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Bioorganic & Medicinal Chemistry
ELSEVIER
Bioorganic & Medicinal Chemistry 12 (2004) 563–570

Investigations using immunization to attenuate the psychoactive effects of nicotine

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Received 22 May 2003; accepted 7 November 2003

Abstract—Despite the enormous health risks, people continue to smoke and use tobacco primarily as a result of nicotine addiction. As part of our immunopharmacotherapy research, the effects of active and passive immunizations on acute nicotine-induced locomotor activity in rats were investigated. To this end, rats were immunized with either a **NIC**-KLH immunoconjugate vaccine designed to elicit an antinicotine immune response, or were administered an antinicotine monoclonal antibody, NIC9D9, prior to a series of nicotine challenges and testing sessions. Vaccinated rats showed a 45% decrease in locomotor activity compared to a 16% decrease in controls. Passive immunization with NIC9D9 resulted in a 66.9% decrease in locomotor activity versus a 3.4% decrease in controls. Consistent with the behavioral data, much less nicotine was found in the brains of immunized rats. The results support the potential clinical value of immunopharmacotherapy for nicotine addiction in the context of tobacco cessation programs.

1. Introduction

Cigarette smoking is the leading preventable risk factor for mortality in developed countries.[1–4] Smokers and other tobacco users acquire their habit in order to maintain nicotine levels in the brain where it exerts powerfully addictive effects.[5,6] Nicotine (Fig. 1) binds to nicotinic cholinergic receptors in the mesolimbic dopamine system, specifically, the nucleus accumbens and the ventral tegmental area, that play a significant role in regulating locomotor activity and drug reinforcement.[7–11] As with other dependence-inducing drugs of abuse, the addictive potential of nicotine is a function of the route of administration by virtue of the rate at which it reaches the brain. Inhalation of cigarette smoke results in a rapid rise in arterial nicotine concentrations and pulmonary delivery to the brain generating an intense euphoric sensation.[12–16] Another determinant of the addictive potential of nicotine is the dose consumed. Several studies of nicotine self-administration in human and nonhuman subjects indicated the onset of nicotine dependence in a dose-related manner.[17–19] These neural and pharmacological features may account for the great difficulty and lack of success for most individuals who try to quit smoking.

Efforts aimed at treating tobacco use (smoking, chewing) include behavioral intervention, group support and pharmacotherapy.[20–25] None of these strategies have proven adequate in fostering tobacco abstinence. Nicotine-replacement therapy, currently the most widely used and perhaps most effective treatment, has had limited success in achieving smoking cessation on a long-term basis, and can have the deleterious effects associated with nicotine.[26–30] In the absence of an effective means to treat nicotine addiction and thus prevent the serious medical consequences of tobacco use, the investigation of new strategies is imperative. Immunopharmacotherapy is an approach that blocks the passage of a drug into the brain by peripherally circulating antibodies, which circumvents the central nervous system and adverse side effects that accompany other pharmacotherapies. Functionally, antibody binding would result in a substantial decrease in the rate of rise of nicotine levels in the brain and in lower doses of nicotine accessing neural reward targets, therefore reducing the addictive potential of the drug. Previous

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doi:10.1016/j.bmc.2003.11.029

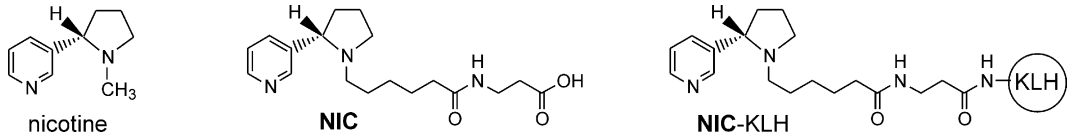

Figure 1. Compounds under discussion.

work from our laboratory,[31–33] followed by other groups,[34–37] showed the efficacy of both active and passive immunization in blocking the psychoactive and reinforcing effects of cocaine in animal models. Subsequently, the development of similar approaches that demonstrated the suppression of the psychoactive and cardiovascular effects of nicotine in rodents were reported.[38–45] We described the development of the hapten **NIC** possessing the (*S*)-(−)-configuration corresponding to natural nicotine and the immunogenic properties of a **NIC**-keyhole limpet hemocyanin (KLH) protein conjugate[46] (Fig. 1). To date, **NIC**-KLH has afforded a reproducible immune response in mice, as well as monoclonal antibodies (mAbs), in particular mAb NIC9D9, with good affinity and specificity for nicotine.

