

# Enhancing the efficacy of a nicotine vaccine

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## **Overview**

Tobacco addiction is the leading cause of preventable death worldwide. Many people continue to smoke cigarettes despite clear detrimental health effects. Available smoking cessation therapies are only partially effective, making new treatment approaches necessary to increase smoking cessation rates. Immunization against nicotine features a different mechanism of action than currently available medications. As a pharmacokinetic antagonist, immunization against nicotine alters distribution, metabolism, and clearance of nicotine to attenuate nicotine-induced behavior in animal models. Nicotine vaccines in clinical trials show efficacy but are limited by the modest and highly variable nicotine-specific antibody (NicAb) concentrations produced. This thesis focuses on ways to improve efficacy of a nicotine vaccine by combining it with additional forms of immunotherapy.

The first aim of this thesis examined the effects of supplementing vaccination against nicotine with individualized doses of Nic311, a nicotine-specific monoclonal antibody. Compared to either immunotherapy alone, combining active and passive immunization produced greater alterations in nicotine pharmacokinetics and nicotine-induced behavior using a locomotor activity model. Only small doses of Nic311 were necessary to supplement vaccine-generated NicAb concentrations to a previously effective threshold. This

decreased cost and use of typically expensive monoclonal antibodies, potentially increasing viability of this approach in a clinical setting.

The second aim of this thesis examined the effects of concurrent administration of two immunologically distinct nicotine immunogens in a bivalent vaccine over a range of vaccine formulations and immunization conditions. Immunogens were co-administered in a bivalent vaccine without compromising immunogenicity of either immunogen when delivered subcutaneously in alum, but not when delivered intraperitoneally in Freund's adjuvant. When combined in alum, immune responses elicited by the two immunogens were largely independent of one another. This suggests that subjects who responded poorly to one immunogen may have responded better to the second, immunologically distinct immunogen in the bivalent vaccine. These results indicate that the bivalent vaccine strategy is a feasible way to increase antibody concentrations above what can be achieved using one immunogen alone, but integrity of the response is highly dependent on vaccine formulation and administration conditions.

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## **List of Abbreviations**

5-HT:	5-hydroxytryptamine (serotonin)
AAV:	adeno-associated virus
ACh:	acetylcholine
ANOVA:	analysis of variance
APC:	antigen-presenting cell (macrophage, dendritic cell, B cell)
B <sub>max</sub> :	maximal ligand binding capacity
BSA:	bovine serum albumin
CRM:	mutant diphtheria toxoid, derived from <i>Corynebacterium diphtheriae</i>
CYP:	cytochrome P-450
Da:	daltons
DA:	dopamine
ELISA:	enzyme-linked immunosorbent assay
FCA:	Freund's complete adjuvant
FIA:	Freund's incomplete adjuvant
GABA:	γ-aminobutyric acid
Glu:	glutamate
i.m.:	intramuscular
i.p.:	intraperitoneal
i.v.:	intravenous
IgA:	immunoglobulin isotype A (α)
IgG:	immunoglobulin isotype G (γ)
IgM:	immunoglobulin isotype M (μ)
K <sub>d</sub> :	equilibrium dissociation constant
KLH:	keyhole limpet hemocyanin, derived from <i>Megathura crenulata</i>
mAb:	monoclonal antibody
MHC:	major histocompatibility complex
NAcc:	nucleus accumbens
nAChR:	nicotinic acetylcholine receptor
NE:	norepinephrine
Nic311:	nicotine-specific monoclonal antibody 311
NicAb:	nicotine-specific antibody
NRT:	nicotine replacement therapy
PCP:	phencyclidine
rEPA:	recombinant exoprotein A, derived from <i>Pseudomonas aeruginosa</i>
s.c.:	subcutaneous
T <sub>h</sub> 1:	T helper cells responsible for the cell-mediated immune response
T <sub>h</sub> 2:	T helper cells responsible for the humoral immune response
TT:	tetanus toxoid, derived from <i>Clostridium tetani</i>
VTA:	ventral tegmental area

# **Chapter 1**

## **Introduction**

**This section represents a summary of background information relevant to the aims in this thesis**

## **1. Tobacco smoking: Why new smoking cessation therapies are needed**

Tobacco smoking is the leading cause of preventable death worldwide. Over 5 million people die annually due to smoking-related causes, including nearly 450,000 in the United States [1]. Despite the clear relationship between cigarette smoking and adverse health effects such as cardiovascular disease, many people continue to smoke. Over 50% of current tobacco smokers in the United States try to quit annually but are largely unsuccessful even with the benefit of available medications [2].

The main drawback of current smoking cessation therapies is that they are only partially effective, achieving smoking cessation rates of approximately 30% at best [3, 4]. In addition, these treatments target receptors or neurotransmitters in the brain, potentially altering normal, endogenous brain processes. This limits the dose of drug that can be used. Although higher doses increase abstinence rates, they also increase the potential for adverse side effects. Available treatments also require frequent dosing for effect. Due to drawbacks such as these, patient compliance and successful quit rates with currently available medications are limited.

To increase smoking cessation rates beyond what can be achieved with current medications, new approaches to smoking cessation therapy are necessary. Ideally, a new approach would feature a peripheral site of action to minimize

centrally mediated adverse effects and would require infrequent dosing to increase patient compliance. Immunization is a treatment concept that fits these parameters and can be targeted against small molecules such as nicotine. Rather than altering nicotine's complex actions in the brain, active immunization (vaccination) against nicotine targets the drug itself and acts as a pharmacokinetic antagonist to limit drug access to the brain in a therapeutically beneficial manner.

## **2. Nicotine**

Several of the nearly 7,000 compounds in tobacco smoke contribute to the addictive qualities of cigarette smoking. However, nicotine is recognized as the primary psychoactive component contributing to tobacco addiction [5]. Addiction is defined by the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (fourth edition) as "a maladaptive pattern of substance use leading to clinically significant impairment or distress," manifested by three or more characteristics including but not limited to: the development of tolerance, the development of withdrawal, the occurrence of failed quit attempts, and/or the reduction in social, occupational, or recreational activities as a direct result of substance use [6]. Although addiction is often used interchangeably with 'substance dependence,' substance dependence is primarily characterized by physiological conditions such as withdrawal and tolerance. Tobacco smoking fits these descriptions as an addiction or substance dependence for several reasons:



acute tolerance develops to many of the subjective and cardiovascular effects of smoking, withdrawal develops upon cessation in chronic smokers, and smokers are often unable to quit despite harmful consequences.

Vaccines against nicotine act as a pharmacokinetic antagonist, altering nicotine pharmacokinetics and nicotine-induced behavior. To understand the design and rationale of a vaccine against nicotine, the pharmacokinetics and mechanism of action of nicotine and how they relate to addiction must first be understood.

### 2.1 Nicotine pharmacokinetics

#### *Nicotine dose*

The dose of nicotine administered and the plasma nicotine concentrations that arise are important for the rewarding or reinforcing qualities of the drug. This makes plasma drug concentration a primary target of immunization. Nicotine-specific antibodies are too large to enter the brain but can bind to nicotine in plasma and decrease the overall dose of drug that enters the brain; this mechanism is discussed in further detail in a later section. In animal studies, the effect of nicotine dose on nicotine self-administration responding is highlighted by an inverted U-shaped curve, where very high and very low doses of drug result in low self-administration response rates (reviewed in [7, 8]). Typically, high nicotine doses are aversive and low nicotine doses do not adequately activate reward and reinforcement circuitry in the brain. This suggests that there is an ideal range

of nicotine doses that produce reward and reinforcement. Decreasing the dose of nicotine able to enter the brain may therefore reduce the rewarding or reinforcing qualities of the drug.

In humans, only about 10% (1 mg) of the 10 mg of nicotine in an average cigarette is absorbed upon smoking [9] (for an extensive review of nicotine pharmacokinetics, see [10]). Smoking one cigarette results in a mean arterial nicotine concentration of 20-50 ng/ml and a mean venous nicotine concentration of 10-30 ng/ml about five minutes after smoking, although a wide range of plasma nicotine concentrations has been reported [11-14]. Nicotine does not appreciably bind plasma proteins (<10% bound in serum) and is small at 162 Da. As a result, most of the nicotine that is absorbed remains unbound and easily able to enter the brain [15].

The entire nicotine dose does not need to be bound for nicotine-specific antibodies to be effective. Drug-specific antibodies have shown efficacy even at antibody-drug molar equivalent ratios as low as 0.01 in overdose/rescue scenarios (i.e. drug is present prior to antibody) or 0.015 in cessation/relapse prevention scenarios (i.e. antibody is present prior to drug) [16, 17]. In acute nicotine administration studies, the binding of nicotine by antibodies slows the rate of nicotine entry into the brain and decreases the overall dose of nicotine that enters the brain. In chronic nicotine administration studies, the total dose of

nicotine administered can overwhelm the population of nicotine-specific antibodies over time, but antibodies are still able to slow the rate of drug entry into brain for a short time after each administration of nicotine. Effectively decreasing the rate and dose of nicotine entering the brain alters two pharmacokinetic parameters important for nicotine's addictive qualities.

### *Nicotine absorption and distribution*

The rate and extent of absorption and distribution of nicotine also play key roles in tobacco addiction. The rapid rate at which nicotine enters the brain contributes to the addictive qualities of the drug, making the rate of drug entry into brain an excellent target for immunization [18, 19]. During smoking, nicotine from tobacco is rapidly absorbed into the pulmonary vein. Nicotine from cigarettes reaches the brain rapidly, as soon as 5-7 seconds after inhalation, reaching a peak brain concentration within 1-5 minutes [13, 20]. Compared to fast infusion of i.v. nicotine (5 seconds), slow infusion of i.v. nicotine (25-100 seconds) in animal models results in lower nicotine self-administration response rates and decreased expression of neuronal activity marker transcription factor c-fos. This suggests that when drug is administered slowly, reward or reinforcement pathways are not activated in the same manner as when nicotine is administered quickly [21] (reviewed in [22]). Studies for other commonly abused drugs such as cocaine have shown a similar relationship between infusion rates and drug self-administration responses [23, 24]. In contrast, some studies indicate that longer

infusion durations produce higher drug self-administration responding rates [25, 26]. However, conditions were not the same across all studies, indicating that factors other than infusion duration also play a significant role in drug-induced behavior.

Despite this conflicting information, many nicotine replacement therapies deliver drug slowly to reduce abuse potential of the medication. Absorption of nicotine from a transdermal nicotine patch is slow, due in part to diffusion of nicotine through the polymer matrix or membranes of the patch and also to slower absorption kinetics of nicotine through dermal tissue (in comparison to when nicotine is inhaled). As a result, nicotine patches are not as reinforcing as cigarettes and do not have significant abuse liability even though they can potentially produce plasma nicotine concentrations similar to those in cigarette smokers [27-29].

#### *Nicotine metabolism*

Rates of nicotine metabolism influence smoking behavior, making nicotine metabolism an additional target of immunization against nicotine. Nicotine is primarily metabolized in the liver by enzymes of the cytochrome P450 family such as CYP2A6, and to a lesser extent CYP2B6 and CYP2E1. CYP2A13, an extrahepatic enzyme nearly identical to CYP2A6 is also involved in nicotine

metabolism, but is primarily responsible for metabolic activation of tobacco-specific carcinogens in lung tissue [30, 31].

Polymorphisms decreasing the rate of nicotine metabolism by CYP2A6 in humans have been correlated with fewer cigarettes smoked per day and fewer withdrawal symptoms than in subjects with normal nicotine metabolism. Similarly, the fraction of smokers with these polymorphisms, considered 'slow metabolizers,' has been shown to decrease with age [32]. Authors of these studies suggested that slow metabolizers were therefore less dependent and/or more likely to quit smoking than 'normal metabolizers' or 'fast metabolizers' [33-35] (for reviews on the effect of CYP2A6 genetic variation on smoking behavior, see [36] and [37]). Nicotine metabolism has been shown to decrease with age, although studies have reported conflicting data on changes in pharmacokinetic parameters such as nicotine clearance [38, 39]. One study reported an inverse relationship between the fraction of smokers considered slow metabolizers and the number of years as a smoker, supporting a potential link between nicotine metabolism and smoking cessation rates [37]. Overall, this suggests that nicotine metabolism is a worthwhile target for nicotine vaccines and that the ability of vaccines to slow nicotine metabolism is of potential benefit in reducing smoking rates.

In humans, 70-80% of nicotine is oxidized to cotinine, which is then further oxidized to 3'-hydroxycotinine prior to elimination. Remaining nicotine is metabolized primarily to nicotine-N-oxide, nornicotine, or nicotine glucuronide in a similar manner. Nicotine metabolites (such as cotinine) generally have low potency at nicotinic acetylcholine receptors compared to their parent compound or if active, are present in inconsequential quantities (such as nornicotine) and therefore have minimal impact on tobacco addiction [40-43]. This somewhat simplifies immunization against nicotine, as a vaccine would only need to target nicotine and not its metabolites; metabolism of several other addictive drugs often results in active metabolites, potentially complicating vaccine design (this subject is described in more detail below).

Humans and rats have similar nicotine pharmacokinetic parameters. Although the proportion of nicotine metabolized to cotinine is somewhat less in rats than in humans (50% versus 80%), this still remains the primary pathway of nicotine metabolism in both species. Somewhat accounting for this difference, a larger fraction of absorbed nicotine is metabolized to nicotine-N-oxide in rats than in humans (for more information on pharmacokinetics of nicotine in rat models, see [44-46]). The half-life of nicotine is also fairly similar between humans and rats (2 hours in humans, 1 hour in rats). Other pharmacokinetic parameters of nicotine such as hepatic and renal clearance rates and steady state volume of distribution are also comparable between humans and rats, making rats an excellent model

to investigate the effects of immunization against nicotine on nicotine pharmacokinetics. This information is summarized in Table 1.

In summary, nicotine is the primary component of cigarettes responsible for the addictive qualities of tobacco smoking, making it a good target for immunization. The dose administered, the rate of administration, and the rate of metabolism all contribute significantly to the addictive qualities of nicotine. As a pharmacokinetic antagonist, vaccination against nicotine targets all three of these parameters to alter nicotine pharmacokinetics and as a result, nicotine-induced behavior.

## 2.2 Nicotine mechanism of action

The ultimate goal of immunization against nicotine is to alter nicotine-induced behavior. Because behavior is a result of both nicotine pharmacokinetics as well as nicotine pharmacodynamics, to further understand the design and rationale of vaccination against nicotine, it is important to understand nicotine's site and mechanism of action.

### *Centrally mediated actions of nicotine*

Nicotine from cigarettes reaches the brain rapidly, binding to nicotinic acetylcholine receptors (nAChRs) located throughout the brain. These receptors are composed of  $\alpha$  ( $\alpha$ 2-10) and  $\beta$  ( $\beta$ 2-4) subunits in a pentameric configuration either as homomers composed of a single  $\alpha$  subtype, or as heteromers

composed of a mixture of  $\alpha$  and  $\beta$  subtypes [47-49]. Upon binding to a nAChR, nicotine induces an initial brief activation period and subsequently a more prolonged desensitization in which the receptor is unresponsive for up to 6 hours [50]. Upon activation, nAChRs modulate synaptic release of neurotransmitters such as dopamine (DA), glutamate (Glu),  $\gamma$ -aminobutyric acid (GABA), acetylcholine (ACh), and serotonin (5-hydroxytryptamine; 5-HT). Nicotinic acetylcholine receptors also modulate influx of sodium and calcium and efflux of potassium.

Nicotine exerts its effects in several brain regions, including but not limited to the ventral tegmental area (VTA) and nucleus accumbens (NAcc). Projections from the VTA to the NAcc are considered part of the mesolimbic system, which is important in appetitive behavior and reward; other brain regions involved in the mesolimbic system include the amygdala and prefrontal cortex [51, 52]. Nicotine binds to nAChRs on dopaminergic neurons in the VTA that project to the NAcc, stimulating DA release in the NAcc. The binding of nicotine to nAChRs on GABA-ergic neurons also stimulates DA release. GABA typically inhibits DA release, but because nAChRs desensitize (and become inactive) shortly after binding nicotine, GABA transmission is halted and DA transmission is no longer inhibited. Accumbal DA release is important for nicotine-related reward and reinforcement (for reviews on the role of DA in drug reward and reinforcement, see [53, 54]). As a primary reinforcer, nicotine establishes other stimuli as secondary (conditioned)



reinforcers, indicating that when repeatedly paired with a particular context or cue, the context or cue also comes to predict nicotine reward as effectively as nicotine itself [55-58]. Rodents without dopaminergic signaling in the NAcc due to chemical or electrolytic lesion do not self-administer nicotine or most other addictive drugs, highlighting the importance of this pathway in reward and reinforcement [59-61].

Nicotine-induced signaling also occurs in several addiction-relevant brain regions outside of the VTA-NAcc tract. Nicotine-related signaling in the medial prefrontal cortex and anterior cingulate cortex affects the emotional response to stimuli such as nicotine-associated contexts or cues. These regions are also important for nicotine-related signaling involved in cognition and decision-making (for extensive reviews of nicotine-related signaling in the brain, see [62-64]). Additionally, stress signaling between the central amygdala, bed nucleus of the stria terminalis, and the hypothalamus plays a significant role in withdrawal and reinstatement/relapse to addictive drugs such as nicotine [65].

The majority of nAChRs found in the brain are of the  $\alpha 4\beta 2^*$  subtype (\* refers to potential additional subunits), which contain a high affinity binding site for nicotine ( $K_d = 2-4$  nM compared to 40-80 nM for lower affinity receptor subtypes) [66]. Nicotine's high affinity for nAChRs is an important consideration in vaccine design. Nicotine-binding affinity of vaccine-generated antibodies must be high

enough to compete with non-specific and nAChR binding of nicotine in the brain. If vaccine-generated antibodies have a low nicotine-binding affinity, higher concentrations of antibodies would be necessary to bind enough nicotine to compete as described above. As a primary limitation of nicotine vaccines is the modest concentrations of antibodies produced, nicotine-binding affinity remains an important characteristic.

#### *Peripherally mediated actions of nicotine*

Although nicotine's psychoactive effects are mediated centrally, nicotine also acts peripherally. In naïve subjects, low doses of nicotine have been shown to constrict blood vessels, increase heart rate, increase myocardial contractility, and increase blood pressure. These effects are primarily due to activation of the sympathetic nervous system and resulting modulation of norepinephrine (NE) release (reviewed in [10, 67]); with repeated exposure to nicotine throughout the day, acute tolerance often develops to these effects.

#### *Maintenance of smoking behavior*

Continual smoking behavior is driven by several different components, including positive affect, negative affect, and secondary (conditioned) reinforcers. In humans, smoking a cigarette often has mood-enhancing effects and can induce drug "liking," satisfaction, and anxiolytic qualities. In addition, nicotine is also thought to increase cognitive function and attention; these cognitive effects are

mediated primarily by desensitization of nicotinic receptors on glutamatergic neurons signaling to the medial prefrontal cortex and orbitofrontal cortex [68-70].

The first cigarette of the day may be important in reinforcing the link between smoking and the positive subjective effects described above. Although nicotine can accumulate in the brain during waking hours, the majority is eventually transported away and metabolized overnight, allowing nAChRs to re-sensitize. By morning, unbound nAChRs are available for activation by nicotine and are able to activate reward-related circuitry again. Nicotine intake from one cigarette can bind the majority of high affinity  $\alpha 4\beta 2$  nAChRs in the brain [50]. Due to the phenomenon of nAChR desensitization, acute tolerance develops to many nicotine-induced positive subjective effects throughout the course of the day. Further smoking behavior releases additional nicotine into the brain but the majority of it is unable to activate already desensitized nAChRs [50]. As discussed below, dependent smokers continue to smoke throughout the day for several other reasons, including prevention of acute withdrawal and the effects of secondary reinforcers such as smoking-associated cues or contexts.

Control of smoking behavior during the day is often transferred from initial enhancement of positive subjective affect to prevention of acute withdrawal or negative affect. In dependent smokers, most nAChRs remain desensitized throughout the day. One reason that smokers continue their nicotine intake

during the day despite this is to prevent receptors from re-sensitizing and precipitating an acute withdrawal syndrome. Abrupt cessation of nicotine administration results in a decrease in reward-related signaling by neurotransmitters such as DA and Glu. During withdrawal, neurotransmitters involved in brain stress systems such as corticotropin-releasing factor and NE manage negative affective states and are responsible for feelings of anhedonia, anxiety, depression, and dysphoria [71-73]. Over the long term, the severity of these symptoms contributes to the lack of successful cessation attempts for most smokers.

Secondary or conditioned reinforcers also influence continual smoking behavior. Conditioned reinforcers, such as contexts and cues continually associated with nicotine, come to predict nicotine reward in a manner similar to administration of nicotine. For example, in rats, cues previously contingent upon nicotine administration were shown to be more likely to induce reinstatement of nicotine self-administration compared to a priming dose of nicotine itself [74]. In humans, non-nicotine factors such as sensory cues play a significant role in the subjective response to smoking as well; subjects that received i.v. nicotine reported less satisfaction or reward than subjects that smoked nicotine-containing or de-nicotinized cigarettes [7, 75-77]. This suggests that exposure to contexts or cues previously associated with smoking plays a key role in maintaining smoking behavior in current smokers.

### **3. Currently marketed therapies**

Currently available medications to assist subjects in quitting smoking include nicotine replacement therapy, bupropion, and varenicline. Combining use of one of these medications with counseling appears to produce the highest smoking cessation rates in humans. As cessation rates in humans are approximately 5-30%, current treatments remain only partially effective at best and leave much room for improvement [4, 78, 79].

#### *3.1 Nicotine replacement therapy*

Nicotine replacement therapy (NRT) is the most commonly used smoking cessation treatment and can be purchased in various forms, such as gum, lozenge, patch, nasal spray, and inhaler. The constant low levels of nicotine provided by NRT address nicotine craving and prevent precipitation of withdrawal [80, 81]. However, NRT does not make cigarette smoking less pleasurable in the event of relapse, nor does it control urges to smoke in response to conditioned cues or contexts [82]. One of the primary factors affecting the success of NRT is the frequent dosing necessary. Because it has such a short half-life, nicotine in gum, lozenge, or spray form must be administered daily or several times per day. This results in poor patient compliance and limited smoking cessation rates. Additionally, some smokers feel that using NRT as a cessation tool merely replaces nicotine in cigarette form with nicotine in another form, and fails to

address addiction to nicotine itself. With abstinence rates averaging only 5-20%, the main point is that current forms of NRT are not very effective [83-85]. For reviews on NRT, see [79, 86].

### 3.2 Bupropion

Initially marketed as an anti-depressant owing to its ability to inhibit NE and DA reuptake, bupropion has also been found to inhibit nAChRs. In humans, bupropion decreases nicotine craving and nicotine abstinence-related anhedonia and dysphoria associated with withdrawal [87-89]. The central site of action limits use of bupropion since its mechanism of action can alter normal endogenous brain function. Higher, more effective doses are limited by adverse side effects such as insomnia, dry mouth, and increased incidence of seizures. Like NRT, bupropion also requires daily dosing, potentially limiting patient compliance. Smoking cessation rates with bupropion are marginally higher than rates in NRT users, but are at best only around 20-30% [4, 78].

### 3.3 Varenicline

Varenicline is the newest of the currently marketed smoking cessation therapies. A cytisine analog with partial agonist activity at high affinity  $\alpha 4\beta 2$  nAChRs, varenicline also has full agonist activity at lower affinity  $\alpha 3\beta 4$  and  $\alpha 7$  nAChRs. The partial agonist activity results in low to moderate stimulation of the  $\alpha 4\beta 2$  nAChRs (approximately 25-50% DA release compared to nicotine), preventing

spontaneous withdrawal (for reviews on varenicline, see [90, 91]). A unique action of varenicline is that by binding nAChRs as its primary mechanism of action, it prevents nicotine binding and therefore prevents nicotine reinforcement and reward in the event of relapse. Unfortunately, like nicotine, varenicline has been shown to up-regulate or increase the number of functional nAChRs, potentiating DA release in the mesolimbic pathway and potentially sustaining neuroadaptations responsible for subjective effects such as craving during nicotine withdrawal [92-94]. In humans, varenicline prevents cue-induced increases in neuronal activity in addiction-relevant brain regions and decreases nicotine craving and pleasure [95-97]. As with other smoking cessation therapies, varenicline acts on receptors in the brain, potentially altering normal brain function. Administration of higher, more effective doses of varenicline is limited by adverse side effects such as severe nausea, skin reactions, depression, and an increased incidence of suicide (although the link between varenicline use and suicide is controversial) [91, 98, 99]. Although varenicline use is associated with a smoking cessation rate of up to 30% by one year, this still leaves much room for improvement [3, 78, 95].

#### 3.4 Treatments currently in development

Several other options for treating nicotine dependence are presently in development. Modulators of DA, 5-HT, NE, GABA, and Glu neurotransmission as well as various receptor agonists and antagonists have been progressing in

animal models (for an extensive review on treatments currently in development for nicotine dependence, see [100] or [101]). Similarly, currently marketed treatments for other, unrelated ailments have been studied for off-label use in nicotine dependence [102] (reviewed in [103]). Although some of these concepts have shown promise in animal models, administration of effective doses has been limited by several potentially serious centrally-mediated adverse side effects; these treatments also require frequent dosing like most currently available medications.

In summary, currently available smoking cessation therapies do not produce substantial cessation rates, highlighting the necessity for a new approach. Smokers who have quit without medication or counseling typically experience a one-year success rate of 3-10%, and those that have combined one of the three available medications with counseling increase this percentage up to only 30% at best [104-109]. Most treatments currently available or in development act on receptors or neurotransmitters in the brain, altering normal brain function and typically only modulating only one of nicotine's many downstream effects. Most treatments also require frequent dosing, sometimes several doses per day, limiting efficacy due to poor patient compliance. A new approach is necessary to develop more effective medications. Ideally, this approach acts peripherally, has few to no adverse side effects, and requires infrequent dosing. Immunization,



which targets the drug rather than the brain, is a treatment concept that fits these guidelines.

