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Active Immunization against Nicotine Dependence

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ABSTRACT

Tobacco smoking is the largest preventable cause of morbidity and premature mortality in the world. Although its medical consequences are well documented, 20-45% of the adult population in the European countries remains tobacco smokers. The drugs presently used in smoking cessation have limited efficiency and, therefore, there is a need for alternative and improved treatments. A novel type of drug in this regard may be provided by using active immunization against nicotine, i.e. nicotine vaccines. The rationale is to generate endogenous anti-nicotine antibodies that bind nicotine in the blood, thereby preventing it from reaching the brain where it exerts its reinforcing effects. Since nicotine is a small molecule, unable to elicit an immune response, it has to be coupled to a carrier protein via a linker.

In this thesis a series of novel nicotine immunogens, including structurally different linkers coupled to the 5- or 6-position of the nicotine molecule and conjugated to a carrier protein, were studied. The amounts of antibodies generated and their selectivity were assessed by ELISA techniques, and all immunogens generated anti-nicotine antibodies that recognized (*S*)-nicotine or nornicotine better than the other nicotine metabolites tested. In addition, no cross-reactivity to endogenous transmitters was detected. *In vivo* voltammetry was used to assess the nicotine-induced increase in dopamine release in the shell region of the nucleus accumbens, a neurochemical correlate to nicotine's rewarding properties, and our work revealed that this effect of nicotine was differentially affected depending on both the immunogen studied and the selectivity of the antibodies. The nicotine immunogen IP18-KLH, showing a favorable antibody selectivity profile, prevented the nicotine-induced dopamine release and, likewise, the reinstatement of nicotine-seeking behavior in previously self-administering rats. These effects of the immunization with IP18-KLH were found to be associated with an altered distribution of nicotine, resulting in elevated nicotine levels in serum, due to antibody binding, and consequently a retarded nicotine distribution to the brain.

Taken together our results provide substantial preclinical support for the potential utility of nicotine vaccines in the treatment of nicotine dependence, notably relapse prevention.

LIST OF PUBLICATIONS

I. de Villiers SH, Lindblom N, Kalayanov G, Gordon S, Malmerfelt A, Johansson AM, Svensson TH (2002) Active Immunization against Nicotine Suppresses Nicotine-Induced Dopamine Release in the Rat Nucleus accumbens Shell. Respiration 69:247-253

II. Lindblom N, de Villiers SH, Kalayanov G, Gordon S, Johansson AM, Svensson TH (2002) Active Immunization against Nicotine Prevents Reinstatement of Nicotine-Seeking Behavior in Rats. Respiration 69:254-260

III. de Villiers SH, Lindblom N, Kalayanov G, Gordon S, Johansson AM, Svensson TH (2004) Active immunization against nicotine alters the distribution of nicotine but not the metabolism to cotinine in the rat. Naunyn Schmiedebergs Arch Pharmacol 370(4):299-304

IV. de Villiers SHL, Lindblom N, Kalayanov G, Gordon S, Baraznenok I, Malmerfelt A, Marcus MM, Johansson AM, Svensson TH (2010) Nicotine hapten structure, antibody selectivity and effect relationships: Results from a nicotine vaccine screening procedure. Vaccine 28:2161-2168

CONTENTS

LIST OF ABBREVIATIONS

ACH	acetylcholine
BSA	bovine serum albumin
c'_{TOT}	total amount of nicotine per gram of brain
c_{blood}	nicotine concentration in blood
c_{tissue}	amount of nicotine per gram of brain tissue
CHO	choline
COT	(*S*)-cotinine
DA	dopamine
DOPAC	dihydroxyphenylacetic acid
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	enzyme-linked immunosorbent assay
FCA	Freund´s complete adjuvant
FIA	Freund´s incomplete adjuvant
FR	fixed ratio
GABA	γ-amino butyric acid
GLY	glycine
HIST	histamine
i.p.	intraperitoneally
i.v.	intravenously
IC_{20}	a concentration giving 20% reduction in response
IC_{50}	a concentration giving 50% reduction in response
IC_{50COT}	a concentration of cotinine giving 50% reduction in response
IC_{50NIC}	a concentration of nicotine giving 50% reduction in response
i.d.	inner diameter,
IgG	immunoglobulin class G
KLH	keyhole limpet hemocyanin
M_w	molecular weight
n	number
NA	(*S*)-noradrenaline
n.d.	not detectable
NIA	niacin (nicotinic acid)
NIC	(*S*)-nicotine
NAC	nucleus accumbens
nAChR	nicotinic acetylcholine receptors
NAC_{shell}	the shell region of the nucleus accumbens
NOR	nornicotine
NOX	(*S*)-nicotine-1´-N-oxide
NRT	nicotine replacement therapy
o.d.	outer diameter
RIA	radioimmunoassay
s.c.	subcutaneously
SEM	standard error of the mean
TT	tetanus toxoid
$(V_F)_B$	the volume fraction blood in brain
$(V_F)_O$	the volume fraction tissue in brain
VTA	ventral tegmental area
5-HT	serotonin

1 INTRODUCTION

1.1 Tobacco smoking

Tobacco smoking is the largest preventable cause of premature mortality in the world (WHO, 2003), and it is a risk factor in six of the eight leading causes of death (WHO, 2008). Over five million people die annually in diseases caused by tobacco smoking, which exceeds the mortality by tuberculosis, HIV/AIDS and malaria combined (Mathers and Loncar, 2006; WHO, 2008).

In 1988 the Surgeon General concluded that tobacco smoking is addictive and that nicotine in the tobacco is causing the addiction (US Department of Health and Human Services, 1988). It was also concluded that nicotine addiction is characterized by regular and compulsive use and that withdrawal symptoms are experienced during abstinence from smoking. A diagnostic tool for nicotine dependence has been defined by the American Psychiatric Association in the "Diagnostic and Statistical Manual of Mental Disorders" (DMS-IV-TR, First and Tasman, 2004, Box 1), and a higher number of criteria met indicates more severe dependence.

Box 1.

Diagnostic criteria for substance dependence - DSM-IV-TR

A maladaptive pattern of substance use, leading to clinically significant impairment, as manifested by 3 (or more) of the following, occurring within a 12-month period:

1. tolerance, as defined by either of the following:
   a) a need for increased amounts of the substance to achieve intoxication or desired effect
   b) diminished effect with continued use of the same amount of the substance
2. withdrawal, as manifested by either of the following:
   a) the characteristic withdrawal syndrome for the substance
   b) the same (or closely related) substance is taken to relieve or avoid withdrawal symptoms
3. the substance is often taken in larger amounts or over a longer period than was intended
4. there is a persistent desire or unsuccessful effort to cut down or control substance use
5. a great deal of time is spent in activities necessary to obtain the substance, use the substance or recover from its effects
6. important social, occupational, or recreational activities are given up or reduced because of substance use
7. the substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance

Unlike many other addictive substances, such as ethanol, smokers transits rapidly from use to dependence, thereby meeting established criteria of tolerance and withdrawal, indicative of physiological dependence.

About 20-45% of the adult populations of the European countries are smokers, Sweden being among the countries with the lowest prevalence, about 17% (WHO, 2008), probably at least in part attributed to the widespread use of Scandinavian snuff (snus). Most smokers begin smoking in the teens, thus 90% starts before the age of 20 (Ernster, 1993) and therefore nicotine dependence has even been described as a pediatric disease (First and Tasman, 2004). The smokers, at least in the developed world, are well aware of the health consequences of tobacco smoking, and about 70% of the smokers actually wish to quit (Center for Disease Control and Prevention, 2002; Thyrian *et al.*, 2008). For example 30% of European smokers report that they are seriously thinking of giving up smoking within the next 6 month (Thyrian *et al.*, 2008).

Smoking cessation is associated with a number of withdrawal symptoms, which also can be diagnosed according to DSM-IV criteria (First and Tasman, 2004, Box 2). Most withdrawal symptoms decline within 4 weeks, although the exact time course is difficult to predict (for review see Hughes, 2007). In addition, nicotine abstinence is associated with craving, which is thought to be a significant factor in relapse. Craving might persist for long periods of time, even years. Indeed, a third of smokers that have remained abstinent for more than one year still relapse 5-10 years after cessation (First and Tasman, 2004).

Box 2.

Diagnostic criteria for nicotine withdrawal - DSM-IV-TR

A. Daily use of nicotine for at least several weeks.

B. Abrupt cessation of nicotine use, or reduction in the amount of nicotine used, followed within 24 hours by 4 (or more) of the following signs:
   1) dysphoric or depressed mood
   2) insomnia
   3) irritability, frustration, or anger
   4) anxiety
   5) difficulty concentrating
   6) restlessness
   7) decreased heart rate
   8) increased appetite or weight gain

C. The symptoms in criterion B cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

D. The symptoms in criterion B are not due to a general medical condition and are not better accounted for by another mental disorder.

In 2000, 41% of the tobacco smokers studied tried to quit (Center for Disease Control and Prevention, 2002) and in a study by Etter *et al*. (1997), Swiss smokers reported a mean number of 1.6 quit attempts during the preceding year. Although many manage to stop smoking temporarily, the majority of self-quitters will relapse within the first week and only 3-5% still remain abstinent after 6-12 months (Hughes *et al.*, 2004). Tobacco dependence is a chronically relapsing disease (Fiore *et al.*, 2000) and smokers may need 5-7 quit attempts before being successful (Hughes, 2000).

1.2 Effects of nicotine

Nicotine, the major addictive component of tobacco, is a naturally occurring alkaloid. It exerts its action by binding to and activating nicotinic acetylcholine receptors (nAChR), ligand gated ion channels consisting of five subunits. Smokers experience intravenously (i.v.) administered nicotine as rewarding, with a euphoriant effect of the same magnitude as morphine or amphetamine, and they often identify the injected nicotine as cocaine (Henningfield, 1984). Nicotine reduces the responsiveness to stress, enhances mood, vigilance and ability to concentrate (for review see Henningfield, 1984) and is often described as both stimulating and calming, in contrast to most addictive drugs that possess only one of these properties. In addition, smokers cannot specifically describe the effect they seek except for liking the taste of the tobacco smoke (Schelling, 1992).

Although nicotine is rewarding, high doses of intravenously administered nicotine in smokers may cause dysphoria manifesting as a temporary feelings of fear or anxiety (Henningfield *et al.*, 1985). The aversive effects of nicotine are obvious for anyone who tries nicotine for the first time and includes dizziness and nausea. Tolerance to these dysphoric effects of nicotine develops rapidly, exemplified by the large number of teenagers who quickly overcome the aversive effects of the first few cigarettes and become regular smokers. In addition, tolerance to the euphoric effect also shows rapid development as smokers commonly describe the first cigarette of the day to be more satisfying than the ones smoked later. Diminished tolerance or resensitization, however, occurs already after a few hours of abstinence, e.g. overnight, allowing for increased feelings of pleasure, and smokers even experience lightheadedness from the first cigarette after some days of abstinence.

Upon cigarette smoking, nicotine levels in arterial blood increases rapidly, thus allowing for delivery to the brain within 10-19 s (Benowitz, 1996), which is even faster than following intravenous administration. In the central nervous system, the pentameric neuronal nAChRs are composed of combinations of α- and β-subunits or α7-subunits alone (for review see Pierce and Kumaresan, 2006), and to date, 12 different subunits has been identified (α2-10 and β2-4), which results in a great diversity of neuronal nAChR subtypes.

Nicotine, like other addictive substances, activates the mesolimbic dopaminergic pathway, which originates in the ventral tegmental area (VTA) and projects to nucleus accumbens (NAC) and other limbic structures, and is involved in

both natural and drug-induced reward-related behavior. The mesolimbic dopamine system is thought to play a fundamental role in the reinforcing effects of nicotine (Corrigall *et al.*, 1992). Systemically administered nicotine increases the firing of mesolimbic dopamine neurons in rats (Grenhoff *et al.*, 1986) as well as dopamine release in the NAC (Imperato *et al.*, 1986), preferably in the shell region (Pontieri *et al.*, 1996; Nisell *et al.*, 1997), that is involved in motivational, emotional and affective processes. These effects of nicotine are thought to be mediated primarily by nAChR in the VTA (Nisell *et al.*, 1994; Fu *et al.*, 2000), that are located on dopaminergic and GABAergic cell bodies (α4α5β2, α4α5α6β2, α7 and α4α5β2, α7, respectively), as well as on glutamatergic and GABAergic nerve terminals (α7 and α4β2*, respectively, for review see Pierce and Kumaresan, 2006).

1.3 Pharmacological treatment of nicotine dependence

It is by now well established that nicotine replacement therapy (NRT) improves the success rates in smoking cessation, and NRT is accordingly recommended as first line treatment by many clinical guidelines (for review see Stead *et al.*, 2008). NRT is available in several administration forms, including chewing gums, lozenges, sublingual tablets, transdermal patches, inhalation cartridges and nasal sprays. The rationale behind this substitution therapy is to deliver nicotine, thus reducing craving and abstinence symptoms, in a way that results in slower absorption and thereby a retarded delivery to the brain, since the time that elapses from administration to effect of addictive drugs has been found to be negatively correlated with their dependence liability. In addition, the presence of nicotine delivered by NRT may reduce the rewarding effect of a relapse cigarette. However, even though NRT increases the probability to quit smoking by 50-70%, the relapse rates are still very high and the vast majority remains smoking.

Bupropion, originally developed as an antidepressant drug, inhibits dopamine and noradrenaline transporters as well as neuronal nAChR, and seems to decrease withdrawal symptoms and reduce craving in smoking cessation (for review see Dwoskin *et al.*, 2006). Bupropion has been shown to double the chance of remaining smoke-free for one year (Jorenby *et al.*, 1999), although a more recent study by McCarthy *et al.* (2008) reported only moderate effects on prolonged abstinence for one year (18% vs. 14% for placebo), despite the high end of treatment abstinence rate, i.e. 50% vs. 26% for placebo. In addition, bupropion has been reported to increase recovery from lapse in women (Wileyto *et al.*, 2005) as well as the lapse to relapse latency (McCarthy *et al.*, 2008), effects that might be contributed to the inhibition of nAChRs by bupropion.

Varenicline is a partial agonist at α4β2 and a full agonist at α7 nicotinic receptors (Mihalak *et al.*, 2006) and was approved for use in smoking cessation by the European Medicines Agency in 2006. It releases dopamine, although to a smaller extent than nicotine, and may thus decrease abstinence-induced craving. In addition, varenicline inhibits binding of nicotine, hence blunting the reward and satisfaction of cigarettes (Coe *et al.*, 2005; Chandler and Rennard, 2010).Varenicline increases the

abstinence rates 2-3-fold compared to placebo (Cahill *et al.*, 2007; Fagerstrom and Hughes, 2008).

Although the pharmacological aids described above have significant clinical effects, especially during ongoing treatment. However, long-term cessation rates are not encouraging and the majority of the smokers will relapse within a year. At present there are no approved pharmacological treatments that have shown clear cut effectiveness in long term relapse prevention.

A novel approach to treat nicotine dependence, i.e. active immunization against nicotine, was therefore developed, since the so called nicotine vaccines might have the theoretical potential to provide long-lasting protection against relapse, i.e. months to years.

1.4 Active immunization - vaccination

Conventional vaccines are generally used to induce immunity against infectious agents, such as bacteria and viruses, or toxins. The first recorded attempts to immunize against infectious diseases were made in the 15th century by the Chinese and Turks who tried to prevent smallpox by administering dried material from scabs from sick individuals into the nostrils or by skin incisions, so called variolation (Goldsby *et al.*, 2003). Although these methods showed some effect many of the immunized individuals contracted the disease. At the end of the 17th century Jenner discovered that infection with cowpox, a non-fatal disease in humans, prevented a subsequent infection with smallpox. This led to safer procedures and became the starting point for the general practice of vaccination.

The principle behind vaccinations is to use the body's ability to build a defence that is specific for a particular antigen, so called acquired or adaptive immunity, and in addition its ability to exhibit immunologic memory, which makes the immune system react faster the second time the subject is exposed to an antigen. The adaptive immune system requires B- lymphocytes, expressing or secreting antibodies (humoral response), and T-lymphocytes (cell-mediated response), expressing the T-cell receptor, as well as antigen-presenting cells. An immunogen is a substance that is able to induce a specific immune response, whereas an antigen is a substance that can interact with antibodies or cell-surface receptors of T-cells. Although all immunogens are also antigens, not all antigens are immunogenic. In order for a substance to be immunogenic it needs to be foreign to the subject, since the immune system is programmed not to respond to self antigens. In addition, it needs to be fairly large, preferably with a molecular size exceeding 100 kDa (Goldsby *et al.*, 2003).

In most vaccines, an adjuvant is often included in the formulation in order to increase the immunogenicity. The mechanisms of action involved are not fully understood, but one common property of adjuvants is to prolong the absorption. Two of the most widely used adjuvants, alum and Freund´s adjuvant, share this property albeit via different mechanisms. Alum, the most commonly used adjuvant in human

vaccines, is an aluminium salt and the immunogen is thought to be adsorbed to its surface, generating a slow-release formulation, which in addition increases the size of the immunogen. Freund´s adjuvant forms an oil-water emulsion with the immunogen, thereby prolonging absorption, and is available in two forms; Freund´s complete (FCA) and Freund´s incomplete (FIA) adjuvant, where FCA in addition to a mineral oil also contains heat-killed *Mycobacteria*.

In most cases, a single administration of a vaccine is not sufficient to induce the required response, and the primary immunization needs to be followed by additional administrations, so called booster immunizations.

1.5 Active immunization against drugs of abuse – background

In 1972, Berkowitz & Spector (1972) found that active immunization with a morphine immunogen could alter the disposition and pharmacologic effects of morphine in mice. This study was, however, not intended to develop a new type of treatment for drug addiction, but the authors hypothesized that it could serve as a model to investigate a potential immune component involved in drug tolerance. A few years later, Bonese *et al*. (1974) reported that active immunization against morphine could prevent the central reinforcing effects of heroin in a rhesus monkey, as assessed by self-administration, although the authors did not propose any potential clinical utility of the vaccination. In the early 1990s, the idea to use an immunological approach to treat cocaine addiction emerged (Bagasra *et al.*, 1992; Landry *et al.*, 1993) and within a few years novel studies appeared, showing that active immunization against cocaine may both reduce the distribution of cocaine to the brain in rodents (Carrera *et al.*, 1995; Fox *et al.*, 1996) and suppress cocaine-induced locomotor activity and stereotyped behavior (Carrera *et al.*, 1995). Moreover, it was shown that passively administered antibodies can inhibit cocaine self-administration in rats (Fox *et al.*, 1996).

However, even though cocaine addiction represents a major public health concern, the medical consequences of tobacco smoking worldwide is far more severe. Therefore, we directed our efforts in the area toward nicotine dependence. In addition, due to the high selectivity of the cocaine antibodies, the cocaine immunized animals were not protected from the effects of amphetamine (Carrera *et al.*, 1995). Thus, a cocaine addict might conveniently switch to another central stimulant drug of abuse (Self, 1995), a behavior that seems considerably less likely among the majority of tobacco smokers. Finally, the molar dose of nicotine ingested by a smoker is lower than the molar dose of cocaine generally ingested by a cocaine addict, hence the chance that sufficient amounts of antibodies could be generated would be higher when using this approach to threat nicotine addiction.