To examine the therapeutic potential of the two methods, active immunization using a vaccine based on **NIC**-KLH and passive administration of NIC9D9, we tested their effects on the psychoactive properties of clinically relevant doses of nicotine using a sensitive locomotor activity assay in rats. Significantly, to further substantiate the antibody-mediated blockade, brain nicotine levels in rats were also measured.

2. Results

2.1. Active immunization with NIC-KLH

The initial preimmunization subcutaneous (sc) nicotine injections resulted in the classic inhibitory effect on locomotor activity induced by acute treatment with the drug (Fig. 2A). Upon repeated administrations of nicotine, tolerance to the initial depressant effect developed and by the sixth nicotine injection the activating effects

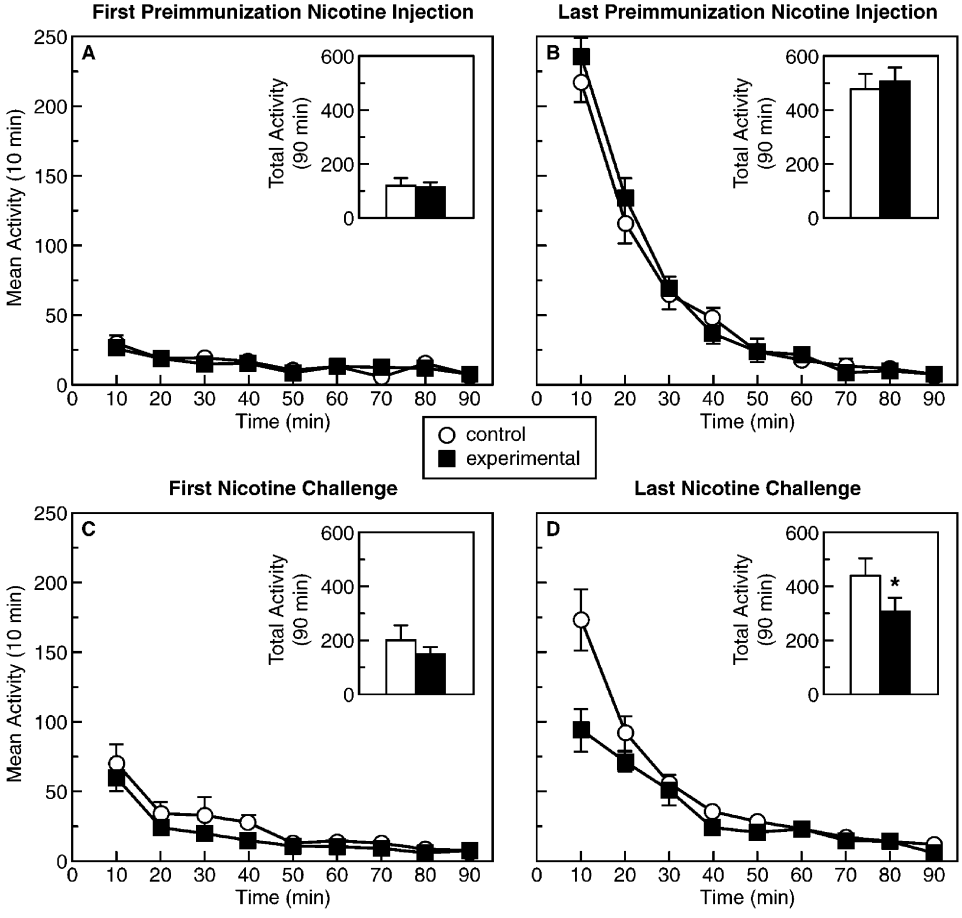

Figure 2. Effect of active immunization with **NIC**-KLH on nicotine-induced rat locomotor activity: (A) The initial preimmunization nicotine injections resulted in the classic inhibitory effect on locomotor activity after acute administration of the drug; (B) With repeated administrations of nicotine, tolerance to the initial depressant effect developed and by the sixth injection the activating effects of nicotine were observed; (C) The first nicotine challenge resulted in a nonsignificant difference in locomotor activity between groups; (D) The last nicotine challenge produced a significant difference in locomotor activity between groups.