### *3.5 Overview of immunization as a treatment for addiction (Figure 1)*

Immunization against nicotine features a completely different approach than available smoking cessation medications. Immunization can be achieved by either active immunization (vaccination) or passive immunization. Active immunization involves administration of an immunogen (a compound which elicits an immune response) directly to a subject; exposure to the immunogen stimulates the subject's immune system to mount a response, resulting in specific antibodies against some component of the immunogen (e.g. nicotine). In contrast, passive immunization refers to the transfer of exogenously derived antibodies to a subject and does not require action from the subject's immune system. Active and passive immunization against nicotine both produce nicotine-specific antibodies. At 150 kDa, these antibodies are too large to cross the blood brain barrier and do not appreciably enter the brain [110]. Nicotine-specific antibodies bind free nicotine in serum. As a result, the rate and extent of nicotine entering the brain is decreased. Binding nicotine in serum also reduces the free nicotine concentration available for metabolism and clearance. Overall, decreasing the metabolism and clearance of nicotine increases its half-life, allowing nicotine to be present in the body for an extended period of time in a manner similar to the 'slow metabolizer' CYP2A6 enzyme variants discussed

previously. In rats, these effects on nicotine pharmacokinetics manifest as alterations in nicotine-induced behaviors such as decreased nicotine self-administration rates and decreased nicotine-induced locomotor activity [111, 112].

#### **4. Active Immunization (Figure 2)**

Active and passive approaches to immunization against nicotine have been studied and both appear to have merit. Active immunization has been the primary focus of immunotherapy against nicotine, due in part to the long-term protection conferred and the excellent safety profile of existing vaccines. Passive immunization is addressed in a subsequent section.

##### 4.1 Immune response produced by active immunization

Active immunization, or vaccination, involves the administration of an immunogenic material to a subject to stimulate the subject's immune system and produce an adaptive immune response. Compounds too small to elicit an immune response themselves, such as nicotine, are coupled to large, foreign 'carrier' proteins by short, multi-carbon 'linker' arms to enhance immune stimulation. The carrier-linker-compound complex forms the immunogen of a conjugate vaccine; the linker-compound portion is considered the hapten (see Figure 3). In conjugate vaccines, the hapten is also considered the antigen, as it is the component of the immunogen that antibodies are typically directed against.

For most nicotine vaccines, the immunogen consists of several nicotine molecules attached to a carrier protein by short 4-10 carbon linkers; this structure is discussed in detail in a later section.

Each component in a conjugate vaccine plays a distinct role. The role of the carrier protein is to facilitate uptake of the hapten (i.e. the antigen) by antigen presenting cells (APCs). Overall, carrier proteins must be large (typically > 10 kDa) to allow phagocytosis of the immunogen by dendritic cells and macrophages or to facilitate cross-linking of the B cell receptor (composed of surface immunoglobulin) to enable uptake by B cells. Carrier proteins must also be foreign, as utilizing a carrier protein that is too similar to endogenous proteins could potentially stimulate an immune response that attacks the host. In addition, fragments of the carrier protein also function as T cell helper epitopes, activating T cells and facilitating activation of B cells. The role of the linker is to extend the compound of interest away from the surface of the carrier protein so that the carrier does not interfere with recognition of the compound by B cell receptors. The role of the hapten is to act as the structure that antibodies are intended to be directed against. In summary, the main goal of immunization with a conjugate vaccine is to produce antibodies directed against the hapten, which contains the compound of interest (e.g. nicotine).

There are several steps involved in the induction of an immune response by a conjugate vaccine. Upon immunization, the immunogen is initially bound and internalized by an APC such as a dendritic cell, macrophage, or hapten-specific B cell. Once inside the APC, the entire immunogen is enveloped in a vesicle and degraded into 13-18 amino acid peptides or fragments; these fragments are considered T cell helper epitopes. One of these epitopes binds to a major histocompatibility complex class II (MHC II) receptor inside the vesicle. This epitope-MHC complex is then exposed on the outside of the APC. The APC is transported from the injection site to secondary lymphoid organs where it binds to the T cell receptor of a nearby CD4+ T cell. The T cell receptor must be specific for the particular epitope that is presented by MHC on the APC. Binding of the T cell receptor and the epitope-MHC complex activates the T cell and induces differentiation and proliferation. Once activated, the T cell can differentiate into different "T-helper" subtypes. T<sub>h</sub>1 cells activate a cell-mediated immune response and T<sub>h</sub>2 cells activate a humoral immune response. T<sub>h</sub>2 cells are important for activation of naïve B cells and subsequent production of hapten-specific antibodies, making them of more interest for conjugate vaccines.

The next stage of immune stimulation activates naïve B cells and produces hapten-specific antibodies designed to bind and inactivate the hapten. The activated CD4+ T cell attracts and activates a naïve B cell. For proper activation, this naïve B cell must have already completed a number of steps: hapten on the

surface of the immunogen must have been bound by the B cell receptors, the immunogen must have been internalized and degraded, a T cell helper epitope from the immunogen must have been bound and presented on the MHC receptor of the B cell, and the epitope-MHC complex must have been presented on the outside of the B cell. The epitope presented by the B cell must be similar to the epitope presented by the APC that initially activated the T cell to allow proper binding between the epitope-specific T cell receptor on the T cell and the epitope-MHC receptor complex on the naïve B cell. Once bound and activated, the B cell can differentiate and proliferate, forming short-lived plasma B cells that produce antibodies to the hapten, or long-lived memory B cells that continue to recognize hapten via B cell receptors.

The isotype of antibodies produced in response to the immunogen depends on a number of additional mechanisms. Initially, plasma B cells produce immunoglobulin isotype M (IgM) antibodies, forming pentamers with low specific binding affinity. After the initial immunization, the majority of memory B cells have B cell receptors that are composed of low affinity surface immunoglobulin similar to IgM. However, a small number of B cells experience class switching and affinity maturation after only one exposure to the immunogen. This allows these B cells to eventually produce antibodies with greater affinity and specificity, increasing the efficacy of the immune response during the second and subsequent immunizations. During class switching,  $T_H2$  cells release cytokines

that signal for recombination of particular regions on genes that encode antibodies; this recombination results in production of different immunoglobulin isotypes such as IgA and IgG. These antibodies have greater specificity and affinity than IgM due to affinity maturation.

Affinity maturation is the result of two processes: somatic hypermutation and clonal selection. Somatic hypermutation involves mutations in particular coding sequences of antibody genes that are responsible for binding antigen; these domains are referred to as the 'complementarity-determining regions.' Clonal selection of B cells involves competition of B cell clones for resources such as antigen; B cells with receptors that have higher affinity for antigen as a result of somatic hypermutation will outcompete B cells with receptors that only have low affinity for antigen. The overall result is production of antibodies with greater affinity. Importance of antibody characteristics such as specificity and affinity is discussed in more detail in the next section.

#### 4.2 Characteristics of an antibody response

The major characteristics defining the ability of antibodies to bind a particular antigen are specificity, affinity, and concentration, all of which generally increase over subsequent immunizations. The specificity of an antibody refers to its ability to bind one particular antigen or hapten compared to other compounds. High specificity is particularly important when the antigen is similar to endogenous

compounds since it may be detrimental to bind and neutralize endogenous neurotransmitters. This is particularly important for nicotine vaccines because antibodies must bind nicotine but not ACh. Affinity is a measure of the strength in which the antibody binds the antigen, often represented by the dissociation constant,  $K_d$ . The dissociation constant corresponds to the concentration of antigen at which 50% of the antibody population is bound. High affinity antibodies ( $K_d$  in the nM range) bind antigen tightly, whereas low affinity antibodies ( $K_d$  in the  $\mu$ M range) bind antigen loosely and are more likely to dissociate from antigen. Greater nicotine-specific antibody concentrations suggest a more robust immune response and are typically able to bind more free nicotine in serum. All three characteristics are necessary for antibodies to effectively alter nicotine pharmacokinetics in a beneficial manner.

#### 4.3 Adjuvants

Adjuvants are immunopotentiators that increase the magnitude of an immune response. These are particularly important for vaccines that produce only modest antibody concentrations when given alone.

Freund's complete adjuvant (FCA) is commonly used in animal studies of vaccines because it induces a robust immune response. FCA is mainly composed of heat-killed mycobacteria (muramyl dipeptide, a peptidoglycan component of cell walls) suspended in mineral oil with the addition of mannide

monooleate as a surfactant to aid in antigen presentation (for a review on Freund's adjuvants, see [113] or [114]). Freund's incomplete adjuvant, FIA, consists of the same components as FCA without the heat-killed mycobacteria. In FCA or FIA, the immunogen is suspended in the aqueous phase of a water-in-oil emulsion. The mechanisms of action of FCA and FIA are not well studied, but emulsifying the immunogen is known to induce prolonged release of the antigen and to increase neutrophil and macrophage recruitment to the injection site. Unfortunately, as FCA and FIA cause local necrotizing dermatitis and granuloma formation at the injection site, in the liver, and in the kidneys, these adjuvants are not acceptable for human use. Despite this, FCA and FIA are still commonly used in animals and remain some of the most effective immunopotentiators available. These adjuvants provide an excellent tool to increase the magnitude of the immune response in early proof-of-principle animal studies.

The variety of adjuvants available for use in humans is relatively small. Nearly all adjuvants used in human vaccines consist of aluminum salts, such as aluminum hydroxide, aluminum phosphate, or potassium aluminum sulfate (alum). Most aluminum salt adjuvants induce an immune response considered moderate at best, particularly when compared to more robust immunopotentiators such as FCA/FIA in animal models. Clinical trials of nicotine and cocaine vaccines containing aluminum salt adjuvants (described in detail below) have resulted in only modest drug-specific antibody concentrations, often partially attributed to the



relatively mild effect of these adjuvants [115, 116]. AS-04, a new aluminum salt adjuvant, is the first adjuvant approved for human use that contains a toll-like receptor-activating component. Activation of toll-like receptors, which typically recognize molecular patterns on foreign pathogens, induces cytokine signaling to enhance activation of the immune response.

The mechanism of action of aluminum salts is different than that of FCA/FIA. It was recently reported that aluminum salt adjuvants increase duration and magnitude of antigen presentation [117, 118]. It has also been shown that aluminum salts induce necrosis in local cells; this produces uric acid and other danger signals, resulting in increased recruitment of immune cells to the injection site [119]. Additionally, studies have reported that aluminum salt adjuvants stimulate antigen uptake through the binding of lipid moieties on dendritic cells, inducing signaling cascades that enhance the immune response [120]. Although use of these adjuvants has produced lower antibody concentrations in animal models of vaccination than other adjuvants, aluminum salts tend to induce fewer local injection site reactions, making them acceptable for human use.

## **5. Immunization against nicotine: Active**

Immunization against nicotine is a novel treatment approach compared to current anti-smoking medications. Advantages of active immunization (vaccination) over currently available smoking cessation therapies include the long-term protection

conferred (as vaccine-generated IgG has a half-life of 1-2 months in humans), the infrequent dosing necessary (on a monthly basis for continual protection), and the historically positive safety profile of currently available vaccines [116, 121, 122]. Perhaps the most important advantage to vaccination is its peripheral site of action. Most smoking cessation medications act within the brain, affecting receptors or transporters that mediate other normal endogenous brain functions such as cognition, memory, and movement. Altering these receptors or transporters also alters related processes and signaling, creating unwanted side effects and limiting the efficacy of medications that act centrally. Because immunization targets the drug rather than the brain, there is no interference with normal brain function and no centrally mediated adverse side effects. However, vaccination also has disadvantages, such as the long period of time required to develop a robust immune response and the high degree of individual variability in vaccine-generated antibody concentrations.

### 5.1 Components of a nicotine vaccine

Vaccine design is highly focused on improving the specificity, affinity, and concentration of vaccine-generated antibodies. All three attributes contribute to the pharmacokinetic antagonism of nicotine. Altering a variety of vaccine components, such as the carrier protein or the linker structure can have significant effects on antibody specificity, affinity, and concentration.

As described previously, the small size of nicotine requires conjugation to a carrier protein to elicit an immune response. A variety of proteins have been conjugated to nicotine, including recombinant exoprotein A (rEPA) of *Pseudomonas Aeruginosa*, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), mutant diphtheria toxoid (CRM), and tetanus toxoid (TT) [123-126]. A relative hierarchy of immunogenicity of carrier proteins is unclear, although KLH remains a common choice due to its large size [127].

Varying linker length, composition, and attachment position to nicotine can greatly affect the immunogenicity of an immunogen. Longer linkers allow more flexibility of the attached nicotine molecule, reducing steric hindrance by the carrier protein and potentially increasing binding of hapten to B cell receptors. One study reported that conjugates with linkers that are 6-8 carbons or more in length produce higher titers of antibodies with greater specificity to nicotine compared to conjugates with shorter linkers [128]. The position on nicotine at which the linker attaches to the drug also plays a significant role in the characteristics of vaccine-generated antibodies. Attaching a linker component to the pyridine ring of nicotine has been suggested to increase the immunogenicity of the pyrrolidine ring and to increase overall nicotine-specific antibody (NicAb) titers. Because most nicotine metabolism occurs on the pyrrolidine ring, presenting the pyrrolidine ring as the 'face' or antibody-binding epitope of the

drug may increase specificity for nicotine and decrease potential cross-reactivity of antibodies with any nicotine metabolites [128, 129].

### 5.2 Characteristics of nicotine-specific antibodies

Immunization against nicotine has produced high affinity antibodies specific for nicotine. Recent generations of nicotine vaccines have generated antibodies with an average nicotine-binding affinity of 10-50 nM, which is similar to the nicotine-binding affinity of nAChRs [123, 130, 131]. Most vaccine-generated antibodies have been highly specific for nicotine, with minimal or no cross-reactivity to metabolites or endogenous compounds such as ACh [125, 128]. Nicotine-specific antibody (NicAb) concentrations produced in rat models can range from 50-1270 µg/ml, but typically average 100-300 µg/ml [131, 132].

### 5.3 Mechanism of action

The primary mechanism of action of immunization against nicotine is that the NicAbs bind nicotine in serum, reducing free drug concentration and preventing or slowing distribution of nicotine to the brain. At 150 kDa, antibodies are too large to cross the blood-brain barrier, therefore nicotine bound by antibody is unable to enter the brain. Because drug-induced reward and reinforcement are somewhat dependent on rate and extent of drug entry into the brain, slowing the rate and reducing the overall dose of the drug entering the brain lessens the ability of the drug to stimulate reward or to reinforce the associations between

drug taking and reward. This is supported by a series of studies that reported inhibition of accumbal DA release, prevention of nicotine-induced reward, and altered nicotine self-administration response rates in rats vaccinated against nicotine [133-135].

#### 5.4 Effects of vaccine-generated nicotine-specific antibodies on nicotine pharmacokinetics

Nicotine-specific antibodies act as a pharmacokinetic antagonist, altering a variety of pharmacokinetic parameters of nicotine. Nicotine-specific antibodies generated by vaccination with the 6-CMUNic-KLH immunogen increased the half-life of nicotine in serum by 6-fold, decreased the volume of distribution of nicotine by 2-4-fold, and decreased the clearance of nicotine by 10-fold [136]. In acute studies, NicAbs have slowed and prevented nicotine from entering the brain, decreasing brain nicotine content by approximately 40-90% depending on nicotine dose and NicAb concentrations [124, 137, 138]. In chronic, long-term studies, the primary mechanism of NicAb action is that antibodies slow accumulation of nicotine in brain. When rats received continuous nicotine infusions of 1 mg/kg/day for 70 days via s.c. osmotic pump (resulting in clinically relevant serum nicotine concentrations of 10-40 ng/ml), total brain nicotine content was decreased by only 29% [139]. However, when the last dose of nicotine in that study was radiolabeled with tritium to enable detection, NicAbs significantly decreased brain levels of the radiolabeled nicotine dose in a manner

similar to that reported in acute nicotine vaccine studies [139]. This highlights that the initial binding and slowing of drug entry to brain by vaccine-generated NicAbs for each nicotine dose is key for the effects of the vaccine during chronic nicotine intake. Additionally, these studies indicate that the vaccine is effective during both acute and chronic nicotine administration protocols.

#### 5.5 Effects of vaccine-generated nicotine-specific antibodies on nicotine-induced behavior

Nicotine vaccines attenuate a variety of nicotine-induced behaviors. A number of different nicotine vaccines have been shown to inhibit development or expression of locomotor sensitization to nicotine when clinically relevant doses of nicotine were administered (0.3-0.5 mg/kg/day, equivalent to 1-2 packs per day) [124, 140, 141]. Although locomotor activity is not an addictive behavior per se, many of the neural pathways involved in locomotor activity overlap with those involved in reward or reinforcement, lending a predictive validity to this model for use as a relatively high-throughput screening tool (reviewed in [142]). Vaccination with the 3'-AmNic-rEPA nicotine immunogen decreased acquisition and maintenance of nicotine self-administration in a protocol that delivered nicotine doses shown to maintain nicotine self-administration behavior in a highly sensitive manner [111]. Similarly, de Villiers et al., 2002 showed that vaccination with the nicotine immunogen IP18-KLH attenuated nicotine-induced DA overflow in the NAcc, inhibiting part of the signaling cascade necessary for the rewarding and

reinforcing qualities of nicotine [133]. This data highlights the ability of nicotine vaccines to reduce the reinforcing effects of nicotine in animal models.

### 5.6 Clinical trials of nicotine vaccines

Four nicotine vaccines have entered into clinical trials: NicVAX (nicotine conjugated to rEPA [3'-AmNic-rEPA] which is also used in the experiments described in the aims of this thesis), Niccine (nicotine conjugated to inactive tetanus toxoid), TA-NIC (nicotine conjugated to cholera toxin subunit B), and Nic-Q $\beta$ , formerly known as Nic-002 (nicotine conjugated to a virus-like particle, the non-infectious coat protein of bacteriophage Q $\beta$ ) [116, 143-147]. Results have been somewhat similar across all nicotine vaccines, although reports from clinical trials involving Niccine and TA-NIC have not been formally published.

Modest and highly variable nicotine-specific antibody (NicAb) concentrations limit the effectiveness of nicotine vaccines in humans. Antibody concentrations produced by nicotine vaccines appear to be dependent on vaccine dose; antibody concentrations have generally increased as vaccine dose increased. However, NicAb concentrations generated by NicVAX plateau at vaccine doses above 200  $\mu$ g. Antibody concentrations did not increase significantly when the dose of NicVAX was increased from 200  $\mu$ g to 400  $\mu$ g [116]. Even with such high vaccine doses, the highest NicAb concentrations in humans that received NicVAX were only approximately 60  $\mu$ g/ml [143]. This is much lower than typical

NicAb concentrations reported in animals (100-300 µg/ml) [112, 130, 131, 137]. Human vaccine doses are much smaller than those used in animals. A vaccine dose of 400 µg in an average 70 kg human yields 5.7 µg vaccine/kg body weight, but a vaccine dose of 25 µg (a typical dose used in animal studies) in an average 300 g rat yields 83.3 µg vaccine/kg body weight. This disparity may contribute to the difference between average NicAb concentrations produced in humans (30-45 µg/ml) and rats (100-300 µg/ml) [131, 143]. Unfortunately, limitations on the amount of aluminum salt adjuvants acceptable for use in humans prevents larger vaccine doses from being administered [148].

Results from clinical trials of NicVAX and Nic-Qβ reported that subjects with the highest antibody concentrations (representing approximately 30% of total subjects receiving nicotine vaccines) had significantly higher smoking cessation rates compared to placebo [143, 144]. Smoking cessation rates in the remaining subjects that received nicotine vaccines were not different from subjects that received placebo vaccines. When smoking cessation rates were averaged across all subjects immunized against nicotine, overall abstinence rates in subjects that received nicotine vaccines were not different than in those that received placebo vaccines [143, 144].

Nicotine vaccines do show some promise in humans. Across most clinical trials, subjects did not increase smoking behavior as a way to compensate for or



overwhelm NicAb concentrations, perhaps owing to the high motivation of most trial participants to quit smoking [116, 144]. In addition, vaccination against nicotine did not precipitate nicotine withdrawal, potentially due to the gradual rise and effect of NicAbs [116]. Across all published clinical trials, there were no significant adverse effects associated with vaccination against nicotine [116, 129, 143, 144, 146].

Overall, clinical trial results suggest that the primary limitations to vaccination against nicotine are the modest NicAb concentrations produced and the high degree of individual variability in these antibody concentrations.

## **6. Active immunization against other drugs of abuse**

Nicotine is not the only widely abused drug that has been studied as a target for immunization. Vaccines against cocaine, methamphetamine, and opioids are also in various stages of development. The design and efficacy of these vaccines have been similar to that of nicotine vaccines; this suggests that the approaches used in the specific aims of this thesis (described in a later section) could potentially be used to benefit not only nicotine vaccines, but other addiction vaccines as well.

### **6.1 Cocaine vaccines**

Like nicotine vaccines, cocaine vaccines have been well studied in animal models but have shown limited efficacy in clinical trials [115, 149-153]. Only one cocaine vaccine, Celtic Pharma's TA-CD (succinylnorcocaine conjugated to CT<sub>B</sub>), has entered clinical trials. In one trial, vaccine doses of 360 µg produced a cocaine-specific antibody response in 98% of immunized subjects, but only about 30% of those subjects reached the targeted effective cocaine-specific antibody concentration of 43 µg/ml [115]. This 'high responder' subset of subjects produced more cocaine-free urine samples (i.e. a reduction in use, not abstinence) during the 16 week immunization protocol than subjects producing lower mean antibody concentrations [115]. However, when all subjects that received the cocaine vaccine were included in analysis, there was not a significant difference in the proportion of cocaine abstinent subjects between those that received the cocaine vaccine and those that received the placebo vaccine [115]. As with nicotine vaccines, efficacy of cocaine vaccines also seems to be limited by modest drug-specific antibody concentrations produced.

### 6.2 Methamphetamine vaccines

Vaccines against methamphetamine have produced variable success in animal models, but are not yet in clinical stages. Metabolites of methamphetamine as well as several similar compounds such as 3,4-methylenedioxymethamphetamine (MDMA, or ecstasy) are psychoactive. The variety of potent metabolites and methamphetamine-like compounds highlights a

challenge not experienced in nicotine vaccine design. With nicotine vaccines, metabolites and similar compounds are of minimal concern; however, antibodies generated by methamphetamine vaccines must have a broader specificity to bind a variety of potent, active targets. Current methamphetamine vaccines have produced moderate levels of antibodies with high affinity for both methamphetamine and amphetamine [154]. Pre-clinical reports of the effects of vaccines on methamphetamine-induced behavior have been mixed, potentially due to compensation and increased drug intake to overwhelm the moderate levels of drug-specific antibodies produced [155-157].

### 6.3 Opioid vaccines

Opioid vaccines were the first substance abuse vaccines studied. Early studies involving heroin and morphine vaccines by both Berkowitz and Bonese instigated interest in addiction vaccines, although investigation of immunization against heroin was halted in the 1970's as other promising opioid addiction treatments such as methadone entered the field [158, 159]. Vaccination against opioids is challenging, because not only does metabolism of heroin yield active metabolites, but there are also several opioid compounds commonly used as antinociceptives. To address this, a variety of immunogens targeting different subsets of similarly structured opioids have been produced, allowing for vaccination against some, but not all opioids [160-163]. Vaccines have been shown to produce antibodies specific for particular opioids, often with minimal

cross-reactivity with other similar compounds [161, 162]. In pre-clinical studies, vaccination against opioids has attenuated a variety of opioid-induced behaviors [162, 164, 165].

Nicotine is a better target for immunization than most other addictive drugs for a variety of reasons. First, typical daily drug doses are much smaller for nicotine, averaging 0.25-0.75 mg/kg/day for smokers compared to 7-10 mg/kg/day for methamphetamine users, 1-7 mg/kg/day for heroin users, and 5-20 mg/kg/day for cocaine users [9, 166-170]. This suggests that a smaller quantity of drug-specific antibodies would be necessary to effectively alter nicotine distribution and nicotine-induced behavior compared to other addictive drugs. In addition, nicotine's metabolites and similar endogenous compounds are largely inactive or have low potency at nAChRs. In contrast, active metabolites of cocaine, methamphetamine, and heroin, as well as active compounds similar to these drugs can potentially complicate vaccine design. Nicotine users are also less likely to switch to other similar drugs if nicotine reward is blunted since metabolites and similar compounds do not have comparable rewarding or reinforcing effects. In contrast, methamphetamine and opioid users are more likely to switch to other drugs since there is a wide library of similar euphoria-inducing compounds. Lastly, because the number of smokers worldwide is substantial, the potential health benefit of a nicotine vaccine greatly outweighs that of vaccines against other addictive drugs.

## **7. Passive immunization**

Passive immunization refers to the passive transfer of pre-formed specific antibodies and is another method of immunization against drugs of abuse that has shown merit in preclinical animal models. Advantages of passive immunization include the immediate effect, as there is no reliance on the host to produce an immune response, as well as the ability to administer very high doses of pre-formed antibodies (up to 4-5g/kg in animal models) without antibody-mediated toxicity [171, 172]. Passive immunization also allows for tight control of antibody concentrations and the ability to pre-select antibodies with high specificity and affinity for the target of choice. As with antibodies generated by vaccination, antibodies delivered via passive immunization (e.g. IgG) bind drug in serum and do not enter the brain. Because passively administered antibodies act peripherally, there are no centrally mediated adverse side effects and no effect on normal brain function. Disadvantages to passive immunization include the relatively short half-life of passively administered antibodies (approximately 7 days in rats, 21 days in humans), as well as the high cost of antibody production (for in-depth reviews on antibody kinetics in humans and rats, see [173] and [174]). In rats, 80 mg/kg of nicotine-specific monoclonal antibody (mAb) Nic311 has been shown to effectively attenuate nicotine-induced behavior. In an average 70-kg human, a comparable effect would require 5600 mg of this mAb. Combining the average mAb production cost of \$1-10/mg with the relatively short

half-life of mAbs in humans currently limits the practicality of using passive immunization against nicotine in a clinical setting [175].

Passive immunization can involve delivery of an entire polyclonal antibody population or of a subset of antibodies selected for a particular characteristic, such as affinity (i.e. monoclonal antibodies). Administration of polyclonal antibodies as a treatment for disease is not as clinically translatable as administration of monoclonal antibodies due to the unpredictability of antibody characteristics such as affinity or specificity in a polyclonal antibody population. Also, production in large-scale quantities is limited for polyclonal antibodies. In contrast, monoclonal antibodies, which stem from one particular B cell clone, can be produced in considerable quantities. Despite high production costs, monoclonal antibodies are currently available or in development to treat autoimmune diseases and several forms of cancer (for more information on polyclonal and monoclonal antibodies, see: [176-178]). Presently, 20 mAbs are available for human use while nearly 500 are in various stages of clinical trials [177].