1.6 Rationale behind active immunization against nicotine dependence

Following cigarette smoking, nicotine is rapidly delivered to the brain were it exerts its reinforcing effects, and a feeling of reward and satisfaction is experienced (Fig 1). The rationale behind active immunization against nicotine dependence is to generate endogenous anti-nicotine antibodies that bind nicotine in the blood, thus preventing it from reaching the brain, and thereby inhibiting the nicotine-induced reinforcement.

Nicotine bound by antibodies is regarded to be inactive, i.e. it cannot bind to the nicotinic receptors, and is unavailable for distribution and elimination. The effect of immunization is primarily decided by the amount of antibodies generated as well as their affinity to nicotine, thus, high titers of highly nicotine-selective antibodies are desirable.

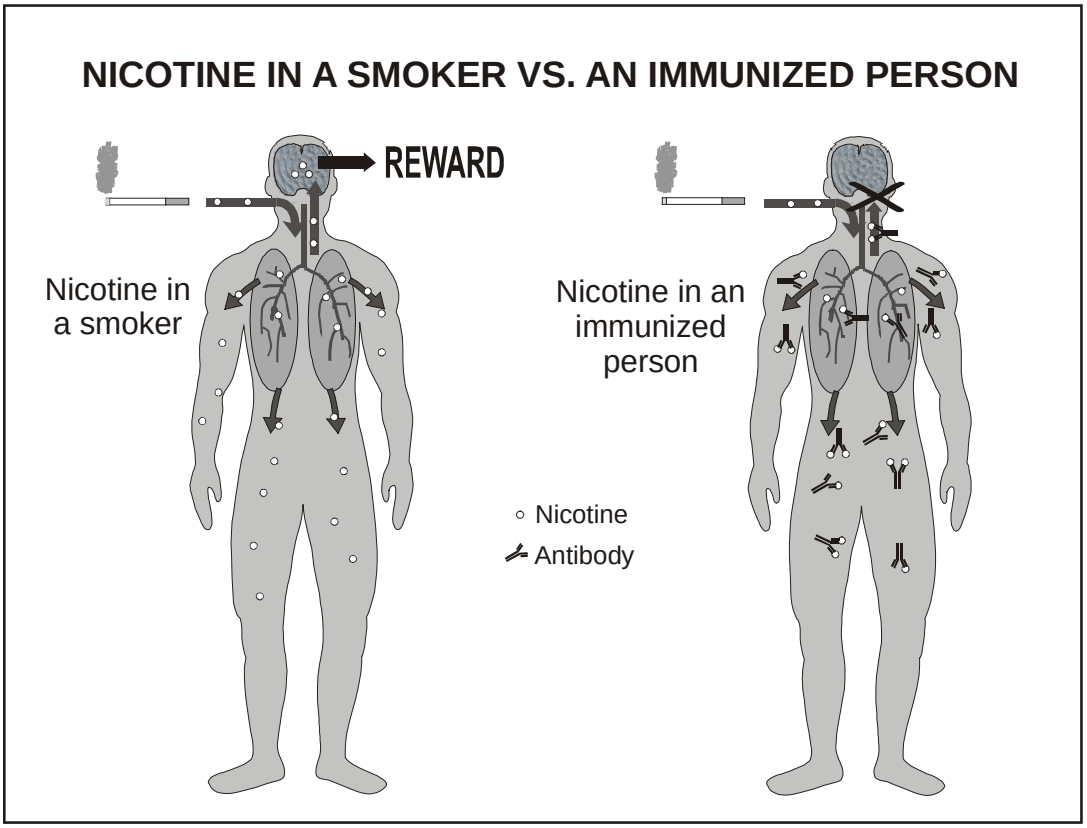


Fig 1. Rational behind active immunization against nicotine dependence.

1.7 Nicotine immunogen design – principal considerations

The nicotine molecule (Fig 2, M_w=162 g/mole) is too small to elicit an immune response on its own, and therefore it needs to be coupled to a larger carrier protein, via a linker, to form a nicotine immunoconjugate (Fig 2).

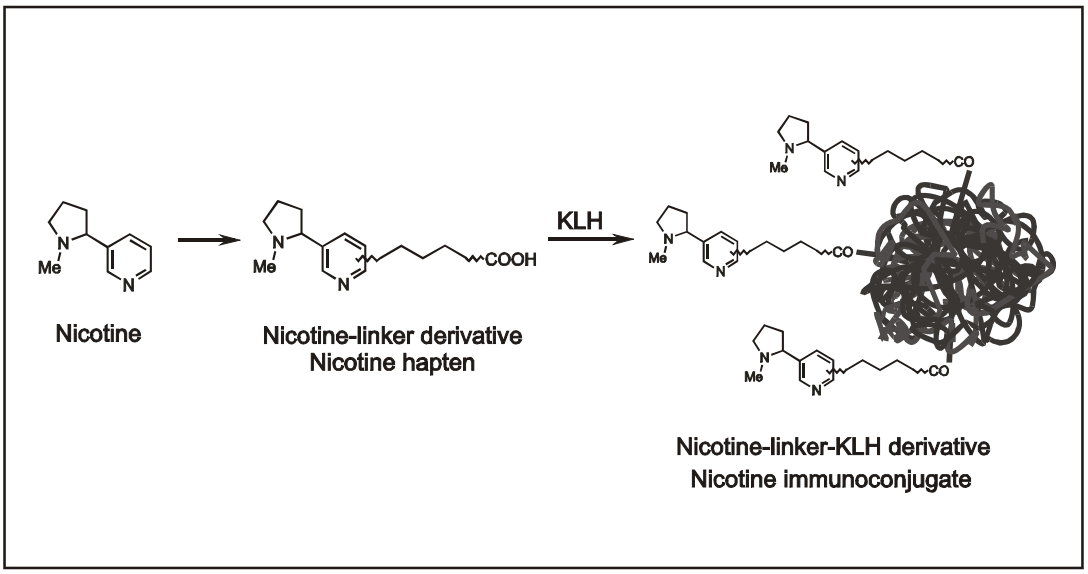


Fig 2. General structure of the nicotine immunoconjugate.

In order to generate antibodies that have high affinity for nicotine, the nicotine part of the immunoconjugate should optimally be as similar as possible to free nicotine, both regarding its three dimensional structure as well as its physical-chemical properties. In addition, the generated antibodies should be selective for nicotine and not recognize nicotine's metabolites, since cross-reactivity with the metabolites, especially the major metabolite cotinine which is present at high levels in smokers, may result in an inadequate number of antibodies left to inactivate nicotine (Carrera *et al.*, 1995). Since the structure of the linker should theoretically influence the properties of the nicotine molecule, the choice of linker as well as its binding site on the nicotine molecule may be of considerable importance.

Nicotine is first metabolized in the non-planar pyrrolidine ring (Fig 3), and in order to increase the antigenicity of this ring, the planar pyridine ring was chosen for linker attachment (Matsukura *et al.*, 1975) of the nicotine haptens included in this thesis (Table 1). The nicotine hapten (nicotine and linker), is coupled to different sites on the carrier protein depending on the functional group of the linker, and this may subsequently affect the amount of haptens that are coupled to the carrier protein, as well as their accessibility by the immune system.

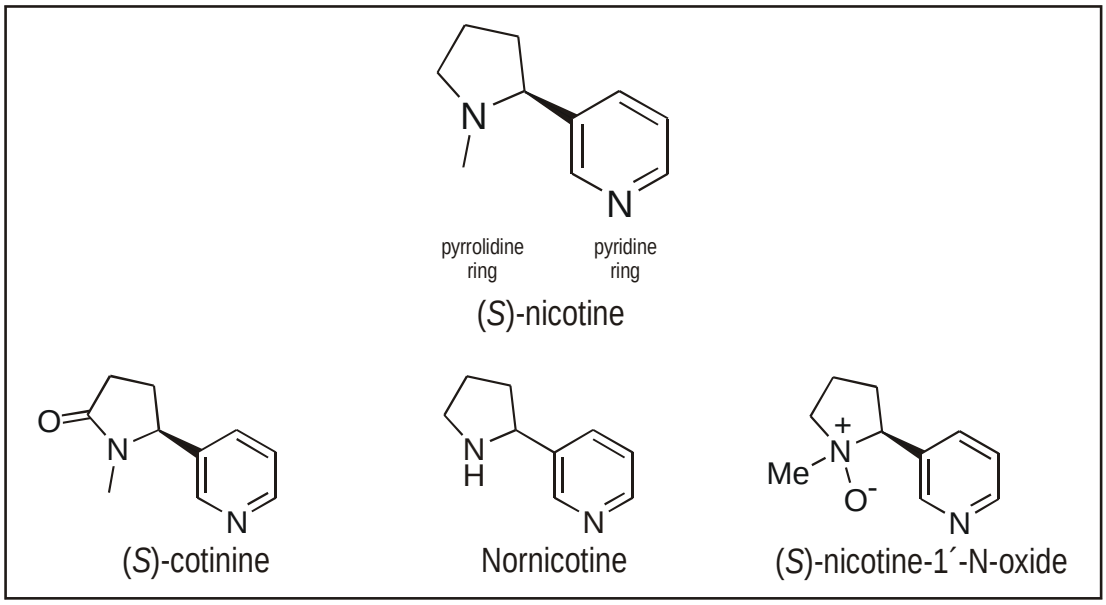


Fig 3. (*S*)-nicotine and some of its metabolites.

The carrier protein should be a protein with immunogenic properties on its own. In addition, it should be fairly uncommon in the normal environment of the immunized subject, since antibodies against the carrier also are generated by the immunization. In the majority of the experiments presented in this thesis, keyhole limpet hemocyanin (KLH), from the giant keyhole limpet *Megathura crenulata*, was used as a carrier protein. The commercially available KLH has strong immunogenic properties and it has been used in several species, including man and rats (Dixon *et al.*, 1966; Curtis and Hersh, 1972). Finally, KLH has been widely used as a carrier protein in hapten immunoconjugates, e.g. for vaccination against cocaine (Bagasra *et al.*, 1992; Carrera *et al.*, 1995) as well as for production of monoclonal anti-nicotine antibodies to be used in enzyme-linked immunosorbent assay (Bjercke *et al.*, 1986).

1.8 Present state of the field

To date there are four nicotine vaccines in clinical phase II trials, TA-NIC by Celtic Pharma (company home page: www.celticpharma.com), NicVAX (Hatsukami et al., 2005; Wagena et al., 2008) by Nabi Biopharmaceuticals (company home page: www.nabi.com), Niccine by Independent Pharmaceutica AB (company home page: www.independentpharma.com) and finally NIC002 (Maurer et al., 2005; Cornuz et al., 2008) (formerly NicQb) by Cytos Biotechnology (company home page: www.cytos.com), which however recently reported that the primary endpoint was not achieved in an interims analysis of their ongoing study. So far all of the nicotine vaccines seem to be well tolerated, and in addition, two of the clinical studies report clinical proof-of-concept in the high antibody responder subgroups.

2 AIMS

The overall aims of the present thesis were to

- evaluate a number of nicotine immunogens as regards their ability to generate high titer nicotine selective antibodies, as assessed by ELISA techniques, as well as their ability to alter the reinforcing effects of nicotine, as assessed by their effect on dopamine release in the NAC_{shell} by means of *in vivo* voltammetry

- study the effect of active immunization in an animal model of relapse

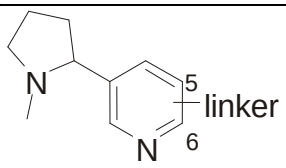
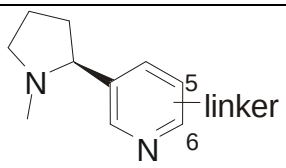
- analyze the effect of active immunization on the distribution of nicotine

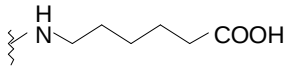
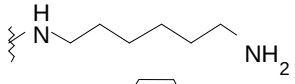
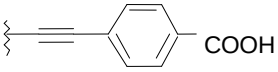
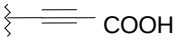
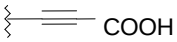
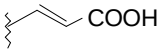
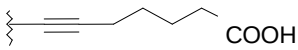
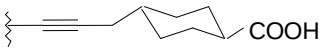
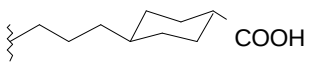
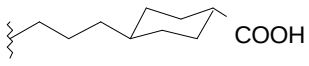
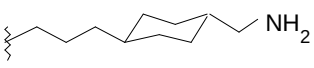
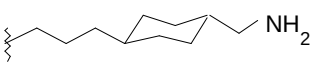
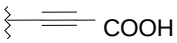
3 MATERIAL AND METHODS

3.1 Nicotine haptens

Nicotine haptens were synthesized at the Department of Organic Pharmaceutical Chemistry, Uppsala University, Uppsala, Sweden. The linkers were attached to the 5- or 6-position of nicotine (racemate) or (*S*)-nicotine (Table 1).

Table 1. The structure of the nicotine haptens

Forms of nicotine	 Racemate	 (S)-enantiomer

Hapten code	Linker structure	Linker position	Form of nicotine
GK14		6-position	Racemate
YH7		6-position	Racemate
GK49		6-position	Racemate
GK56		5-position	Racemate
GK60		6-position	Racemate
GK62		5-position	Racemate
GK81		6-position	Racemate
GK83		6-position	Racemate
IP18		6-position	Racemate
IP31		6-position	(*S*)-enantiomer
SG62		5-position	(*S*)-enantiomer
IB87		6-position	(*S*)-enantiomer
SG50		6-position	(*S*)-enantiomer

3.2 Synthesis of the nicotine hapten-carrier protein immunoconjugates

The respective nicotine haptens were conjugated to keyhole limpet hemocyanin (KLH) or tetanus toxoid (TT) using carbodiimide-mediated reactions.

3.2.1 Carboxyl nicotine haptens (GK14, GK49, GK56, GK60, GK62, GK81, GK83, IP18, IP31 and SG50)

A solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma-Aldrich, Stockholm, Sweden, 9 molar equivalents compared to the nicotine hapten) in distilled H_2O was added to an mixture of each nicotine hapten (0.01 g) and H_2O and kept at 0 ^oC. After 10 min, a solution of KLH (Calbiochem, La Jolla, CA, 0.01 g) or TT (SSI, Copenhagen, Denmark) in distilled water (1.0 ml) was added and the mixture was stirred at 0 °C for 10 min, and thereafter at ambient temperature over night.

3.2.2 Amine nicotine haptens (YH7, SG62 and IB87)

A solution of KLH or TT (0.01 g) in distilled water (1.0 ml) was added to a mixture of each nicotine hapten (0.01 g) and H_2O at 0 °C. Subsequently a solution of EDC (9 molar equivalents compared to the nicotine hapten) in distilled H_2O was added. The mixtures were stirred at 0 °C for 10 min, and thereafter at ambient temperature over night.

During the reactions, pH was checked and adjusted to pH 4.5-6 with HCl if needed. The reaction mixtures were either purified on a Sephadex G-25, PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) or dialyzed in a Spectra/Por 4 membrane (cut-off 12-14 kDa) presoaked in distilled water against an excess volume (approx. 5 l) of physiological saline for 24 h, during which time the saline was changed twice. Immunoconjugates purified on column were freeze-dried, weighed and stored at 4-8 ºC. From the dialyzed immunoconjugates, a sample of known volume was freeze-dried and weighed in order to determine concentration. The immunoconjugate solution was aliquoted and stored at 4-8 ºC.

3.3 Vaccine formulations

3.3.1 FCA/FIA

The nicotine immunoconjugates (hapten-KLH or hapten-TT) were dissolved, if necessary, and diluted in distilled water or physiological saline to a concentration of 0.5 mg/ml. The solution was subsequently emulsified with an equal volume Freund´s complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA, Boule Nordic AB, Huddinge, Sweden or Difco Laboratories, Detroit, MI, USA) using two connected glass syringes (Hamilton Bonaduz AG, Switzerland). The plunges of the syringes were pushed back and forth until the viscosity greatly increased. Since it is of great importance that the emulsion is stable, the stability was occasionally tested by putting a drop of the emulsion on a water surface and the drop should not dissolve. The

nicotine immunoconjugate-adjuvant emulsions were used immediately. FCA was only used for primary immunizations. Immunizations with Freund´s adjuvant were administered intraperitoneally (i.p.) in 0.4 ml.

3.3.2 Alum

Formulations were performed according to a down-scaled modified protocol from SSI (Copenhagen, Denmark). Nicotine immunoconjugates (hapten-KLH or hapten-TT) were dissolved, if necessary, and diluted in physiological saline to a concentration of 400 µg/ml. Aluminum hydroxide, 2 ml, 5mg/ml (SSI, Copenhagen, Denmark) and 43.75 µl 0.1 N NaOH were added. The volume was adjusted to 5 ml with physiological saline and the solution was mixed. After confirming that the pH was approximately 6.5-7, the mixture was stored at 4-8 ºC until use. Alum immunizations were administered subcutaneously (s.c.) in a volume of 0.5 ml or 0.25 ml (1 µg dose).

3.3.3 Without adjuvant

Nicotine immunoconjugates (hapten-KLH or hapten-TT) were dissolved, if necessary, and diluted in physiological saline to a concentration of 200 µg/ml (100 µg immunoconjugate/dose). For nasal administration (50 µg hapten-TT per dose), the immunoconjugate was dissolved in physiological saline to a concentration of 830 µg/ml.

3.4 Animals

All studies were approved by, and conducted according to guidelines of, the local animal ethics committee (Stockholms Norra Försöksdjursetiska nämnd) and the Karolinska Institutet, Sweden.

3.4.1 In vitro experiments, in vivo voltammetry and nicotine distribution

Male Wistar rats (BK, Sollentuna, Sweden) were used in the experiments. The animals typically weighed 200-220 g at the start of the experiments and were housed 3-5 in Makrolon 4 cages in a temperature-controlled environment with free access to food and water. Before the start of immunizations or experiments, animals were allowed a minimum of one week to adapt to their new environment.

3.4.2 Nicotine-seeking behavior

Male Wistar rats (BK, Sollentuna, Sweden) were used in the experiments and arrived at the department at the age of 6 weeks. They were kept in a temperature-controlled environment on a reversed light-dark cycle (lights on 6 p.m. to 6 a.m.). Before the start of the experiments, animals were allowed a minimum of one week to adapt to their new environment and where then housed 4-5 in Makrolon 4 cages with free access to food and water. During the experimental phase rats where housed

singly in elevated Makrolon 3 cages, had free access to water and received 20 g food per day. Experiments were performed on 5 days per week between 7 a.m. and 5 p.m., during the rats' active phase.

3.5 Immunization protocols

For an overview of immunization doses, schedules, adjuvants and routes of administration see Table 2.

3.6 ELISA (enzyme-linked immunosorbent assay)

Sera from immunized rats were analyzed on ELISA-plates coated with the same hapten used for immunization, but conjugated to bovine serum albumin (BSA, Sigma-Aldrich, Stockholm, Sweden). On all plates a series of controls were included; positive control, negative control and controls for unspecific binding by the secondary antibody to the hapten-BSA coating and the plastic of the plate, respectively. The wells of the positive controls were coated with the carrier protein used at immunization and the negative control wells were coated with BSA, and only a single dilution (1:125) of each serum was used. Unspecific binding to the coating was tested by excluding the sera and exchanging with PBS, and the unspecific binding to the plate was tested by excluding the coating and serum. ELISA-plates (Labsystems, Stockholm, Sweden) were coated over night at 8°C or for one hour at 37°C with 10 μg/ml of hapten-BSA conjugate, coupled according to the same method used for the preparation of the KLH and TT conjugates (see above). When needed, plates were blocked with 3% BSA in PBS for 1 h in 37°C. After an extensive wash, seven dilutions of serum in steps of five (1:25, 1:125, 1:625, 1:3125, 1:15625, 1:78125 and 1:390625) were added and the plates incubated at 37°C. Serum samples from animals used in the *in vivo* voltammetry or distribution experiments were collected before administration of nicotine. The plates were washed again and after addition of the secondary antibody, an alkaline phosphatase conjugated goat anti-rat IgG (Sigma-Aldrich, Stockholm, Sweden), they were further incubated at 37°C. The enzyme substrate *p*-nitrophenyl phosphate (Sigma-Aldrich, Stockholm, Sweden) produces a colored end product that can be read spectro-photometrically at 405 nm (Fig 4).