of the drug were observed (Fig. 2B). The first nicotine challenge resulted in a nonsignificant difference in locomotor activity [$F(1,14) = 3.125$; $P < 0.099$] (Fig. 2C). This result may be interpreted as a reemergence of the initial depressant effect of nicotine in control rats, but not experimental rats. The last nicotine challenge produced a significant difference between groups in locomotor activity [$F(1,14) = 6.392$; $P < 0.024$]. Compared to baseline values, experimental rats as a group showed a 45% decrease in the ambulatory measure (crossovers) (**NIC**-KLH: 542.63 ± 59.85; 307.38 ± 49.13), whereas controls showed a decrease of 16% (KLH: 516.63 ± 64.81; 437.50 ± 61.48) (Fig. 2D). These results indicated that a vaccine using **NIC**-KLH significantly suppressed the psychomotor effects of nicotine as compared to controls.

2.2. Passive immunization with NIC9D9

Behavior elicited by preimmunization nicotine injections followed the same biphasic pattern as in the active immunization study. Again, repeated administrations of nicotine resulted in tolerance to the initial depressant effect and by the sixth nicotine injection the activating effects of the drug were observed (Fig. 3A). This last preimmunization nicotine challenge resulted in a nonsignificant difference in locomotor activity [$F(1,14) = 0.18$; $P < 0.895$]. Intravenous (iv) infusion of NIC9D9 (5 mg/kg) in the experimental group resulted in a significant decrease in locomotor activity compared to controls during the first 20 min of the session [$F(1,14) = 6.757$; $P < 0.020$] (Fig. 3B). This effect was optimized by increasing doses of the mAb. After infusion of NIC9D9 (25 mg/kg), differences in locomotion between groups were observed throughout the first 40 min of the session [$F(1,14) = 18.214$; $P < 0.0008$] (Fig. 3C). The maximum effect was obtained upon treatment with the highest mAb dose (50 mg/kg) where differences in the ambulatory measure persisted throughout the first 40 min of the session [$F(1.14) = 37.376$; $P < 0.000$] (Fig. 3-D) In addition, a dramatic decrease during the initial 20 min of testing of the ambulatory locomotor responses were observed. Compared to baseline values, experimental rats as a group showed a 66.9% decrease in locomotor activity (NIC9D9: 703.38 ± 111.88; 308.75 ± 57.51), whereas controls showed a decrease of 3.4% (saline: 688.13 ± 111.10; 740 ± 117.62). These results indicated that passive immunization with NIC9D9 dose-dependently suppressed the psychomotor effects of nicotine as compared to controls.