### 7.1 Passive immunization against nicotine

Passive immunization has been examined as a treatment for addiction for several decades but has only recently been used to target nicotine. In one study, polyclonal IgG developed from vaccination with the 3'-AmNic-rEPA immunogen

was administered in rats to compare effects of passive immunization with active immunization; results were comparable between the two approaches, which suggested that nicotine-specific antibodies (NicAbs) act similarly regardless of source [124]. Monoclonal antibodies (mAbs) against nicotine, such as Nic311 ( $K_d = 60$  nM), have produced dose-dependent effects in animal models, sequestering nicotine in serum and decreasing distribution of nicotine to brain [179]. Nicotine-specific mAbs have also attenuated nicotine-induced locomotor activity and nicotine discrimination [140, 141, 180, 181]. These effects appear to be somewhat dependent on mAb affinity for nicotine, as Keyler et al., 2005 showed that mAbs with higher nicotine-binding affinity produced greater effects on distribution of nicotine in serum and brain than mAbs with lower nicotine-binding affinity [179].

New approaches to passive immunization against nicotine continue to develop in pre-clinical animal models. Recently, the cDNA for production of nicotine-specific mAb Nic9D9 was encapsulated in an adeno-associated viral vector (AAV) and delivered in mice, mediating persistent and continual host expression of the Nic9D9 mAb [180]. Monoclonal antibodies produced by this gene attenuated nicotine-induced cardiovascular effects and locomotor activity in mice when tested 7 weeks after immunization [180]. This approach to passive immunization addresses the limitation of short half-life in mAbs, potentially increasing translation of passive immunization against nicotine to a clinical setting.

## 7.2 Passive immunization against cocaine

Monoclonal antibodies against cocaine have also shown efficacy in animal models. Most recently, when anti-cocaine mAb GNC92H2 was delivered in an AAV vector and inserted into the host genome, high concentrations of high affinity (6 nM for cocaine) cocaine-specific antibodies were produced for 6 months following immunization [182]. Much like immunization with Nic9D9-AAV, using an AAV vector for cocaine mAb delivery required only one immunization and resulted in long-term effects. Gene therapy may become a more popular immunization approach with further development of AAV vectors as delivery vehicles.

Catalytic antibodies represent a different approach to passive immunization, harnessing naturally occurring enzymes designed to catalyze the transformation of drug to inactive metabolites. As with cocaine vaccines or traditional cocaine mAbs (i.e. IgG), catalytic antibodies against cocaine have also decreased cocaine distribution to brain and attenuated cocaine-induced behavior [183, 184]. This approach is of less interest for nicotine, as nicotine is not metabolized by simple esterases as in the case of cocaine, but highlights that the field of passive immunization as a potential treatment for addiction is not limited to using only vaccine-generated mAbs.



### 7.3 Passive immunization against methamphetamine

Monoclonal antibodies against methamphetamine have also shown some promise in preclinical animal models. Like methamphetamine vaccines, mAbs have produced mixed effects on methamphetamine-induced locomotor activity and self-administration [185-188]. Passive immunization against methamphetamine in rats has indicated that methamphetamine IgG mAbs have a much shorter half-life (<4 days) than most other IgG mAbs directed against addictive drugs (6-8 days). Authors of these studies have speculated that this discrepancy may be due to mAb binding of methamphetamine transition state analogs or recycling by FcRn receptors, both resulting in lower circulating mAb concentrations available to bind drug [189, 190]. The short half-life of these antibodies currently hinders further methamphetamine mAb development.

### 7.4 Passive immunization against phencyclidine

Antibodies against phencyclidine (PCP) have been a potential treatment against PCP overdose for several decades [16, 191, 192]. Studies have reported that even low doses of anti-PCP antibodies, at antibody-drug molar equivalent ratios of 0.25 and even as low as 0.01, can prevent PCP-induced toxicity when pre-administered and can rescue from PCP-induced toxicity when administered immediately after the drug [16, 193, 194]. Although the precise mechanism behind the function of anti-PCP antibodies at such low antibody-drug molar equivalent ratios is unknown, it is speculated that the antibodies preferentially

draw PCP out of the brain faster than other tissues since PCP equilibration between serum and brain is faster than between serum and other tissues [16, 195].

Currently, clinical translation of passive immunization as a treatment for addiction is limited. Together, the short half-lives and the high production cost of these antibodies limit the practicality of using passive immunization in humans. Although the cost may be less prohibitive in overdose situations requiring a single dose of mAb, the cost of even a single dose remains high.

#### **8. Specific aims of this thesis**

The antibody response generated by nicotine vaccines, while offering long-term protection, is also highly variable due to its dependence on the ability of host cells respond effectively to vaccine components. Clinical trials of nicotine vaccines exemplify this, as vaccine efficacy is limited primarily by the modest and highly variable NicAb concentrations produced. Combining active immunization against nicotine with an additional treatment may circumvent the current limits of vaccination; however, current literature on such approaches is minimal. This thesis presents two approaches to produce higher and less variable NicAb concentrations.

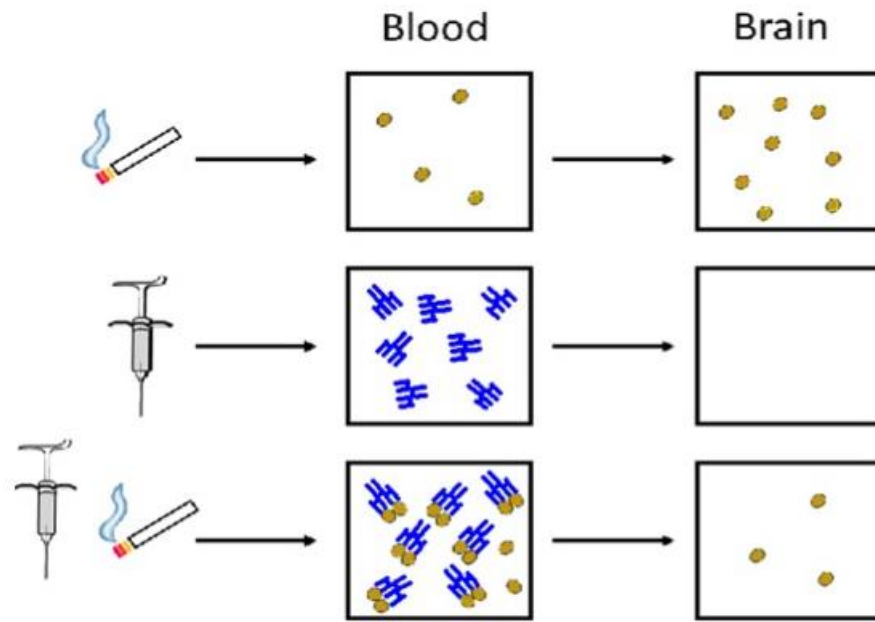
The first approach to producing higher and less variable NicAb concentrations combined vaccination against nicotine with passive immunization in an individualized manner. Only two studies exploring the effects of combining active and passive immunization have been published. These studies combined large, fixed doses of monoclonal or catalytic antibodies with vaccination [141, 196]. Because the high cost of monoclonal antibodies makes use of large, fixed mAb doses less viable in a clinical setting, it was important to adapt this approach using an individualized manner. Individualizing mAb administration by administering just enough mAb to boost vaccine-generated NicAb concentrations to reach an effective threshold could potentially decrease the cost and use of the mAb. This strategy was further investigated in the first specific aim of this thesis.

The second approach to producing higher and less variable NicAb concentrations co-administered two immunologically distinct nicotine vaccines as a bivalent vaccine across a range of formulation and administration conditions. Literature on multivalent vaccines targeting small molecules has been minimal. One study that explored the potential benefits of a bivalent opioid vaccine combined two opioid immunogens, but these immunogens targeted different compounds rather than the same one, and it is important to know whether multiple immunogens can be combined to produce antibodies specific to a single target as small as nicotine [160]. A separate study examined the effects of combining two immunologically distinct nicotine immunogens into a bivalent

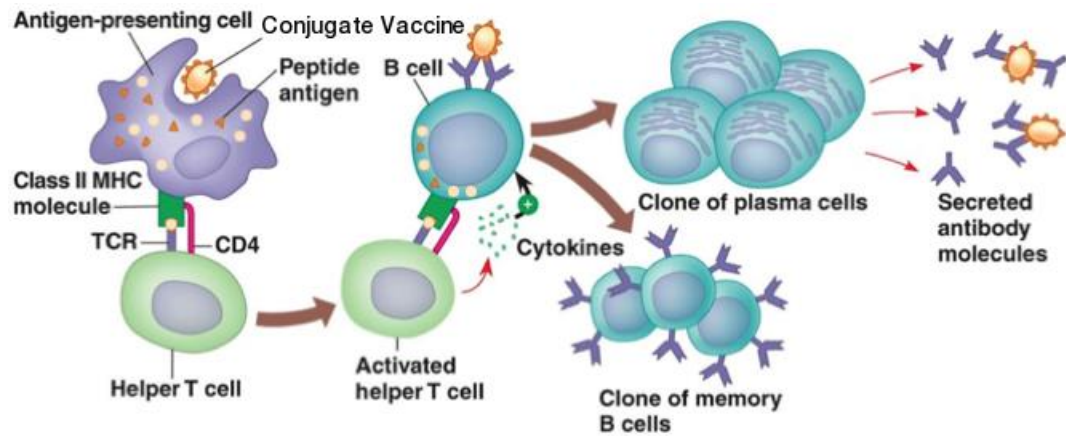
nicotine vaccine. However, doses of immunogens were not matched between the monovalent and bivalent vaccines, making it unclear whether the increased NicAb concentrations in rats that received bivalent vaccines were due to the combination of immunogens or simply to higher immunogen doses [197]. To determine whether combining two immunogens is more effective than simply increasing the dose of one immunogen, it was important to compare NicAb characteristics generated by monovalent and bivalent vaccines at matched total immunogen doses. Additionally, to investigate whether immunogenicity of either immunogen is compromised upon concurrent administration in a bivalent vaccine, it was important to compare NicAb titers and concentrations generated by immunogens in monovalent vaccines to their dose-matched contribution in bivalent vaccines. A bivalent vaccine strategy incorporating these ideas was explored further in the second aim of this thesis.

		Human (70 kg)	Rat (0.3 kg)
Total Clearance	$Cl_t$ (L/hr/kg)	$1.1 \pm 0.3^a$	$2.9 \pm 0.4^b$
Hepatic Clearance	$Cl_h$ (L/hr/kg)	$0.9 \pm 0.2^a$ ( $f = 0.82$ )	$2.7 \pm 0.5^b$ ( $f = 0.93$ )
Renal Clearance	$Cl_r$ (L/hr/kg)	$0.2 \pm 0.1^a$ ( $f = 0.18$ )	$0.2 \pm 0.1^b$ ( $f = 0.07$ )
Volume of Distribution	$V_{d,ss}$ (L/kg)	$2.6 \pm 0.8^a$	$3.7 \pm 0.6^b$
Elimination Half-life	$t_{1/2\beta}$ (hr)	$2 \pm 0.7^a$	$0.9 \pm 0.1^b$
% Bound in Plasma	(%)	$4.9^a$	$9.0 \pm 0.5^c$
Fraction Unbound	$f_u$	$0.95^a$	$0.91^c$

**Table 1.** Nicotine pharmacokinetic parameters in the human and in the rat.  
<sup>a</sup>[15], <sup>b</sup>[46], <sup>c</sup>[198]

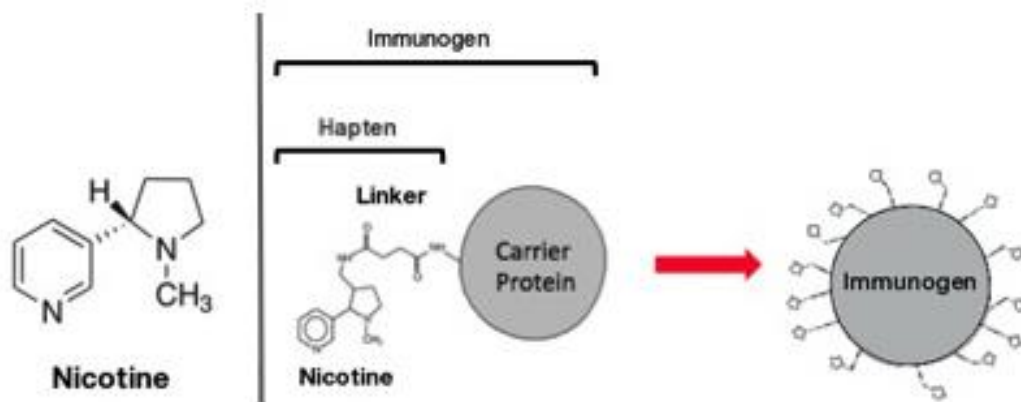


**Figure 1.** Overview of the effect of immunization on nicotine distribution to brain.



**Figure 2.** Overview of immune response generated by a conjugate vaccine.

This figure has been adapted for this thesis from: Campbell NA & Reece JB (2007). *Biology*. Benjamin Cummings Publishing Co, San Francisco.



**Figure 3.** Components of a conjugate vaccine.



## **Chapter 2**

### **Overall goals and aims**

**This section introduces the specific aims of this thesis and explains how subsequent chapters (3 and 4) address the aims of this thesis**

## **1. Overall goal**

The overall goal of this thesis was to improve efficacy of a nicotine vaccine by increasing NicAb concentrations and decreasing overall variability using clinically relevant strategies. The specific goals of this thesis explored the combination of a monovalent vaccine against nicotine with either passive immunization or with another monovalent nicotine vaccine in animal models. Efficacy of supplementing vaccination with a monoclonal antibody in an individualized manner was determined using a nicotine-induced locomotor activity model and measuring alterations in nicotine pharmacokinetics. The immune response generated by a bivalent nicotine vaccine across varying vaccine formulation and administration conditions was characterized using several immunoassays. These studies explored the immunologic, pharmacokinetic, and behavioral effects of combining vaccination with additional immunotherapy and provide insight into methods by which nicotine vaccine efficacy can be increased. Both approaches have potential for translation to a clinical setting and can be easily adapted to improve efficacy of other addiction vaccines as well.

## **2. Specific aims and hypotheses**

Specific aim #1 was to adapt a combination immunotherapy protocol into a more clinically translatable approach. Although combining vaccination and passive immunization against nicotine using monoclonal antibody Nic311 has previously shown to be more effective than vaccination alone, use of a monoclonal antibody

remains limited by high production cost [141, 175]. Therapeutic drug monitoring is a treatment approach in which drug levels are continuously monitored and therapy is administered on an individualized basis; this approach is commonly used in clinical settings. The combination immunotherapy protocol was adapted to apply this strategy. Vaccine-generated antibody concentrations were continuously monitored and Nic311 was administered on an individual basis as necessary to reach a target total antibody concentration previously determined to show efficacy in a locomotor model. Nicotine-induced locomotor sensitization was used to study the behavioral effects of this approach. The hypothesis tested was that combining vaccination against nicotine with small doses of Nic311 administered on an individualized basis would prevent sensitization to nicotine's locomotor activity enhancing effects and decrease nicotine distribution to brain more effectively than vaccination alone.

Specific aim #2 was to expand previous findings regarding a bivalent nicotine vaccine to include a range of immunogen doses and vaccine formulation and administration conditions that more accurately reflect clinical immunization procedures. A previous study suggested that vaccine efficacy was enhanced when two immunologically distinct nicotine immunogens were combined into a bivalent vaccine [197]. However, vaccine doses were not matched between the bivalent and monovalent vaccines, making it unclear whether the enhanced efficacy of the bivalent vaccine was due to the combination of immunogens or

simply to larger immunogen doses. In addition, the previous study was designed in a proof-of-concept manner and utilized a vaccine formulation and administration condition that was not acceptable for use in humans. The previous approach was therefore extended to include a range of immunogen doses to compare monovalent and bivalent vaccines at equivalent total immunogen doses as well as to examine any potential compromise in immunogenicity when equivalent immunogen doses are delivered either alone as a monovalent vaccine or combined with another immunogen as a component of a bivalent vaccine. Vaccines were delivered using formulation and administration conditions that are commonly used in animals as well formulation and administration conditions commonly used in humans. The hypothesis tested was that a bivalent vaccine would produce higher nicotine-specific antibody (NicAb) levels than equivalent doses of either monovalent vaccine in both animal- and human-relevant conditions.

### **3. Summary of how chapters 3 and 4 address these aims**

Chapter 3 includes data that addresses aim 1. The effect of combining vaccination against nicotine with small, individualized doses of Nic311 was investigated using a locomotor activity model and measuring nicotine distribution in brain and serum. Potential alterations in sensitization to nicotine-induced locomotor activity were studied to measure effects of this approach on nicotine-induced behavior. Locomotor activity is a well-characterized model that shows

predictive validity for other, more robust behavioral models that directly measure drug-induced reward or reinforcement. In addition, many of the neural pathways involved in locomotor activity overlap with those involved in reward or reinforcement [142]. Effects of the combination of treatments were compared with vaccination alone and with small, yoked doses of Nic311 alone. Animals receiving the combination of treatments received vaccine and small, individualized doses of Nic311 administered as necessary to reach a target antibody concentration previously shown to be effective in attenuating nicotine-induced locomotor sensitization. Brain and serum nicotine concentrations as well as NicAb concentrations were also measured to correlate the immunologic, pharmacokinetic, and behavioral effects of this approach.

Chapter 4 includes data that addresses aim 2. The immune responses generated by two separate monovalent vaccines administered over a range of immunogen doses were compared to those generated by a bivalent nicotine vaccine administered over a range of total immunogen doses. This study characterized the immune response produced by these vaccines when vaccines were formulated in alum and administered s.c. and also when vaccines were formulated in Freund's adjuvant and administered i.p. These two immunization models were used because they allowed for comparison of typical clinical immunization procedures with immunization conditions commonly used in animals. The focus of this study was to determine whether or not two

immunologically distinct immunogens could be combined under a variety of conditions without compromising the immunogenicity of either immunogen. NicAb concentrations, titers, and affinities were measured to determine immunologic efficacy of vaccines.

## **Chapter 3**

**Combined active and passive immunization against nicotine: Minimizing monoclonal antibody requirements using a target antibody concentration strategy**

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## **Overview**

Nicotine vaccines have shown preliminary evidence of efficacy for enhancing smoking cessation rates, but the serum nicotine-specific antibody (NicAb) concentrations produced are highly variable and many subjects do not develop effective levels. As an alternative to vaccination, passive immunization with nicotine-specific monoclonal antibodies could produce more uniform serum NicAb concentrations, but its use is limited by their high cost and shorter elimination half-life. This study investigated supplementing vaccination with monoclonal antibodies in a targeted fashion to increase vaccine efficacy while minimizing the required monoclonal antibody dose. Rats were vaccinated and then given individualized supplemental doses of the nicotine-specific monoclonal antibody Nic311 to achieve a target total serum NicAb concentration known to be effective for blocking locomotor sensitization (LMS) to nicotine. Rats received vaccine, Nic311, both, or neither, followed by 0.3 mg/kg nicotine s.c. for 10 days to produce LMS. Combination immunotherapy completely blocked the development of LMS, while monotherapy with vaccine or Nic311 alone were only minimally effective. Lower brain nicotine levels were associated with reduced locomotor activity averaged over days 7-10. Despite its greater efficacy, combination immunotherapy did not reduce the variability in the resulting total serum NicAb concentrations. Variability in total serum NicAb concentrations was contributed to by both vaccine-generated antibody and by Nic311. These data show that combination immunotherapy, using a Nic311 dose that is by itself only



minimally effective, can substantially enhance nicotine vaccine efficacy. However, variability in serum NicAb levels with combination immunotherapy may make translation of this approach challenging.

## 1. Introduction

Immunization is being studied as a potential treatment for drug addiction. Vaccination with a suitably designed drug-protein conjugate vaccine stimulates the production of drug-specific antibodies that reduce or slow drug distribution to brain and attenuate their behavioral effects [169, 199-201]. Vaccines directed against nicotine and cocaine have entered clinical trials and have provided early evidence of efficacy with no important side effects [115, 143, 144]. Vaccination for the treatment of nicotine or other drug addictions has a number of attractive features. Because the antibodies generated are highly specific for the target drug and do not bind endogenous compounds or structures, they appear to be quite safe. In principle, even very high antibody concentrations should be well tolerated. Vaccine-generated antibody is long lasting so that, in contrast to most other addiction medications, daily dosing is not required. In clinical trials, 3-5 initial monthly injections followed by a booster dose months later provided a sustained antibody response. Immunotherapies for opiate, amphetamine, and phencyclidine abuse are also being developed [190, 202, 203].

Efficacy of addiction vaccines in animals is closely correlated with the serum concentration of drug-specific antibody present; initial observations suggest the same in humans. Two common themes have emerged from clinical trials. First, efficacy of vaccination is largely confined to subjects achieving the highest serum antibody concentrations [115, 143, 144]. In a phase II trial of a nicotine vaccine,

efficacy for enhancing smoking cessation rates was entirely attributable to the 30% of subjects with the highest serum nicotine-specific antibody (NicAb) concentrations [143]. Second, the serum drug-specific antibody concentrations achieved are highly variable and substantially lower than those achieved in animals [115, 143, 144, 204]. Providing a robust and reproducible immune response with consistently high serum antibody concentrations has emerged as the principle challenge for successful translation of addiction immunotherapy into clinical use.

Immunization may be achieved actively through vaccination or passively through the administration of drug-specific monoclonal antibodies. Clinical trials have focused on vaccination because of its excellent safety profile, long duration of action, and relatively low cost. Passive immunization with drug-specific monoclonal antibodies provides efficacy in animals similar to that of vaccination and has advantages that could address the limitations of vaccination. Passive immunization allows for control of the antibody dose and more uniform initial serum antibody concentrations [179]. Because monoclonal antibodies are well tolerated [205], high doses can be administered to achieve higher serum antibody concentrations than can be produced by vaccination. However, passive immunization is substantially more expensive than vaccination. In addition, the serum half-life of passively administered IgG is shorter than IgG generated by vaccination (3 weeks v. several months or longer in humans) [122], and even

humanized or fully human monoclonal antibodies may themselves be immunogenic [206].

An alternative to using vaccination or passive immunization alone is to combine these treatments in an effort to exploit the advantages of each while minimizing their limitations. Combining these treatments could provide higher serum NicAb concentrations than vaccination alone while decreasing the cost and use of the monoclonal antibody, because smaller doses would be necessary than if used alone. In a proof-of-concept study, Roiko, et al. (2008) showed that combining vaccination with a fixed and partially effective dose of the nicotine-specific monoclonal antibody Nic311 produced significantly higher total NicAb concentrations, greater reductions of nicotine distribution to brain, and greater attenuation of locomotor sensitization to nicotine than vaccination alone [141]. Because there was considerable variability in vaccine-generated NicAb concentrations, some rats achieved an effective serum NicAb concentration with vaccination alone and presumably did not require combined therapy for efficacy. This finding suggests that instead of administering a fixed dose of Nic311 to all subjects, an individualized targeted approach to Nic311 supplementation could be used to selectively supplement only those subjects requiring it while tailoring the Nic311 dose to their vaccine-generated NicAb response.

The purpose of the current study was to assess the feasibility and efficacy of a target antibody concentration strategy for supplementing vaccination against nicotine with Nic311. The vaccination schedule was chosen to provide sub-maximal efficacy to avoid a ceiling effect that could mask an increase in efficacy due to supplemental Nic311 administration. Each vaccinated animal received only enough Nic311 to increase its serum NicAb level to the anticipated effective target concentration. Immunization effects were evaluated by measuring serum NicAb concentrations, LMS to nicotine, and serum and brain nicotine concentrations.

## **2. Materials and methods**

### 2.1 Animals

Male Holtzman Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 275-300 g were individually housed in temperature- and humidity-controlled rooms and maintained on a 12 h light/dark cycle, with testing taking place during hours 4-6 of the light phase. Animals were restricted to 18 g/day of rat chow to minimize weight gain and prevent catheter migration. All protocols were approved by the Minneapolis Medical Research Foundation Animal Care and Use Committee and were in accordance with National Institute of Health guidelines.

### 2.2 Vaccine

The nicotine immunogen 3'-AmNic-rEPA is a 3'-aminomethyl-nicotine hapten conjugated via a succinic acid linker to the carrier protein recombinant *Pseudomonas* exoprotein A. This immunogen generates antibodies that have a high affinity for nicotine ( $K_d = 20$  nM) and <1% cross-reactivity with similar compounds including acetylcholine, the major nicotine metabolites cotinine and nicotine-*N*-oxide, and other neurotransmitters and medications [124]. Control immunogen was the carrier protein rEPA alone. The vaccine dose was 25  $\mu$ g of immunogen in complete Freund's adjuvant for the initial immunization and incomplete Freund's adjuvant for subsequent immunizations in a volume of 0.4 ml.

### 2.3 Monoclonal antibody

The monoclonal antibody Nic311 was prepared from mice immunized with 3'-AmNic-rEPA. Nic311 was purified by protein G chromatography to  $\geq 95\%$  protein content with endotoxin levels of  $< 0.2$  enzyme unit/mg and diluted in phosphate-buffered saline. Nic311 has previously been characterized as an IgG<sub>1k</sub> with  $K_d = 60$  nM for nicotine and  $<1\%$  cross-reactivity with cotinine, nicotine-N-oxide or acetylcholine [207]. Control IgG was human polyclonal IgG (Gammagard; Baxter Healthcare Corp., Westlake Village, CA) that does not bind nicotine or influence nicotine pharmacokinetics or behavior [179].

### 2.4 Nicotine

(-)-Nicotine Bitartrate (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline and adjusted to a pH of 7.4 with 1M NaOH. Nicotine doses are expressed as weight of the base.

Serum and brain nicotine levels were measured by solvent extraction of serum or tissue followed by gas chromatography with nitrogen-phosphorus detection [208]. Brain nicotine concentrations were corrected for brain blood content [138].

### 2.5 Immunologic assays

Serum nicotine-specific antibody (NicAb) concentrations produced by vaccination were measured by ELISA using 3'-AmNic-polyglutamate as the coating antigen and goat anti-rat horseradish peroxidase as the detecting antibody [209]. Nic311 concentrations were determined using goat anti-mouse IgG horseradish peroxidase as the detecting antibody [179]. Because Nic311 levels in the combination immunotherapy group were measured in the presence of antibodies generated by vaccination, these levels were corrected for the minimal (3.2%) cross reactivity of vaccine-generated antibodies with the coating antigen used to quantitate Nic311.

## 2.6 Locomotor activity

Locomotor activity sessions were conducted in open-field activity chambers (MED Associates, St. Albans, VT) each measuring 43 cm x 43 cm. Horizontal activity was measured using a 16 x 16 photocell array placed 2.5 cm above the chamber floor. Interruptions in photocell transmission were measured as horizontal activity and were recorded using open-field activity software (MED Associates). Chambers were placed inside sound-attenuating boxes with ambient lighting and exhaust fans to provide white noise.