Titers were determined as the inverse of the dilution giving a positive signal, i.e. the mean signal measured from control sera plus two standard deviations (Equation 1). The titer limits were set for each separate run. In some cases, where the response to the immunizations was very strong, another higher titer limit was used in order to be able to make comparisons between different groups. This higher limit, denoted "titer high limit", is calculated as the mean signal measured from control sera plus six standard deviations (Equation 2).

Equation 1 $$Titer\ limit = mean + 2SD$$

Equation 2 $$Titer\ high\ limit = mean + 6SD$$

Table 2. Immunization schedules.

Experiments	**Immunogens**	**Primary immunization, day 0**			**Booster immunization(s)**			
		Dose	Adjuvant	Volume and route of administration	Day(s)	Dose	Adjuvant	Volume and route of administration
Effects of nicotine haptens In vivo voltammetry	Hapten-KLH	100 µg	FCA	0.4 mL, i.p	13 or 14	100 µg	FIA	0.4 mL, i.p
Effects of carrier proteins	IP18-KLH	100 µg	FCA	0.4 mL, i.p	14	100 µg	FIA	0.4 mL, i.p
	IP18-TT	100 µg	FCA	0.4 mL, i.p	14	100 µg	FIA	0.4 mL, i.p
Effects of adjuvants	IP31-TT	100 µg	FCA	0.4 mL, i.p	14	100 µg	FIA	0.4 mL, i.p
		100 µg	alum	0.5 mL, s.c.	14	100 µg	alum	0.5 mL, s.c.
		100 µg	---	0.5 mL, s.c.	14	100 µg	---	0.5 mL, s.c.
		10 µg	alum	0.5 mL, s.c.	14	10 µg	alum	0.5 mL, s.c.
Effects of immunization schedules and routes	IP31-TT	50 µg	---	2x30 µl, nasally	26, 48, 68, 89	50 µg	---	2x30 µl, nasally
		50 µg	alum	0.5 mL, s.c.	No booster immunization			
		50 µg	alum	0.5 mL, s.c.	21, 42	50 µg	alum	0.5 mL, s.c.
		50 µg	alum	0.5 mL, s.c.	21, 42	50 µg	---	2x30 µl, nasally
		1 µg	alum	0.25 mL, s.c.	No booster immunization			
		1 µg	alum	0.25 mL, s.c.	73, 94, 115, 136	50 µg	---	2x30 µl, nasally
		1 µg	alum	0.25 mL, s.c.	22, 48	1 µg	alum	0.25 mL, s.c.
Nicotine-seeking behavior	IP18-KLH	100 µg	FCA	0.4 mL, i.p	7, 28	100 µg	FIA	0.4 mL, i.p
Nicotine distribution	IP18-KLH	100 µg	FIA	0.4 mL, i.p	21 or 22	100 µg	FIA	0.4 mL, i.p

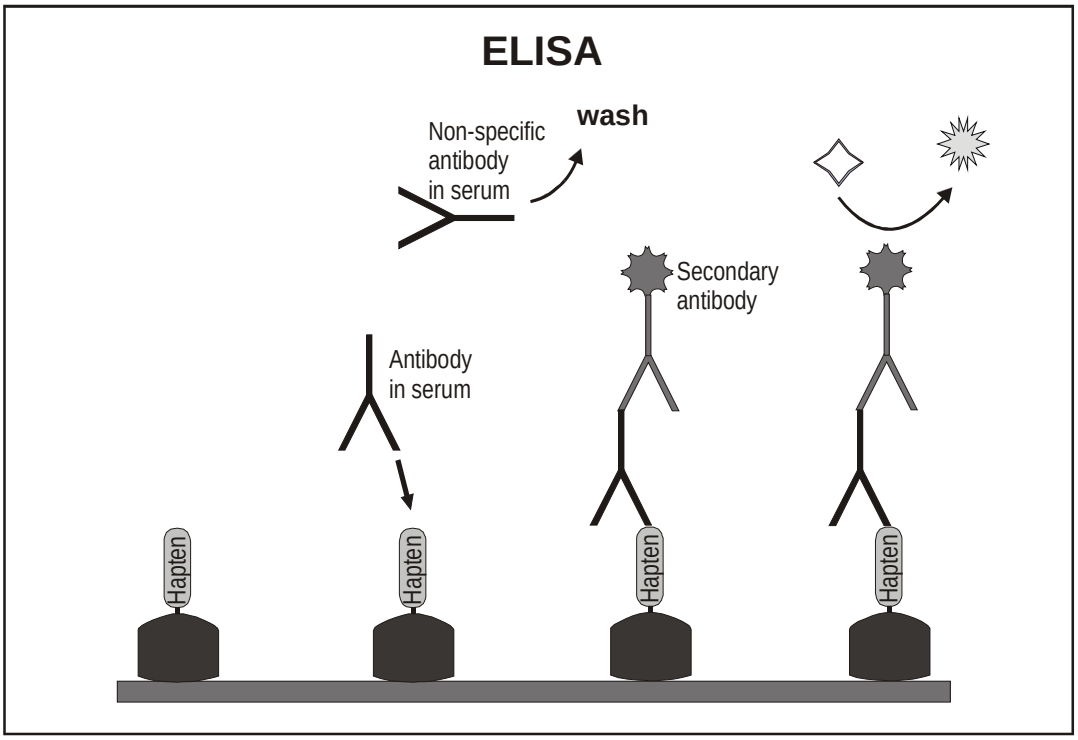


Fig 4. Enzyme-linked immunosorbent assay (ELISA).

3.7 Competitive ELISA

The competitive ELISA was performed as the ordinary ELISA with the exception that only a single dilution of serum was used (Fig 5). The competitors [all from Sigma-Aldrich, Stockholm, Sweden except (*S*)-nicotine-1´-N-oxide that was synthesized from (*S*)-nicotine at the department of Organic Pharmaceutical Chemistry, Uppsala University] were added to the wells prior to addition of the sera. Competitors were tested in different concentrations. Maximum absorbance in the assay was determined as the mean absorbance from wells not containing any competitor, or from wells containing very low concentrations of competitors that did not show inhibition, and set to 100%. Selectivity was determined in two ways; i) the IC_{20} corresponds to the concentration of competitor resulting in 20% decrease in absorbance, ii) the IC_{50}, i.e. the concentration resulting in 50% decrease in absorbance, was determined by non-linear regression using an equation describing one-site competition (Graph Pad Prism 4, GraphPad Software, Inc).

During the course of the screening process, the chosen serum dilutions varied between 16-98% of the maximum absorption in the ELISA. In some cases, the cross-reactivity measured in the competitive ELISA was adjusted with respect to the antibody titer as follows; for each run of the ELISA, a titer limit, i.e. the mean signal measured from control sera plus two standard deviations, was determined. These limits were assumed to correspond to equal amounts of antibodies, even though it is not possible to determine the exact quantity by our assay. The total amount of antibodies in non-diluted sera would then be the amount of antibodies at the titer limit multiplied by the titer. The total amount of antibodies was then divided by the inverse

of the serum dilution used in the competitive ELISA to provide an estimate of the quantity of antibodies in the competition assay. The IC_{20} or IC_{50} for each inhibitor was corrected to correspond to the concentration necessary to inhibit the amount of antibodies at the titer limit, i.e. by dividing by the fraction of the antibody amount in the competitive ELISA and the amount of antibody at the titer limit.

For example, if the amount of antibodies at the titer limit is assumed to be X, the titer for a sample is 50000 and the dilution at which the competitive ELISA is run is 1:5000, the total quantity of antibodies in non-diluted serum will be 50000X. The amount of antibodies in the competitive ELISA will be 50000X/5000=10X and the IC_{50} needs to be divided by 10 in order to correspond to the concentration necessary to inhibit the amount of antibodies at the titer limit.

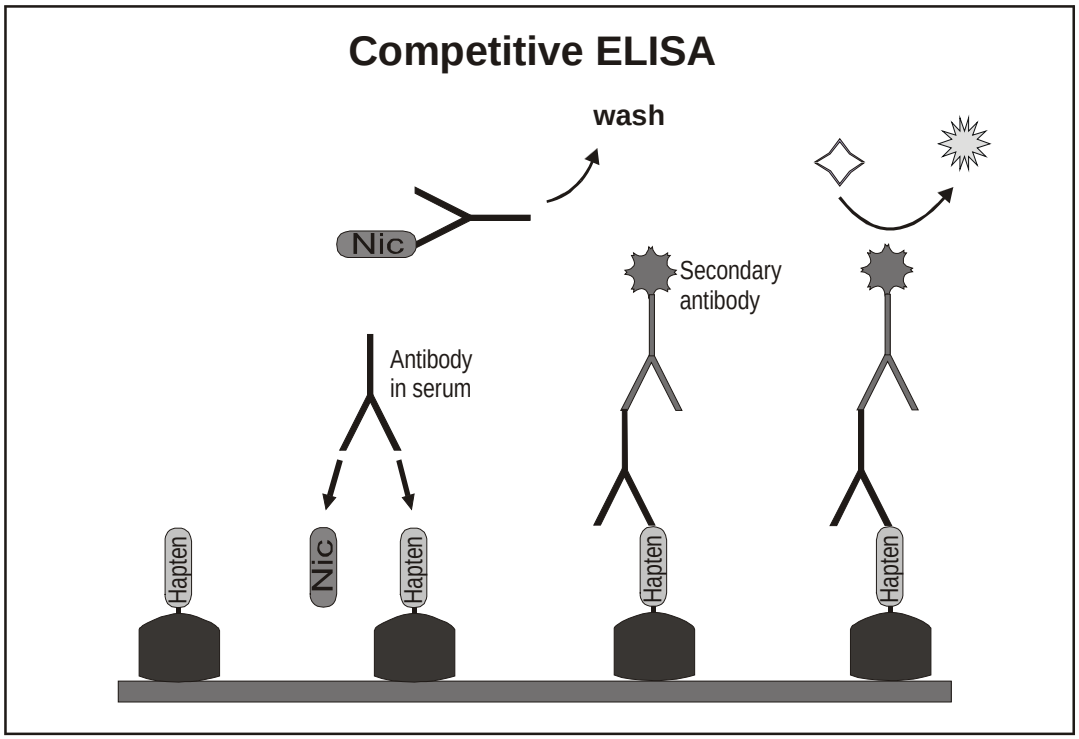


Fig 5. Competitive ELISA.

3.8 *In vivo* voltammetry

Voltammetric measurements of dopamine release in the shell region of the nucleus accumbens (NAC_{shell}) were performed 3-7 days post-immunization. Rats were pre-treated with pargyline (Sigma-Aldrich, Stockholm, Sweden, 75 mg/kg i.p.), to prevent formation of dihydroxyphenylacetic acid (DOPAC) and thus minimizing the interference of DOPAC with the catechol peak (Gonon et al., 1984; Gonon, 1988). The rats were then anesthetized with chloral hydrate (400 mg/kg i.p., Fluka, Buchs, Switzerland) and a catheter was placed in the femoral vein for continuous infusion of chloral hydrate (100 mg/kg/h) during the experiment. A tracheal tube and a jugular vein catheter were inserted and a blood sample for antibody analysis was collected.

Carbon fibre electrodes were prepared and treated as described by Gonon (1988) and Marcus et al. (1996). The active part of the electrode was 12 μm thick and 500 μm long. Electrodes were positioned at the coordinates: AP= +1.6 and ML= ±0.8 (from bregma) for the NACshell (Paxinos and Watson, 1986). The tip of the electrode was placed 6.5-7.0 mm below cortical surface, and differential normal pulse voltammetry was used to record voltammograms (every min) with parameters as described previously (Gonon et al., 1984; Gonon, 1988).

After a stable baseline was observed, the animal was injected intravenously (i.v.) via the jugular catheter with saline and thereafter nicotine [(S)-nicotine hydrogen tartrate salt (Sigma-Aldrich, Stockholm, Sweden) in physiological saline, pH adjusted to 7.20-7.40 with sodium hydroxide] was administered in incremental doses (6, 12, 24 and 48 μg/kg expressed as free base) at 10 min intervals. At the end of the experiment, rats were sacrificed, brains removed, and the position of the electrode was verified histologically.

3.9 Nicotine-seeking behavior

3.9.1 Apparatus

Food shaping as well as nicotine self-administration were performed in operant chambers placed in sound-attenuating boxes (Med Associates, St. Albans, Vt., USA). Boxes were equipped with two levers with associated cue lights, a house light, a tone alert, and a drug delivery infusion system. Upon responding on the active lever, the house light was inactivated. The cue light placed above the active lever as well as the tone alert were activated for 1 s during which the nicotine infusion was delivered in a volume of 32 μl. Nicotine reinforcement was followed by a 1-minute time-out period during which there was no consequence upon responding. As the time-out period ended, the house light was reactivated. Experimental parameters were controlled and recorded by a Med-PC software (Med Associates) running on a personal computer connected to the experimental boxes via an interface module (Med Associates).

3.9.2 Food shaping

Before the first food shaping sessions were performed, rats were left without food for approximately 24 h. During food shaping rats were gradually trained in three phases to press a lever in order to receive 45 mg pellets (Bilaney Consultants, Düsseldorf, Germany) as reinforces. As they had acquired the operant task, rats were surgically implanted with a jugular vein catheter.

3.9.3 Surgery

For a detailed description of the method see Lindblom *et al.* (2002). Rats were anesthetized with pentobarbital (50 mg/kg i.p.) and given buprenorphinum (0.05 mg/kg s.c.) as an analgesic before surgery. A thin siliastic catheter (i.d. 0.30 mm, o.d. 0.64 mm, Sikema, Stockholm, Sweden) was inserted into the jugular vein and tunneled under the skin running from the neck to the head, where it was connected to

a C313G adapter (Bilaney Consultants), to which the nicotine infusion system was connected later on. The adapter was mounted and attached to the skull and the incisions were closed. After surgery rats were administered antibiotic (ampicillin 100 mg/ml, 0.05 ml i.v.) once daily for one week to optimize recovery. Furthermore, the analgesic buprenorphinum (0.05 mg/ml/kg s.c.) was administered twice a day for 5 days to minimize discomfort during recovery from the surgery. Rats were administered heparin twice daily for the duration of the experiment in order to keep the catheter from occluding.

3.9.4 Acquisition of nicotine self-administration

During acquisition of nicotine self-administration, rats were gradually trained to lever press for intravenous nicotine infusions (0.03 mg/kg/infusion) on a fixed ratio (FR) from FR1 to FR3. Rats had to meet certain criteria in order to progress during their acquisition. Thus, to proceed from FR1 to FR2 rats had to take 25 infusions within 3 h. To go on to FR3 rats had to press to get 15 infusions within 2 h for 2 consecutive days. The final criterion for stable nicotine self-administration i.e. maintenance, was a 1-hour nicotine intake that did not vary more than 15% between 3 consecutive sessions. As the rats had stabilized their nicotine intake, after approximately 5 weeks, they were immunized (Table 2) and put on extinction of nicotine self-administration.

3.9.5 Extinction of nicotine self-administration

The daily 1-hour extinction sessions were initiated by administration of a passive infusion of saline, signaled by the cue light and tone alert being activated for 1 s. Lever presses had no consequence for the entire session. The extinction procedure takes approximately 25 days to complete, and extinguished self-administration behavior was characterized by total lever presses not exceeding 8 presses/h for 2 consecutive days.

3.9.6 Reinstatement of nicotine-seeking behavior

Reinstatement sessions were initiated by a passive infusion of nicotine, and similarly to the extinction sessions, the cue light and tone alert that were activated for 1 s signaled infusion. Lever presses had no consequence for the entire session.

3.10 Assessments of nicotine distribution

Nicotine distribution experiments were performed 7-11 days after the booster immunization. The animals were randomized in two groups, and nicotine distribution was studied either 3 min or 60 min after nicotine administration. Animals were anesthetized with 8% chloral hydrate and a catheter was placed in the jugular vein. For animals in the 60 min group a catheter was placed also in the femoral vein. Blood samples for ELISA measurements were drawn. (*S*)-Nicotine (nicotine hydrogen tartrate salt, Sigma-Aldrich, Stockholm, Sweden), 15 μg/kg (3 min group) or 30

µg/kg (60 min group) expressed as free base and pH adjusted to 7.20-7.40, was administered via the jugular catheter.

For the 3 min group, animals were decapitated 3 minutes after nicotine administration. Trunk blood was collected in heparinized tubes and the brains removed, rinsed in distilled water and stored at -20°C. For the 60 min group, a blood sample was taken via the femoral catheter 3 minutes after nicotine administration. The animals were decapitated 60 minutes after nicotine administration, trunk blood collected and brains removed and frozen. Blood samples were centrifuged at 2500 rpm (Beckman rotor GH 3.7) and plasma collected and stored at -20°C.

3.10.1 Preparation of brain samples

Brain nicotine was extracted according to a modified method outlined by Hieda et al. (1997). Brains were thawed and divided by a sagittal section. One hemisphere was used for the analysis and its weight was noted. Brain samples were digested in 5 vol of 1 M NaOH at ambient temperature over night and then sonicated for 10-15 seconds. The pH was adjusted to below pH 4 with 1 M sulfuric acid. The brain solutions were vortexed with 3 ml toluene/1-butanol (7:3) and then centrifuged at 2000 rpm (Beckman rotor GH 3.7) for 20 min. The aqueous phase was decanted and stored at -20°C.

3.10.2 Analyses of nicotine and cotinine

Nicotine and cotinine analyses were performed at the Bioanalytical laboratory at Consumer Healthcare, Pharmacia, Helsingborg, Sweden. Nicotine and cotinine and their internal standards, N-methylanabasine and N-ethylnorcotinine were analyzed on a HP 5890A gas chromatograph equipped with capillary columns, CP-sil-8 CB 25m x 32mm id (Chrompack). A single step liquid-liquid extraction with toluene/1-butanol, after addition of 5 M NaOH, preceded the analyses. Nicotine and cotinine were detected by means of a nitrogen sensitive detector with high selectivity and sensitivity.

3.10.3 Correction for brain blood content

The nicotine concentration in the aqueous phase obtained after extraction of the brain samples was used to calculate total brain nicotine concentration, as follows; Aqueous nicotine concentration (ng/ml) was multiplied by the total volume of the water phase, which gives total nicotine (ng) in the brain sample. Total nicotine was then divided by the weight of the brain sample to obtain the total amount of nicotine per gram of brain (ng/g, c'_{TOT}).

Brain blood contents were corrected for, using a method described by Khor *et al*. (Khor and Mayersohn, 1991; Khor *et al.*, 1991), based on distribution coefficients. Briefly, the volume fraction blood in brain $[(V_F)_B]$ was multiplied by the nicotine concentration in blood (ng/ml, c_{blood}), and the result was subtracted from the total amount of nicotine per gram of brain (c'_{TOT}), and the total divided by the volume

fraction organ [$(V_F)_O$], i.e. including the cells and interstitial fluid of the organ but not the blood, see equation 3. The value used for the volume fraction blood in brain [$(V_F)_B$] was 0.037 (Khor *et al.*, 1991) and for $(V_F)_O$ 0.963 [i.e. $(V_F)_O$=1-$(V_F)_B$]. For c'_{TOT} and c_{blood} individual values were used.

