efficacy in altering nicotine pharmacokinetics and reinforcement remains constant. Further studies are needed to corroborate this finding. If this hypothesis is confirmed clinically, this provides a guideline to determine how much supplementation is required to reach a certain threshold for effective serum NicAb levels.

3.3 Passive immunization as a means of rapidly initiating immunotherapy

Another limitation of vaccination is the 6-8 week delay in generating effective serum NicAb levels. The use of passive immunization at the beginning of a vaccination regimen could provide short-term protection during the delay in generating vaccine-elicited serum NicAb levels. This would be expensive since larger doses of NicAbs would be required, but would be less expensive compared to passive immunization as a monotherapy. One consideration is that passive immunization may reduce the efficacy of a nicotine vaccine

if administered in this manner. Passively administered NicAbs may bind the nicotine vaccine immunogen, and prevent it from being available for antigenic processing, resulting in a diminished immune response. This question needs to be addressed before the combination of passive immunization and vaccination would be used clinically.

3.4 Combining immunotherapy with other medication classes

An additional potential clinical use of passive immunization (and also vaccination) is in combination with one of the currently available pharmacotherapies for smoking cessation. Immunotherapy alters nicotine's pharmacokinetics and reinforcing properties, but may not reduce craving when nicotine is no longer present. Therapies such as bupropion or varenicline can attenuate craving, and provide additive effects in attenuating nicotine's effects in the brain. Immunotherapy (active or passive) and these pharmacotherapies thus appear to have complementary actions, and further studies are needed to characterize how vaccination or passive immunization could be combined with other medication classes.

4. Passive immunization as an experimental tool

Passive immunization provides an experimental tool to study immunotherapy's mechanism of action. For example, passive immunization allows the study of dose-effect relationships, whereas this is challenging to do using vaccination. Passive immunization also can be used as an experimental tool to examine the role of drug pharmacokinetics in addiction. Studies in this thesis demonstrated that reducing nicotine distribution to the

brain attenuated nicotine's psychoactive effects in a locomotor sensitization model, but did not precipitate a nicotine withdrawal syndrome. The use of passive immunization enabled the detection of this novel finding, and indicates a need for further study of the role of brain nicotine levels and nicotine withdrawal.

5. Concluding remarks

Tobacco addiction is the result of a complex set of environmental, behavioral, and pharmacological factors. Each of these factors can be altered to attenuate the addictiveness of tobacco. This thesis provides additional evidence to support the role of nicotine pharmacokinetics in tobacco addiction. Altering nicotine pharmacokinetics attenuates nicotine's addictive properties and can serve as a strategy to help individuals quit smoking. Passive immunization provides a safe and effective strategy to attenuate nicotine's addictive effects, and has the potential to serve in multiple ways as an additional clinical tool to help individuals quit smoking.

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