## 2.7 Experimental protocol

### *2.7.1 Overview*



Five groups of rats (n = 8-10 per group) were used (Table 1). A non-immunized nicotine only group served as a positive control to demonstrate LMS to nicotine in the absence of immunotherapy, and a non-immunized saline only group served as a negative control. Those groups receiving vaccine had serum NicAb concentrations measured prior to beginning the LMS protocol to assure that they were within the desired range. Rats in the combination group were supplemented with Nic311 after vaccination and prior to the LMS protocol as needed in order to reach their target total serum NicAb concentration. Nic311 doses for rats in the Nic311 alone group were matched to the Nic311 doses administered to the combination group.

### *2.7.2 Vaccination*

Rats were immunized with 3'-AmNic-rEPA or control vaccine on days 0, 21, and 42 (Fig 1). One week after the third vaccine dose, an indwelling catheter was implanted in the right jugular vein and blood was obtained to measure vaccine-generated serum NicAb concentrations prior to starting the LMS protocol. Animals with a pre-LMS NicAb concentration of 100-150  $\mu\text{g/ml}$  began the LMS protocol one week after blood sampling. Animals with pre-LMS vaccine-generated NicAb concentrations  $>150 \mu\text{g/ml}$  were monitored every two weeks until NicAb levels had decreased to within the desired range before starting the LMS protocol. Animals with vaccine-generated NicAb concentrations  $<100 \mu\text{g/ml}$

received a fourth vaccine dose on day 63 and serum NicAb concentrations were again determined one week later and monitored until in the desired range.

The pre-LMS NicAb concentration range of 100-150 µg/ml was selected because, based on an expected decline in serum NicAb over the study period (unpublished data), it would result in a post-LMS serum NicAb concentration range of 80-120 µg/ml. This post-LMS NicAb concentration range was shown in pilot studies to produce minimal attenuation of LMS to nicotine. A partial effect from vaccination was necessary so that any additional effect of Nic311 in the combination immunotherapy group could be detected.

### *2.7.3 Passive immunization*

Rats in the combination immunotherapy group received Nic311 when the vaccine-generated pre-LMS serum NicAb was within the desired range as above. Nic311 doses were calculated to provide a target post-LMS total serum NicAb concentration (vaccine-generated NicAb + Nic311) of 200 µg/ml, based on pilot data suggesting that this total serum NicAb concentration should be sufficient to markedly suppress LMS to nicotine. Nic311 doses were calculated as a proportionality based on preliminary data showing that a dose of 27 mg/kg produces a serum Nic311 concentration of 100 µg/ml 24 hours after dosing. The difference between each rat's measured vaccine-generated serum NicAb concentration and the target of 200 µg/ml was used to determine the required

Nic311 dose. Rats began the first locomotor activity test session 60 minutes after receiving their initial supplemental dose of Nic311. Because Nic311 has a half-life of 7 days in the rat, an additional 50% of the initial Nic311 dose was administered on day 7 of the LMS protocol to maintain the desired total serum NicAb concentration [141]. Rats were studied in 6 cohorts of 6-12 animals each using a balanced design. Rats in the Nic311 only group of each cohort received a dose of Nic311 matching the mean of the individual Nic311 doses administered to rats in the combination immunotherapy group of that cohort (cohorts 1-6 received 25, 32, 22, 32, 30.5, and 30 mg/kg Nic311, respectively).

#### *2.7.4 Locomotor activity measurement*

The locomotor sensitization protocol consisted of two habituation sessions in the locomotor chambers followed by 10 consecutive test sessions; all sessions lasted 30 minutes each. All animals received saline s.c. immediately prior to the habituation sessions. During test sessions, the saline control group continued to receive saline while all other groups received 0.3 mg/kg nicotine s.c. [141]. Following the tenth test session (40 min after nicotine dosing), rats received pentobarbital 50 mg/kg to confirm catheter patency and were sacrificed with brain and serum samples collected for analysis.

#### 2.8 Statistical analyses

Serum NicAb concentrations, serum nicotine levels, and brain nicotine levels were analyzed using separate one-way ANOVAs followed by Bonferroni's post test for between-group comparisons. Locomotor activity was measured as total horizontal distance traveled over each 30-min session. Data during habituation and test sessions were analyzed using separate two-way ANOVAs with group as a between-subjects factor and day as a within-subjects factor. The primary outcome for this study was locomotor activity on days 7-10 as used previously [141]. To confirm that the non-immunized nicotine control group exhibited sensitization, a paired-samples t-test was used to compare activity in this group during the first (test day 1) and final (mean of test days 7-10) days of sensitization. Mean distance traveled over test days 7-10 was compared among groups using a one-way ANOVA followed by Bonferroni's post-test. Within-session data on test days 1 and 10 were analyzed as 5-minute blocks using separate two-way ANOVAs with group as a between-subjects factor and time as a within-subjects factor, followed by Bonferroni's post-test for between-group comparisons.

Relationships between total serum NicAb concentrations, serum nicotine levels, brain nicotine levels, and mean locomotor activity over days 7 to 10 were analyzed using linear regression with Pearson's correlation coefficient for normally distributed data or Spearman's correlation coefficient for non-normally distributed data.

### 2.9 Exclusions

Two rats receiving only Nic311 and one rat receiving the combination of treatments were excluded from all analyses due to technical problems. Two non-immunized rats were excluded only from serum nicotine concentration analyses due to loss of sample.

### **3. Results**

#### **3.1 Serum antibody concentrations**

Six of 10 rats in the vaccine only group had post-LMS serum NicAb concentrations within the intended range of 80-120 µg/ml. The mean serum NicAb concentration for this group of  $81 \pm 24$  µg/ml was somewhat lower than anticipated but still within the intended range (Table 2). Seven of 9 rats in the combination immunotherapy group had post-LMS total serum NicAb concentrations (vaccine-generated NicAb + Nic311) of  $>160$  µg/ml and the mean concentration of  $192 \pm 75$  µg/ml was close to the targeted 200 µg/ml. The individual contributions of vaccination and Nic311 to the total serum NicAb concentration in the combination group are shown in Table 2. There was considerable variability in the total serum NicAb concentration in the combination group (range 58-274 µg/ml). The Nic311 alone group did not have a specific intended serum NicAb range since Nic311 doses in this group were matched to those in the combination group. The mean Nic311 dose administered to the combination and Nic311 groups was  $30 \pm 4$  mg/kg (range 21-38 mg/kg).

#### **3.2 Locomotor activity**

##### ***3.2.1 Sensitization to nicotine (Fig 2a)***

During habituation there was a significant effect of day ( $p = 0.0004$ ), reflecting a decrease in activity over the habituation sessions for all groups, but no effect of group or interaction. Over all 10 days of sensitization there was a significant

effect of day ( $p = 0.009$ ) and group ( $p = 0.04$ ) and interaction between the two ( $p = 0.005$ ). The non-immunized nicotine control group exhibited a significant increase in activity between the first and final days of testing ( $p = 0.02$ ), confirming that this nicotine dosing regimen produced sensitization.

### *3.2.2 Treatment effects on days 7-10 (Fig 2b)*

There was an effect of treatment ( $p = 0.007$ ) on activity averaged over days 7-10. Only the non-immunized saline control and combination immunotherapy groups showed significantly lower mean activity scores than the non-immunized nicotine control group; the monotherapy groups (vaccine only and Nic311 only) did not differ from the non-immunized nicotine control group. The 3 treatment groups (vaccine only, Nic311 only, combination immunotherapy) did not differ from each other.

### *3.2.3 Within session effects (Fig 3)*

Within session analysis on *day 1* showed no effects of treatment, but an effect of time ( $p < 0.0001$ ) and interaction ( $p = 0.002$ ). Over the first 5-minute block of the session, the non-immunized saline control group showed significantly greater activity than all other groups, showing an initial suppression of activity in animals receiving nicotine (Fig 3a). Within session analysis of *day 10* indicated a significant effect of group ( $p = 0.02$ ), time ( $p < 0.0001$ ), and interaction ( $p < 0.0001$ ). Over the first 5-minute block, activity in the non-immunized nicotine

control group was greater than in all other groups (Fig 3b). Activity levels in the combination immunotherapy group and the non-immunized saline control group were lower than either of the monotherapy groups.

### 3.3 Serum and brain nicotine concentrations

The combination immunotherapy and vaccine alone groups had higher serum nicotine concentrations and lower brain nicotine concentrations than the non-immunized nicotine control group (Fig 4 and Table 2). Serum and brain nicotine levels in the Nic311 alone group did not differ from the non-immunized nicotine control group. The brain nicotine level in the combination immunotherapy group was lower than that of the Nic311 only group or the non-immunized nicotine control group ( $p < 0.05$ ). The difference in brain nicotine levels between the combination immunotherapy and vaccine alone groups approached significance ( $p = 0.07$ ).

### 3.4 Correlations

Higher serum NicAb concentrations were associated with larger effects on nicotine distribution. There was a significant negative correlation between serum NicAb and brain nicotine concentrations (Fig 5). There was a trend toward a positive correlation between serum NicAb and serum nicotine concentrations overall; however, these correlations for each individual treatment group were highly significant (Fig 5a). Nicotine concentrations were correlated with the mean



distance traveled across days 7 to 10 (Fig 6), with lower serum levels and higher brain levels associated with greater distance traveled. There was no correlation between serum NicAb concentration and distance traveled on days 7-10.

#### **4. Discussion**

Combination immunotherapy using a target serum NicAb concentration strategy provided substantially greater attenuation of LMS to nicotine than vaccination alone. Enhanced efficacy was achieved using a mean supplemental Nic311 dose that was by itself only minimally effective. These data support the potential use of targeted combination immunotherapy to improve the efficacy of vaccination against nicotine while minimizing the required monoclonal antibody dose.

The use of drug-specific monoclonal antibodies alone to block the behavioral effects of addictive drugs has been well studied in rodents and is remarkably effective, but high doses are generally needed [140, 141, 184, 188, 189, 207]. Nic311 doses required to attenuate or block LMS, nicotine discrimination, or the re-acquisition of nicotine self-administration in rats when Nic311 is used alone have ranged from 80-160 mg/kg [141], unpublished data]. The primary impediment to using monoclonal antibodies as a monotherapy for addiction is the cost of such high doses. The use of similar doses of monoclonal antibodies has clinical precedent in the treatment of some cancers or immunological disorders, but is less appealing for a widespread problem such as tobacco addiction [210, 211]. The possible need for repeated administration of monoclonal antibody to sustain its effects could further increase the required dose. Occasional immune responses to the monoclonal antibody, even if it is humanized, may also reduce

its efficacy [206, 212]. Minimizing the required monoclonal antibody dose through targeted combination immunotherapy could address these concerns to some extent. While the mean initial Nic311 dose of 30 mg/kg used in this study was still appreciable, it was lower than the 80 mg/kg used in a prior study of combination immunotherapy in which supplemental Nic311 was administered as a fixed dose rather than in a targeted manner [141].

Nic311 is a murine monoclonal antibody and would need to be humanized or replaced with a fully human monoclonal to be suitable for clinical use. Nevertheless Nic311 provides a convenient experimental tool to investigate the combination immunotherapy concept and how to optimize it. Extrapolation of required antibody doses from rats to humans is difficult but it is possible that the Nic311 doses required by smokers would be lower than those suggested by the rat model. Clinical trials of nicotine vaccines show that smoking cessation can be enhanced with lower vaccine-generated serum NicAb concentrations (40-100 µg/ml) than are typically required in rat behavioral studies of nicotine vaccines (100-300 µg/ml) [111, 116, 141, 143]. It is also possible that the current study overestimated the required Nic311 dose because immunotherapy produced essentially complete blockade of LMS; a lower Nic311 dose might have been effective as well.

Blockade of LMS by combination immunotherapy in this study was essentially complete, producing activity levels comparable to that of saline controls. It is interesting that both Nic311 alone and vaccine alone were only marginally effective, yet the serum NicAb concentrations in the Nic311 alone group were nearly twice those of vaccine-generated NicAb in the vaccine alone group. This apparent difference in potency between Nic311 and vaccine-generated antibodies was likely due to their different affinities for nicotine (Nic311  $K_d = 60$  nM; vaccine-generated NicAb  $K_d = 20$  nM) [124, 179].

A limitation of the experimental design is that vaccine-generated serum NicAb concentrations were selected to be within a relatively narrow range so that vaccine alone would have the desired minimal efficacy in the LMS model. As a result, the twofold range of Nic311 doses required in the combination immunotherapy group was not as wide as might otherwise have been the case. It could be argued that a fixed Nic311 dose of 30 mg/kg (the mean dose required) would have produced the same result. However, it is unlikely that a fixed dose approach would be as useful if the vaccine-generated NicAb concentrations had been as variable as the antibody levels reported in nicotine vaccine clinical trials.

The trends in the pharmacokinetic data were consistent with the LMS data but by several analyses did not reach statistical significance. This result was

anticipated and is not surprising. The primary outcome measure for this study was LMS and the protocol was designed to optimize this behavioral measure rather than effects on drug levels, which are greatest shortly (1-25 min) after a nicotine dose and are less apparent with repeated or chronic nicotine dosing [139, 213]. Nevertheless, the trends in the serum and brain nicotine levels were consistent with the behavioral data. In addition, expected correlations were observed between the serum NicAb and nicotine concentrations as well as between serum or brain nicotine concentrations and distance traveled in the LMS protocol across days 7-10.

Despite its efficacy, the targeted combination immunotherapy approach did not reduce overall variability in the total serum NicAb concentrations measured at the end of the LMS protocol (range 58-274  $\mu\text{g/ml}$ ). Variability in the vaccine-generated contribution to the serum NicAb concentrations was expected, but variability in Nic311 levels was also considerable. Nic311 concentration variability likely represented individual differences in both the volume of distribution and clearance of Nic311 over the 10 day protocol. The elimination half-life of Nic311 in rats is 7 days so that the 10 day protocol was long enough for differences in Nic311 elimination to have a substantial effect [141]. The elimination half-life of monoclonal antibodies in humans is 3 weeks [214], longer than in rats, but because the usual duration of treatment for tobacco dependence

(i.e. with counseling or marketed medications) is 6-12 weeks or longer, variability in monoclonal antibody elimination could be an issue for human use as well.

The current study extends earlier findings on combination immunotherapy by showing that an individualized target concentration strategy can be used to produce complete blockade of LMS to nicotine and that this can be achieved using a Nic311 dose that is by itself only minimally effective. This approach to immunotherapy has the potential to produce greater efficacy than is possible with vaccination alone while minimizing the Nic311 dose required. A possible limitation to this approach is that the resulting total serum NicAb concentrations are highly variable and may require monitoring to assure they remain above the targeted level.

Acknowledgements: Supported by NIH grants R01 DA10714 and T32 DA07097, and the Minneapolis Medical Research Foundation Translational Addiction Research Program.

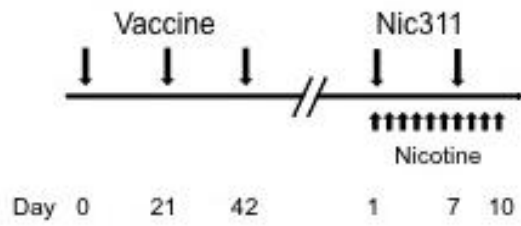
<b>Group</b>	<b>Active Immunization (Vaccine)</b>	<b>Passive Immunization (Nic311)</b>	<b>Nicotine</b>
Nicotine control	-	-	+
Vaccine only	+	-	+
Nic311 only	-	+	+
Combination	+	+	+
Saline control	-	-	-

**Table 1.** Group Design



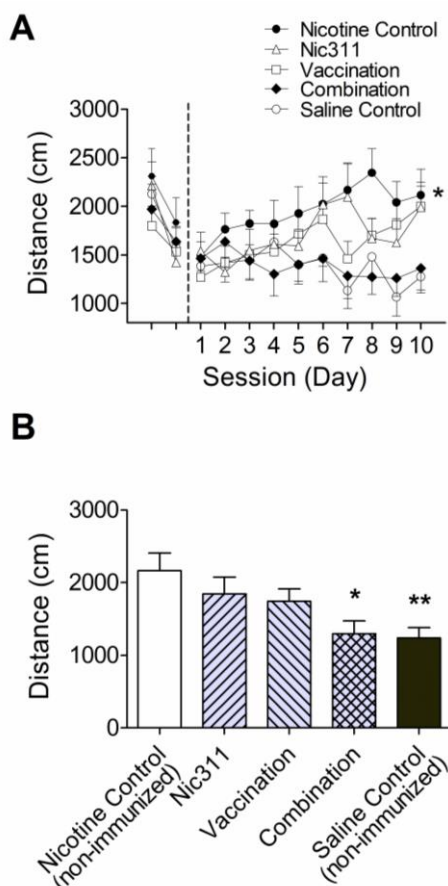
	<i>Vaccine</i> ( $\mu\text{g/ml}$ )	<i>Nic311</i> ( $\mu\text{g/ml}$ )	<i>Total</i> ( $\mu\text{g/ml}$ )	<i>Serum</i> ( $\text{ng/ml}$ )	<i>Brain</i> ( $\text{ng/g}$ )
Nicotine control	--	--	--	70 $\pm$ 10	380 $\pm$ 80
Vaccine only	81 $\pm$ 24	--	81 $\pm$ 24 (39 – 106)	580 $\pm$ 260	260 $\pm$ 50
Nic311 only	--	150 $\pm$ 32	150 $\pm$ 32 (116 – 199)	340 $\pm$ 80	290 $\pm$ 130
Combination	83 $\pm$ 39 (32 – 144)	109 $\pm$ 45 (26 – 144)	192 $\pm$ 75 (58 – 274)	720 $\pm$ 450	160 $\pm$ 90
Saline control	--	--	--	--	--

**Table 2.** Serum NicAb and nicotine concentrations, mean  $\pm$  SD (range)



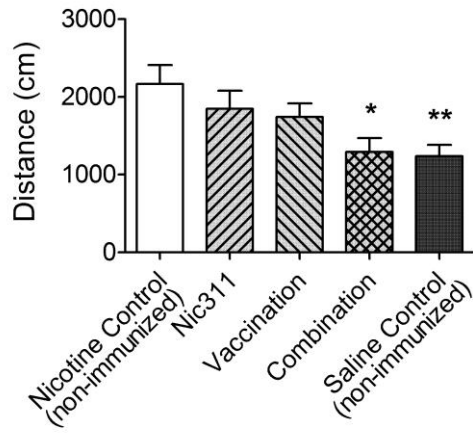
**Figure 1.** Schedule of treatments.

Animals were assessed for serum NicAb level starting at 1 week after the third vaccine dose. If serum NicAb levels were below the intended range after the third vaccine dose, a fourth dose was administered at day 63. When the serum NicAb level was within the intended range, animals underwent 2 days of habituation, received the first dose of Nic311, and began nicotine dosing for the LMS protocol.



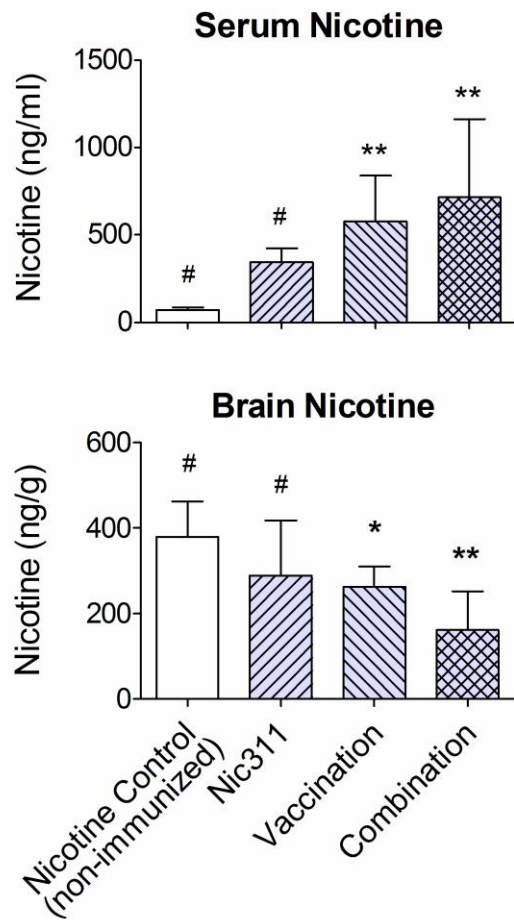
**Figure 2.** Locomotor activity as measured across all days for the entire 30-minute session or as measured by the mean distance traveled over days 7-10.

a) Locomotor activity (mean  $\pm$  SE) measured across all days for the entire 30 minute session. Initial unmarked days are non-nicotine habituation sessions and days 1 through 10 are nicotine test sessions. The non-immunized nicotine control group showed sensitization to nicotine, \*  $p < 0.05$ , mean across days 7-10 compared to day 1. b) Effects of treatment on the primary outcome, mean distance traveled over the 30 minute session across days 7-10 (mean  $\pm$  SE), \*  $p < 0.05$ , \*\*  $p < 0.001$  compared to non-immunized nicotine control group.



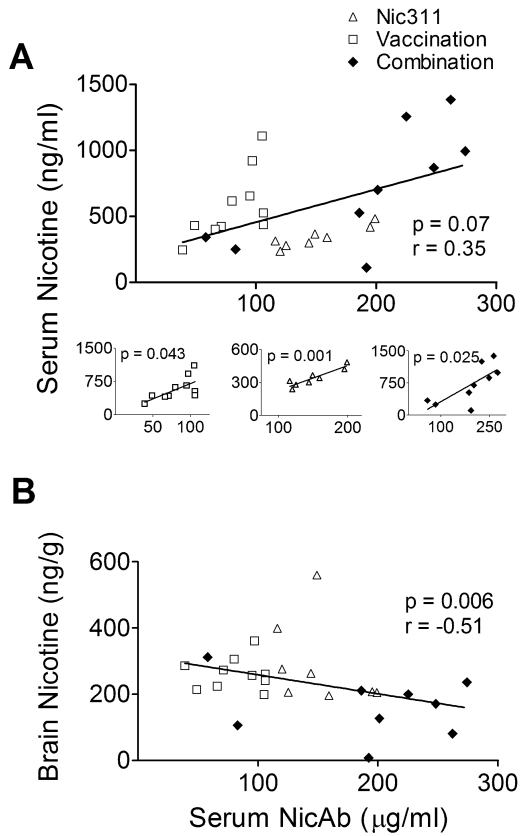
**Figure 3.** Within-session locomotor activity (mean  $\pm$  SE) in 5-minute blocks on days 1 and 10.

a) Day 1; All groups receiving nicotine had lower activity than the saline control group, reflecting an initial decrease in activity due to nicotine, \*  $p < 0.05$ . b) Day 10; All immunized groups as well as the non-immunized saline control group had lower locomotor activity than the non-immunized nicotine alone group, \*  $p < 0.05$ . The combination immunotherapy and non-immunized saline control groups had lower activity than either of the monotherapy groups, #  $p < 0.05$ .



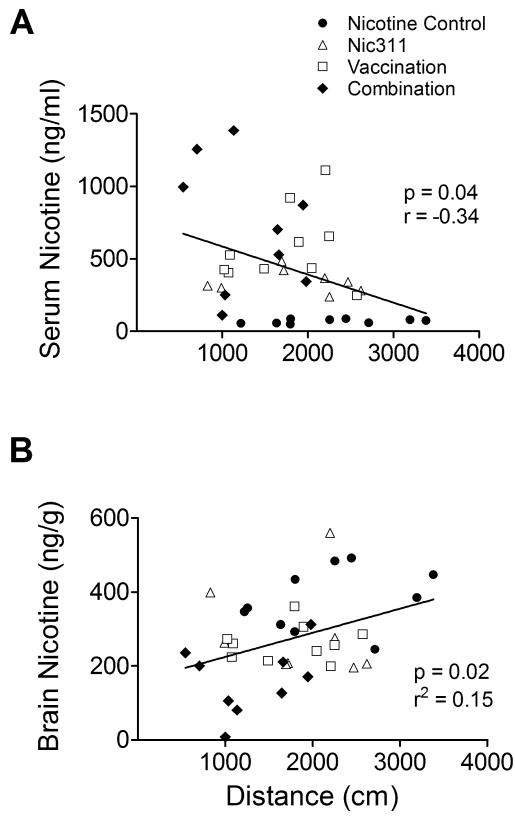
**Figure 4.** Nicotine concentrations obtained 40 min after the final nicotine dose of the LMS protocol (mean  $\pm$  SD).

\*  $p < 0.05$ , \*\*  $p < 0.001$  compared to non-immunized nicotine control group; #  $p < 0.05$  compared to combination group.



**Figure 5.** Relationship of serum or brain nicotine concentrations to serum NicAb concentration.

- a) Relationship of serum nicotine concentration to serum NicAb concentration across all groups; smaller figures show the relationship of serum nicotine concentration to serum NicAb concentration within individual treatment groups.
- b) Relationship of brain nicotine concentration to serum NicAb concentration.



**Figure 6.** Relationship of mean distance traveled on days 7 through 10 to serum (a) and brain (b) nicotine concentrations.

## **Chapter 4**

### **Immunogenicity of individual immunogens in a bivalent nicotine vaccine differs according to vaccine formulation and administration conditions**

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Marco Pravetoni

Paul R. Pentel

This manuscript is in preparation to be submitted for publication in *PLoS One* and has been adapted for this thesis.



## Overview

Structurally distinct nicotine immunogens can elicit independent antibody responses against nicotine when administered concurrently. Co-administering different nicotine immunogens together as a multivalent vaccine could be a useful way to generate higher antibody levels than with monovalent vaccines alone. The immunogenicity and additivity of monovalent and bivalent vaccines was studied across a range of immunogen doses, adjuvants, and routes to assess its generality. Rats were vaccinated with total immunogen doses of 12.5 - 100  $\mu\text{g}$  of 3'-aminomethyl nicotine conjugated to recombinant *Pseudomonas* exoprotein A (3'-AmNic-rEPA), 6-carboxymethylureido nicotine conjugated to keyhole limpet hemocyanin (6-CMUNic-KLH), or both. Vaccines were administered s.c. in alum or i.p. in Freund's adjuvant at matched total immunogen doses. When administered s.c. in alum, the contributions of the individual immunogens to total nicotine-specific antibody (NicAb) titers and concentrations (as measured by ELISA) were preserved across a range of doses. However, when administered i.p. in Freund's adjuvant, the contributions of the individual immunogens to total NicAb titers and concentrations at some doses were compromised. The affinities of antibodies generated by the individual immunogens (as measured by equilibrium dialysis) were similar when immunogens were administered separately or together regardless of immunogen dose, adjuvant, or route of administration. Patterns of vaccine immunogenicity differed markedly between the two adjuvants and routes of administration;

vaccines administered s.c. in alum produced higher affinity antibodies whereas those administered i.p. in Freund's adjuvant produced higher concentrations of antibodies (as measured by equilibrium dialysis). These results support the potential strategy of combining nicotine vaccines to achieve higher antibody levels than can be obtained with monovalent vaccines alone and suggest that alum is better suited to this approach.