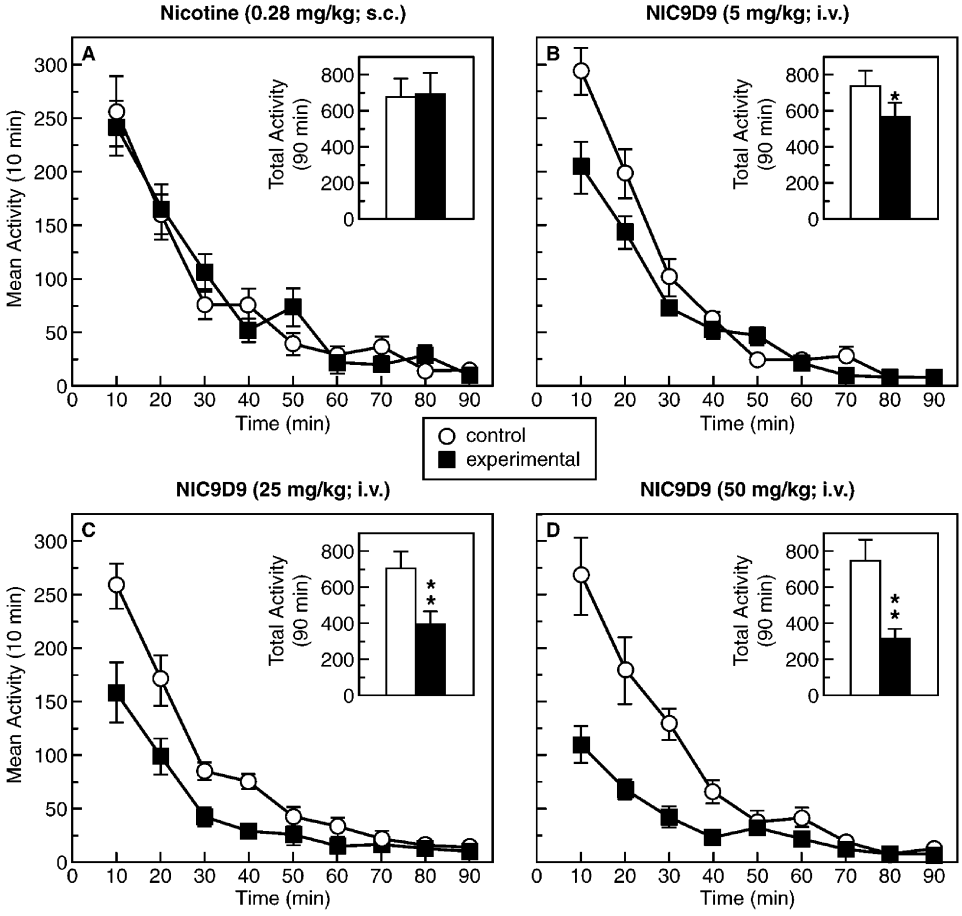

Figure 3. Effect of passive immunization with mAb NIC9D9 on nicotine-induced rat locomotor activity: (A) Tolerance to the initial depressant effect of nicotine developed by the sixth injection and then the activating effects of the drug were observed. This last preimmunization nicotine challenge resulted in a nonsignificant difference in locomotor activity between groups; (B) Administration of NIC9D9 at the lowest dose resulted in a significant decrease in locomotor activity compared to controls during the first 20 min of the session; (C) At an increased dose of NIC9D9, differences in locomotor activity between groups were observed throughout the first 40 min of the session; (D) At the highest dose of NIC9D9, differences in locomotor activity were most significant and persisted throughout the first 40 min of the session.

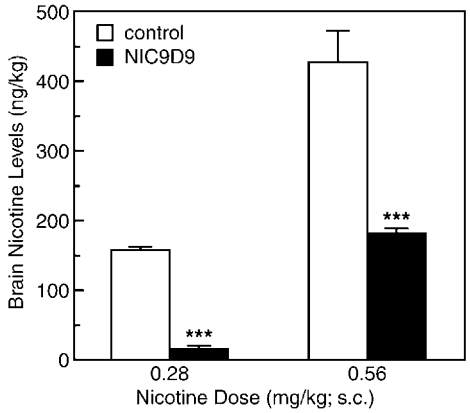

Figure 4. Effect of passive immunization with mAb NIC9D9 on brain nicotine levels in rats. Measurements in both experimental and control rats were obtained after three min of a single dose of the indicated amount of nicotine.

2.3. Nicotine levels in the brain

Administration of NIC9D9 (30 mg/kg; iv) significantly reduced nicotine distribution to the brain at both 0.28 and 0.56 mg/kg sc doses of nicotine. Compared to saline-treated controls, brain tissue from passively immunized rats revealed an 89.8% decrease in nicotine concentration at the 0.28 mg/kg dose [$F(1,6) = 44.18$; $P < 0.0001$] and 57.8% at the 0.56 mg/kg dose [$F(1,6) = 20.912$; $P < 0.004$] (Fig. 4). Further analysis revealed a nonsignificant treatment–dose interaction [$F(1,6) = 3.665$; $P < 0.1041$].

3. Discussion

Traditionally, vaccines have been used for protection against a variety of infectious diseases. Yet, 30 years ago, a vaccine concept was described for the potential treatment of morphine and heroin addiction[47–49] and digoxin toxicity.[50,51] However, only recently has the strategy of immunopharmacotherapy evolved and gained interest for targeting health problems such as drug abuse.[52–54] Over the past decade, a number of reports have established the feasibility of immunopharmacotherapy for treating cocaine,[31–37] nicotine,[38–45] and methamphetamine and phencyclidine[55–60] addictions. Our vaccine comprised of the immunoconjugate **NIC**-KLH formulated with an adjuvant has elicited reproducible, albeit moderate, antinicotine polyclonal antibody titers in mice. The murine immune response also allowed the ensuing development of mAb NIC9D9 that demonstrated good affinity ($K_d = 200$ nM) and specificity for nicotine. Most important, the rat behavioral data presented herein lends credence to our hypothesis that actively generated antinicotine antibodies and the passive administration of an antinicotine mAb each offer potential therapeutic value as tobacco cessation treatments.