Equation 3 $$c_{tissue} = \frac{c'_{TOT} - (V_F)_B \times c_{blood}}{(V_F)_O}$$

4 RESULTS AND DISCUSSION

4.1 Antibody response as measured by ELISA

When immunizing against nicotine dependence, it is not the nicotine immunoconjugate *per se* that alters the central dependence producing effects of nicotine, but the anti-nicotine antibodies generated by the immunizations. The amounts of generated antibodies are depending on, not only the nicotine hapten, but also the carrier protein to which the hapten is conjugated, the adjuvant used, as well as the administered dose of immunogen and immunization schedule.

4.1.1 Antibody response – Effects of different nicotine haptens

Blood samples were in general collected 3-7 days after the second immunization, with the exception of the GK14-KLH immunized group where blood samples were collected day 18, 19 or 47 post-immunization. All tested nicotine immunogens generated measurable titers of antibodies against nicotine as assessed by ELISA (Table 3). There were statistical differences, evaluated by the Kruskal-Wallis test [$H(12)=49.43$, $p<0.001$], between the immunogens generating the lowest compared to the ones generating the highest antibody responses.

Following immunization with GK49-KLH and GK62-KLH, although there were measurable antibody titers, all rats had titers below 8000 and were defined as non-responders. The GK49 hapten comprises an aromatic ring in the linker, which could interfere with the conjugation of the hapten to the carrier protein, and thereby result in fewer haptens per carrier protein molecule and a lower antibody titer. The GK62 hapten comprises a short linker with two double bond carbon atoms, and it could be hypothesize that the limited length is the reason for the low antibody titer. However, the GK56, GK60 and SG50 [the (*S*)-enantiomer of GK60] haptens have equally few carbon atoms in the linker, although connected via a triple bond, and immunization with all these hapten-immunoconjugates resulted in high titers.

4.1.2 Antibody response – Effects of different carrier proteins

In order to evaluate the effect of different carrier proteins, the IP18 hapten was coupled to either tetanus toxoid (TT) or keyhole limpet hemocyanin (KLH). Animals in the IP18-TT group were sacrificed and blood samples collected 7 days after the second immunization and in the IP18-KLH group, blood samples were collected 3-5 days after the second immunization. Due to the high antibody response in the tetanus toxoid group, the titer high limit (see Material and methods) was used to define titers. Immunizations with IP18-TT generated antibody titers that were significantly higher than when the hapten was coupled to KLH (Mann-Whitney $U=0$, $p=0.004$, Fig 6, panel A). Although both carriers are good immunogens (Bergey and Etris, 1934; Dixon *et al.*, 1966; Curtis and Hersh, 1972; Maclennan *et al.*, 1973), the formalin treatment of the tetanus toxoid may contribute to the superior immunogenicity by increasing the stability of the toxoid (Porro *et al.*, 1980).

Table 3. Mean nicotine antibody titers.

Nicotine immunogen	Mean titer[i] (mean±SEM)	Number of animals	Non-responders (Titer<8000)
GK14-KLH	142,125 ± 57,975	8	0
YH7-KLH	85,833 ± 27,794	6	0
GK49-KLH	1500 ± 224	6	6
GK56-KLH	42,500 ± 7779	6	0
GK60-KLH	39,833 ± 12,103	6	2
GK62-KLH	1000 ± 516	6	6
GK81-KLH	8167 ± 2548	6	2
GK83-KLH	63,333 ± 15,019	6	0
IP18-KLH	112,444 ± 51,567	18	2
IP31-KLH	166,250 ± 52,442	12	0
SG62-KLH	110,667 ± 48,484	12	2
IB87-KLH	213,917 ± 77,366	12	1
SG50-KLH	274,667 ± 80,730	12	0

[i] Non-responders are included in the calculation of the mean.

4.1.3 Antibody response – Effects of different adjuvants

The antibody response was assessed in rats immunized with IP31-TT (100 µg) in Freund´s adjuvant, adsorbed to alum or with no adjuvant. In addition, a lower dose of immunoconjugate (10 µg) on alum was also investigated. Rats were sacrificed and blood samples collected 7-10 days after the second immunization. Due to the high antibody response, the titer high limit (see Material and methods) was used to define titers (Fig 6, panel B). The Kruskal-Wallis test showed a significant difference between groups [H(3)=11.03, p=0.012]. Immunization with the immunoconjugate alone, without adjuvant, generated a mean antibody titer of 16,500 [titer high limit, corresponding to 127,000 using the more conventional titer limit (mean of the controls + 2SD)], suggesting the nicotine immunoconjugate to be highly immunogenic on its own, probably due to the very high immunogenicity of the TT

(see above). The addition of alum significantly increased the titer more than 10-fold (p=0.047) and there was no difference in the antibody responses generated by the Freund´s or alum immunizations (100 µg dose, p=1.0), although Freund´s seemed to cause larger variations in the immune response. This is probably due to the more complicated mixing procedure involved in the formulation with Freund´s adjuvant, since an emulsion needs to be formed, and, in addition, the more complicated injection of the viscous emulsion. The lower 10 µg dose of IP31-TT on alum generated a subsequently lower antibody response, and the decrease of the response was of the same magnitude as the decrease of the dose. In addition, the lower dose in combination with alum generates an antibody titer equal to the higher 100 µg dose given without adjuvant.

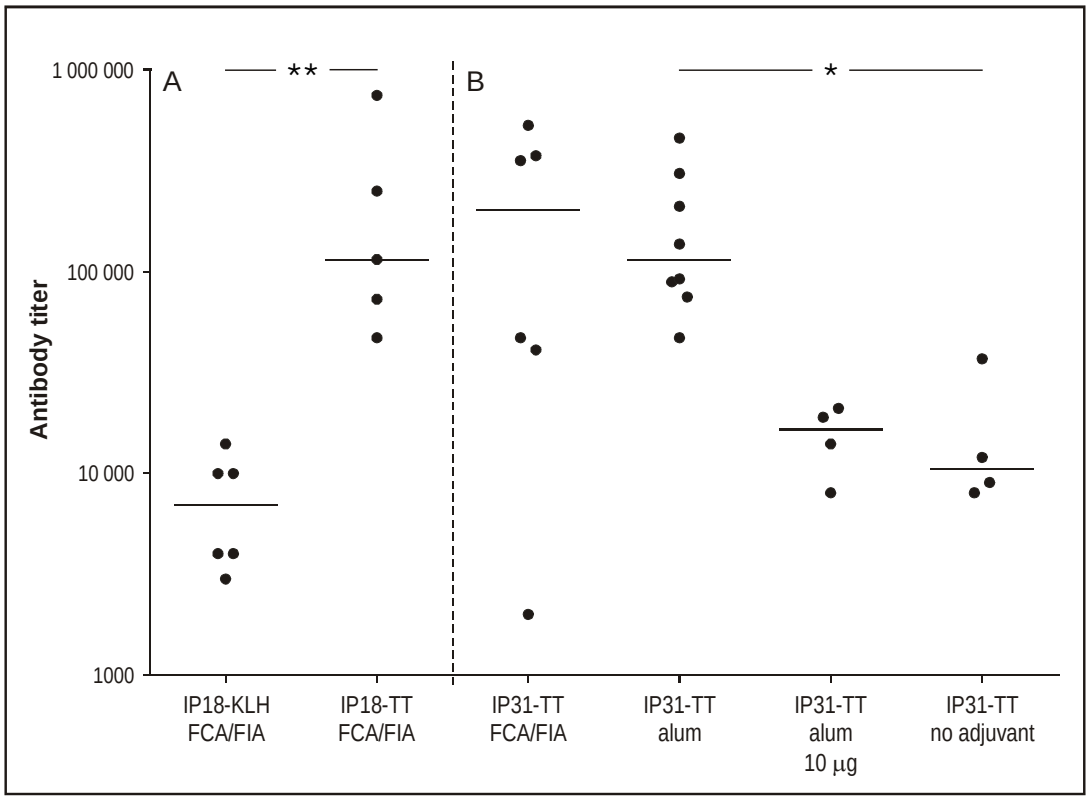


Fig 6. Individual antibody titer (defined by titer high limit) following immunization with different nicotine immunogens. All immunizations were given in a dose of 100 µg, except one group receiving a 10 µg dose. The horizontal lines represent the median titers, and the *asterisks* represent a significant difference between groups, * $p<0.05$ and ** $p<0.01$.

4.1.4 Antibody response over time – Effects of different immunization schedules and routes of administration

The aim of this study was to investigate the development of the antibody response over time, measured by ELISA, after immunizing male Wistar rats with the nicotine immunogen IP31-TT. The immunogen was administered either subcutaneously in alum in doses of 1 µg or 50 µg, or nasally, without any adjuvant, in a dose of 50 µg. We assessed both the primary antibody response and the effects of

booster immunizations. Since TT was used as carrier protein and this result in higher titers, the titer high limit was used to define the titers.

The antibody titer following a nasally administered primary immunization was below detection limit day10 post-immunization. Subsequent booster immunizations were administered in the same manner day 26, 48, 68 and 89, and generated low but measurable antibody titers. Peak titers of about 900 where reached day 50-60, i.e. after the third immunization. Antibody titers after a single subcutaneous immunization of 1 µg or 50 µg immunogen also seemed to reach its peak about day 50-60 (Fig 7). The higher dose gave a mean antibody titer that was approximately 4-fold higher than that obtained by immunization with the low (1 µg) dose.

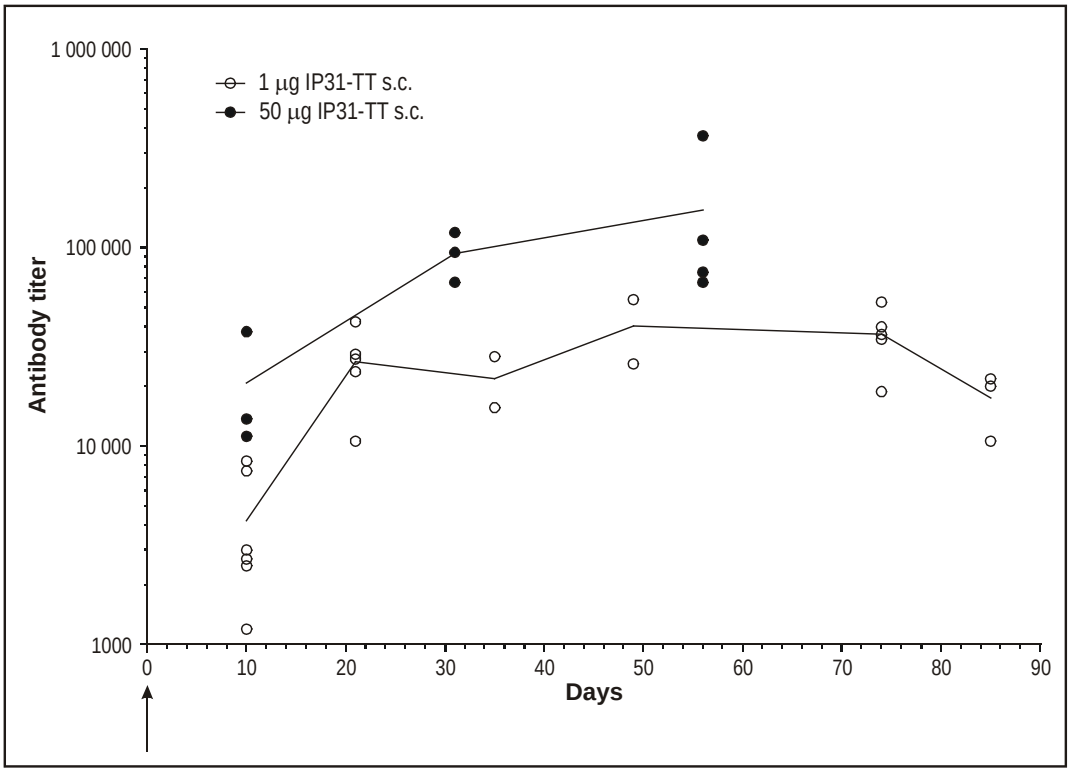


Fig 7. Antibody titers (titer high limit) after a single subcutaneous immunization with 1 µg (open circles) or 50 µg (closed circles) IP31-TT. Circles represent individual titers and lines represent the group mean.

When the primary immunization of 50 µg IP31-TT in alum subcutaneously was followed by nasally administered booster immunizations (50 µg dose), administered day 21 and 42, i.e. given before the initial antibody response had reached its peak, the antibody response seemed to level out earlier, resulting in lower titers than after a single immunization (Fig 8). In contrast, booster immunizations with a 50 µg s.c. dose of the immunogen on alum given at the corresponding time points, augmented the antibody response compared to a single immunization. This difference in booster effect might be due to the route of administration *per se*, as the nasal immunization may switch antibody production from IgG, which is measured by our ELISA, to secretory antibodies. Moreover, no adjuvants were used in the nasal immunizations in contrast to the subcutaneous immunizations. Finally, when giving the immunogen

nasally, a large part of the dose might end up in the gastro-intestinal tract, which may cause tolerance (for review see Burks *et al.*, 2008), and consequently, the amount of immunogen that is absorbed by the mucous membrane of the nose and thereby becomes available to activate the immune system might be lower, resulting in a lower active or "bioavailable" dose.

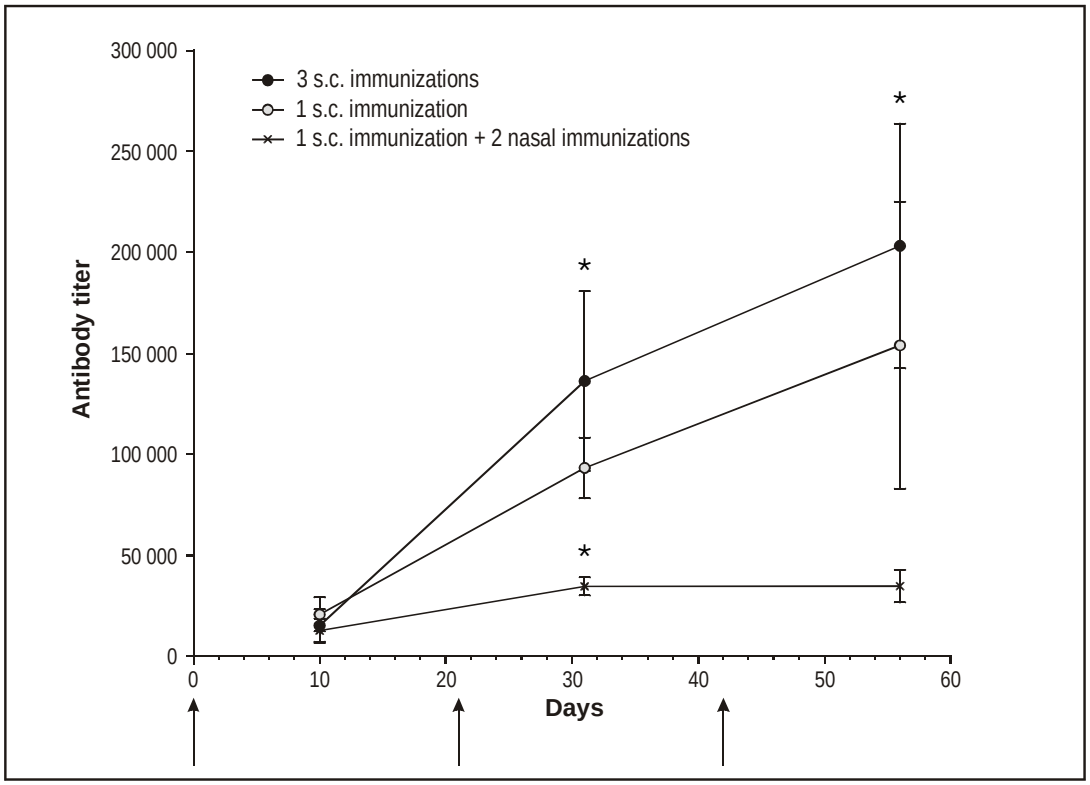


Fig 8. Antibody titers (mean±SEM) after three subcutaneous immunizations day 0, 21 and 42 (closed circles), one subcutaneous immunization day 0 (open circles) or one subcutaneous immunization day 0 followed by nasal boosters day 21 and 42 (crosses). All immunizations

This interpretation was supported by an experiment where we administered a small dose of 1 µg IP31-TT in alum s.c. at day 0, 22 and 48, in which the antibody titer seemed to decrease more rapidly after the second and third immunizations compared to that obtained by a single immunization (Fig 9). These results may imply that booster immunizations, whether nasally given or by administration of low subcutaneous doses of the immunogen can cause tolerance and thereby lower antibody titers, at least when administered before peak levels of antibodies have been reached. In contrast, when a primary immunization of 1 µg IP31-TT on alum s.c. was followed by nasally administered boosters, given day 73, 94, 115 and 136, i.e. after peak titers been reached, the antibodies were maintained at peak level, being significantly higher than after a single immunization alone (Fig 10).

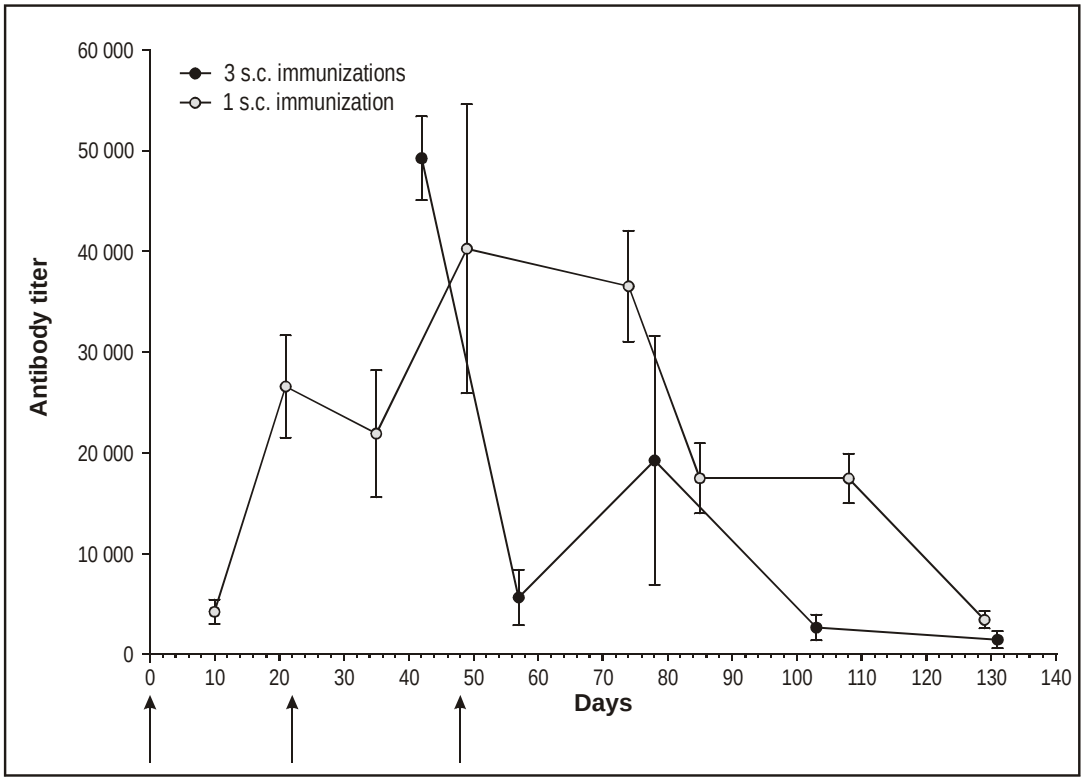


Fig 9. Antibody titers (mean±SEM) after one immunization day 0 (open circles) or three immunizations day 0, 22 and 48 (closed circles). All immunizations were given subcutaneously in a dose of 1 µg.