## 1. Introduction

Immunization is being developed as a treatment for drug addiction for various drugs of abuse, including nicotine. Immunization with a nicotine-protein conjugate vaccine produces highly specific antibodies to nicotine. These antibodies bind and sequester drug in serum, preventing or slowing drug distribution to brain and attenuating drug-induced behavioral effects (for reviews, see [169, 215, 216]).

Vaccines against nicotine and cocaine have entered clinical trials as potential adjuncts for treatment of these addictions, showing preliminary evidence of efficacy with minimal side effects [115, 143, 144]. Although not yet in clinical trials, immunotherapies against opioids, methamphetamine, and phencyclidine are also in development [161, 190, 202, 203]. The efficacy of these vaccines in both animals and humans is closely correlated with the concentration of serum drug-specific antibodies produced. In clinical trials of one nicotine vaccine the 30% of subjects producing the highest serum nicotine-specific antibody (NicAb) levels had higher smoking cessation rates compared to controls. However, there was no difference in smoking cessation rates when all subjects were included in analyses because of the overall low and variable NicAb levels [116, 143, 144]. The low and variable NicAb levels produced remain the primary challenge for successfully translating vaccination against nicotine into clinical use.

One potential strategy to increase serum NicAb concentrations is to combine two or more vaccines against nicotine into a multivalent vaccine. Combination of vaccines in a multivalent format is a well-established approach for infectious disease vaccines, showing that it is possible to combine several structurally distinct vaccines with little to no compromise in immunogenicity of each component. The goal when combining vaccines for infectious diseases is to achieve a broad specificity, such as targeting several serotypes in the influenza vaccine. Combining vaccines also allows administration of several unrelated vaccines at once, such as diphtheria, tetanus, and pertussis. In contrast, the goal for extending this multivalent approach to nicotine is to achieve a greater response to the single target of nicotine. In a previous study using a bivalent nicotine vaccine, two nicotine-protein conjugate immunogens with linkers placed at different positions on the nicotine molecule were used [197]. This approach presented different nicotine epitopes for B cell recognition. Concurrent administration of the two immunogens produced NicAbs with only marginal cross-reactivity, presumably representing only minimally overlapping B cell populations [197]. This showed that despite the small size of nicotine (162 daltons) it was possible to design distinct nicotine haptens that stimulate independent B cell responses.

The current study extends this approach in order to assess its generality. Monovalent and bivalent vaccines were compared across a range of matched

total immunogen doses administered either s.c. in alum or i.p. in Freund's adjuvant. Administration s.c. in alum was studied because of its clinical relevance, and administration i.p. in Freund's was studied because of its common use in animal models. The goals of this study were to determine whether the individual vaccines fully retain their immunogenicity when combined, and whether there is any difference in response to the individual or combined immunogens between vaccination s.c. in alum and i.p. in Freund's. Immunogenicity was assessed as serum NicAb titer, concentration, and affinity.

## **2. Materials and methods**

### **2.1. Animals**

Male Holtzman Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 275-300 g were double-housed in temperature- and humidity-controlled rooms and maintained on a 12 h light/dark cycle. Animals received unrestricted water and food. Protocols were approved by the Minneapolis Medical Research Foundation Animal Care and Use Committee and were in accordance with National Institute of Health guidelines.

### **2.2. Study design and vaccination protocol**

Two parallel experiments were performed; the first experiment administered vaccines s.c. in alum and the second experiment administered vaccines i.p. in Freund's adjuvant. In the first experiment, all vaccines contained 0.2 ml of immunogen(s) in PBS and 0.2 ml of alum (Alhydrogel [Al(OH)<sub>3</sub>], E. M. Sergeant Chemical Co., Clifton NJ), yielding a final injection volume of 0.4 ml and a final aluminum concentration of 1.25 mg/ml. When two immunogens were included in a dose, they were added to alum at the same time. Tubes containing vaccines were gently inverted and allowed to sit at room temperature for 30 minutes. Rats were vaccinated every three weeks (days 0, 21, 42, 63) for a total of 4 immunizations. Vaccines were administered s.c. in the upper back. On day 70, rats were anesthetized with Innovar (fentanyl/droperidol) and sacrificed and trunk blood was harvested for ELISA and equilibrium dialysis.

Groups of 12 rats received 3'-AmNic-rEPA alone, 6-CMUNic-KLH alone, or both (in the bivalent vaccine), each at total immunogen doses of 12.5 µg, 25 µg, 50 µg, or 100 µg for a total of 12 groups. In the bivalent groups, the total immunogen dose reflected the sum of individual immunogen doses used (i.e. 6.25 µg of 3'-AmNic-rEPA + 6.25 µg of 6-CMUNic-KLH = 12.5 µg total immunogen dose).

In the second experiment, equal volumes of immunogen(s) in PBS and Freund's adjuvant were combined for a final injection volume of 0.4 ml. Freund's complete adjuvant (EMD Millipore, Billerica MA) was used for the first immunization, and Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis MO) was used for all subsequent immunizations. As with vaccines administered s.c. in alum, when two immunogens were included for bivalent vaccine preparation, they were added to Freund's adjuvant at the same time. Tubes containing vaccines were mixed on a vortex instrument for 10 minutes prior to injection. Vaccines were administered i.p. The vaccination protocol and experimental design was otherwise identical to that of the first experiment, which utilized alum as an adjuvant.

### 2.3. Immunogens

The nicotine immunogen 3'-AmNic-rEPA consists of 3'-aminomethyl-nicotine conjugated to the carrier protein recombinant *Pseudomonas* exoprotein A. Antibodies that are produced by 3'-AmNic-rEPA have a high affinity for nicotine

( $K_d = 10\text{-}19\text{ nM}$ ) and marginal (<1%) cross-reactivity with similar compounds including acetylcholine, nicotine metabolites cotinine and nicotine-*N*-oxide, and other neurotransmitters [124, 139]. The nicotine immunogen 6-CMUNic-KLH consists of 6-carboxymethylureido nicotine conjugated to the carrier protein keyhole limpet hemocyanin [131]. Antibodies that are generated by 6-CMUNic-KLH also have high nicotine-binding affinity ( $K_d = 29\text{-}71\text{ nM}$ ) and high specificity for nicotine similar to that of 3'-AmNic-rEPA [125, 138].

#### 2.4. Enzyme-linked immunosorbent assay (ELISA)

Serum nicotine-specific antibody (NicAb) concentrations and titers produced by vaccination were measured by ELISA using 3'-AmNic-polyglutamate or 6-CMUNic-BSA as the coating antigens and goat anti-rat horseradish peroxidase as the detecting antibody [209]. NicAb concentrations were measured against a standard curve derived from previously characterized serum NicAb samples. Cross-reactivity in the ELISA results was determined by measuring 3'-AmNic-rEPA-generated antibodies against a 6-CMUNic-BSA coating antigen and measuring 6-CMUNic-KLH-generated antibodies against a 3'-AmNic-polyglutamate coating antigen [197]. Cross-reactivity between the two immunogens across all vaccination conditions was less than 10%. The cross-reactivity from these assays was used to correct serum NicAb concentrations in the bivalent group.



### 2.5. Equilibrium dialysis

Equilibrium dialysis was used to measure antibody affinity and also provided a second means of estimating NicAb concentration from maximum nicotine binding capacity. Six of 12 serum samples from each vaccine group receiving a total immunogen dose of 12.5 or 100  $\mu\text{g}$  were analyzed. Nic311, a previously characterized IgG1 <sub>$\kappa$</sub>  monoclonal antibody derived from immunization of mice with 3'-AmNic-rEPA (production and purification described in [179] and [181]) served as a positive control across all equilibrium dialysis runs. Serum samples from vaccinated rats were diluted to a NicAb concentration of approximately 100  $\mu\text{g}/\text{mL}$  and added to the serum side of 96-well equilibrium dialysis plates (MWCO 10 kDa; the Nest Group Inc, Southborough MA). The dialysate side consisted of non-immune (blank) rat serum containing unlabeled nicotine ((-)-nicotine hydrogen tartrate salt; Sigma-Aldrich, St. Louis MO) along with  $^3\text{H}$ -nicotine (81.7  $\mu\text{Ci}/\text{mL}$ ; PerkinElmer, Boston MA) at concentrations of 1-1024 nM. Plates were placed on a shaker for 72 hours at room temperature. The radiolabeled nicotine was used to estimate nicotine concentrations in serum and dialysate. Nicotine-binding affinity of NicAbs (represented by antibody dissociation constant  $K_d$ ) and maximum nicotine-binding capacity of antibodies ( $B_{\text{max}}$ ) were measured from saturation curves using Prism 6.0 (GraphPad Software Inc., La Jolla CA). NicAb concentration was calculated from  $B_{\text{max}}$ , assuming a molecular weight of 150 kD for IgG and 2 nicotine-specific binding sites per molecule.

## 2.6. Statistical analyses

Effects of vaccine group and total immunogen dose were examined using two-way ANOVA followed by Holm-Sidak's post-test. Vaccine-generated NicAb concentrations and titers were compared across vaccine groups, within individual matched immunogen doses and also across immunogen doses, within vaccine groups using one-way ANOVA followed by Bonferroni's post-test. The relationship between serum NicAb concentrations generated by individual immunogens within the bivalent group was compared using correlation analysis.

### **3. Results**

#### ***3.1 Experiment 1: Vaccines administered s.c. in alum adjuvant***

##### ***3.1.1 Serum antibody titers as measured by ELISA (Fig 1A)***

The analysis corresponding to Figure 1 considers the total dose of immunogen administered to each group. For example, rats in the 25 µg bivalent group received 12.5 µg of each individual immunogen. Total serum nicotine-specific antibody (NicAb) titer or concentration for the bivalent group refers to the sum of titers or concentrations against 3'-AmNic-rEPA and 6-CMUNic-KLH.

There was a main effect of vaccine group and of immunogen dose on total NicAb titers ( $p < 0.001$  for both). When compared within each vaccine group, total NicAb titers increased with increasing total immunogen dose. Total NicAb titers in the bivalent group were significantly higher than those in the monovalent 3'-AmNic-rEPA group ( $p < 0.001$ ) but did not differ from NicAb titers in the monovalent 6-CMUNic-KLH group.

##### ***3.1.2 Serum antibody concentrations as measured by ELISA (Fig 1B)***

In animals administered vaccines s.c. in alum, NicAb concentrations generally paralleled the NicAb titers. There was a main effect of both vaccine group and immunogen dose on total NicAb concentrations ( $p < 0.001$  for both). Similar to the NicAb titers, when compared within each vaccine group, NicAb concentrations increased with increasing total immunogen dose. Total NicAb

concentrations in the bivalent group were significantly higher than those in the monovalent 3'-AmNic-rEPA group ( $p < 0.001$ ) but again did not differ from those in the monovalent 6-CMUNic-KLH group. Overall, serum NicAb concentrations were significantly different between the monovalent 3'-AmNic-rEPA and monovalent 6-CMUNic-KLH groups ( $p < 0.05$ ).

In summary, these analyses comparing the immunogenicity of vaccines administered s.c. in alum at matched total immunogen doses indicate that the bivalent vaccine produced higher NicAb titers and concentrations than monovalent 3'-AmNic-rEPA but not higher than monovalent 6-CMUNic-KLH. Variability was high among all vaccine groups, for both NicAb titers and concentrations.

The bivalent group received only half of the dose of each individual immunogen to reflect a total immunogen dose that when combined is matched to monovalent vaccines (i.e. 25  $\mu\text{g}$  of bivalent = 12.5  $\mu\text{g}$  of 3'-AmNic-rEPA and 12.5  $\mu\text{g}$  of 6-CMUNic-KLH). Because of this, additional analyses of these data were performed (below). These additional analyses provide a measure of whether the immunogenicity of the individual immunogens was retained when they were co-administered as components of the bivalent vaccine.

### *3.1.3 Contributions of individual components in bivalent group as measured by ELISA (Fig 2A,B)*

This figure compares given doses of immunogen when each particular dose was administered alone or as a component of the bivalent vaccine in rats immunized s.c. in alum as measured by NicAb titers (Fig 2A, left) and concentrations (Fig 2B, right). For example, NicAb titers generated by 12.5 µg of monovalent 3'-AmNic-rEPA vaccine were compared to the NicAb titers generated by 12.5 µg of 3'-AmNic-rEPA when delivered as a component of the 25 µg bivalent vaccine. Data are from the same animals as in Figure 1 but are displayed differently in order to clarify whether interference occurred when monovalent vaccines were combined. NicAb titers or concentrations for both immunogens did not differ whether immunogens were given alone or co-administered.

### *3.1.4 Observed and expected serum antibody titers and concentrations in bivalent group as measured by ELISA (Fig 3A, B)*

Observed and expected NicAb titers (Fig 3A) and concentrations (Fig 3B) in the bivalent groups were compared at matched total doses. Data in this figure are from the same animals as in previous figures, but represented as observed and expected NicAb levels, where observed values are those measured in the bivalent group and expected values are the sum of those measured in the corresponding monovalent vaccine groups (e.g. 25 µg of expected = 12.5 µg of monovalent 3'-AmNic-rEPA and 12.5 µg of monovalent 6-CMUNic-KLH). Representing the data in

this manner allowed for further exploration of potential additivity or compromise in immunogenicity when immunogens were combined. Observed and expected NicAb titers or concentrations did not differ across all immunogen doses.

### *3.1.5 Correlation of individual immunogens within the bivalent vaccine group*

*(Fig 4A)*

Overall serum NicAb concentrations produced by 3'-AmNic-rEPA and 6-CMUNic-KLH when co-administered in bivalent vaccines (as measured by ELISA) were not significantly correlated ( $r^2 = 0.06$ ,  $p = 0.11$ ). One point was excluded from the figure to aid in visual representation of data because the 6-CMUNic-KLH concentration was 0  $\mu\text{g}/\text{mL}$  but this point was included as its actual (unaltered) value in all correlations and analyses.

### *3.1.6 Antibody affinity and NicAb concentration as measured by equilibrium*

*dialysis (Fig 5)*

This figure shows NicAb nicotine-binding affinity (A) and concentration (as calculated from  $B_{\text{max}}$ ; B). NicAb affinity generated by vaccines administered s.c. in alum was similar, regardless of vaccine group or total immunogen dose (12.5  $\mu\text{g}$  vs. 100  $\mu\text{g}$ ). There was a main effect of immunogen dose on NicAb concentration ( $p < 0.05$ ), but NicAb concentrations did not differ across vaccine groups.

## 3.2 Experiment 2: Vaccines administered i.p. in Freund's adjuvant

### 3.2.1 Serum antibody titers as measured by ELISA (Fig 6A)

There was a main effect of vaccine group ( $p < 0.05$ ) and immunogen dose ( $p < 0.001$ ) on total NicAb titer. When compared within each vaccine group, total NicAb titers decreased with increasing total immunogen dose. When pooled across doses, total NicAb titers were significantly higher in the bivalent group than the monovalent 6-CMUNic-KLH group ( $p < 0.05$ ). Total NicAb titers did not differ between the bivalent group and the monovalent 3'-AmNic-rEPA group.

### 3.2.2 Serum antibody concentrations as measured by ELISA (Fig 6B)

There was a main effect of vaccine group ( $p < 0.001$ ) and immunogen dose ( $p < 0.01$ ) on total NicAb concentrations. However, when compared within each vaccine group, total NicAb concentrations did not indicate a clear relationship with total immunogen dose. Total NicAb concentrations in the bivalent group were significantly higher than in the monovalent 3'-AmNic-rEPA group, but did not differ compared to NicAb concentrations in the monovalent 6-CMUNic-KLH group ( $p < 0.001$ ). NicAb concentrations in the monovalent 6-CMUNic-KLH group were significantly higher than those in the monovalent 3'-AmNic-rEPA group ( $p < 0.001$ ).

In summary, these analyses comparing the immunogenicity of vaccines administered i.p. in Freund's adjuvant at matched total immunogen doses

indicate that the bivalent vaccine produced higher NicAb titers and concentrations than one or the other monovalent vaccines, but not both. As with vaccines administered s.c. in alum, vaccines administered i.p. in Freund's also generated considerable variability among all vaccine groups, as evidenced by both NicAb titers and concentrations.

### *3.2.3 Contributions of individual components in bivalent group as measured by ELISA (Fig 7A, B)*

As in figure 2, this figure compares given doses of immunogen when each particular dose was administered alone or as a component of the bivalent vaccine in rats immunized i.p. in Freund's adjuvant as measured by NicAb titers (Fig 7A, left) and concentrations (Fig 7B, right). Analyses in this figure were similar to those performed for figure 2. In rats administered 3'-AmNic-rEPA, there was a main effect of vaccine group ( $p < 0.001$ ) and immunogen dose ( $p < 0.01$ ) on NicAb titers. Rats that received 3'-AmNic-rEPA produced higher titers when the immunogen was administered alone as a monovalent vaccine compared to when the immunogen was administered as a component of the bivalent vaccine. Regarding NicAb concentrations, there was a significant effect of vaccine group ( $p < 0.001$ ) but not immunogen dose in rats administered 3'-AmNic-rEPA. In rats that received 6-CMUNic-KLH, there was a main effect of both vaccine group ( $p < 0.01$ ) and immunogen dose ( $p < 0.001$ ) on NicAb titers. However, there were no differences in NicAb concentrations generated by 6-CMUNic-KLH regardless of



whether the immunogen was administered alone as a monovalent vaccine or as a component of the bivalent vaccine.

#### *3.2.4 Observed and expected serum antibody titers and concentrations in bivalent group as measured by ELISA (Fig 8A, B)*

Observed and expected total NicAb titers (Fig 8A) and concentrations (Fig 8B) in the bivalent groups were also compared. Data in this figure are displayed similarly to data in figure 3, but instead reflect observed and expected NicAb titers and concentrations for vaccines administered i.p. in Freund's. There was a main effect of vaccine group ( $p < 0.001$ ) and immunogen dose ( $p < 0.001$ ) as well as an interaction between the two ( $p < 0.01$ ) on total NicAb titers. The observed and expected NicAb concentrations were not significantly different at any dose.

#### *3.2.5 Correlation of individual immunogens within the bivalent vaccine group (Fig 4B)*

Individual NicAb concentrations produced by 3'-AmNic-rEPA and 6-CMUNic-KLH (ELISA) across all immunogen doses are positively correlated, although this correlation accounted for only a small portion of variability ( $r^2 = 0.37$ ,  $p < 0.001$ ). Two points were altered on the figure as they had 3'-AmNic-rEPA concentrations of 0  $\mu\text{g/mL}$ . These points were instead represented as having 3'-AmNic-rEPA concentrations of 10  $\mu\text{g/mL}$  to aid in visual representation of data. These two points were included in all correlations and analyses as their actual (unaltered) values.

### 3.2.6 *Antibody affinity and NicAb concentration as measured by equilibrium dialysis (Fig 9)*

This figure shows NicAb nicotine-binding affinity (A) and concentration (calculated from  $B_{max}$ ; B). There was a significant interaction between vaccine group and immunogen dose ( $p < 0.05$ ) on NicAb affinity, but when pooled, affinities did not differ when compared across vaccine group or immunogen dose. NicAb concentrations also did not differ across vaccine group or immunogen dose.

#### **4. Discussion**

This study reports that two immunologically distinct nicotine vaccines can be co-administered s.c. in alum over a range of immunogen doses without compromising NicAb titers, concentration, or affinity. In addition, antibody responses generated by 3'-AmNic-rEPA were largely independent of those generated by 6-CMUNic-KLH in animals that received the bivalent vaccine. Formulation of vaccine is important, as interference between immunogens occurred in bivalent vaccines administered i.p. in Freund's adjuvant. In summary, administration of a bivalent nicotine vaccine s.c. in alum may be one way to move beyond the modest antibody concentrations produced by monovalent nicotine vaccines in a clinically relevant and generalizable manner.

Most nicotine vaccine studies have measured NicAb titers or concentrations across individual animals. However, nicotine-binding affinity of NicAbs has only been measured from pooled sera or reported for a limited number of animals. Previous studies have not investigated potential effects of immunogen dose, adjuvant, or route of administration on affinity of NicAbs in individual animals, so it was pertinent to do so in this study. Individual variability in nicotine-binding affinity in NicAbs was considerable across all immunization conditions.

Vaccine groups were compared at matched total immunogen doses to assess any potential additivity in immune responses generated by 3'-AmNic-rEPA and 6-

CMUNic-KLH. However, additivity did not occur under any conditions examined in this study. The ability to detect additivity was limited due to the high variability across all endpoints and to the unequal efficacy of the immunogens. As a result, monovalent vaccines were compared to their dose-matched contributions in the bivalent vaccine to uncover any potential interference between immunogens when co-administered. Although additivity was not observed, concurrent administration of immunogens s.c. in alum yielded no compromise in immunogenicity of immunogens.

The current study revealed that immunogenicity of individual vaccine components was unaffected when immunogens were co-administered in bivalent vaccines formulated in alum. Vaccines against infectious agents have previously been combined in alum without interference between individual components. Nevertheless, a few studies have reported a decrease in antibodies generated by these vaccines when co-administered [217, 218] (for reviews, see [219, 220]). In these studies, interference may have been caused by incompatible preservatives or by a non-adjuvanted vaccine displacing an alum-adsorbed vaccine from its adjuvant upon combination [217, 218]. As vaccine formulation and administration conditions were different in these studies compared to the current study, these potential explanations do not seem to apply to the current results.

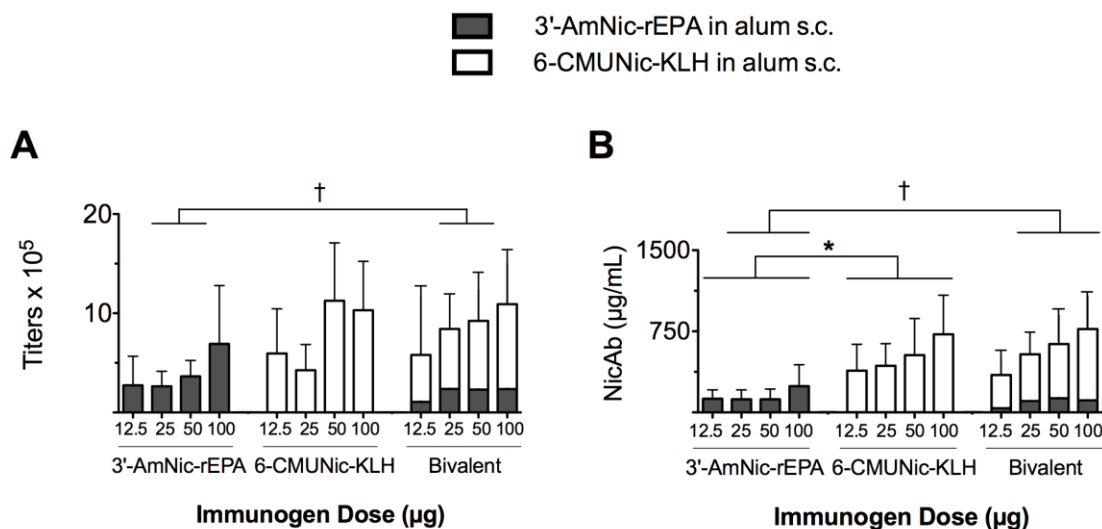
In the current study, vaccines administered s.c. in alum produced modest concentrations of higher affinity antibodies and vaccines administered i.p. in Freund's produced high concentrations of antibodies with lower nicotine-binding affinity. This finding is supported by other nicotine vaccine studies that reported trends where vaccines also produced high concentrations of lower affinity NicAbs or modest concentrations of higher affinity NicAbs [130-132]. One possible explanation is that the higher NicAb concentrations generated by vaccines administered i.p. in Freund's represents expansion of a larger diversity of B cell clones. Presumably a number of these clones produce low affinity antibodies, contributing to the lower mean affinity in these NicAb populations than was observed in NicAbs generated by vaccines administered s.c. in alum. The differing patterns in immunogenicity may also be due to the way the vaccines are formulated. Freund's adjuvant consists largely of mineral oil with supplementary components added to increase its immunopotentiative effect; immunogens in saline are mixed with Freund's to create a water-in-oil emulsion. In contrast, when alum is used, immunogens are adsorbed to the adjuvant, rather than simply being mixed together as with Freund's. This adsorption characteristic of alum may limit the potential for protein-protein interaction or other mechanisms of interference between the two immunogens.

One limitation to this study was that vaccines administered i.p. in alum or s.c. in Freund's adjuvant were not compared. As a result, separate contributions of

adjuvant and route of administration to immunogenicity could not be measured. However, the focus of this study was to examine common routes of vaccine administration in humans and in animals. Delivering vaccines i.p. in alum or s.c. in Freund's is less common and was therefore of less interest for this study.

It is reasonable to question whether or not the bivalent approach offers additional benefit compared to simply increasing immunogen dose of a monovalent vaccine. In the current study, increasing the dose of monovalent vaccines administered s.c. in alum increased NicAb titers and concentrations. However, results from a clinical trial of NicVAX, a nicotine vaccine containing the 3'-AmNic-rEPA immunogen used in the current study, indicated that NicAb concentrations plateau near an immunogen dose of 200 µg in humans. In that study, increasing the immunogen dose to 400 µg did not produce significantly greater NicAb concentrations [116, 143]. Even with such high immunogen doses, the highest NicAb concentrations in humans (60 µg/ml) have been much lower than NicAb concentrations reported in this study, likely due to the 10-20-fold difference between typical human and animal vaccine doses [116, 143]. Limitations on the amount of alum acceptable in humans prevent delivery of vaccine doses as high as those delivered in animals [148]. In situations where increasing the immunogen dose is either not possible or does not provide additional benefit, multivalent vaccines offer a useful way to increase antibody levels over what can be achieved by a monovalent vaccine alone.