Immunization of rats with **NIC**-KLH yielded titers of 1:3200 to 1:6400. Even though these values are not indicative of a robust immune response, the rats demonstrated resistance to the increases in locomotor activation upon acute nicotine injection (0.28 mg/kg; sc). This effect was most notable upon the last nicotine challenge where a significant difference in ambulatory behavior was observed during the first 20 min of the testing session (Fig. 2D). These data are consistent with reports by others where modest titers produced comparable locomotor activity results after nicotine administration.[45] The absence of behavioral differences recorded after the first nicotine challenge (Fig. 2C) may have a two-fold explanation. First, given the time-period elapsed since the previous nicotine injection (35 days), it is possible that the lack of a differential effect was due to the classic hypomotility resulting from initial nicotine exposure characteristic of the biphasic psychomotor effects of nicotine[61–65] in both control (KLH) and experimental (**NIC**-KLH) groups. Alternatively, while the absences of increased ambulatory values may be thus explained in the control group, this same behavioral pattern in the experimental rats may obey a true immune-mediated suppression of locomotor activity. Since the third nicotine challenge produced differences in locomotor output, the latter explanation would seem more plausible and supports the capacity of the vaccine to block both the hypostimulatory and hyperstimulatory effects of nicotine.

Passive immunization with NIC9D9 yielded a greater significant difference in behavioral profile between groups (Fig. 3). At the lowest dose (5 mg/kg; iv), ambulatory values were observed to modestly differ only within the first 20 min of the session (Fig. 3B). This effect followed a true dose-response pattern as the dose of NIC9D9 was increased with the greatest differences in locomotor behavior recorded at the highest dose (50 mg/kg) (Fig. 3D). The suppressive effect of this passive immunization was not only quantitative (>10-fold in the initial 20 min of the session), but also qualitative, as it lasted well into the fourth 10 min interval or twice as long as with the 5 mg/kg dose. An important observation regarding this finding is the dose-time interaction in which the linear correlation between dose and longevity of effect suggest a true dose–response event. The dose-dependent efficacy of NIC9D9 satisfies a crucial feature in the criteria for pharmacotherapy, since it allows controlled and strategized application in the clinical setting. Notably, the results herein are the first report of an antinicotine mAb to suppress nicotine effects in animal studies. In contrast with the pattern of suppression observed after active immunization, the interpretation of these data is less prone to speculation regarding the biphasic psychoactive effects of nicotine.[61–65] Unlike with the active immunization protocol, passive immunization allows direct assessment of the psychoactive properties of nicotine in a time-continuum which avoids the caveat of a possible reemergence of the suppressive effects of a first-time exposure. Therefore, the behavioral blockade observed herein is not only solidly conclusive, but also is the most dramatic immune-mediated nicotine suppression reported to date.

The behavioral pharmacology of nicotine effects in animals faithfully models the human condition of

nicotine addiction.[66,67] In our investigations, the nicotine dose regimen used was designed both to elicit experimentally useful levels of locomotor activity in rats and to simulate typical human consumption. The nicotine dose (0.28 mg/kg, sc) was equivalent to that delivered by several cigarettes and is a dose that generally results in venous plasma nicotine concentrations of 10–40 ng/mL, and up to 10-fold higher arterial concentrations, in humans and rats.[5,6,68–70] Since immunopharmacotherapy must sufficiently block peripherally circulating nicotine from accessing the brain to reduce the psychoactive effects, it is the key feature of a model for this treatment intervention. However, precisely what fraction must be blocked and in what time period to ultimately attenuate the reinforcing and addictive effects of the drug is unknown. Particularly in smokers, the most critical interaction is likely to be the binding of some threshold amount of an initial nicotine bolus that enters pulmonary arterial circulation and reaches the brain within 15–60 seconds, although the binding of subsequent peak plasma nicotine should also be important. One can estimate that 50–90% of plasma nicotine was bound by the 25–50 mg/kg dose range of NIC9D9 in our locomotor activity experiments with rats. This correlated with the analysis of nicotine levels in cerebral tissue after nicotine injection (0.28 mg/kg) that revealed considerably less nicotine in rats immunized with NIC9D9 (30 mg/kg) compared to those of controls. Combined with the active immunization results, the data provide a two-fold revelation. The immune-mediated blockade of even a small fraction of nicotine, as in the low titer immune response or lowest dose of NIC9D9, affords observable attenuation of psychoactive effects. Yet, on the other hand, only a small fraction of a typical dose of nicotine that remains unbound can enter the brain to induce to some degree these same effects.