Interestingly, even after a single immunization by a low dose of the IP31-TT immunogen, high titers (above 1:10,000 defined by titer high limit, mean of controls + 6SD), persisted for 4 months and subsequently remained on a mean titer level of 1:3,000 for additionally 2 months. At the end of the experiment, i.e. 238 days or 8 months after immunization, all animals still had measurable titers (mean 1:1,000).

In conclusion, all nicotine haptens coupled to a carrier protein generated anti-nicotine antibodies, although a few haptens induced very low antibody titers. The choice of the carrier protein may be more important than the adjuvant for generating high antibody titers. IP31-TT administered without any adjuvant generated antibody titers in the same magnitude as IP18-KLH in Freund´s, and in addition, IP18 conjugated to TT in Freund´s increased the antibody response 33-fold (mean) compared to the KLH-conjugate. Since the IP31 and the IP18 are structurally similar, IP31 being the (*S*)-enantiomer of the racemate IP18, and the titer after immunization with the haptens conjugated to TT and given with Freund´s are in the same magnitude, this effect is in all probability not due to the different haptens, but rather the carrier protein. We expected the alum adjuvant to be inferior compared to Freund´s adjuvant, but when immunizing with 100 µg of TT-conjugate, it resulted in very high titers of the same magnitude regardless of which adjuvant that was used. If anything, the alum immunizations seemed to cause less variation of the titer than Freund´s.

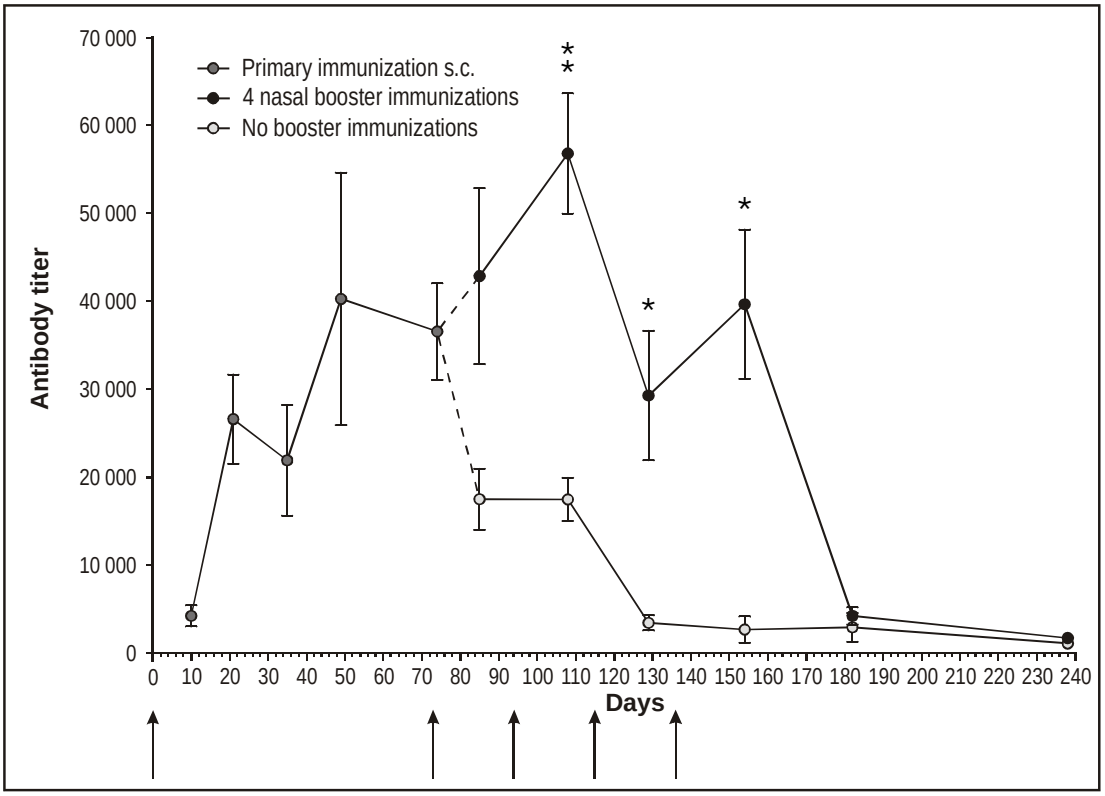


Fig 10. Antibody titers (mean±SEM) after one subcutaneous immunization day 0 (grey circles) followed by either no booster (open circles) or nasally administered boosters day 73, 94, 115 and 136 (closed circles). *Asterisks* represent statistical difference between the groups (* $p<0.05$, ** $p<0.01$).

4.2 Antibody selectivity as measured by competitive ELISA

After establishing that the nicotine immunogens could generate anti-nicotine antibodies, we investigated the antibodies selectivity for nicotine and potential cross-reactivity with nicotine's metabolites or endogenous substances, by means of competitive ELISA. Since all sera contain different concentrations of antibodies, and during the course of the project, the chosen serum dilutions used in the competitive ELISA varied between 16-98% of the maximum absorption in the ELISA, the competitive ELISA have consequently been run at different antibody levels. This difference, and the fact that there is a correlation between the concentration of competitor needed to inhibit antibody binding and the concentration of antibodies, makes quantitative comparisons between groups immunized with different immunogens difficult. Competitive ELISA results, in general, in a more qualitative measurement of the antibody selectivity, and allow for quantitative assessments only within groups. In order to overcome this weakness of the method, the cross-reactivity measured in the competitive ELISA were, in some cases, adjusted to correspond to the concentration necessary to inhibit the amounts of antibodies at the titer limit (see Materials and methods), thereby resulting in more quantitative measurements and allowing for evaluations of differences in antibody selectivity also between groups (Tables 5, 6 and 7).

Table 4. Selectivity of antibodies generated after immunization with different nicotine immunogens as measured by competitive ELISA. IC_{20} represents the concentration of competitor that results in 20% inhibition of the maximum response.

IC_{20} (mM)	NIC	COT	NOR	NOX	NIA	ACH	NA	DA	5-HT	HIST	GLY	CHO	GABA
YH7-KLH	0.16	5.0	0.28	22	32								
IP18-KLH	0.084	n.d.	1.4	2.8		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
IP31-KLH	0.19	n.d.	7.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SG62-KLH	1.2	n.d	6.3	n.d		n.d	n.d	n.d	n.d	n.d	n.d		
IB87-KLH	0.24	n.d	3.2	n.d		n.d	n.d	n.d	n.d	n.d	n.d		
IP18-TT	0.4	n.d.	2.0	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		

NIC:(*S*)-nicotine, COT: (*S*)-cotinine, NOR: nornicotine, NOX: (*S*)-nicotine-1´-N-oxide, NIA: niacin (nicotinic acid), ACH: acetylcholine, NA: (*S*)-noradrenaline, DA: dopamine, 5-HT: serotonin, HIST: histamine, GLY: glycine, CHO: choline, GABA: -amino butyric acid

4.2.1 Antibody selectivity – Effects of different nicotine haptens

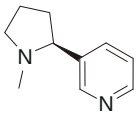
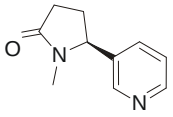
The antibodies generated by all immunogens were more selective for (*S*)-nicotine than for any of the metabolites of nicotine (Tables 4, 6 and 7), with the exceptions of GK56-KLH and GK60-KLH. These immunogens, which comprise short linkers, generated antibodies with either higher selectivity for the minor metabolite nornicotine than (*S*)-nicotine (GK60-KLH, about 3-fold) or an equal nornicotine-nicotine selectivity (GK56-KLH). Moreover, antibodies generated by all immunogens were less selective for the major metabolite (*S*)-cotinine and the minor metabolite (*S*)-nicotine-1´-N-oxide than for nornicotine. Nornicotine may contribute to the dependence producing properties of tobacco smoking (Bardo *et al.*, 1999), and thus it may be advantageous if the antibodies to some degree inactivate also nornicotine.

Table 5. IC_{20} for nicotine adjusted to correspond to the concentration necessary to inhibit the amount of antibodies at the titer limit.

	Adjusted IC_{20NIC} (mM)
IP18-KLH	0.007
IP31-KLH	0.0018
IP18-TT	0.003

There were a great range in the nicotine selectivity, and a higher selectivity for (*S*)-nicotine by the antibodies may indicate that the nicotine part of the used nicotine hapten possesses a three dimensional structure that is more similar to free (*S*)-nicotine. Consistently, antibodies generated by immunization with (*S*)-enantiomeric hapten immunoconjugates were found to be more selective for (*S*)-nicotine than antibodies generated by racemic hapten immunoconjugates (Tables 5 and 6). This difference is, at least in part, probably due to the enantiopure immunoconjugates being more structurally homogeneous than the racemic ones and can also be assumed to possess approximately twice as many (*S*)-nicotine sites as the racemic immunoconjugates. This is specifically exemplified by the lower IC_{20} (adjusted value) for the (*S*)-enantiomeric hapten immunogen IP31-KLH compared to its racemic counterpart IP18-KLH (Table 5). The (*S*)-enantiomeric hapten immunogens, IP31-KLH, SG62-KLH and IB87-KLH, as well as the racemic hapten immunogens YH7-KLH and IP18-KLH, generated antibodies with the highest nicotine selectivity. The linkers of these immunogens display a greater flexibility close to the nicotine molecule, since this part of the linker comprises single-bonded atoms, and this flexibility may also contribute to the generation of more nicotine selective antibodies. The YH7, although, comprises a nitrogen atom next to the nicotine molecule, which seem to result in antibodies that cross-reacts to a greater extent with the metabolites of nicotine.

Table 6. IC_{50}-values for (*S*)-nicotine and (*S*)-cotinine. The IC_{50}-values are corrected to correspond to the concentration necessary to inhibit the amount of antibodies at the titer limit.

Nicotine immunogen	n	IC_{50} (*S*)-nicotine (mM, mean±SEM)  (*S*)-nicotine	IC_{50} (*S*)-cotinine (mM, mean±SEM)  (*S*)-cotinine	$\frac{IC_{50COT}}{IC_{50NIC}}$
GK56-KLH	6	0.850 ± 0.382	7.183 ± 2.731	8.5
GK60-KLH	4	0.615 ± 0.173	10.066 ± 2.346	16.4
GK81-KLH	4	0.165 ± 0.076	4.343 ± 1.407	26.3
GK83-KLH	5	0.273 ± 0.092[a]	2.266 ± 0.474	8.3
IP31-KLH	6	0.014 ± 0.007	1.967[b]	136.8
SG62-KLH	6	0.144 ± 0.081	2.293 ± 1.949[c]	16.0
IB87-KLH	6	0.020 ± 0.009	1.108 ± 0.486[d]	56.0

[a] n=4, one rat excluded due to technical errors
[b] n=1, in five samples no inhibition were detected
[c] n=2, in four samples no inhibition were detected
[d] n=3, in three samples no inhibition were detected

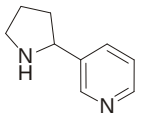
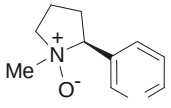
The linkers of the remaining immunogens included in the present selectivity study, comprise a triple bond attached directly to the nicotine molecule and are, consequently, more rigid in this part of the linker. Among these nicotine immunogens, GK81-KLH generated the most nicotine-selective antibodies and the linker of GK81 also comprises a longer flexible part, although it is directed towards the carrier protein. The antibodies generated by immunogens comprising shorter linkers, i.e. GK56-KLH and GK60-KLH, were less nicotine-selective than antibodies generated by immunogens with longer linkers, suggesting shorter rigid linkers to be inferior in this regard. This may be so since the shorter and more rigid linkers will probably not present the nicotine part of the hapten to the immune system as efficiently as the longer flexible linkers.

Binding of a linker to the 6-position of the nicotine molecule generated antibodies with higher nicotine selectivity than binding of the corresponding linker to the 5-position, as exemplified by GK60 compared to GK56, and IB87 compared to SG62. In addition, the ratio of the IC_{50} for (*S*)-cotinine and (*S*)-nicotine (Table 6) is greater when the linker is attached to the 6-position compared to the 5-position of nicotine. Nicotine is, to a major extent, metabolized to cotinine, and due to its long half-life (Benowitz *et al.*, 1983), the serum levels of cotinine in smokers are much

higher than the levels of nicotine. Therefore it is of great importance that the antibodies only to a very small extent cross-react with cotinine. Taken together, our data propose that the 6-position is more preferable for linker attachment.

Importantly, there were no detectable level of inhibition for the brain neurotransmitters acetylcholine, noradrenaline, dopamine, serotonin, glycine, choline, GABA or histamine by the generated antibodies (Table 4), even at a concentration of the competitors (10 mM), which exceeds the physiological plasma levels more than 30-fold (Barnes *et al.*, 1982; Kagedal and Goldstein, 1988; Maes *et al.*, 1998; Croonenberghs *et al.*, 2000; Rang *et al.*, 2007). The antibodies generated by YH7-KLH cross-reacted (max. concentration in assay 60 mM) to some extent with the vitamin niacin, which seems not to be present in plasma (Lang *et al.*, 2010), whereas antibodies generated by IP31-KLH (max. concentration in assay 10 mM), showing a more favorable selectivity profile, did not recognize niacin.

Table 7. IC_{50}-values for nornicotine and (*S*)-nicotine-1´-N-oxide. The IC_{50}-values are corrected to correspond to the concentration necessary to inhibit the amount of antibodies at the titer limit.

Nicotine immunogen	n	IC_{50} nornicotine (mM, mean±SEM)  Nornicotine	IC_{50} (*S*)-nicotine-1´-N-oxide (mM, mean±SEM)  (*S*)-nicotine-1´-N-oxide
GK56-KLH	6	0.830 ± 0.259	24.395 ± 8.361[a]
GK60-KLH	4	0.219 ± 0.059	11.770 ± 6.228[b]
GK81-KLH	4	1.141 ± 0.455	18.328 ± 3.684
GK83-KLH	5	0.447 ± 0.109	5.647 ± 1.170
IP31-KLH	6	0.227 ± 0.110[a]	0.335 ± 0.152[a]
SG62-KLH	6	0.571 ± 0.312[a]	1.443 ± 0.764[a]
IB87-KLH	6	0.139 ± 0.071	0.324 ± 0.152

[a] n=5, in one sample no inhibition was detected

[b] n=2, the highest concentration of competitor was excluded due to lack of sufficient amounts of (*S*)-nicotine-1´-N-oxide, no inhibition were detected

4.2.2 Antibody selectivity – Effects of different carrier proteins

When comparing the selectivity of antibodies generated by immunizations with IP18 coupled either to the carrier protein KLH or to TT (Table 4), it seemed that the IP18-TT antibodies were less selective for (*S*)-nicotine (about 4.8-fold), nornicotine

(about 1.4-fold) and (*S*)-nicotine-1´-N-oxide (not detectable) than the IP18-KLH generated antibodies. Since the antibody titers generated by the TT-conjugate were much higher than those generated by the KLH-conjugate, and the competitive ELISA run at a higher antibody concentration for the IP18-TT sera, this difference, in all probability, rather reflects a difference in titer than in selectivity. When the IC_{20} were corrected to correspond to the concentration necessary to inhibit the amount of antibodies at the titer limit (see Materials and methods), the IC_{20} for (*S*)-nicotine were instead in the same range (Table 5), i.e. 0.007 mM and 0.003 mM for antibodies generated by IP18-KLH and IP18-TT, respectively. Moreover, the antibodies generated by the TT-conjugate seemed more selective for nornicotine than the antibodies induced by the KLH-conjugate, i.e. the IC_{20} for nornicotine were 0.016 mM and 0.117 mM, respectively. Importantly, the selectivity profiles were similar after immunization with both conjugates; antibodies showed no cross-reactivity for the major nicotine metabolite (*S*)-cotinine or the endogenous transmitters.

In conclusion, even small changes in the structure of the nicotine hapten may greatly influence the selectivity of the generated antibodies, whereas the carrier protein seems to only have a minor effect on the antibody selectivity. A high selectivity to nicotine may be achieved by attachment of the semi-flexible linker to the 6-position of nicotine with the flexibility of the linker directed towards the nicotine molecule.

4.3 *In vivo* voltammetry

After establishing the ability of the nicotine immunoconjugates to generate nicotine selective antibodies, we wanted to investigate whether active immunization against nicotine could prevent the central effects of nicotine that is considered critical to its dependence producing properties. *In vivo* voltammetry was used to assessed the effect of four intravenous nicotine doses (6, 12, 24 and 48 μg/kg) on dopamine output in NAC_{shell}.

In rats immunized with GK56-KLH, GK60-KLH, GK81-KLH, GK83-KLH, IP31-KLH, SG62-KLH and IB87-KLH, and in controls there were seemingly dose-dependent increases in nicotine-induced dopamine output in the NAC_{shell} following the doses 6-24 μg/kg i.v. (Fig 11 A-D), even though the magnitude of the response differed between groups. For some groups the dose-dependent increase extended also to the highest dose (48 μg/kg, i.v.), whereas in other groups, including the controls, the highest dose caused a smaller dopamine release than the previous one, resulting in a bell-shaped dose-response curve. The attenuated effect following the highest nicotine dose may reflect desensitisation of the nicotine receptors.