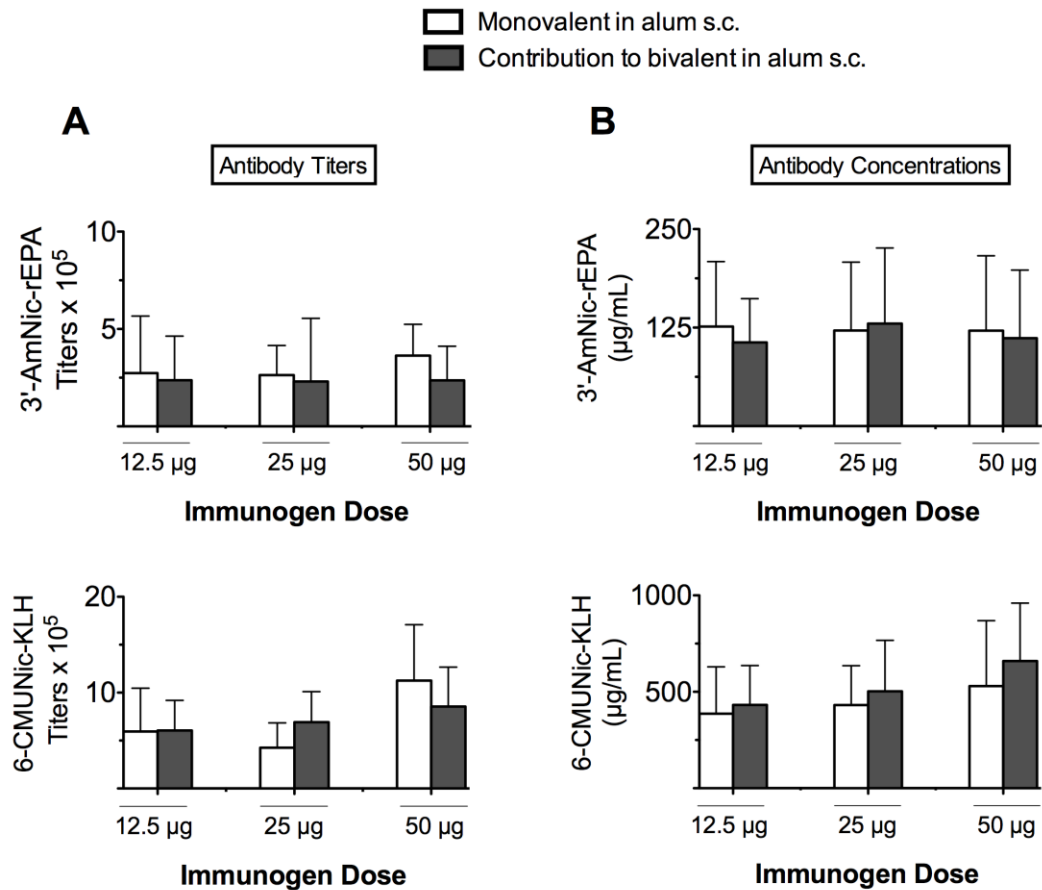
Correlation analysis revealed that the antibody responses generated by 3'-AmNic-rEPA and 6-CMUNic-KLH were largely independent of one another. This supports findings in a previous study that indicated that these immunogens were immunologically distinct and stimulated different B cell populations [197]. In animals that received the bivalent vaccine s.c. in alum, those that responded weakly to one immunogen tended to produce a stronger response to the second immunogen. The potential of a second immunogen to rescue weak- or non-responders provides an additional benefit to using a bivalent nicotine vaccine.



**Figure 1.** Serum nicotine-specific antibody (NicAb) titers (A) and concentrations (B) of rats immunized with monovalent or bivalent vaccines s.c. in alum (mean  $\pm$  SD).

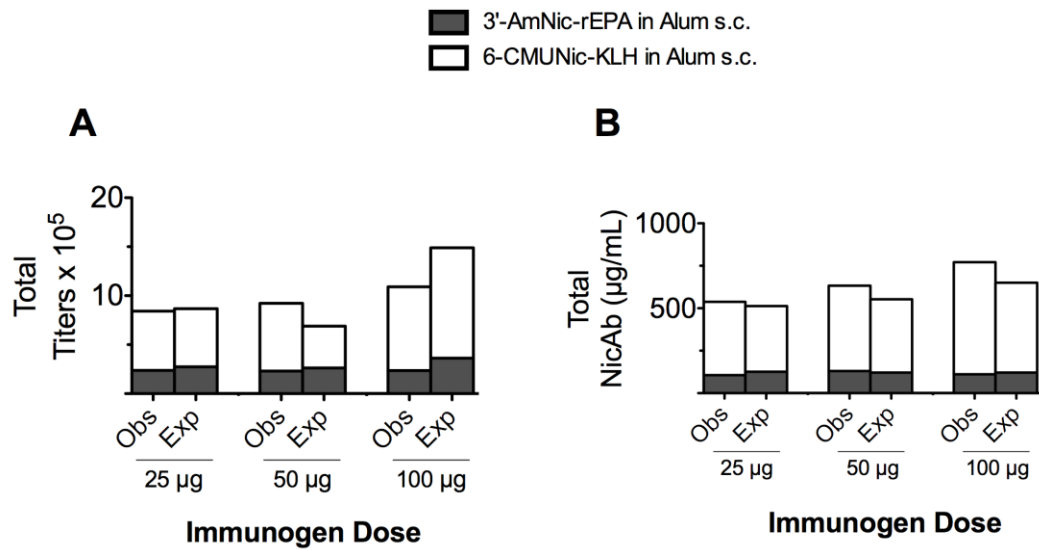
There were main effects of immunogen dose and vaccine group on both NicAb titers and concentrations ( $p < 0.001$  for all). Individual comparisons show that NicAb concentrations generated by monovalent 3'-AmNic-rEPA and those generated by monovalent 6-CMUNic-KLH were different across all immunogen doses; \*  $p < 0.05$  at matched total immunogen dose. The bivalent group produced significantly higher total NicAb titers and concentrations compared to monovalent 3'-AmNic-rEPA, but not compared to monovalent 6-CMUNic-KLH; †  $p < 0.05$  at matched total immunogen dose.





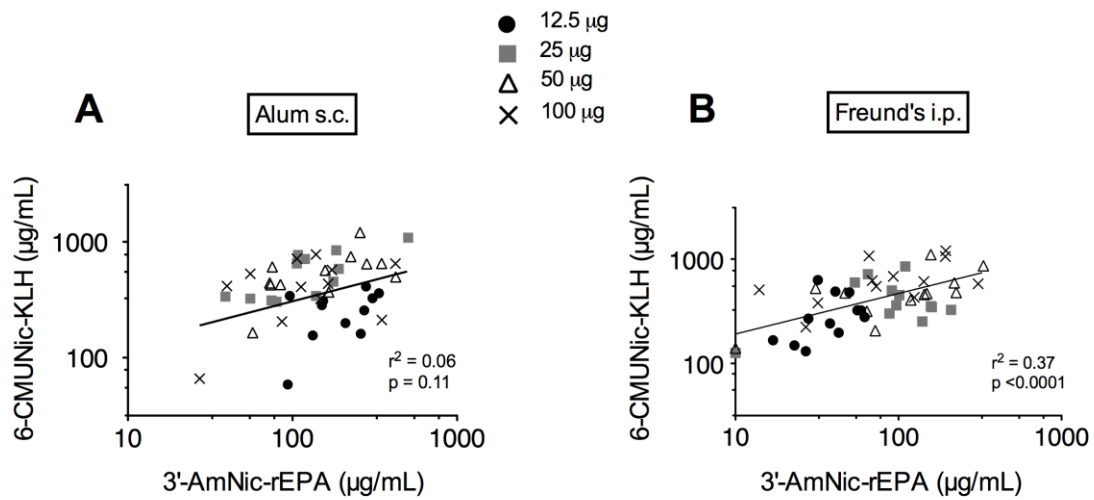
**Figure 2.** 3'-AmNic-rEPA and 6-CMUNic-KLH titers (A) and concentrations (B) in monovalent groups and their individual dose-matched contributions in rats immunized s.c. in alum (mean  $\pm$  SD).

NicAb titers and concentrations as measured by ELISA did not differ between administration of immunogens as a monovalent vaccine or as bivalent vaccine components, suggesting no interference occurred when immunogens were co-administered.



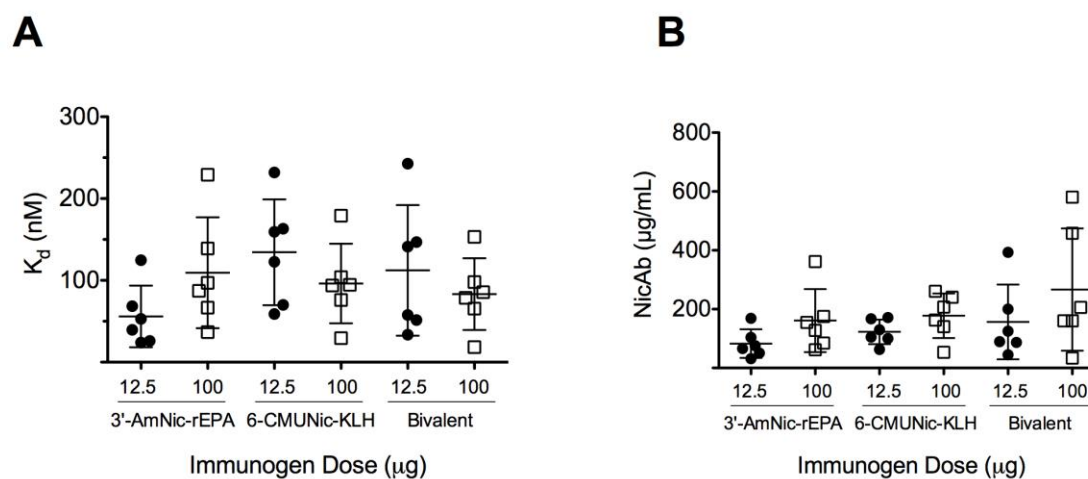
**Figure 3.** Observed and expected NicAb titers (A) and concentrations (B) in immunized s.c. in alum (mean).

Observed values are those measured in the bivalent group and expected values are the sum of those measured in the corresponding monovalent vaccine groups. There were no differences between total observed and total expected NicAb titers or concentrations, again indicating immunogenic interference did not occur.



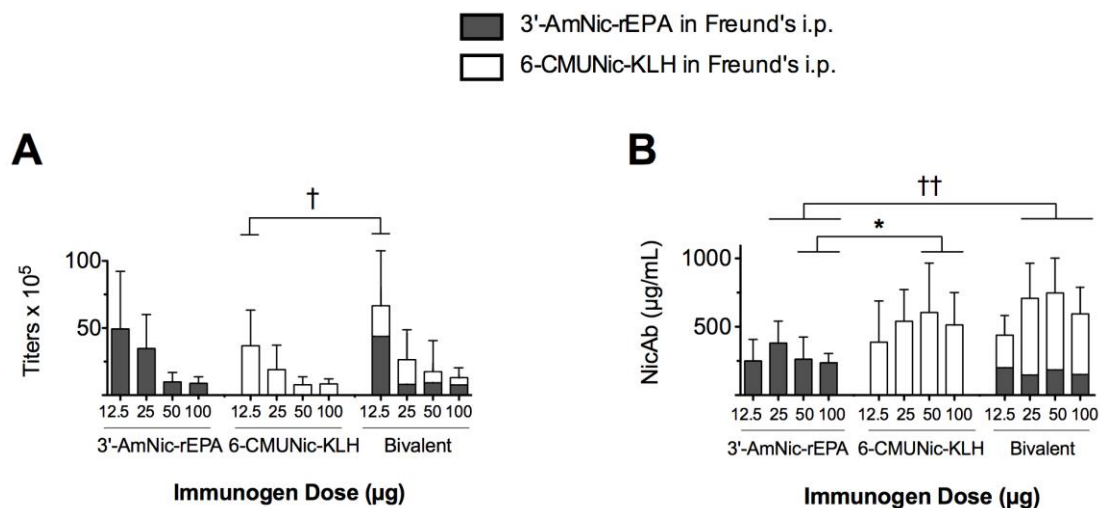
**Figure 4.** Correlation between serum NicAb concentrations produced by 3'-AmNic-rEPA and by 6-CMUNic-KLH in rats immunized with bivalent vaccines s.c. in alum (A) or i.p. in Freund's adjuvant (B).

Data indicates that there was minimal or no association between antibody responses generated by 3'-AmNic-rEPA and 6-CMUNic-KLH in the bivalent groups. In rats administered the bivalent vaccine s.c. in alum (A), serum NicAb concentrations attributable to 3'-AmNic-rEPA were not correlated with those attributable to 6-CMUNic-KLH;  $r^2 = 0.06$ ,  $p = 0.11$ . In rats that received the vaccine i.p. in Freund's (B), serum NicAb concentrations attributable to 3'-AmNic-rEPA were positively correlated with those attributable to 6-CMUNic-KLH, although this correlation accounted for only a small portion of variability;  $r^2 = 0.37$ ,  $p < 0.001$ .



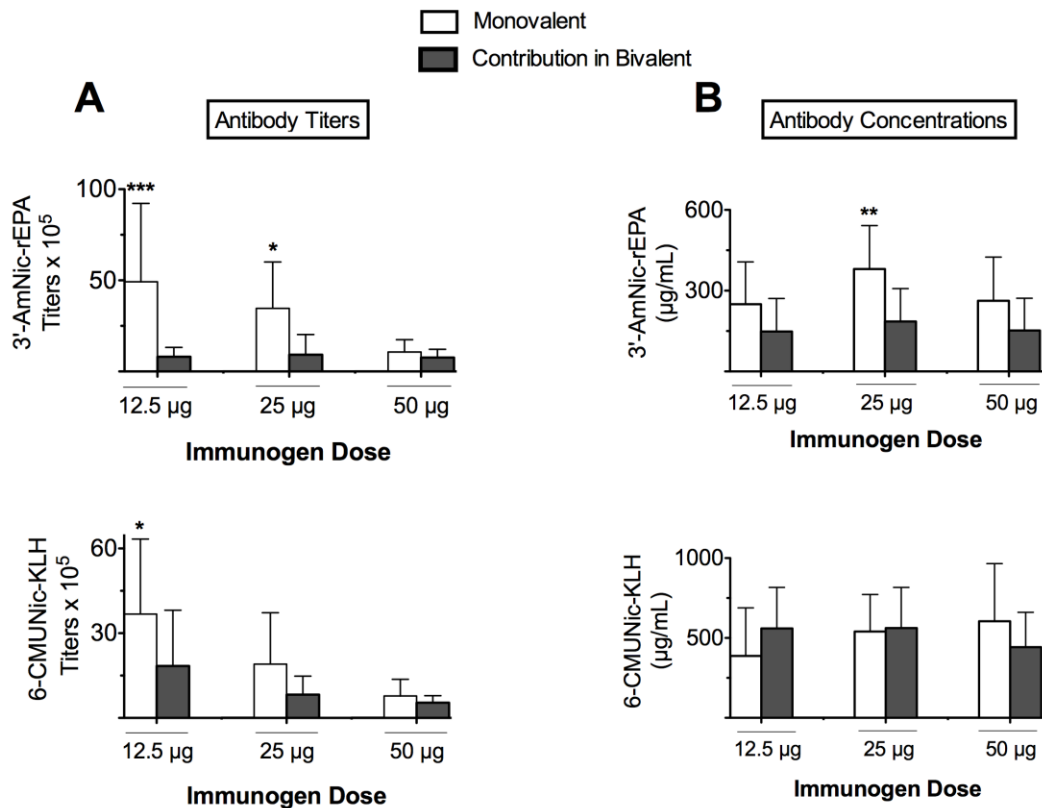
**Figure 5.** NicAb nicotine-binding affinity (A) and concentration (B) in rats immunized s.c. in alum (mean  $\pm$  SD).

Nicotine-binding affinity as measured by equilibrium dialysis is represented as equilibrium dissociation constant,  $K_d$ , where a lower  $K_d$  indicates higher affinity. NicAb concentration, also measured by equilibrium dialysis, was calculated from nicotine-binding capacity ( $B_{max}$ ). Nicotine-binding affinity of NicAbs did not differ across total immunogen dose or vaccine group. There was a main effect of total immunogen dose on NicAb concentration ( $p < 0.05$ ), indicating that NicAb concentrations increased as total immunogen dose increased. As with affinity, NicAb concentrations also did not differ between vaccine groups. Together, this suggests that affinity and concentration were not compromised when immunogens were co-administered.



**Figure 6.** Serum nicotine-specific antibody (NicAb) titers (A) and concentrations (B) of rats immunized with monovalent or bivalent vaccines i.p. in Freund's adjuvant (mean ± SD).

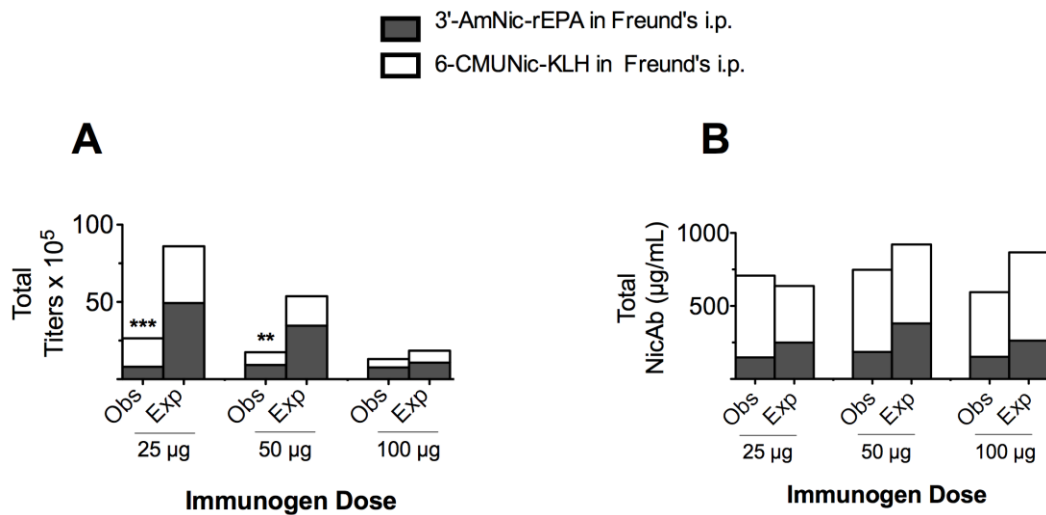
There were main effects of immunogen dose ( $p < 0.01$ ) and vaccine group ( $p < 0.05$ ) on total NicAb titer and concentration as measured by ELISA. Individual comparisons indicate that NicAb titers in rats that received the bivalent vaccine were higher than those that received the monovalent 6-CMUNic-KLH vaccine, but only at a dose of 12.5 µg; †  $p < 0.05$  at matched total immunogen dose. When compared at individual total immunogen doses, the bivalent vaccine produced higher NicAb concentrations at some immunogen doses than monovalent 3'-AmNic-rEPA; ††  $p < 0.01$  at matched total immunogen dose. Similarly, animals that received monovalent 6-CMUNic-KLH produced higher NicAb concentrations at some immunogen doses than animals that received monovalent 3'-AmNic-rEPA; \*  $p < 0.05$  at matched total immunogen dose.



**Figure 7.** 3'-AmNic-rEPA and 6-CMUNic-KLH titers (A) and concentrations (B) as measured by ELISA in monovalent groups and their individual dose-matched contributions in rats immunized i.p. in Freund's adjuvant (mean  $\pm$  SD).

There were main effects of vaccine group and immunogen dose for both 3'-AmNic-rEPA and 6-CMUNic-KLH titers ( $p < 0.01$  for all). Individual comparisons indicate that NicAb titers were lower in rats that received either immunogen as a component of the bivalent vaccine than when the same immunogen was received as a monovalent vaccine, particularly at lower doses; \*  $p < 0.05$ , \*\*\*  $p < 0.001$  compared to dose-matched contribution in bivalent group. There was a main effect of vaccine

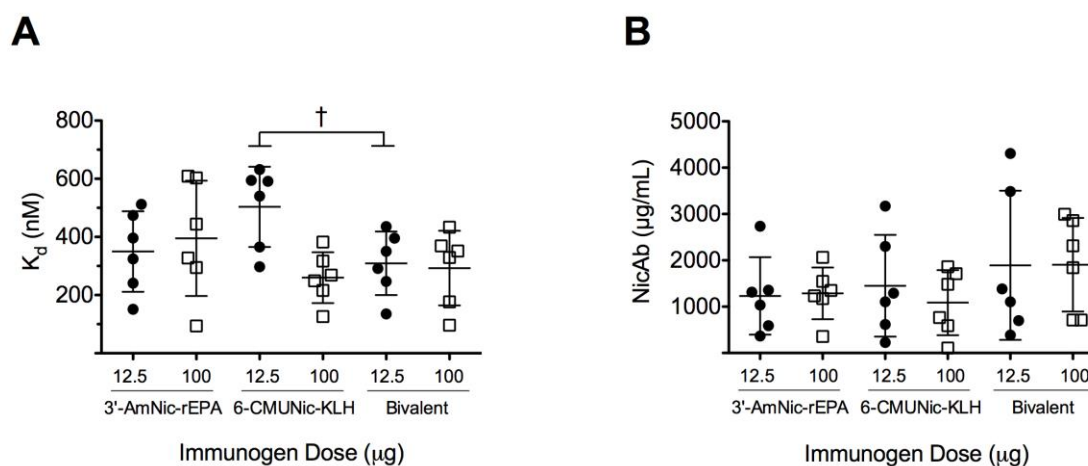
group for NicAb concentrations ( $p < 0.001$ ) as well, but it was only significant for NicAb concentrations generated by 3'-AmNic-rEPA and not those generated by 6-CMUNic-KLH. This is supported by individual comparisons that indicate that NicAb concentrations generated by 3'-AmNic-rEPA were lower when the immunogen was administered as a component of the bivalent vaccine compared to when administered alone as a monovalent vaccine; \*\*  $p < 0.01$  compared to dose-matched contribution in bivalent group.



**Figure 8.** Observed and expected NicAb titers (A) and concentrations (B) in rats immunized i.p. in Freund's adjuvant (mean).

As in figure 3, observed values are those measured in the bivalent group and expected values are the sum of those measured in the corresponding monovalent vaccine groups. There were main effects of vaccine group and dose as well as an interaction between the two ( $p < 0.01$ ) when comparing NicAb titers. Individual comparisons indicate that observed NicAb titers were less than expected at some doses; \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared to expected NicAb titer. Observed and expected NicAb concentrations did not differ at any immunogen dose.





**Figure 9.** NicAb nicotine-binding affinity (A) and concentration (B) in rats immunized i.p. in Freund's adjuvant (mean  $\pm$  SD).

Nicotine-binding affinity and NicAb concentration were measured by equilibrium dialysis and are represented as described for figure 5. There was an interaction between vaccine group and total immunogen dose ( $p < 0.05$ ) for nicotine-binding affinity, but neither produced main effects alone. An individual comparison indicates that animals that received the bivalent vaccine produced NicAbs with a significantly higher nicotine-binding affinity (represented by a lower  $K_d$ ) than animals that received monovalent 6-CMUNic-KLH, but this difference was only significant at a total immunogen dose of 12.5  $\mu\text{g}$ ; †  $p < 0.05$  at matched total immunogen dose. NicAb concentrations from animals receiving vaccines i.p. in Freund's adjuvant were not different across total immunogen doses and vaccine groups.

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## **Chapter 5**

### **Conclusion**

**This section provides concluding remarks and addresses how the aims of this thesis contribute to the development and clinical use of nicotine and other addiction vaccines**

## **1. Introduction**

The overall goal of this thesis was to enhance nicotine vaccine efficacy by increasing NicAb concentrations and decreasing overall variability using clinically relevant approaches. To achieve this, a monovalent nicotine vaccine was combined with passive immunization against nicotine in the first aim and combined with an additional monovalent nicotine vaccine to produce a bivalent vaccine in the second aim. Both strategies used vaccine designs and immunization protocols commonly used in clinical settings.

## **2. Results and implications**

### *2.1 Aim 1: Combination of active and passive immunization*

The first aim combined active and passive immunization, focusing on ways to make this strategy more clinically feasible. Previous studies have indicated that NicAbs generated by a nicotine vaccine or by passive immunization were largely equivalent, regardless of source. The combination of treatments reached the overall target NicAb concentration using an average dose of monoclonal antibody nearly two-thirds less than was used in a previous combination immunotherapy study. The combination of treatments produced greater alterations in nicotine pharmacokinetics and nicotine-induced behavior than either treatment alone [139, 141]. However, the addition of a monoclonal antibody did not decrease variability in overall NicAb concentrations as hoped. The variability in Nic311 NicAb concentrations obscured potentially significant differences in total NicAb

concentrations between rats receiving the combination of treatments and rats receiving Nic311 only. Previous studies with Nic311 indicated that variability in Nic311 NicAb concentrations would be relatively low, although these studies only examined acute pharmacokinetics of a single dose of Nic311. As a result, it was not possible to effectively predict the increased variability in Nic311 NicAb concentrations that occurred over the course of the ten-day, multi-dose protocol used in this aim.

This aim determined that monitoring NicAb concentrations after vaccination and supplementing vaccine-generated NicAb concentrations with individualized doses of Nic311 as necessary was a beneficial way to increase NicAb concentrations to an effective threshold. The individualized treatment design used in this aim, therapeutic drug monitoring, is a common approach in clinical settings and is often used for treatment of various forms of cancer. In therapeutic drug monitoring, blood levels of particular biomarkers or drugs are continuously monitored and the course of treatment is dependent on those values. As one particular dose of a drug is not always appropriate for all subjects, many treatments are moving forward in this individualized manner.

This treatment design could be adapted for use in a clinical setting as a way to increase effective NicAb concentrations. However, the use of a monoclonal antibody as a supplement in this protocol is currently somewhat limited by

production cost of monoclonal antibodies such as Nic311. Although the mAb dose necessary in this protocol was greatly decreased, it would still remain expensive to administer a dose of 30 mg/kg of Nic311. This strategy may become more clinically translatable as production costs for monoclonal antibodies decrease with the advance of necessary technology.

### 2.2 Aim 2: Bivalent nicotine vaccine

The second aim of this thesis focused on optimizing vaccine administration and formulation conditions necessary for efficacy of a bivalent nicotine vaccine. This study determined that two immunologically distinct immunogens could be combined over a range of immunogen doses without compromising key contributors to vaccine efficacy, such as NicAb titers, concentration, and affinity. Formulation of vaccines is important, as the integrity of these responses was preserved only for vaccines administered s.c. in alum.

The profile of the immune response differed markedly between vaccines administered s.c. in alum and those administered i.p. in Freund's adjuvant. Vaccines delivered s.c. in alum produced modest concentrations of higher affinity NicAbs. In contrast, vaccines administered i.p. in Freund's adjuvant produced high concentrations of lower affinity NicAbs. Other nicotine vaccine studies have shown similar relationships, where vaccines generated modest concentrations of higher affinity NicAbs or high concentrations of lower affinity NicAbs [130-132].