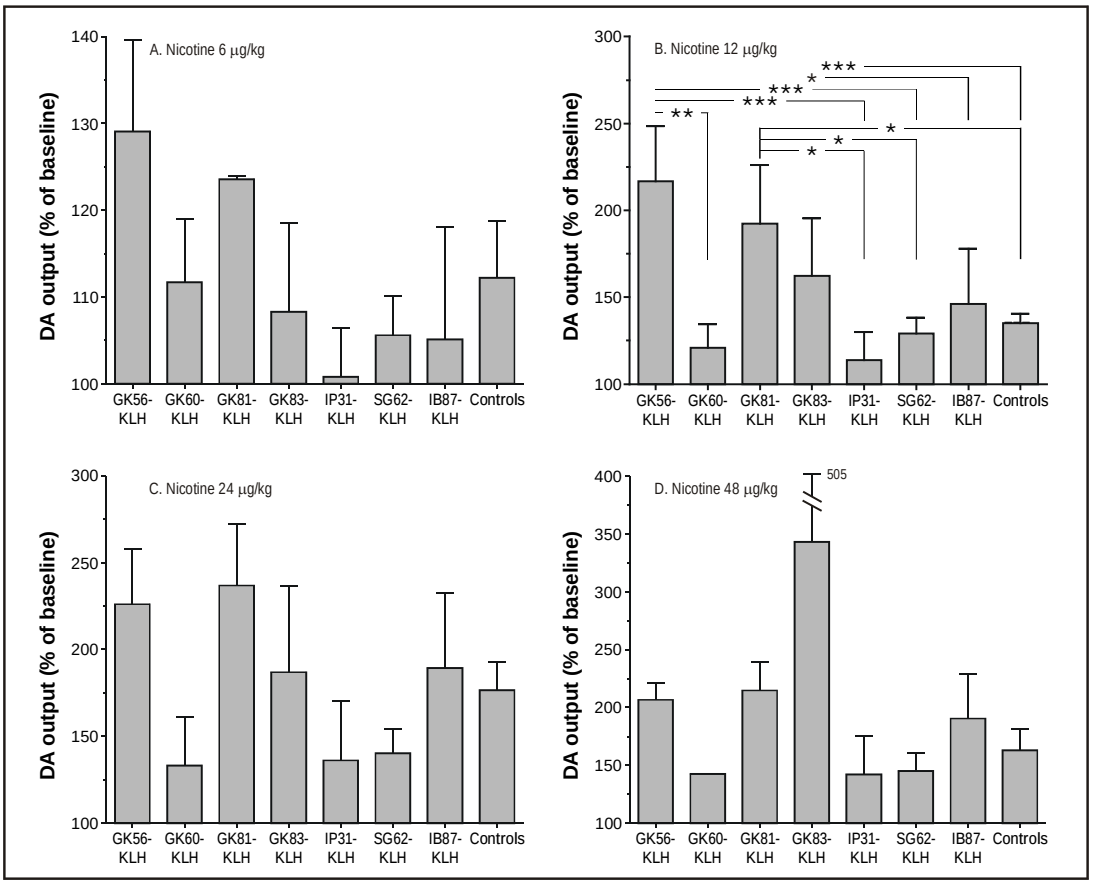


Fig 11 A-D. Dopamine (DA) output in the nucleus accumbens shell (NAC_{shell}) expressed as percent of baseline levels, assessed by *in vivo* voltammetry in immunized rats and controls after administration of nicotine 6 (panel A), 12 (panel B), 24 (panel C) and 48 (panel D) μg/kg i.v (incremental doses). Statistical evaluation was performed by means of one-way ANOVA followed by LSD test. The *asterisks* represent a significant difference between groups, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

We expected that all immunogens that generated anti-nicotine antibodies, would cause a decrease in the nicotine-induced dopamine release compared to controls, as measured by *in vivo* voltammetry. Surprisingly, the effects of the immunizations on the nicotine-induced dopamine release in the NAC_{shell} did not result in a consistent decrease but instead varied depending on the immunogens used, despite the fact that anti-nicotine antibodies were induced by all immunogens.

In animals immunized with IP18-KLH, the immunogen showing the highest selectivity for nicotine in the initial screening, there was no significant effect on dopamine release in NAC following intravenous nicotine administration in doses of 6, 12, 24 μg/kg (Fig 12), while in controls, nicotine induced a dose-dependent significant increase in the dopamine output in this brain area. After the second nicotine dose of 12 μg/kg (i.e. a cumulative dose of 18 μg/kg), which approximately corresponds to the amount of nicotine obtained from smoking one cigarette, there was a significant difference ($p=0.029$) in the mean dopamine release between the animals immunized with the IP18-KLH immunogen in Freund´s adjuvant and controls treated with Freund´s adjuvant alone (Fig 13). In controls, the effect of nicotine on dopamine overflow was significant already within the first minute after the nicotine injection,

whereas this rapid action, in contrast, was prevented in animals immunized with IP18-KLH. The increase in dopamine release in immunized rats following the largest dose of nicotine (48 μg/kg, approximately corresponding to the nicotine dose delivered by 3 cigarettes, Benowitz and Jacob P, 1984) was not directly associated with the nicotine injection, but appeared somewhat later.

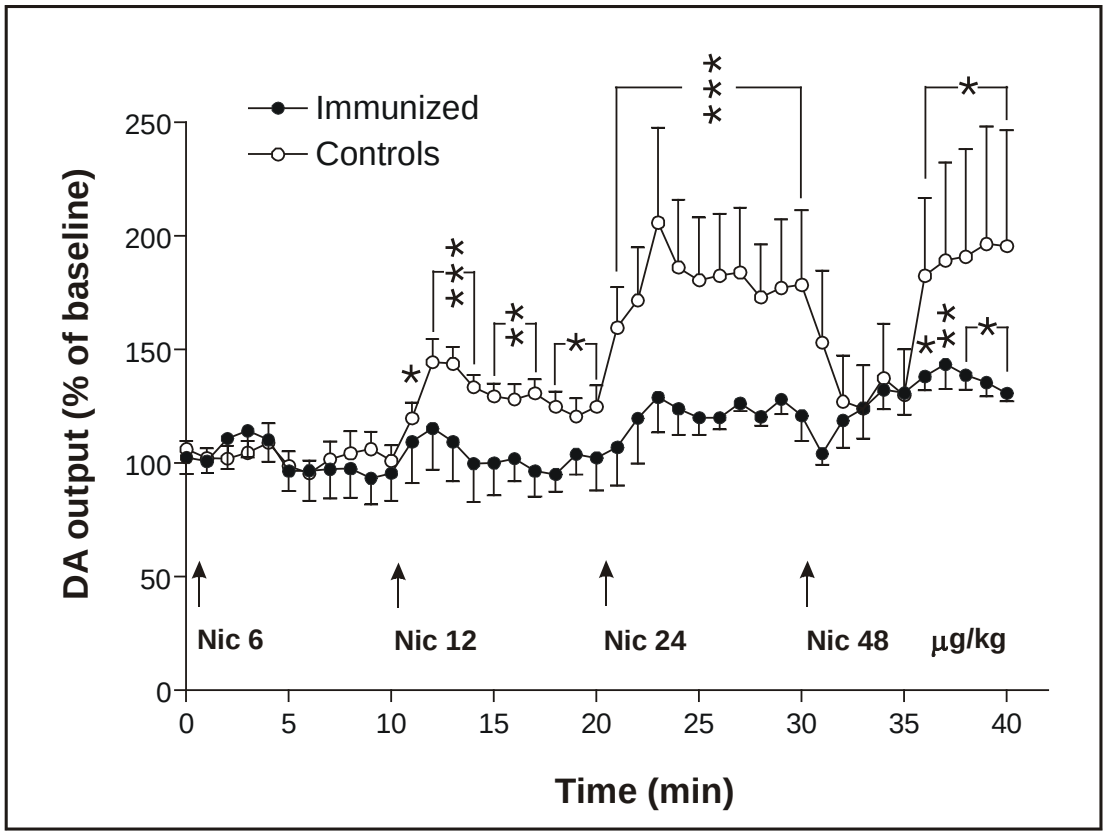


Fig 12. Nicotine-induced dopamine release in the NAC_{shell} was studied using *in vivo* voltammetry in animals immunized with IP18-KLH in Freund´s adjuvant (n=4-7, closed circles) and controls treated with Freund´s adjuvant (n=11-13, open circles) alone. Statistical evaluation was performed by means of one-way ANOVA followed by Newman-Keuls test for appropriate post hoc comparisons. *Asterisks* indicate statistically significant difference form baseline. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

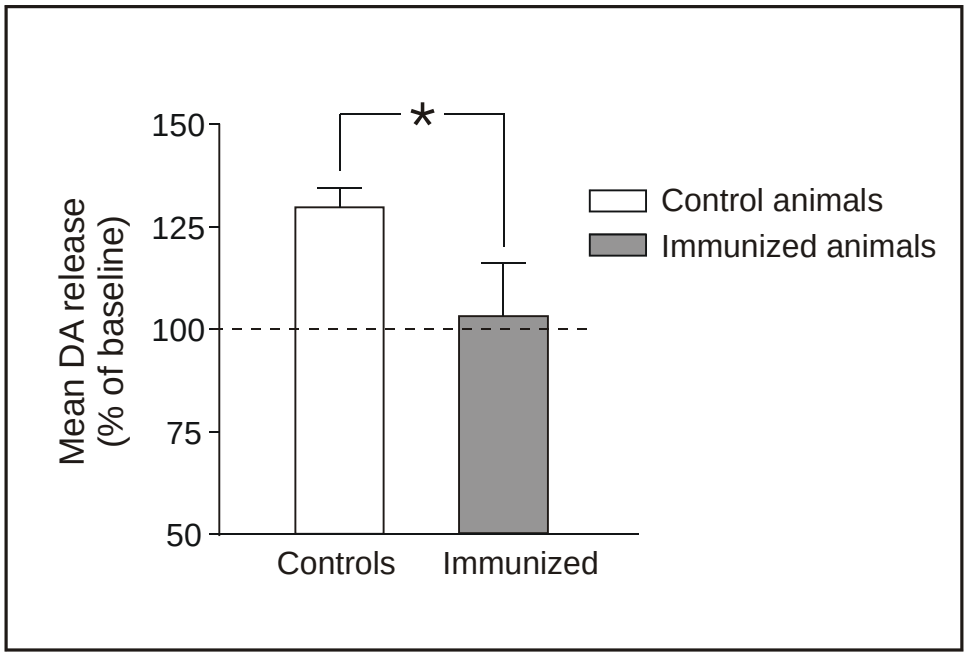


Fig 13. Mean dopamine overflow in NAC_{shell} after a nicotine dose of 12 µg/kg, i.e. a cumulative dose of 18 µg/kg, as measured by *in vivo* voltammetry. Statistical evaluation was performed by means of Students t-test. The *asterisk* represents a significant difference between IP18-KLH immunized animals (n=5, closed bar) and control immunized animals (n=13, open bar), * p=0.029.

4.4 Nicotine-seeking behavior

Nicotine dependence is a chronically relapsing disorder (Fiore *et al.*, 2000), and tobacco smokers need about 5-7 quit-attempts in order to succeed in remaining smoke-free for one year (Hughes, 2000). There are at present no approved pharmacological aids to prevent relapse. Active immunization against nicotine has the potential to provide a long-term protection against relapse. In previously self-administering rats that undergoes extinction, a small priming dose of nicotine has been shown to reinstate the nicotine-seeking behavior (Chiamulera *et al.*, 1996), mimicking the drug-induced relapse situation in humans. Therefore we assessed whether immunization with IP18-KLH, i.e. the nicotine immunogen showing the most promising profile in our screening process, could prevent reinstatement of nicotine-seeking induced by two different priming doses in rats following extinguished nicotine self-administration behavior.

In controls (not immunized or with a titer <1:3000) a priming dose of 1 µg/kg i.v. significantly reinstated nicotine-seeking behavior, whereas a 30 µg/kg dose did not (Fig 14). This is consistent with results from Chiamulera *et al.* (1996), which showed significant increase in responding following priming doses of 1, 3 and 10 µg/kg but not 30 µg/kg. Indeed, the lowest dose induced the highest increase in responding. It has been hypothesized (Corrigall and Coen, 1989; Chiamulera *et al.*, 1996) that the lack of response following the 30 µg/kg dose could be due to that the dose in itself might induce sufficient reward and thus reduce the craving for more drug.

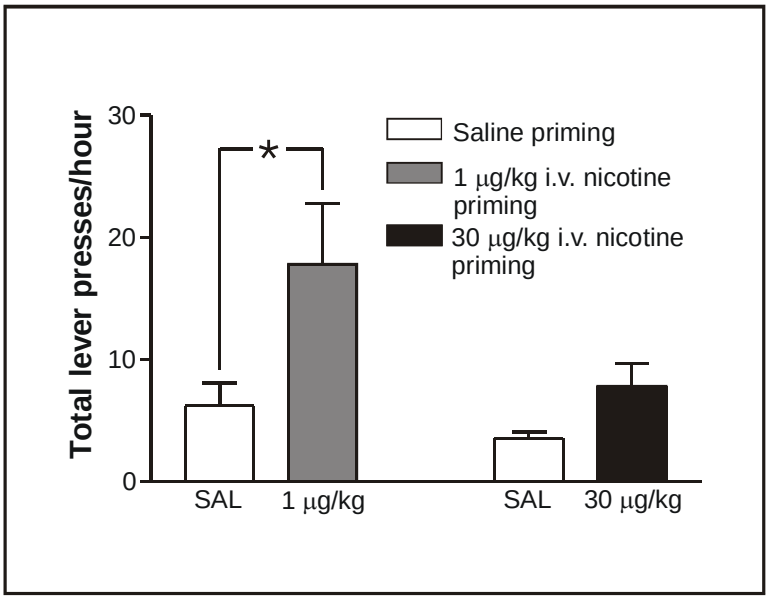


Fig 14. Reinstatement of nicotine-seeking behavior in rats with low or no titers of antibodies (<1:3000). A small priming dose of nicotine (1 μg/kg i.v.) significantly reinstates nicotine-seeking behavior, whereas the higher dose (30 μg/kg i.v.) does not. Data are expressed as mean number of lever presses ±SEM. * p=0.0459.

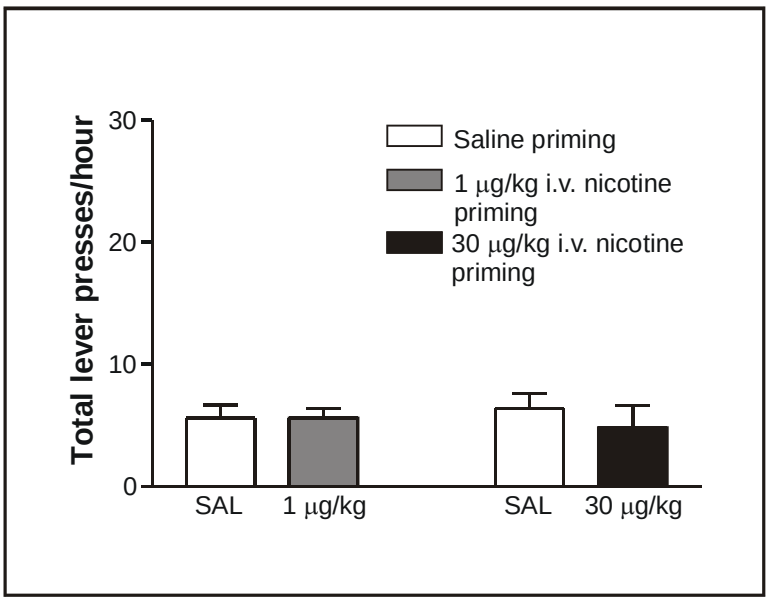


Fig 15. Blockade of nicotine-seeking behavior in rats with high titers of antibodies (>1:10,000). Neither the smaller (1 μg/kg i.v.) nor the higher (30 μg/kg i.v.) priming dose of nicotine reinstates nicotine-seeking behavior. Data are expressed as mean number of lever presses ±SEM.

In contrast to controls, the 1 μg/kg dose did not reinstate nicotine-seeking (Fig 15) in rats immunized with IP18-KLH, indicating that immunized animals have sufficient amount of antibodies to inactivate the small priming dose. Since our results from *in vivo* voltammetry indicated that a large dose of nicotine, albeit very high, might overcome the protective effect by the antibodies generated by IP18-KLH and induce dopamine release in the NAC_{shell}, and nicotine-seeking behavior may be

reinstated by very small doses of nicotine, we hypothesized that the higher priming dose might reinstate nicotine seeking in immunized rats. However, the 30 µg/kg priming dose had no effect on responding in immunized animals, indicating that antibodies generated by IP18-KLH may abolish the reinforcing effect of the higher priming dose as well.

4.5 Nicotine distribution

The previous results obtained after immunization with IP18-KLH, showing a favorable profile of antibody titers and selectivity, as well as an ability of the generated antibodies to prevent the reinforcing effects of nicotine, and, in addition, to prevent reinstatement of nicotine-seeking behavior in an animal model of relapse, made this nicotine immunogen a good candidate for further studies. Since an altered distribution of nicotine is the actual mechanism of action of nicotine vaccines, we decided to investigate the effects of immunization with IP18-KLH in this regard. In this experiment we choose nicotine doses that had been used previously by others (Hieda *et al.*, 1997, 1999; Keyler *et al.*, 1999; Pentel *et al.*, 2000), and, in addition, had been shown to have no effect on the dopamine release in NAC_{shell} in rats immunized with IP18-KLH (de Villiers *et al.*, 2002).

Three minutes after administration of 15 µg/kg (*S*)-nicotine, the mean serum nicotine level in rats immunized with IP18-KLH were 73.70±23.6 ng/ml (mean±SEM), i.e. 6.6-fold higher than in controls (11.06±0.6 ng/ml, p=0.0088). Serum nicotine was also significantly higher in immunized animals compared to controls after a 30 µg/kg (*S*)-nicotine dose both 3 min (4.8-fold, p=0.0003) and 60 min (4.2-fold, p=0.0007) after administration (Fig 16). The serum nicotine levels in control rats 3 min after nicotine administration corresponds very well with the serum level in humans five minutes after smoking 1-3 cigarettes (Benowitz *et al.*, 1988, 1989). The increase in serum nicotine levels in immunized animals is in the same range as what has been observed by others, using different immunoconjugate vaccines (Hieda *et al.*, 1997, 1999; Keyler *et al.*, 1999; Pentel *et al.*, 2000) and may in all probability be due to an increased plasma protein binding of nicotine by the antibodies, that results in a decreased tissue distribution of nicotine. Importantly, the attenuated distribution of nicotine in immunized animals resulted in significant reduction of brain nicotine levels (corrected for blood content) by 39.4 % (p=0.0013) in immunized animals compared to the controls, 3 min after receiving a 15 µg/kg intravenous (*S*)-nicotine dose. This is in the same range as the 36% reduction that previously has been reported by Hieda *et al*. (1999) after immunization with nicotine immunogen CMUNic, but lower than that observed by Pentel *et al*. (2000), i.e. a 64% reduction 3 minutes after nicotine administration in immunized rats.

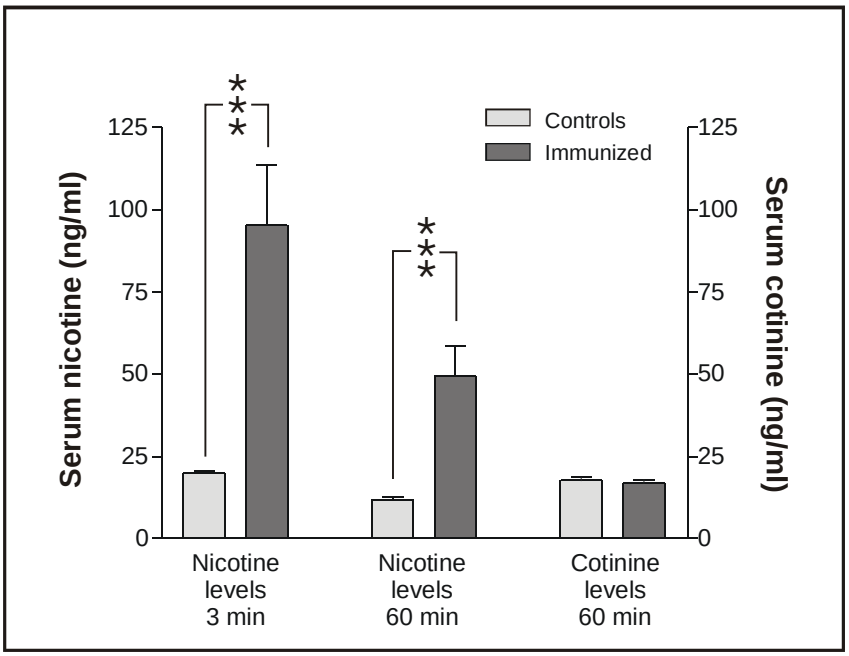


Fig 16. Mean serum nicotine levels 3 and 60 minutes and mean cotinine levels 60 minutes after administration of an intravenous nicotine dose of 30 µg/kg in IP18-KLH immunized rats. Statistical evaluation was performed by means of Students t-test. The *asterisks* represent a significant difference between IP18-KLH immunized animals (n=7 in the 3 min sample and n=8 in the 60 min samples, closed bars) and control immunized animals (n=9, open bars), *** $p < 0.001$.

Importantly, our study demonstrate that one hour after the 30 µg/kg intravenous (*S*)-nicotine dose there was still a significant reduction (34.4%, p=0.0087) in brain nicotine levels in the immunized animals compared to controls, which was similar to that observed after 3 minutes. Consequently, the reduction in brain nicotine levels obtained is robust over different doses and time intervals, lasting for at least 60 min. There was, as expected, a positive correlation between serum nicotine levels and brain nicotine levels in control rats 3 minutes after drug administration (r^2=0.44). Conversely, in animals immunized with IP18-KLH the serum nicotine levels were negatively correlated to brain nicotine levels (r^2=0.88, Fig 17), and somewhat surprisingly, this correlation was stronger than the positive correlation between antibody titers and brain nicotine levels (r^2=0.57). In immunized rats, increased serum nicotine reflects increased nicotine binding by the antibodies, indicating that not only the amount of antibodies, but also their affinity for nicotine is important for the reduced distribution of nicotine to the brain.

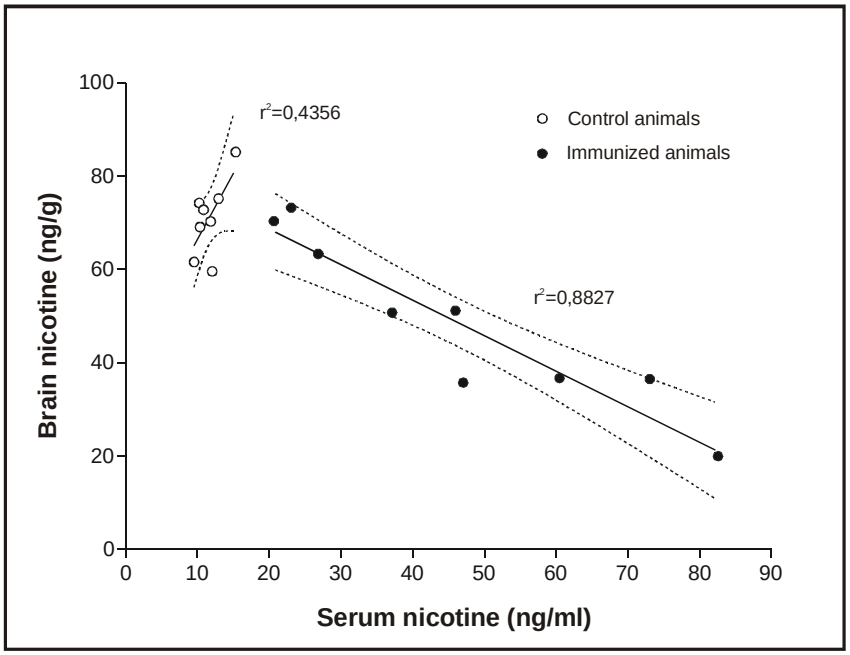


Fig 17. Correlation between serum nicotine and brain nicotine levels three minutes after a 15 μg/kg intravenous nicotine dose in IP18-KLH immunized rats (n=9, closed circles) and control rats (n=8, open circles), respectively. Solid lines represent lines of regression and dotted lines the 95% confidence intervals.

The levels of the major nicotine metabolite cotinine found at 60 min after nicotine administration were virtually identical in immunized animals and controls, which suggests that the metabolism to cotinine remained unaffected by immunization. This is somewhat surprising since active immunization with the nicotine immunoconjugate CMUNic has previously been reported to prolong the half-life of nicotine in rats (Keyler *et al.*, 1999), an effect that also resulted in decreased cotinine levels in the immunized rats. The relatively low nicotine dose and limited time period of measurement might contribute to the lack of difference in cotinine levels in our study. However, in a study by Lindblom *et al.* (2005), there were no differences in cotinine levels in rats immunized by the IP18-KLH immunogen and controls even after chronic administration of a high dose of nicotine. The present data set on serum concentrations of nicotine, which from a pharmacokinetic standpoint is quite limited, taken together with the lack of difference in cotinine levels in immunized rats and controls following single as well as chronic nicotine administration, may indicate that immunization with IP18-KLH does not prolong the half-life of nicotine, although the effect of IP18-KLH immunization on the elimination of nicotine needs to be further investigated.

5 GENERAL DISCUSSION

Active immunization with a hapten immunogen against a small molecule, such as nicotine, differs from conventional vaccinations against infections agents, like bacteria or viruses, or toxins. The challenges for conventional vaccines lie in achieving a non-toxic and non-infectious immunogen which still display epitopes similar enough to the natural antigen so that it may be recognized by the immune system. The generation of high titers of antibodies is not as vital for conventional vaccines as for immunizations against small molecules, since the conventional antigens usually are larger proteins which, unlike nicotine, are immunogenic themselves. In addition, the binding of antibodies to conventional antigens can rely on the combined strength of multiple bond interaction, i.e. the avidity, whereas antibodies binding to the small nicotine molecule in all probability can solely rely on the affinity of a single bond. Finally, the multi-epitopes of the conventional antigens allow the formation of immunoconjugates which activates other parts of the immune system, causing inflammation and neutralization, while the main objective of vaccination against nicotine is to inactivate the drug by reducing its distribution.

These differences make the course of development different between vaccines against small molecules and conventional vaccines. In addition, the methods used to assess the efficacy differ, both as regards the choice of methods as well as what is measured by conventional methods such as ELISA. In ELISA the antigen is absorbed to the plastic of the plate, and with conventional vaccines the protein antigen can be used as it is, subsequently being detected by antibodies in serum, whereas a small molecule, such as nicotine, needs to be conjugated to a protein in order to be adsorbed to the plate, similarly as for immunization. Commonly, the protein BSA is used in the hapten conjugate in the ELISA in order to prevent binding of antibodies directed towards the carrier protein used in the immunogen.

However, the nicotine hapten used for immunization is in general the same as that also used in the ELISA (e.g. Hieda *et al.*, 1997; Keyler *et al.*, 1999; Cerny *et al.*, 2002; de Villiers *et al.*, 2002). Since the attachment of a linker to nicotine will influence the three-dimensional structure of the nicotine molecule, it will thus differ from free nicotine. A consequence of using the ELISA method under these circumstances may be that antibodies against the nicotine hapten are detected, that may also include antibodies recognizing the linker and not free nicotine. This notion is supported by Hieda *et al*. (1997) reporting a greater antibody selectivity for the hapten used in the immunization than for nicotine. In addition, these authors (Hieda *et al.*, 2000) and others (Cerny *et al.*, 2002; Lindblom *et al.*, 2005) have reported ELISA to be unaffected by high serum concentrations of nicotine.

These observations suggest that the predictive value of ELISA measurements, when assessing the effects of immunization, may be associated with certain limitations. Methods using free nicotine, such as radioimmunoassay (RIA) and equilibrium dialysis, might tentatively provide a higher reliability in this regard. Preliminary results from our laboratory indicate that this is the case. The above discussed limitations of the ELISA might, at least in part, be the reason why, in our studies, the negative correlations between the antibody titers and dopamine release in

NAC_{shell} as well as the brain nicotine levels are not as pronounced as one might expect. However, the effects of antibodies generated by nicotine vaccination should in all probability depend on both the amounts of antibodies achieved as well as the strength of their binding to nicotine.

Taking both antibody titer and nicotine selectivity into account, as exemplified by the strong negative correlations between $Log(Titer/IC_{50NIC})$ and the nicotine-induced dopamine release (Fig 18) or between the serum nicotine levels and brain nicotine levels in immunized animals (Fig 17), gives a higher predictive value as regards the results of immunization. This conclusion is supported by a study by Keyler *et al.* (2005) on nicotine distribution following passive administration of monoclonal antibodies, which showed that increased nicotine affinity and antibody titers were associated with increased serum nicotine and decreased brain nicotine levels.

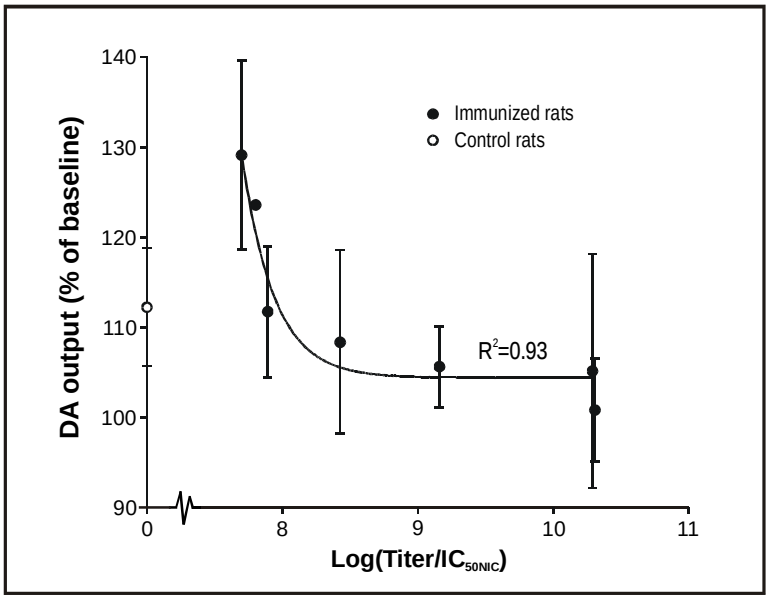


Fig 18. The logarithm of the fraction of the antibody titer and the IC_{50} for nicotine [$Log(titer/IC_{50NIC})$] is nonlinearly and negatively correlated with the dopamine overflow (% of baseline levels) in the NAC_{shell} induced by the intravenous administration of 6 µg/kg nicotine. Closed circles represent groups of rats immunized with different immunogens, and the open circle represents the control group.

Surprisingly, the selectivity profile of the antibodies seemed to be more important than we anticipated. Especially the cross-reactivity with cotinine by the antibodies generated by some of the immunogens seemed to negatively influence the ability of the antibodies to suppress the reinforcing effects of nicotine. Since the serum cotinine levels are higher than the nicotine levels in smokers, and a higher concentration results in a higher antibody binding, even a small cross-reactivity by the antibodies to cotinine might result in a substantial binding and, hence, fewer antibodies would be available to inhibit the effects of nicotine. However, our results indicate that cross-reactivity to cotinine not only prevents the expected decrease in nicotine-induced dopamine release after immunization, but even may cause an

enhanced dopamine release compared to controls (Fig 19). Cotinine has been shown to counteract the effect of nicotine, possibly by desensitizing the receptors (Sziraki *et al.*, 1999; Oliver *et al.*, 2007; Buccafusco *et al.*, 2007), and inactivation of cotinine by the antibodies may thus augment the effect of nicotine on the receptors.

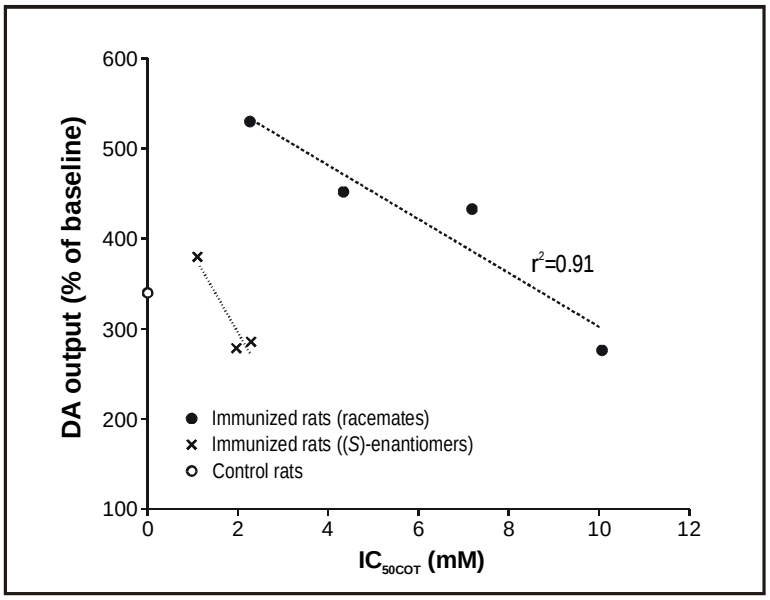


Fig 19. The IC_{50} for (*S*)-cotinine is negatively correlated (p=0.047) with the total dopamine overflow (% of baseline levels) in the NAC_{shell} induced by the intravenous administration of the doses 24 and 48 µg/kg of nicotine, in rats immunized with racemic immunogens GK56-KLH, GK60-KLH, GH81-KLH and GK83-KLH. The (*S*)-nicotine immunogens (IP31-KLH, SG62-KLH and IB87-KLH) were not included in these correlations since inhibition could not be detected in more than half of the samples, and therefore the IC_{50}-values are not representative for the groups.

Our results on nicotine-seeking behavior as well as nicotine-induced dopamine release in rats immunized with IP18-KLH indicates that the antibodies generated are sufficient to block the effect of at least 30-42 µg/kg nicotine administered intravenously, corresponding to that ingested by smoking 2-3 cigarettes. When investigating the distribution of nicotine in intravenous doses of 15 and 30 µg/kg in rats immunized with IP18-KLH, we observed that although there were significant reductions in brain nicotine levels compared to controls, a substantial amount of nicotine was still present in the brains of the immunized animals. This may seem surprising since the reinforcing effects of nicotine in these doses seemed to be abolished in the immunized animals. Our findings suggest that the amount of nicotine that is present in the brain 3 minutes after its administration in IP18-KLH immunized rats (43.1 ± 6.4 ng/g, 15 µg/kg dose) is not sufficient to effectively activate the central dopaminergic reward pathway, while the brain levels of nicotine in controls (71.0 ± 2.9 ng/g) are. These finding might be interpreted to imply that the effect of the immunization could be surmountable by increasing the dose of nicotine. Indeed, in the *in vivo* voltammetry experiments, administration of the highest nicotine dose (48 µg/kg, i.v.) caused a slight increase in accumbal dopamine overflow also in immunized animals.

However, in these experiments the rats had received a total nicotine dose of 90 µg/kg within 30 minutes, which corresponds to the amount of nicotine ingested by smoking six cigarettes. A major difference is, though, that in contrast to control rats, where nicotine caused a significant increase in dopamine release already within the first minute following its administration, the increased dopamine release in immunized rats was not associated in time with the intravenous injection of nicotine, but was clearly delayed and appeared several minutes later. The attenuated nicotine distribution caused by the antibodies in actively immunized animals may therefore not only affect the amount of nicotine that reaches the brain, but importantly also its rate of delivery. Since systemic nicotine exerts a very fast effect on brain transmitter systems, and the time of onset of the rewarding effect of an addictive drug is directly related to its dependence liability, the rate of nicotine delivery to the brain will also play a critical role (Henningfield and Keenan, 1993). Consequently, both a slower rate of nicotine delivery to the brain, as well as a reduced amount of nicotine delivered, will probably contribute to prevent the rewarding effects of nicotine in animals immunized with IP18-KLH.

In support of this contention Kantak *et al.* (2000), investigating the effect of a cocaine vaccine on cocaine self-administration, showed that after immunization the cocaine dose-response curve was shifted downward. Thus, the effect of the immunization was clearly not easily surmountable by increasing doses of cocaine, in contrast to the effect of a dopamine receptor antagonist on cocaine self-administration, which caused a shift to the right of the cocaine dose-response curve. The authors accordingly concluded that the efficacy of the cocaine vaccine may to a significant extent reside in a pharmacokinetic mechanism, i.e. the reduction in the rate of drug entry into the brain. The fact that the dependence liability of NRT, which generates a considerably slower absorption of nicotine to the blood than smoking, is indeed very low (Henningfield and Keenan, 1993; West *et al.*, 2000), lends further support for this assumption.

Judging from the clinical trials of the different nicotine vaccines (see Introduction), the major problem when using these vaccines in humans seems to be to reach sufficient antibody titers, since clinical proof of concept has been established only in subjects with high antibody titers but not in subjects with low antibody titers. In addition, throughout the clinical trials there has been a continual shift in the protocols towards the use of both higher antigen doses as well as more intense immunization schedules.

However, the present experimental study shows that not only the titers achieved but also the selectivity profile of the generated antibodies represent crucial factors to obtain optimal effect of immunization against nicotine. Moreover, this study allows the conclusions that even small changes in the site of the linker attachment on the nicotine molecule, as well as the structure of the linker, may greatly influence the selectivity of the antibodies and the central neurobiological effects of nicotine that are considered critical for its dependence producing properties. Clearly, high titers of antibodies, with high nicotine affinity and low or no cotinine affinity should be advantageous. Our work propose that this goal may be achieved by attachment of the

semi-flexible linker to the 6-position of nicotine with the flexibility of the linker directed towards the nicotine molecule.

The nicotine immunogen IP18-KLH fulfils the above described criteria, and immunization with IP18-KLH has been shown to prevent the rewarding effects of nicotine, as indicated by our neurochemical data using *in vivo* voltammetry as well as our behavioral results, using the reinstatement of nicotine-seeking behavior in an animal model of relapse. In addition, immunization with IP18-KLH did not precipitate withdrawal in chronically nicotine-treated rats and finally, the nicotine withdrawal reaction was attenuated following immunization (Lindblom *et al.*, 2005).

Consequently the present set of studies suggests that immunization with a nicotine immunogen such as IP18-KLH might be used in smoking cessation. Immunization of smokers would be expected to generate antibodies with a favorable selectivity profile, which may persist over a sustained period of time. Our data also suggest that immunization with IP18-KLH might prevent the rewarding effects of a relapse cigarette and thus could be used to provide long-lasting protection in this regard. Needless to say, although this thesis provide an experimental rational and, in certain aspects, even proof of concept for its clinical utility, only studies in human smokers can determine its efficacy.

6 SUMMARY

The present study was undertaken to develop a method of active immunization against nicotine as a potential treatment for nicotine dependence, in particular relapse prevention, and allows for the following conclusions:

- Active immunization of rats with the synthesized nicotine immunoconjugates can generate high titers of nicotine selective antibodies.

- The titer of the generated antibodies seems to be mainly determined by the choice of carrier protein, however also the dose of immunogen, choice of adjuvant, route of administration and immunization schedule influences the antibody response.

- The structure of the nicotine hapten greatly influences the nicotine selectivity of the antibodies as well as the cross-reactivity with major metabolites of nicotine.

- The effects of immunization on nicotine-induced dopamine release in reward-related areas in brain depend on the titer and nicotine selectivity of the generated antibodies, as well as their cross-reactivity with cotinine, the major metabolite of nicotine.

- Immunization with IP18-KLH, which showed an advantageous antibody selectivity profile, prevents the rewarding effects of nicotine assessed both by neurochemical and behavioral methodologies, i.e. *in vivo* voltammetry and reinstatement of nicotine-seeking behavior in an animal model of relapse.

- These effects of the IP18-KLH immunization seem to be due to an altered distribution of nicotine by the antibodies, resulting in a retarded nicotine delivery to the brain.

Taken together, the results of this study provide significant preclinical support for the utility of active immunization against nicotine in smoking cessation, in particular relapse prevention.

7 REFERENCES

Bagasra O, Forman LJ, Howeedy A, and Whittle P (1992) A potential vaccine for cocaine abuse prophylaxis. *Immunopharmacology* **23**: 173-179.

Bardo MT, Green TA, Crooks PA, and Dwoskin LP (1999) Nornicotine is self-administered intravenously by rats. *Psychopharmacology* **146**: 290-296.