One possible explanation for this finding is that the higher NicAb titers and concentrations generated by vaccines administered i.p. in Freund's represent expansion of a larger diversity of B cell clones. Presumably a number of these clones produced low affinity antibodies, contributing to the lower mean affinity observed in NicAb populations generated by vaccines administered i.p. in Freund's compared to those generated by vaccines administered s.c. in alum.

The approach used in this aim, combination of monovalent vaccines into a multivalent vaccine, is commonly used in clinical settings. In multivalent vaccines against infectious diseases, vaccines are often combined either for convenience of administration (e.g. measles-mumps-rubella and diphtheria-tetanus-pertussis) or to provide broad protection against several serotypes of one pathogen (e.g. rabies and influenza). In contrast, the goal for applying this multivalent approach to nicotine vaccines was instead to achieve a greater response to the single target of nicotine.

Just as in the first aim, the study design used in this aim also employed a more individualized approach. All subjects may not respond in the same manner to one particular vaccine or vaccine component, as evidenced by the great variability in immune response generated by nicotine vaccines in clinical trials. Antibody responses produced by 3'-AmNic-rEPA and 6-CMUNic-KLH in the bivalent vaccine when administered s.c. in alum were largely independent of one another,

indicating that subjects that did not respond well to one immunogen responded better to the second, immunologically distinct immunogen. The bivalent approach did not decrease the overall variability in NicAb concentrations since variability was still considerable across all vaccine groups. However, as there were no vaccine non-responders (i.e. NicAb concentrations of zero) in rats that received the bivalent vaccine, this strategy may also reduce the total number of vaccine non-responders in a clinical setting.

The generalizable approach used in this aim is another method that may surmount the limited antibody concentrations produced by monovalent nicotine vaccines and can potentially be translated to benefit other small molecule vaccines as well.

### **3. Future pathways for nicotine vaccines**

Overall, this thesis explored ways of combining vaccination against nicotine with additional immunotherapies to increase vaccine efficacy. Supplementation of vaccination with passive immunization in an individualized manner and co-administration of two nicotine immunogens in a bivalent vaccine are only two effective strategies of many.

The combination of vaccination and passive immunization could be further explored using a different dosing schedule. This protocol would deliver a



nicotine-specific mAb early in the vaccination schedule to increase NicAb concentrations and provide immediate benefit while vaccine-generated antibody concentrations were still low. Once vaccine-generated antibody concentrations reached an effective threshold, mAb administration would be tapered off. Effects of this approach would be studied on nicotine pharmacokinetics and nicotine-induced behavior. This strategy would provide the benefit of immediate effect while limiting the cost and use of the monoclonal antibody.

Additionally, the contributions of NicAb affinity and concentration to the overall efficacy of a bivalent vaccine could be further explored. Better optimization of immunization protocols may allow for a comparison of the effects of high versus low mean nicotine-binding affinity and high versus low NicAb concentration. Ideally, a therapeutic drug monitoring approach would be used to measure and subdivide animals into groups based on vaccine-generated NicAb concentrations and nicotine-binding affinity of the NicAbs during the immunization protocol. Effects of high versus low nicotine-binding affinity and NicAb concentration on nicotine pharmacokinetics and nicotine-induced behavior would be measured. This approach would potentially explain the relative importance of two key antibody characteristics, benefitting future vaccine development efforts.

#### **4. Conclusion**

Vaccination against nicotine has been shown to alter nicotine pharmacokinetics and to attenuate nicotine-induced behavior in animals and humans. Because of the substantial variability and modest response to vaccination, efficacy in human subjects has been limited. Combining vaccination against nicotine with additional immunotherapies (active or passive) can increase antibody concentrations over what can be produced by vaccination with one nicotine immunogen alone. The combination approaches used in this thesis have the potential to be used clinically as a safe and effective way of bettering nicotine vaccine efficacy and helping individuals achieve their goal of quitting smoking.

## References

- [1] WHO. WHO report on the global tobacco epidemic, 2009. Geneva: World Health Organization; 2008.
- [2] Prevention CfDCa. Quitting smoking among adults -- United States, 2001-2010. *Morbidity and Mortality Weekly Report*. 2011;60:1513-19.
- [3] Garrison GD, Dugan SE. Varenicline: a first-line treatment option for smoking cessation. *Clin Ther*. 2009;31:463-91.
- [4] Jorenby DE, Leischow SJ, Nides MA, Rennard SI, Johnston JA, Hughes AR, et al. A controlled trial of sustained-release bupropion, a nicotine patch, or both for smoking cessation. *N Engl J Med*. 1999;340:685-91.
- [5] CDC. 2010 Surgeon General's report—How tobacco smoke causes disease: The biology and behavioral basis for smoking-attributable disease. 2010.
- [6] *Diagnostic and Statistical Manual of Mental Disorders (DSM-IV)*. 4th ed. Washington, DC: American Psychiatric Association; 1994.
- [7] Rose JE, Corrigan WA. Nicotine self-administration in animals and humans: similarities and differences. *Psychopharmacology*. 1997;130:28-40.
- [8] Matta SG, Balfour DJ, Benowitz NL, Boyd RT, Buccafusco JJ, Caggiula AR, et al. Guidelines on nicotine dose selection for in vivo research. *Psychopharmacology (Berl)*. 2007;190:269-319.
- [9] Benowitz NL, Jacob P, 3rd. Daily intake of nicotine during cigarette smoking. *Clin Pharmacol Ther*. 1984;35:499-504.
- [10] Hukkanen J, Jacob P, 3rd, Benowitz NL. Metabolism and disposition kinetics of nicotine. *Pharmacol Rev*. 2005;57:79-115.
- [11] Gourlay SG, Benowitz NL. Arteriovenous differences in plasma concentration of nicotine and catecholamines and related cardiovascular effects after smoking, nicotine nasal spray, and intravenous nicotine. *Clin Pharmacol Ther*. 1997;62:453-63.
- [12] Abobo CV, Ma J, Liang D. Effect of menthol on nicotine pharmacokinetics in rats after cigarette smoke inhalation. *Nicotine Tob Res*. 2012;14:801-8.
- [13] Rose JE, Mukhin AG, Lokitz SJ, Turkington TG, Herskovic J, Behm FM, et al. Kinetics of brain nicotine accumulation in dependent and nondependent smokers assessed with PET and cigarettes containing <sup>11</sup>C-nicotine. *Proc Natl Acad Sci U S A*. 2010;107:5190-5.
- [14] Rose JE, Behm FM, Westman EC, Coleman RE. Arterial nicotine kinetics during cigarette smoking and intravenous nicotine administration: implications for addiction. *Drug Alcohol Depend*. 1999;56:99-107.
- [15] Benowitz NL, Jacob P, 3rd, Jones RT, Rosenberg J. Interindividual variability in the metabolism and cardiovascular effects of nicotine in man. *J Pharmacol Exp Ther*. 1982;221:368-72.

- [16] Laurenzana EM, Gunnell MG, Gentry WB, Owens SM. Treatment of adverse effects of excessive phencyclidine exposure in rats with a minimal dose of monoclonal antibody. *J Pharmacol Exp Ther*. 2003;306:1092-8.
- [17] Tuncok Y, Hieda Y, Keyler DE, Brown S, Ennifar S, Fattom A, et al. Inhibition of nicotine-induced seizures in rats by combining vaccination against nicotine with chronic nicotine infusion. *Exp Clin Psychopharmacol*. 2001;9:228-34.
- [18] de Wit H, Dudish S, Ambre J. Subjective and behavioral effects of diazepam depend on its rate of onset. *Psychopharmacology (Berl)*. 1993;112:324-30.
- [19] Henningfield JE, Keenan RM. Nicotine delivery kinetics and abuse liability. *J Consult Clin Psychol*. 1993;61:743-50.
- [20] Berridge MS, Apana SM, Nagano KK, Berridge CE, Leisure GP, Boswell MV. Smoking produces rapid rise of [<sup>11</sup>C]nicotine in human brain. *Psychopharmacology (Berl)*. 2010;209:383-94.
- [21] Samaha AN, Yau WY, Yang P, Robinson TE. Rapid delivery of nicotine promotes behavioral sensitization and alters its neurobiological impact. *Biol Psychiatry*. 2005;57:351-60.
- [22] Samaha AN, Robinson TE. Why does the rapid delivery of drugs to the brain promote addiction? *Trends Pharmacol Sci*. 2005;26:82-7.
- [23] Samaha AN, Mallet N, Ferguson SM, Gonon F, Robinson TE. The rate of cocaine administration alters gene regulation and behavioral plasticity: implications for addiction. *J Neurosci*. 2004;24:6362-70.
- [24] Balster RL, Schuster CR. Fixed-interval schedule of cocaine reinforcement - Effect of dose and infusion duration. *Journal of the Experimental Analysis of Behavior*. 1973;20:119-29.
- [25] Sorge RE, Clarke PB. Rats self-administer intravenous nicotine delivered in a novel smoking-relevant procedure: effects of dopamine antagonists. *J Pharmacol Exp Ther*. 2009;330:633-40.
- [26] Fowler CD, Kenny PJ. Intravenous nicotine self-administration and cue-induced reinstatement in mice: effects of nicotine dose, rate of drug infusion and prior instrumental training. *Neuropharmacology*. 2011;61:687-98.
- [27] Benowitz NL. Nicotine replacement therapy. What has been accomplished--can we do better? *Drugs*. 1993;45:157-70.
- [28] West R, Hajek P, Foulds J, Nilsson F, May S, Meadows A. A comparison of the abuse liability and dependence potential of nicotine patch, gum, spray and inhaler. *Psychopharmacology (Berl)*. 2000;149:198-202.
- [29] Le Houezec J. Role of nicotine pharmacokinetics in nicotine addiction and nicotine replacement therapy: a review. *Int J Tuberc Lung Dis*. 2003;7:811-9.
- [30] Jalas JR, Ding X, Murphy SE. Comparative metabolism of the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol by rat cytochrome P450 2A3 and human cytochrome P450 2A13. *Drug Metab Dispos*. 2003;31:1199-202.
- [31] Murphy SE, Raulinaitis V, Brown KM. Nicotine 5'-oxidation and methyl oxidation by P450 2A enzymes. *Drug Metab Dispos*. 2005;33:1166-73.

- [32] Schoedel KA, Hoffmann EB, Rao Y, Sellers EM, Tyndale RF. Ethnic variation in CYP2A6 and association of genetically slow nicotine metabolism and smoking in adult Caucasians. *Pharmacogenetics*. 2004;14:615-26.
- [33] O'Loughlin J, Paradis G, Kim W, DiFranza J, Meshefedjian G, McMillan-Davey E, et al. Genetically decreased CYP2A6 and the risk of tobacco dependence: a prospective study of novice smokers. *Tob Control*. 2004;13:422-8.
- [34] Strasser AA, Benowitz NL, Pinto AG, Tang KZ, Hecht SS, Carmella SG, et al. Nicotine metabolite ratio predicts smoking topography and carcinogen biomarker level. *Cancer Epidemiol Biomarkers Prev*. 2011;20:234-8.
- [35] Rubinstein ML, Benowitz NL, Auerback GM, Moscicki AB. Rate of nicotine metabolism and withdrawal symptoms in adolescent light smokers. *Pediatrics*. 2008;122:e643-7.
- [36] Mroziewicz M, Tyndale RF. Pharmacogenetics: a tool for identifying genetic factors in drug dependence and response to treatment. *Addict Sci Clin Pract*. 2010;5:17-29.
- [37] Malaiyandi V, Sellers EM, Tyndale RF. Implications of CYP2A6 genetic variation for smoking behaviors and nicotine dependence. *Clin Pharmacol Ther*. 2005;77:145-58.
- [38] Molander L, Hansson A, Lunell E. Pharmacokinetics of nicotine in healthy elderly people. *Clin Pharmacol Ther*. 2001;69:57-65.
- [39] Gourlay SG, Benowitz NL. The benefits of stopping smoking and the role of nicotine replacement therapy in older patients. *Drugs Aging*. 1996;9:8-23.
- [40] Zevin S, Jacob P, Geppetti P, Benowitz NL. Clinical pharmacology of oral cotinine. *Drug Alcohol Depend*. 2000;60:13-8.
- [41] Ghosheh O, Dwoskin LP, Li WK, Crooks PA. Residence times and half-lives of nicotine metabolites in rat brain after acute peripheral administration of [2'-(14)C]nicotine. *Drug Metab Dispos*. 1999;27:1448-55.
- [42] Teng L, Crooks PA, Buxton ST, Dwoskin LP. Nicotinic-receptor mediation of S(-)-nornicotine-evoked -3H-overflow from rat striatal slices preloaded with -3H-dopamine. *J Pharmacol Exp Ther*. 1997;283:778-87.
- [43] Clark MS, Rand MJ, Vanov S. Comparison of pharmacological activity of nicotine and related alkaloids occurring in cigarette smoke. *Arch Int Pharmacodyn Ther*. 1965;156:363-79.
- [44] Kyerematen GA, Taylor LH, deBethizy JD, Vesell ES. Pharmacokinetics of nicotine and 12 metabolites in the rat. Application of a new radiometric high performance liquid chromatography assay. *Drug Metab Dispos*. 1988;16:125-9.
- [45] Adir J, Miller RP, Rotenberg KS. Disposition of nicotine in the rat after intravenous administration. *Res Commun Chem Pathol Pharmacol*. 1976;13:173-83.
- [46] Plowchalk DR, Andersen ME, Debethizy JD. A physiologically based pharmacokinetic model for nicotine disposition in the Sprague-Dawley rat. *Toxicology and Applied Pharmacology*. 1992;116:177-88.

- [47] Tuesta LM, Fowler CD, Kenny PJ. Recent advances in understanding nicotinic receptor signaling mechanisms that regulate drug self-administration behavior. *Biochem Pharmacol.* 2011;82:984-95.
- [48] Baddick CG, Marks MJ. An autoradiographic survey of mouse brain nicotinic acetylcholine receptors defined by null mutants. *Biochem Pharmacol.* 2011;82:828-41.
- [49] Grady SR, Salminen O, Lavery DC, Whiteaker P, McIntosh JM, Collins AC, et al. The subtypes of nicotinic acetylcholine receptors on dopaminergic terminals of mouse striatum. *Biochem Pharmacol.* 2007;74:1235-46.
- [50] Brody AL, Mandelkern MA, London ED, Olmstead RE, Farahi J, Scheibal D, et al. Cigarette smoking saturates brain alpha 4 beta 2 nicotinic acetylcholine receptors. *Arch Gen Psychiatry.* 2006;63:907-15.
- [51] Alcaro A, Huber R, Panksepp J. Behavioral functions of the mesolimbic dopaminergic system: an affective neuroethological perspective. *Brain Res Rev.* 2007;56:283-321.
- [52] Kelley AE, Berridge KC. The neuroscience of natural rewards: relevance to addictive drugs. *J Neurosci.* 2002;22:3306-11.
- [53] Volkow ND, Li TK. Drug addiction: the neurobiology of behaviour gone awry. *Nat Rev Neurosci.* 2004;5:963-70.
- [54] Hyman SE. Addiction: a disease of learning and memory. *Am J Psychiatry.* 2005;162:1414-22.
- [55] Caggiula AR, Donny EC, White AR, Chaudhri N, Booth S, Gharib MA, et al. Cue dependency of nicotine self-administration and smoking. *Pharmacol Biochem Behav.* 2001;70:515-30.
- [56] Palmatier MI, Evans-Martin FF, Hoffman A, Caggiula AR, Chaudhri N, Donny EC, et al. Dissociating the primary reinforcing and reinforcement-enhancing effects of nicotine using a rat self-administration paradigm with concurrently available drug and environmental reinforcers. *Psychopharmacology (Berl).* 2006;184:391-400.
- [57] Palmatier MI, Matteson GL, Black JJ, Liu X, Caggiula AR, Craven L, et al. The reinforcement enhancing effects of nicotine depend on the incentive value of non-drug reinforcers and increase with repeated drug injections. *Drug Alcohol Depend.* 2007;89:52-9.
- [58] Chaudhri N, Caggiula AR, Donny EC, Booth S, Gharib M, Craven L, et al. Operant responding for conditioned and unconditioned reinforcers in rats is differentially enhanced by the primary reinforcing and reinforcement-enhancing effects of nicotine. *Psychopharmacology (Berl).* 2006;189:27-36.
- [59] Corrigall WA, Franklin KB, Coen KM, Clarke PB. The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. *Psychopharmacology.* 1992;107:285-9.
- [60] Roberts DC, Koob GF. Disruption of cocaine self-administration following 6-hydroxydopamine lesions of the ventral tegmental area in rats. *Pharmacol Biochem Behav.* 1982;17:901-4.

- [61] Alderson HL, Parkinson JA, Robbins TW, Everitt BJ. The effects of excitotoxic lesions of the nucleus accumbens core or shell regions on intravenous heroin self-administration in rats. *Psychopharmacology (Berl)*. 2001;153:455-63.
- [62] Feltenstein MW, See RE. The neurocircuitry of addiction: an overview. *Br J Pharmacol*. 2008;154:261-74.
- [63] Markou A. Review. Neurobiology of nicotine dependence. *Philos Trans R Soc Lond B Biol Sci*. 2008;363:3159-68.
- [64] Benowitz NL. Neurobiology of nicotine addiction: implications for smoking cessation treatment. *Am J Med*. 2008;121:S3-10.
- [65] Bruijnzeel AW. Tobacco addiction and the dysregulation of brain stress systems. *Neurosci Biobehav Rev*. 2012;36:1418-41.
- [66] U.S. Department of Health and Human Services. The health consequences of smoking: nicotine addiction, a report of the Surgeon General. DHHS. 1988:374-443.
- [67] Benowitz NL. Clinical pharmacology of nicotine: implications for understanding, preventing, and treating tobacco addiction. *Clin Pharmacol Ther*. 2008;83:531-41.
- [68] Levin ED, Briggs SJ, Christopher NC, Rose JE. Persistence of chronic nicotine-induced cognitive facilitation. *Behav Neural Biol*. 1992;58:152-8.
- [69] Myers CS, Taylor RC, Moolchan ET, Heishman SJ. Dose-related enhancement of mood and cognition in smokers administered nicotine nasal spray. *Neuropsychopharmacology*. 2008;33:588-98.
- [70] Levin ED, Cauley M, Rezvani AH. Improvement of attentional function with antagonism of nicotinic receptors in female rats. *Eur J Pharmacol*. 2013.
- [71] Bruijnzeel AW, Prado M, Isaac S. Corticotropin-releasing factor-1 receptor activation mediates nicotine withdrawal-induced deficit in brain reward function and stress-induced relapse. *Biol Psychiatry*. 2009;66:110-7.
- [72] Bruijnzeel AW, Ford J, Rogers JA, Scheick S, Ji Y, Bishnoi M, et al. Blockade of CRF1 receptors in the central nucleus of the amygdala attenuates the dysphoria associated with nicotine withdrawal in rats. *Pharmacol Biochem Behav*. 2012;101:62-8.
- [73] Marcinkiewicz CA, Prado MM, Isaac SK, Marshall A, Rylkova D, Bruijnzeel AW. Corticotropin-releasing factor within the central nucleus of the amygdala and the nucleus accumbens shell mediates the negative affective state of nicotine withdrawal in rats. *Neuropsychopharmacology*. 2009;34:1743-52.
- [74] LeSage MG, Burroughs D, Dufek M, Keyler DE, Pentel PR. Reinstatement of nicotine self-administration in rats by presentation of nicotine-paired stimuli, but not nicotine priming. *Pharmacol Biochem Behav*. 2004;79:507-13.
- [75] Rose JE, Behm FM, Westman EC, Johnson M. Dissociating nicotine and nonnicotine components of cigarette smoking. *Pharmacol Biochem Behav*. 2000;67:71-81.

- [76] Brauer LH, Behm FM, Lane JD, Westman EC, Perkins C, Rose JE. Individual differences in smoking reward from de-nicotinized cigarettes. *Nicotine Tob Res.* 2001;3:101-9.
- [77] Rose JE. Nicotine and nonnicotine factors in cigarette addiction. *Psychopharmacology (Berl).* 2006;184:274-85.
- [78] Jorenby DE, Hays JT, Rigotti NA, Azoulay S, Watsky EJ, Williams KE, et al. Efficacy of varenicline, an alpha4beta2 nicotinic acetylcholine receptor partial agonist, vs placebo or sustained-release bupropion for smoking cessation: a randomized controlled trial. *JAMA.* 2006;296:56-63.
- [79] Raupach T, van Schayck CP. Pharmacotherapy for Smoking Cessation Current Advances and Research Topics. *CNS Drugs.* 2011;25:371-82.
- [80] Fant RV, Owen LL, Henningfield JE. Nicotine replacement therapy. *Prim Care.* 1999;26:633-52.
- [81] Waters AJ, Shiffman S, Sayette MA, Paty JA, Gwaltney CJ, Balabanis MH. Cue-provoked craving and nicotine replacement therapy in smoking cessation. *J Consult Clin Psychol.* 2004;72:1136-43.
- [82] Henningfield JE. Reducing the urge to smoke. *Chest.* 1987;92:963.
- [83] Shiffman S, Rolf CN, Hellebusch SJ, Gorsline J, Gorodetzky CW, Chiang YK, et al. Real-world efficacy of prescription and over-the-counter nicotine replacement therapy. *Addiction.* 2002;97:505-16.
- [84] Lerman C, Kaufmann V, Rukstalis M, Patterson F, Perkins K, Audrain-McGovern J, et al. Individualizing nicotine replacement therapy for the treatment of tobacco dependence: a randomized trial. *Ann Intern Med.* 2004;140:426-33.
- [85] Aubin HJ, Bobak A, Britton JR, Oncken C, Billing CB, Jr., Gong J, et al. Varenicline versus transdermal nicotine patch for smoking cessation: results from a randomised open-label trial. *Thorax.* 2008;63:717-24.
- [86] Malarcher A. Quitting smoking among adults --- United States, 2001-2010. *Morbidity and Mortality Weekly Report: Centers for Disease Control and Prevention;* 2011. p. 1513-19.
- [87] Culbertson CS, Bramen J, Cohen MS, London ED, Olmstead RE, Gan JJ, et al. Effect of bupropion treatment on brain activation induced by cigarette-related cues in smokers. *Arch Gen Psychiatry.* 2011;68:505-15.
- [88] Shiffman S, Johnston JA, Khayrallah M, Elash CA, Gwaltney CJ, Paty JA, et al. The effect of bupropion on nicotine craving and withdrawal. *Psychopharmacology (Berl).* 2000;148:33-40.
- [89] Durcan MJ, Deener G, White J, Johnston JA, Gonzales D, Niaura R, et al. The effect of bupropion sustained-release on cigarette craving after smoking cessation. *Clin Ther.* 2002;24:540-51.
- [90] Rollema H, Chambers LK, Coe JW, Glowa J, Hurst RS, Lebel LA, et al. Pharmacological profile of the alpha4beta2 nicotinic acetylcholine receptor partial agonist varenicline, an effective smoking cessation aid. *Neuropharmacology.* 2007;52:985-94.
- [91] Fagerstrom K, Hughes J. Varenicline in the treatment of tobacco dependence. *Neuropsychiatr Dis Treat.* 2008;4:353-63.



- [92] Hussmann GP, Turner JR, Lomazzo E, Venkatesh R, Cousins V, Xiao Y, et al. Chronic sazetidine-A at behaviorally active doses does not increase nicotinic cholinergic receptors in rodent brain. *J Pharmacol Exp Ther*. 2012;343:441-50.
- [93] Turner JR, Castellano LM, Blendy JA. Parallel anxiolytic-like effects and upregulation of neuronal nicotinic acetylcholine receptors following chronic nicotine and varenicline. *Nicotine Tob Res*. 2011;13:41-6.
- [94] Sallette J, Bohler S, Benoit P, Soudant M, Pons S, Le Novere N, et al. An extracellular protein microdomain controls up-regulation of neuronal nicotinic acetylcholine receptors by nicotine. *J Biol Chem*. 2004;279:18767-75.
- [95] Gonzales D, Rennard SI, Nides M, Oncken C, Azoulay S, Billing CB, et al. Varenicline, an alpha4beta2 nicotinic acetylcholine receptor partial agonist, vs sustained-release bupropion and placebo for smoking cessation: a randomized controlled trial. *JAMA*. 2006;296:47-55.
- [96] Franklin T, Wang Z, Suh JJ, Hazan R, Cruz J, Li Y, et al. Effects of varenicline on smoking cue-triggered neural and craving responses. *Arch Gen Psychiatry*. 2011;68:516-26.
- [97] West R, Baker CL, Cappelleri JC, Bushmakin AG. Effect of varenicline and bupropion SR on craving, nicotine withdrawal symptoms, and rewarding effects of smoking during a quit attempt. *Psychopharmacology (Berl)*. 2008;197:371-7.
- [98] Gunnell D, Irvine D, Wise L, Davies C, Martin RM. Varenicline and suicidal behaviour: a cohort study based on data from the General Practice Research Database. *BMJ*. 2009;339:b3805.
- [99] MHRA. Varenicline: adverse psychiatric reactions, including depression. *Drug Safety Update*. 2008;2:2.
- [100] Xi ZX, Spiller K, Gardner EL. Mechanism-based medication development for the treatment of nicotine dependence. *Acta Pharmacol Sin*. 2009;30:723-39.
- [101] Heidbreder C. Novel pharmacotherapeutic targets for the management of drug addiction. *Eur J Pharmacol*. 2005;526:101-12.
- [102] Liu X, Palmatier MI, Caggiula AR, Sved AF, Donny EC, Gharib M, et al. Naltrexone attenuation of conditioned but not primary reinforcement of nicotine in rats. *Psychopharmacology (Berl)*. 2009;202:589-98.
- [103] Harmey D, Griffin PR, Kenny PJ. Development of novel pharmacotherapeutics for tobacco dependence: progress and future directions. *Nicotine Tob Res*. 2012;14:1300-18.
- [104] Fiore MC. US public health service clinical practice guideline: treating tobacco use and dependence. *Respir Care*. 2000;45:1200-62.
- [105] Rubinstein ML, Benowitz NL, Auerback GM, Moscicki AB. A randomized trial of nicotine nasal spray in adolescent smokers. *Pediatrics*. 2008;122:e595-600.
- [106] Doggrell SA. Which is the best primary medication for long-term smoking cessation--nicotine replacement therapy, bupropion or varenicline? *Expert Opin Pharmacother*. 2007;8:2903-15.