Barnes PJ, Ind PW, and Brown MJ (1982) Plasma histamine and catecholamines in stable asthmatic subjects. *Clinical Science (London, England)* **62**: 661-665.

Benowitz NL (1996) Pharmacology of nicotine: addiction and therapeutics. *Annual Review of Pharmacology and Toxicology* **36**: 597-613.

Benowitz NL and Jacob P 3 (1984) Daily intake of nicotine during cigarette smoking. *Clinical Pharmacology and Therapeutics* **35**: 499-504.

Benowitz NL, Kuyt F, Jacob P 3, Jones RT, and Osman AL (1983) Cotinine disposition and effects. *Clinical Pharmacology and Therapeutics* **34**: 604-611.

Benowitz NL, Porchet H, and Jacob P 3 (1989) Nicotine dependence and tolerance in man: pharmacokinetic and pharmacodynamic investigations. *Progress in Brain Research* **79**: 279-287.

Benowitz NL, Porchet H, Sheiner L, and Jacob P 3 (1988) Nicotine absorption and cardiovascular effects with smokeless tobacco use: comparison with cigarettes and nicotine gum. *Clinical Pharmacology and Therapeutics* **44**: 23-28.

Bergey DH and Etris S (1934) Immunization of Humans with Alum Precipitated Tetanus Toxoid. *American Journal of Public Health and the Nation's Health* **24**: 582-586.

Berkowitz B and Spector S (1972) Evidence for active immunity to morphine in mice. *Science* **178**: 1290-1292.

Bjercke RJ, Cook G, Rychlik N, Gjika HB, Van Vunakis H, and Langone JJ (1986) Stereospecific monoclonal antibodies to nicotine and cotinine and their use in enzyme-linked immunosorbent assays. *Journal of Immunological Methods* **90**: 203-213.

Bonese KF, Wainer BH, Fitch FW, Rothberg RM, and Schuster CR (1974) Changes in heroin self-administration by a rhesus monkey after morphine immunisation. *Nature* **252**: 708-710.

Buccafusco JJ, Shuster LC, and Terry Jr AV (2007) Disconnection between activation and desensitization of autonomic nicotinic receptors by nicotine and cotinine. *Neuroscience Letters* **413**: 68-71.

Burks AW, Laubach S, and Jones SM (2008) Oral tolerance, food allergy, and immunotherapy: implications for future treatment. *Journal of Allergy and Clinical Immunology* **121**: 1344-1350.

Cahill K, Stead LF, and Lancaster T (2007) Nicotine receptor partial agonists for smoking cessation. *Cochrane Database of Systematic Reviews* **1**: CD006103.

Carrera MR, Ashley JA, Parsons LH, Wirsching P, Koob GF, and Janda KD (1995) Suppression of psychoactive effects of cocaine by active immunization. *Nature* **378**: 727-730.

Center for Disease Control and Prevention (2002) Cigarette Smoking Among Adults - United States, 2000. *Morbidity and Mortality; Weekly Report* **51**: 642-645.

Cerny EH, Lévy R, Mauel J, Mpandi M, Mutter M, Henzelin-Nkubana C, Patiny L, Tuchscherer G, and Cerny T (2002) Preclinical development of a vaccine 'against smoking'. *Onkologie* **25**: 406-411.

Chandler MA and Rennard SI (2010) Smoking cessation. *Chest* **137**: 428-435.

Chiamulera C, Borgo C, Falchetto S, Valerio E, and Tessari M (1996) Nicotine reinstatement of nicotine self-administration after long-term extinction. *Psychopharmacology* **127**: 102-107.

Coe JW, Brooks PR, Vetelino MG, Wirtz MC, Arnold EP, Huang J, Sands SB, Davis TI, Lebel LA, Fox CB, Shrikhande A, Heym JH, Schaeffer E, Rollema H, Lu Y, Mansbach RS, Chambers LK, Rovetti CC, Schulz DW, Tingley FD 3, and O'Neill BT (2005) Varenicline: an alpha4beta2 nicotinic receptor partial agonist for smoking cessation. *Journal of Medicinal Chemistry* **48**: 3474-3477.

Cornuz J, Zwahlen S, Jungi WF, Osterwalder J, Klingler K, van Melle G, Bangala Y, Guessous I, Muller P, Willers J, Maurer P, Bachmann MF, and Cerny T (2008) A vaccine against nicotine for smoking cessation: a randomized controlled trial. *PLoS One* **3**: e2547.

Corrigall WA and Coen KM (1989) Nicotine maintains robust self-administration in rats on a limited-access schedule. *Psychopharmacology* **99**: 473-478.

Corrigall WA, Franklin KB, Coen KM, and Clarke PB (1992) The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. *Psychopharmacology* **107**: 285-289.

Croonenberghs J, Delmeire L, Verkerk R, Lin AH, Meskal A, Neels H, Van der Planken M, Scharpe S, Deboutte D, Pison G, and Maes M (2000) Peripheral markers of serotonergic and noradrenergic function in post-pubertal, caucasian males with autistic disorder. *Neuropsychopharmacology* **22**: 275-283.

Curtis JE and Hersh EM (1972) The human secondary immune response to Keyhole limpet haemocyanin. *Clinical and Experimental Immunology* **10**: 171-177.

de Villiers SH, Lindblom N, Kalayanov G, Gordon S, Malmerfelt A, Johansson AM, and Svensson TH (2002) Active immunization against nicotine suppresses nicotine-induced dopamine release in the rat nucleus accumbens shell. *Respiration; International Review of Thoracic Diseases* **69**: 247-253.

Dixon FJ, Jacot-Guillarmod H, and McConahey PJ (1966) The antibody responses of rabbits and rats to hemocyanin. *Mian Yi Xue za Zhi* **97**: 350-355.

Dwoskin LP, Rauhut AS, King-Pospisil KA, and Bardo MT (2006) Review of the pharmacology and clinical profile of bupropion, an antidepressant and tobacco use cessation agent. *CNS Drug Reviews* **12**: 178-207.

Ernster VL (1993) Women and smoking. *American Journal of Public Health* **83**: 1202-1204.

Etter JF, Perneger TV, and Ronchi A (1997) Distributions of smokers by stage: international comparison and association with smoking prevalence. *Preventive Medicine* **26**: 580-585.

Fagerstrom K and Hughes J (2008) Varenicline in the treatment of tobacco dependence. *Neuropsychiatr Dis Treat* **4**: 353-363.

Fiore MC, Bailey WC, Cohen SJ, Dorfman SF, Goldstein MG, Gritz ER and et al (2000) *Treating Tobacco Use and Dependence. Clinical Practice Guideline*, U.S. Department of Health and Human Services. Public Health Service. June 2000, Rockville, MD.

First MB and Tasman A (2004) *DSM-IV-TR Mental Disorders, Diagnosis, Etiology & Treatment*, Wiley, Chichester.

Fox BS, Kantak KM, Edwards MA, Black KM, Bollinger BK, Botka AJ, French TL, Thompson TL, Schad VC, Greenstein JL, Gefter ML, Exley MA, Swain PA, and Briner TJ (1996) Efficacy of a therapeutic cocaine vaccine in rodent models. *Nature Medicine* **2**: 1129-1132.

Fu Y, Matta SG, Gao W, and Sharp BM (2000) Local alpha-bungarotoxin-sensitive nicotinic receptors in the nucleus accumbens modulate nicotine-stimulated dopamine secretion in vivo. *Neuroscience* **101**: 369-375.

Goldsby RA, Kindt TJ, Osborne BA and Kuby J (2003) *Immunology, 5th ed*, W. H. Freeman and Company, New York.

Gonon F, Buda M, and Pujol JF (1984) Treated carbon fiber electrodes for measuring catechols and ascorbic acid, in *Measurement of neurotransmittor release in vivo* (Marsden CA ed) pp 153-171, Wiley, Chichester.

Gonon FG (1988) Nonlinear relationship between impulse flow and dopamine released by rat midbrain dopaminergic neurons as studied by in vivo electrochemistry. *Neuroscience* **24**: 19-28.

Grenhoff J, Aston-Jones G, and Svensson TH (1986) Nicotinic effects on the firing pattern of midbrain dopamine neurons. *Acta Physiologica Scandinavica* **128**: 351-358.

Hatsukami DK, Rennard S, Jorenby D, Fiore M, Koopmeiners J, de Vos A, Horwith G, and Pentel PR (2005) Safety and immunogenicity of a nicotine conjugate vaccine in current smokers. *Clinical Pharmacology and Therapeutics* **78**: 456-467.

Henningfield JE (1984) Pharmacologic basis and treatment of cigarette smoking. *Journal of Clinical Psychiatry* **45**: 24-34.

Henningfield JE and Keenan RM (1993) Nicotine delivery kinetics and abuse liability. *Journal of Consulting and Clinical Psychology* **61**: 743-750.

Henningfield JE, Miyasato K, and Jasinski DR (1985) Abuse liability and pharmacodynamic characteristics of intravenous and inhaled nicotine. *Journal of Pharmacology and Experimental Therapeutics* **234**: 1-12.

Hieda Y, Keyler DE, Ennifar S, Fattom A, and Pentel PR (2000) Vaccination against nicotine during continued nicotine administration in rats: immunogenicity of the vaccine and effects on nicotine distribution to brain. *International Journal of Immunopharmacology* **22**: 809-819.

Hieda Y, Keyler DE, Vandevoort JT, Kane JK, Ross CA, Raphael DE, Niedbalas RS, and Pentel PR (1997) Active immunization alters the plasma nicotine concentration in rats. *Journal of Pharmacology and Experimental Therapeutics* **283**: 1076-1081.

Hieda Y, Keyler DE, VanDeVoort JT, Niedbala RS, Raphael DE, Ross CA, and Pentel PR (1999) Immunization of rats reduces nicotine distribution to brain. *Psychopharmacology* **143**: 150-157.

Hughes JR (2000) New treatments for smoking cessation. *CA* **50**: 143-51 quiz 152-5.

Hughes JR (2007) Effects of abstinence from tobacco: valid symptoms and time course. *Nicotine & Tobacco Research* **9**: 315-327.

Hughes JR, Keely J, and Naud S (2004) Shape of the relapse curve and long-term abstinence among untreated smokers. *Addiction* **99**: 29-38.

Imperato A, Mulas A, and Di Chiara G (1986) Nicotine preferentially stimulates dopamine release in the limbic system of freely moving rats. *European Journal of Pharmacology* **132**: 337-338.

Jorenby DE, Leischow SJ, Nides MA, Rennard SI, Johnston JA, Hughes AR, Smith SS, Muramoto ML, Daughton DM, Doan K, Fiore MC, and Baker TB (1999) A controlled trial of sustained-release bupropion, a nicotine patch, or both for smoking cessation. *New England Journal of Medicine* **340**: 685-691.

Kagedal B and Goldstein DS (1988) Catecholamines and their metabolites. *Journal of Chromatography* **429**: 177-233.

Kantak KM, Collins SL, Lipman EG, Bond J, Giovanoni K, and Fox BS (2000) Evaluation of anti-cocaine antibodies and a cocaine vaccine in a rat self-administration model. *Psychopharmacology* **148**: 251-262.

Keyler DE, Hieda Y, st Peter J, and Pentel PR (1999) Altered disposition of repeated nicotine doses in rats immunized against nicotine. *Nicotine & Tobacco Research* **1**: 241-249.

Keyler DE, Roiko SA, Benlhabib E, LeSage MG, St Peter JV, Stewart S, Fuller S, Le CT, and Pentel PR (2005) Monoclonal nicotine-specific antibodies reduce nicotine distribution to brain in rats: dose- and affinity-response relationships. *Drug Metabolism and Disposition* **33**: 1056-1061.

Khor SP, Bozigian H, and Mayersohn M (1991) Potential error in the measurement of tissue to blood distribution coefficients in physiological pharmacokinetic modeling. Residual tissue blood. II. Distribution of phencyclidine in the rat. *Drug Metabolism and Disposition* **19**: 486-490.

Khor SP and Mayersohn M (1991) Potential error in the measurement of tissue to blood distribution coefficients in physiological pharmacokinetic modeling. Residual tissue blood. I. Theoretical considerations. *Drug Metabolism and Disposition* **19**: 478-485.

Landry DW, Zhao K, Yang GX, Glickman M, and Georgiadis TM (1993) Antibody-catalyzed degradation of cocaine. *Science* **259**: 1899-1901.

Lang R, Wahl A, Skurk T, Yagar EF, Schmiech L, Eggers R, Hauner H, and Hofmann T (2010) Development of a hydrophilic liquid interaction chromatography-high-performance liquid chromatography-tandem mass spectrometry based stable isotope dilution analysis and pharmacokinetic studies on bioactive pyridines in human plasma and urine after coffee consumption. *Analytical Chemistry* **82**: 1486-1497.

Lindblom N, de Villiers SH, Kalayanov G, Gordon S, Johansson AM, and Svensson TH (2002) Active immunization against nicotine prevents reinstatement of nicotine-seeking behavior in rats. *Respiration; International Review of Thoracic Diseases* **69**: 254-260.

Lindblom N, de Villiers SH, Semenova S, Kalayanov G, Gordon S, Schilstrom B, Johansson AM, Markou A, and Svensson TH (2005) 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 Archives of Pharmacology* **372**: 182-194.

Maclennan R, Levine L, Newell KW, and Edsall G (1973) The early primary immune response to absorbed tetanus toxoid in man: A study of the influence

of antigen concentration, carrier concentration, and sequence of dosage on the rate, extent, and persistence of the immune response to one and to two doses of toxoid. *Bulletin of the World Health Organization* **49**: 615-626.

Maes M, Verkerk R, Vandoolaeghe E, Lin A, and Scharpe S (1998) Serum levels of excitatory amino acids, serine, glycine, histidine, threonine, taurine, alanine and arginine in treatment-resistant depression: modulation by treatment with antidepressants and prediction of clinical responsivity. *Acta Psychiatrica Scandinavica* **97**: 302-308.

Marcus MM, Nomikos GG, and Svensson TH (1996) Differential actions of typical and atypical antipsychotic drugs on dopamine release in the core and shell of the nucleus accumbens. *European Neuropsychopharmacology* **6**: 29-38.

Mathers CD and Loncar D (2006) Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Medicine* **3**: e442.

Matsukura S, Sakamoto N, Imura H, Matsuyama H, Tamada T, Ishiguro T, and Muranaka H (1975) Radioimmunoassay of nicotine. *Biochemical and Biophysical Research Communications* **64**: 574-580.

Maurer P, Jennings GT, Willers J, Rohner F, Lindman Y, Roubicek K, Renner WA, Muller P, and Bachmann MF (2005) A therapeutic vaccine for nicotine dependence: preclinical efficacy, and Phase I safety and immunogenicity. *European Journal of Immunology* **35**: 2031-2040.

McCarthy DE, Piasecki TM, Lawrence DL, Jorenby DE, Shiffman S, Fiore MC, and Baker TB (2008) A randomized controlled clinical trial of bupropion SR and individual smoking cessation counseling. *Nicotine & Tobacco Research* **10**: 717-729.

Mihalak KB, Carroll FI, and Luetje CW (2006) Varenicline is a partial agonist at alpha4beta2 and a full agonist at alpha7 neuronal nicotinic receptors. *Molecular Pharmacology* **70**: 801-805.

Nisell M, Marcus M, Nomikos GG, and Svensson TH (1997) Differential effects of acute and chronic nicotine on dopamine output in the core and shell of the rat nucleus accumbens. *Journal of Neural Transmission* **104**: 1-10.

Nisell M, Nomikos GG, and Svensson TH (1994) Systemic nicotine-induced dopamine release in the rat nucleus accumbens is regulated by nicotinic receptors in the ventral tegmental area. *Synapse* **16**: 36-44.

Oliver JL, Pashmi G, Barnett P, Mettens P, Biemans R, Monteyne P, Palmantier R, Gallagher T, Ramaya S, and Wonnacott S (2007) Development of an anti-cotinine vaccine to potentiate nicotine-based smoking cessation strategies. *Vaccine* **25**: 7354-7362.

Paxinos G and Watson C (1986) *The rat brain in stereotaxic coordinates*, 2nd ed. Academic Press, London.

Pentel PR, Malin DH, Ennifar S, Hieda Y, Keyler DE, Lake JR, Milstein JR, Basham LE, Coy RT, Moon JW, Naso R, and Fattom A (2000) A nicotine conjugate vaccine reduces nicotine distribution to brain and attenuates its behavioral and cardiovascular effects in rats. *Pharmacology, Biochemistry, and Behavior* **65**: 191-198.

Pierce RC and Kumaresan V (2006) The mesolimbic dopamine system: the final common pathway for the reinforcing effect of drugs of abuse? *Neuroscience and Biobehavioral Reviews* **30**: 215-238.

Pontieri FE, Tanda G, Orzi F, and Di Chiara G (1996) Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. *Nature* **382**: 255-257.

Porro M, Saletti M, Nencioni L, Tagliaferri L, and Marsili I (1980) Immunogenic correlation between cross-reacting material (CRM197) produced by a mutant of Corynebacterium diphtheriae and diphtheria toxoid. *Journal of Infectious Diseases* **142**: 716-724.

Rang HP, Dale MM, Ritter JM and Flower RJ (2007) *Rang and Dale´s Pharmacology, 6th ed*, Churchill Livingstone Elsevier.

Schelling TC (1992) Addictive drugs: the cigarette experience. *Science* **255**: 430-433.

Self DW (1995) Drug addiction. Cocaine abuse takes a shot. *Nature* **378**: 666-667.

Stead LF, Perera R, Bullen C, Mant D, and Lancaster T (2008) Nicotine replacement therapy for smoking cessation. *Cochrane Database of Systematic Reviews* **1**: CD000146.

Sziraki I, Sershen H, Benuck M, Lipovac M, Hashim A, Cooper TB, Allen D, and Lajtha A (1999) The effect of cotinine on nicotine- and cocaine-induced dopamine release in the nucleus accumbens. *Neurochemical Research* **24**: 1471-1478.

Thyrian JR, Panagiotakos DB, Polychronopoulos E, West R, Zatonski W, and John U (2008) The relationship between smokers' motivation to quit and intensity of tobacco control at the population level: a comparison of five European countries. *BMC Public Health* **8**: 2.

US Department of Health and Human Services (1988) *The health consequences of smoking: Nicotine Addiction, a report of the Surgeon General (Publication no. CDC 88-8406)*, Public Health Service, Centers for Disease Control, Center for Health Promotion and Education, Office on Smoking and Health, Rockville, Maryland.

Wagena EJ, de Vos A, Horwith G, and van Schayck CP (2008) The immunogenicity and safety of a nicotine vaccine in smokers and nonsmokers: results of a randomized, placebo-controlled phase 1/2 trial. *Nicotine & Tobacco Research* **10**: 213-218.

West R, Hajek P, Foulds J, Nilsson F, May S, and Meadows A (2000) A comparison of the abuse liability and dependence potential of nicotine patch, gum, spray and inhaler. *Psychopharmacology* **149**: 198-202.

WHO (2003) *The World health report: 2003 Shaping the future*, World Health Organization, Geneva.

WHO (2008) *Report on the Global Tobacco Epidemic, 2008: The MPOWER package*, World Health Organization, Geneva.

Wileyto EP, Patterson F, Niaura R, Epstein LH, Brown RA, Audrain-McGovern J, Hawk Jr LW, and Lerman C (2005) Recurrent event analysis of lapse and recovery in a smoking cessation clinical trial using bupropion. *Nicotine & Tobacco Research* **7**: 257-268.