- [107] Melvin CL, Dolan-Mullen P, Windsor RA, Whiteside HP, Jr., Goldenberg RL. Recommended cessation counselling for pregnant women who smoke: a review of the evidence. *Tob Control*. 2000;9:III80-4.
- [108] Jorenby DE, Smith SS, Fiore MC, Hurt RD, Offord KP, Croghan IT, et al. Varying nicotine patch dose and type of smoking cessation counseling. *Jama*. 1995;274:1347-52.
- [109] Hughes JR, Keely J, Naud S. Shape of the relapse curve and long-term abstinence among untreated smokers. *Addiction*. 2004;99:29-38.
- [110] Satoskar SD, Keyler DE, LeSage MG, Raphael DE, Ross CA, Pentel PR. Tissue-dependent effects of immunization with a nicotine conjugate vaccine on the distribution of nicotine in rats. *Int Immunopharmacol*. 2003;3:957-70.
- [111] LeSage MG, Keyler DE, Hieda Y, Collins G, Burroughs D, Le C, et al. Effects of a nicotine conjugate vaccine on the acquisition and maintenance of nicotine self-administration in rats. *Psychopharmacology (Berl)*. 2006;184:409-16.
- [112] Cornish KE, Harris AC, LeSage MG, Keyler DE, Burroughs D, Earley C, et al. Combined active and passive immunization against nicotine: minimizing monoclonal antibody requirements using a target antibody concentration strategy. *Int Immunopharmacol*. 2011;11:1809-15.
- [113] Stills HF, Jr. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. *ILAR J*. 2005;46:280-93.
- [114] Adam A, Petit JF, Lefrancier P, Lederer E. Muramyl peptides. Chemical structure, biological activity and mechanism of action. *Mol Cell Biochem*. 1981;41:27-47.
- [115] Martell BA, Orson FM, Poling J, Mitchell E, Rossen RD, Gardner T, et al. Cocaine vaccine for the treatment of cocaine dependence in methadone-maintained patients a randomized, double-blind, placebo-controlled efficacy trial. *Arch Gen Psychiatry*. 2009;66:1116-23.
- [116] Hatsukami DK, Rennard S, Jorenby D, Fiore M, Koopmeiners J, de Vos A, et al. Safety and immunogenicity of a nicotine conjugate vaccine in current smokers. *Clin Pharmacol Ther*. 2005;78:456-67.
- [117] Ghimire TR, Benson RA, Garside P, Brewer JM. Alum increases antigen uptake, reduces antigen degradation and sustains antigen presentation by DCs in vitro. *Immunol Lett*. 2012;147:55-62.
- [118] Spreafico R, Ricciardi-Castagnoli P, Mortellaro A. The controversial relationship between NLRP3, alum, danger signals and the next-generation adjuvants. *Eur J Immunol*. 2010;40:638-42.
- [119] Kool M, Soullie T, van Nimwegen M, Willart MA, Muskens F, Jung S, et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp Med*. 2008;205:869-82.
- [120] Flach TL, Ng G, Hari A, Desrosiers MD, Zhang P, Ward SM, et al. Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. *Nat Med*. 2011;17:479-87.

- [121] Maurer P, Jennings GT, Willers J, Rohner F, Lindman Y, Roubicek K, et al. A therapeutic vaccine for nicotine dependence: preclinical efficacy, and phase I safety and immunogenicity. *Eur J Immunol.* 2005;35:2031-40.
- [122] Bazin-Redureau MI, Renard CB, Scherrmann JG. Pharmacokinetics of heterologous and homologous immunoglobulin G, F(ab')<sub>2</sub> and Fab after intravenous administration in the rat. *Journal of Pharmacy and Pharmacology.* 1997;49:277-81.
- [123] Moreno AY, Azar MR, Warren NA, Dickerson TJ, Koob GF, Janda KD. A Critical Evaluation of a Nicotine Vaccine within a Self-Administration Behavioral Model. *Mol Pharm.* 2010;7:431-41.
- [124] Pentel PR, Malin DH, Ennifar S, Hieda Y, Keyler DE, Lake JR, et al. A nicotine conjugate vaccine reduces nicotine distribution to brain and attenuates its behavioral and cardiovascular effects in rats. *Pharmacol Biochem Behav.* 2000;65:191-8.
- [125] Hieda Y, Keyler DE, Vandevort JT, Kane JK, Ross CA, Raphael DE, et al. Active immunization alters the plasma nicotine concentration in rats. *J Pharmacol Exp Ther.* 1997;283:1076-81.
- [126] Meijler MM, Matsushita M, Altobelli LJ, 3rd, Wirsching P, Janda KD. A new strategy for improved nicotine vaccines using conformationally constrained haptens. *J Am Chem Soc.* 2003;125:7164-5.
- [127] Harris JR, Markl J. Keyhole limpet hemocyanin (KLH): a biomedical review. *Micron.* 1999;30:597-623.
- [128] de Villiers SHL, Lindblom N, Kalayanov G, Gordon S, Baraznenok I, Malmerfelt A, et al. Nicotine hapten structure, antibody selectivity and effect relationships: Results from a nicotine vaccine screening procedure. *Vaccine.* 2010;28:2161-8.
- [129] Escobar-Chavez JJ, Dominguez-Delgado CL, Rodriguez-Cruz IM. Targeting nicotine addiction: the possibility of a therapeutic vaccine. *Drug Des Devel Ther.* 2011;5:211-24.
- [130] Moreno AY, Azar MR, Koob GF, Janda KD. Probing the protective effects of a conformationally constrained nicotine vaccine. *Vaccine.* 2012;30:6665-70.
- [131] Pravetoni M, Keyler DE, Pidaparathi RR, Carroll FI, Runyon SP, Murtaugh MP, et al. Structurally distinct nicotine immunogens elicit antibodies with non-overlapping specificities. *Biochem Pharmacol.* 2012;83:543-50.
- [132] Lockner JW, Ho SO, McCague KC, Chiang SM, Do TQ, Fujii G, et al. Enhancing nicotine vaccine immunogenicity with liposomes. *Bioorg Med Chem Lett.* 2013.
- [133] de Villiers SH, Lindblom N, Kalayanov G, Gordon S, Malmerfelt A, Johansson AM, et al. Active immunization against nicotine suppresses nicotine-induced dopamine release in the rat nucleus accumbens shell. *Respiration.* 2002;69:247-53.
- [134] Lindblom N, de Villiers SH, Semenova S, Kalayanov G, Gordon S, Schilstrom B, et al. Active immunisation against nicotine blocks the reward facilitating effects of nicotine and partially prevents nicotine withdrawal in the rat

as measured by dopamine output in the nucleus accumbens, brain reward thresholds and somatic signs. *Naunyn Schmiedeberg's Arch Pharmacol.* 2005;372:182-94.

[135] Lindblom N, De Villiers SH, Kalayanov G, Gordon S, Johansson AM, Svensson TH. Active Immunization against Nicotine Prevents Reinstatement of Nicotine-Seeking Behavior in Rats. *Respiration.* 2002;69:254-60.

[136] Keyler DE, Hieda Y, St Peter J, Pentel PR. Altered disposition of repeated nicotine doses in rats immunized against nicotine. *Nicotine Tob Res.* 1999;1:241-9.

[137] Pravetoni M, Keyler DE, Raleigh MD, Harris AC, LeSage MG, Mattson CK, et al. Vaccination against nicotine alters the distribution of nicotine delivered via cigarette smoke inhalation to rats. *Biochem Pharmacol.* 2011;81:1164-70.

[138] Hieda Y, Keyler DE, VanDeVoort JT, Niedbala RS, Raphael DE, Ross CA, et al. Immunization of rats reduces nicotine distribution to brain. *Psychopharmacology (Berl).* 1999;143:150-7.

[139] Hieda Y, Keyler DE, Ennifar S, Fattom A, Pentel PR. Vaccination against nicotine during continued nicotine administration in rats: immunogenicity of the vaccine and effects on nicotine distribution to brain. *Int J Immunopharmacol.* 2000;22:809-19.

[140] Carrera MR, Ashley JA, Hoffman TZ, Isomura S, Wirsching P, Koob GF, et al. Investigations using immunization to attenuate the psychoactive effects of nicotine. *Bioorg Med Chem.* 2004;12:563-70.

[141] Roiko SA, Harris AC, Keyler DE, LeSage MG, Zhang Y, Pentel PR. Combined active and passive immunization enhances the efficacy of immunotherapy against nicotine in rats. *J Pharmacol Exp Ther.* 2008;325:985-93.

[142] Vezina P, McGehee DS, Green WN. Exposure to nicotine and sensitization of nicotine-induced behaviors. *Prog Neuropsychopharmacol Biol Psychiatry.* 2007;31:1625-38.

[143] Hatsukami DK, Jorenby DE, Gonzales D, Rigotti NA, Glover ED, Oncken CA, et al. Immunogenicity and smoking-cessation outcomes for a novel nicotine immunotherapeutic. *Clin Pharmacol Ther.* 2011;89:392-9.

[144] Cornuz J, Zwahlen S, Jungi WF, Osterwalder J, Klingler K, van Melle G, et al. A vaccine against nicotine for smoking cessation: A randomized controlled trial. *Plos One.* 2008;3.

[145] Cerny T. Anti-nicotine vaccination: where are we? *Recent Results Cancer Res.* 2005;166:167-75.

[146] Trial watch: Xenova's TA-NIC vaccine shows promise. *Expert Rev Vaccines.* 2004;3:386.

[147] Swedish anti-nicotine vaccine to be tested in Nordic countries. Stockholm: Associated Free Press; 2008.

[148] WHO. Requirements for Diphtheria, Tetanus, Pertussis and Combined Vaccines. World Health Organization Technical Report Series 800. 1990.

- [149] Fox BS, Kantak KM, Edwards MA, Black KM, Bollinger BK, Botka AJ, et al. Efficacy of a therapeutic cocaine vaccine in rodent models. *Nature Medicine*. 1996;2:1129-32.
- [150] Carrera MR, Ashley JA, Parsons LH, Wirsching P, Koob GF, Janda KD. Suppression of psychoactive effects of cocaine by active immunization. *Nature*. 1995;378:727-30.
- [151] Kantak KM, Collins SL, Bond J, Fox BS. Time course of changes in cocaine self-administration behavior in rats during immunization with the cocaine vaccine IPC-1010. *Psychopharmacol*. 2001;153:334-40.
- [152] Haney M, Gundersen EW, Jiang HP, Collins ED, Foltin RW. Cocaine-Specific Antibodies Blunt the Subjective Effects of Smoked Cocaine in Humans. *Biological Psychiatry*. 2010;67:59-65.
- [153] Hicks MJ, De BP, Rosenberg JB, Davidson JT, Moreno AY, Janda KD, et al. Cocaine analog coupled to disrupted adenovirus: a vaccine strategy to evoke high-titer immunity against addictive drugs. *Mol Ther*. 2011;19:612-9.
- [154] Carroll FI, Abraham P, Gong PK, Pidaparathi RR, Blough BE, Che YN, et al. The Synthesis of Haptens and Their Use for the Development of Monoclonal Antibodies for Treating Methamphetamine Abuse. *J Med Chem*. 2009;52:7301-9.
- [155] Miller ML, Moreno AY, Aarde SM, Creehan KM, Vandewater SA, Vaillancourt BD, et al. A Methamphetamine Vaccine Attenuates Methamphetamine-Induced Disruptions in Thermoregulation and Activity in Rats. *Biol Psychiatry*. 2012.
- [156] Duryee MJ, Bevins RA, Reichel CM, Murray JE, Dong Y, Thiele GM, et al. Immune responses to methamphetamine by active immunization with peptide-based, molecular adjuvant-containing vaccines. *Vaccine*. 2009;27:2981-8.
- [157] Shen XY, Kosten TA, Lopez AY, Kinsey BM, Kosten TR, Orson FM. A vaccine against methamphetamine attenuates its behavioral effects in mice. *Drug Alcohol Depend*. 2012.
- [158] Bonese KF, Wainer BH, Fitch FW, Rothberg RM, Schuster CR. Changes in heroin self-administration by a rhesus monkey after morphine immunization. *Nature*. 1974;252:708-10.
- [159] Berkowitz B, Spector S. Evidence for active immunity to morphine in mice. *Science*. 1972;178:1290-2.
- [160] Pravetoni M, Raleigh MD, Le Naour M, Tucker AM, Harmon TM, Jones JM, et al. Co-administration of morphine and oxycodone vaccines reduces the distribution of 6-monoacetylmorphine and oxycodone to brain in rats. *Vaccine*. 2012;30:4617-24.
- [161] Pravetoni M, Le Naour M, Harmon TM, Tucker AM, Portoghese PS, Pentel PR. An oxycodone conjugate vaccine elicits drug-specific antibodies that reduce oxycodone distribution to brain and hot-plate analgesia. *J Pharmacol Exp Ther*. 2012;341:225-32.
- [162] Stowe GN, Vendruscolo LF, Edwards S, Schlosburg JE, Misra KK, Schulteis G, et al. A vaccine strategy that induces protective immunity against heroin. *J Med Chem*. 2011;54:5195-204.

- [163] Stowe GN, Schlosburg JE, Vendruscolo LF, Edwards S, Misra KK, Schulteis G, et al. Developing a vaccine against multiple psychoactive targets: a case study of heroin. *CNS Neurol Disord Drug Targets*. 2011;10:865-75.
- [164] Li QQ, Luo YX, Sun CY, Xue YX, Zhu WL, Shi HS, et al. A morphine/heroin vaccine with new hapten design attenuates behavioral effects in rats. *J Neurochem*. 2011;119:1271-81.
- [165] Anton B, Leff P. A novel bivalent morphine/heroin vaccine that prevents relapse to heroin addiction in rodents. *Vaccine*. 2006;24:3232-40.
- [166] Matsushita M, Hoffman TZ, Ashley JA, Zhou B, Wirsching P, Janda KD. Cocaine catalytic antibodies: the primary importance of linker effects. *Bioorg Med Chem Lett*. 2001;11:87-90.
- [167] Vocci FJ, Montoya ID. Psychological treatments for stimulant misuse, comparing and contrasting those for amphetamine dependence and those for cocaine dependence. *Curr Opin Psychiatry*. 2009;22:263-8.
- [168] Kantak KM. Vaccines against drugs of abuse: a viable treatment option? *Drugs*. 2003;63:341-52.
- [169] Moreno AY, Janda KD. Immunopharmacotherapy: Vaccination strategies as a treatment for drug abuse and dependence. *Pharmacol Biochem Behav*. 2009;92:199-205.
- [170] Rook EJ, Huitema AD, van den Brink W, van Ree JM, Beijnen JH. Population pharmacokinetics of heroin and its major metabolites. *Clin Pharmacokinet*. 2006;45:401-17.
- [171] Keyler DE, Salerno DM, Murakami MM, Ruth G, Pentel PR. Rapid administration of high-dose human antibody Fab fragments to dogs: pharmacokinetics and toxicity. *Fundam Appl Toxicol*. 1991;17:83-91.
- [172] Keyler DE, Shelver WL, Landon J, Sidki A, Pentel PR. Toxicity of high doses of polyclonal drug-specific antibody Fab fragments. *Int J Immunopharmacol*. 1994;16:1027-34.
- [173] Waldmann TA, Strober W. Metabolism of immunoglobulins. *Prog Allergy*. 1969;13:1-110.
- [174] Koleba T, Ensom MH. Pharmacokinetics of intravenous immunoglobulin: a systematic review. *Pharmacotherapy*. 2006;26:813-27.
- [175] Lipman NS, Jackson LR. Hollow fibre bioreactors: an alternative to murine ascites for small scale (<1 gram) monoclonal antibody production. *Res Immunol*. 1998;149:571-6.
- [176] Peterson E, Owens SM, Henry RL. Monoclonal antibody form and function: Manufacturing the right antibodies for treating drug abuse. *Aaps Journal*. 2006;8:E383-E90.
- [177] Wang W, Wang EQ, Balthasar JP. Monoclonal antibody pharmacokinetics and pharmacodynamics. *Clin Pharmacol Ther*. 2008;84:548-58.
- [178] Lipman NS, Jackson LR, Trudel LJ, Weis-Garcia F. Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. *ILAR J*. 2005;46:258-68.

- [179] Keyler DE, Roiko SA, Benlhabib E, Lesage MG, St Peter JV, Stewart S, et al. Monoclonal nicotine-specific antibodies reduce nicotine distribution to brain in rats: Dose- and affinity-response relationships. *Drug Metab Disposition*. 2005;33:1056-61.
- [180] Hicks MJ, Rosenberg JB, De BP, Pagovich OE, Young CN, Qiu JP, et al. AAV-Directed Persistent Expression of a Gene Encoding Anti-Nicotine Antibody for Smoking Cessation. *Science Translational Medicine*. 2012;4.
- [181] LeSage MG, Shelley D, Pravetoni M, Pentel PR. Enhanced attenuation of nicotine discrimination in rats by combining nicotine-specific antibodies with a nicotinic receptor antagonist. *Pharmacol Biochem Behav*. 2012;102:157-62.
- [182] Rosenberg JB, Hicks MJ, De BP, Pagovich O, Frenk E, Janda KD, et al. AAVrh.10-mediated expression of an anti-cocaine antibody mediates persistent passive immunization that suppresses cocaine-induced behavior. *Hum Gene Ther*. 2012;23:451-9.
- [183] Brimijoin S, Gao Y, Anker JJ, Gliddon LA, Lafleur D, Shah R, et al. A cocaine hydrolase engineered from human butyrylcholinesterase selectively blocks cocaine toxicity and reinstatement of drug seeking in rats. *Neuropsychopharmacology*. 2008;33:2715-25.
- [184] Norman AB, Norman MK, Buesing WR, Tabet MR, Tsibulsky VL, Ball WJ. The effect of a chimeric human/murine anti-cocaine monoclonal antibody on cocaine self-administration in rats. *J Pharmacol Exp Ther*. 2009;328:873-81.
- [185] Byrnes-Blake KA, Laurenzana EM, Carroll FI, Abraham P, Gentry WB, Landes RD, et al. Pharmacodynamic mechanisms of monoclonal antibody-based antagonism of (+)-methamphetamine in rats. *Eur J Pharmacol*. 2003;461:119-28.
- [186] Byrnes-Blake KA, Laurenzana EM, Landes RD, Gentry WB, Owens SM. Monoclonal IgG affinity and treatment time alters antagonism of (+)-methamphetamine effects in rats. *Eur J Pharmacol*. 2005;521:86-94.
- [187] McMillan DE, Hardwick WC, Li M, Gunnell MG, Carroll FI, Abraham P, et al. Effects of murine-derived anti-methamphetamine monoclonal antibodies on (+)-methamphetamine self-administration in the rat. *J Pharmacol Exp Ther*. 2004;309:1248-55.
- [188] Daniels JR, Wessinger WD, Hardwick WC, Li M, Gunnell MG, Hall CJ, et al. Effects of anti-phencyclidine and anti-(+)-methamphetamine monoclonal antibodies alone and in combination on the discrimination of phencyclidine and (+)-methamphetamine by pigeons. *Psychopharmacology*. 2006;185:36-44.
- [189] Gentry WB, Laurenzana EM, Williams DK, West JR, Berg RJ, Terlea T, et al. Safety and efficiency of an anti-(+)-methamphetamine monoclonal antibody in the protection against cardiovascular and central nervous system effects of (+)-methamphetamine in rats. *Int Immunopharmacol*. 2006;6:968-77.
- [190] Laurenzana EM, Hendrickson HP, Carpenter D, Peterson EC, Gentry WB, West M, et al. Functional and biological determinants affecting the duration of action and efficacy of anti-(+)-methamphetamine monoclonal antibodies in rats. *Vaccine*. 2009;27:7011-20.

- [191] McClurkan MB, Valentine JL, Arnold L, Owens SM. Disposition of a monoclonal anti-phencyclidine Fab fragment of immunoglobulin G in rats. *J Pharmacol Exp Ther.* 1993;266:1439-45.
- [192] Lacy HM, Gunnell MG, Laurenzana EM, Owens SM. Engineering and characterization of a mouse/human chimeric anti-phencyclidine monoclonal antibody. *Int Immunopharmacol.* 2008;8:1-11.
- [193] Proksch JW, Gentry WB, Owens SM. Anti-phencyclidine monoclonal antibodies provide long-term reductions in brain phencyclidine concentrations during chronic phencyclidine administration in rats. *J Pharmacol Exp Ther.* 2000;292:831-7.
- [194] Hubbard JJ, Laurenzana EM, Williams DK, Gentry WB, Owens SM. Chronic anti-phencyclidine monoclonal antibody therapy decreases phencyclidine-induced in utero fetal mortality in pregnant rats. *Int Immunopharmacol.* 2011;11:2181-7.
- [195] Valentine JL, Owens SM. Antiphencyclidine monoclonal antibody therapy significantly changes phencyclidine concentrations in brain and other tissues in rats. *J Pharmacol Exp Ther.* 1996;278:717-24.
- [196] Carroll ME, Zlebnik NE, Anker JJ, Kosten TR, Orson FM, Shen X, et al. Combined cocaine hydrolase gene transfer and anti-cocaine vaccine synergistically block cocaine-induced locomotion. *Plos One.* 2012;7:e43536.
- [197] Keyler DE, Roiko SA, Earley CA, Murtaugh MP, Pentel PR. Enhanced immunogenicity of a bivalent nicotine vaccine. *Int Immunopharmacol.* 2008;8:1589-94.
- [198] Miller RP, Rotenberg KS, Adir J. Effect of dose on the pharmacokinetics of intravenous nicotine in the rat. *Drug Metab Dispos.* 1977;5:436-43.
- [199] LeSage MG, Keyler DE, Pentel PR. Current status of immunologic approaches to treating tobacco dependence: vaccines and nicotine-specific antibodies. *AAPS J.* 2006;8:E65-75.
- [200] Elkashef A, Biswas J, Acri JB, Vocci F. Biotechnology and the treatment of addictive disorders - New opportunities. *Biodrugs.* 2007;21:259-67.
- [201] Orson FM, Kinsey BM, Singh RAK, Wu Y, Gardner T, Kosten TR. Substance Abuse Vaccines. *Addiction Reviews* 2008. Oxford: Blackwell Publishing; 2008. p. 257-69.
- [202] Pitas G, Laurenzana EM, Williams DK, Owens SM, Gentry WB. Anti-phencyclidine monoclonal antibody binding capacity is not the only determinant of effectiveness, disproving the concept that antibody capacity is easily surmounted. *Drug Metab Dispos.* 2006;34:906-12.
- [203] Anton B, Salazar A, Flores A, Matus M, Marin R, Hernandez JA, et al. Vaccines against morphine/heroin and its use as effective medication for preventing relapse to opiate addictive behaviors. *Hum Vaccines.* 2009;5:214-29.
- [204] Pentel PR, Keyler DE. Vaccines to treat drug addiction. In: Levine MM, editor. *New Generation Vaccines.* 3rd ed. New York: Dekker; 2004. p. 1057-66.
- [205] Hardin JS, Wessinger WD, Wenger GR, Proksch JW, Laurenzana EM, Owens SM. A single dose of monoclonal anti-phencyclidine IgG offers long-term



- reductions in phencyclidine behavioral effects in rats. *J Pharmacol Exp Ther.* 2002;302:119-26.
- [206] Getts DR, Getts MT, McCarthy DP, Chastain EML, Miller SD. Have we overestimated the benefit of human(ized) antibodies? *Mabs.* 2010;2:682-94.
- [207] Pentel PR, Dufek MB, Roiko SA, LeSage MG, Keyler DE. Differential effects of passive immunization with nicotine-specific antibodies on the acute and chronic distribution of nicotine to brain in rats. *J Pharmacol Exp Ther.* 2006;317:660-6.
- [208] Jacob P, 3rd, Wilson M, Benowitz NL. Improved gas chromatographic method for the determination of nicotine and cotinine in biologic fluids. *J Chromatogr.* 1981;222:61-70.
- [209] Keyler DE, Shoeman D, Lesage MG, Calvin AD, Pentel PR. Maternal vaccination against nicotine reduces nicotine distribution to fetal brain in rats. *J Pharmacol Exp Ther.* 2003;305:587-92.
- [210] Tabrizi MA, Roskos LK. Preclinical and clinical safety of monoclonal antibodies. *Drug Discovery Today.* 2007;12:540-7.
- [211] Dierickx D, Delannoy A, Saja K, Verhoef G, Provan D. Anti-CD20 monoclonal antibodies and their use in adult autoimmune hematological disorders. *Am J Hematol.* 2010;86:278-91.
- [212] Presta LG. Engineering of therapeutic antibodies to minimize immunogenicity and optimize function. *Advanced Drug Delivery Reviews.* 2006;58:640-56.
- [213] Satoskar SD, Keyler DE, LeSage MG, Raphael DE, Ross CA, Pentel PR. Tissue-dependent effects of immunization with a nicotine conjugate vaccine on the distribution of nicotine in rats. *Int Immunopharmacol.* 2003;3:957-70.
- [214] Vincenti F, Kirkman R, Light S, Bumgardner G, Pescovitz M, Halloran P, et al. Interleukin-2-receptor blockade with daclizumab to prevent acute rejection in renal transplantation. *New England Journal of Medicine.* 1998;338:161-5.
- [215] Shen XY, Orson FM, Kosten TR. Vaccines against drug abuse. *Clin Pharmacol Ther.* 2012;91:60-70.
- [216] Raupach T, Hoogsteder P, van Schayck CP. Nicotine vaccines to assist with smoking cessation: Current status of research. *Drugs.* 2012;72:e1-e16.
- [217] Halperin SA, Langley JM, Eastwood BJ. Effect of inactivated poliovirus vaccine on the antibody response to Bordetella pertussis antigens when combined with diphtheria-pertussis-tetanus vaccine. *Clinical Infectious Diseases.* 1996;22:59-62.
- [218] Pichichero ME, Passador S. Administration of combined diphtheria and tetanus toxoids and pertussis vaccine, hepatitis B vaccine, and Haemophilus influenzae type b (hib) vaccine to infants and response to a booster dose of hib conjugate vaccine. *Clinical Infectious Diseases.* 1997;25:1378-84.
- [219] Vidor E. The nature and consequences of intra- and inter-vaccine interference. *Journal of Comparative Pathology.* 2007;137:S62-S6.
- [220] Insel RA. Potential alterations in immunogenicity by combining or simultaneously administering vaccine components. In: Williams JC, Goldenthal

KL, Burns DL, Lewis BP, editors. Combined Vaccines and Simultaneous Administration: Current Issues and Perspectives 1995. p. 35-47.