The experiments herein only addressed nicotine psychoactive effects as modeled by locomotor behavior in rats, and not conditions of nicotine reinforcement and relapse. Clearly, the results provide substantial support for nicotine immunopharmacotherapy that warrants further study. But, most important, is whether the pharmacodynamics of nicotine can be adequately controlled by immunopharmacotherapy during human consumption of nicotine, as in smoking, to suppress reinforcement and maintain abstinence. Given the feasibility of implementing nicotine vaccines and mAbs for treating nicotine addiction, their safety and effectiveness need to be established in clinical trials in target populations. Vaccines using nicotine immunoconjugates exemplified by **NIC**-KLH and/or a human mAb having properties similar to NIC9D9 could be useful in smoking/tobacco cessation programs. Studies in this direction continue in our laboratory.

4. Experimental

4.1. Animals

Male albino Wistar rats (250–350 g; Charles River, Kingston, NY) were housed in groups of two, had *ad libitum* access to food and water, and were maintained in a temperature (22 °C) and light (12-h light/dark cycle) controlled environment. Separate groups of rats were randomly assigned into control or experimental groups ($n = 8$). Behavioral performance in the task was compared within groups (assay), as well as between groups (treatment).

4.2. Locomotor activity testing

4.2.1. Active immunization. After a 90 min period of habituation preceded by a sc saline injection (1 mL/kg), animals received a sc injection of (−)-nicotine hydrogen tartrate salt (Sigma Chem. Co.) at 0.28 mg/kg mixed in saline solution (bolus 1 mL/kg) and their locomotor responses measured during a 90 min session. Following a five-day pretreatment with sc nicotine (0.5 mg/kg), rats were tested in photocell cages after receiving an equal dose of sc nicotine (0.5 mg/kg) to determine preimmunization drug response (baseline). This dose of nicotine is an intermediate dose that produces a significant locomotor activation. Based on locomotor activity scores, animals were assigned to the experimental or control group in ranking order. Animals then were subjected to the corresponding immunization protocol. Three days after the final boost of **NIC**-KLH or KLH, the animals were challenged with systemic nicotine and their locomotor responses recorded (first nicotine challenge). Rats received four consecutive additional nicotine challenges on days five through eight post-immunization.

4.2.2. Passive immunization. The effects on acute nicotine-induced psychomotor response using passive immunization with mAb NIC9D9 was investigated. Male Wistar rats were prepared with intrajugular catheters and allowed a seven-day recovery period. All animals were treated with sc nicotine (0.28 mg/kg; 1 mL/kg) for five consecutive days and observed in photocell cages after administration to determine preimmunization drug response (baseline). On the test day, experimental animals were infused iv with either 5, 25 or 50 mg/kg of NIC9D9 in a volume of approximately 1.5 mL/kg. Control rats were treated with an equivalent volume of physiological saline (iv). Thirty min after immunization, all animals received a sc nicotine injection (0.28 mg/kg) and their locomotor responses were assessed for the next 90 min. All locomotor activity test sessions were preceeded by a 90 min habituation session following a sc saline injection (1 mL/kg).

4.3. Locomotor activity apparatus

Locomotor activity was measured in a bank of 16 wire cages, each cage 20 cm high×25 cm wide×36 cm long with two horizontal infrared beams across the long axis 2 cm above the floor. Total photocell beam interruptions and crossovers, the number of times breaking one photocell beam that is followed directly by breaking the other photocell beam, were recorded by a computer every 10 min. Background noise was provided by a white noise generator.

4.4. Immunization procedure

Rats were immunized with intraperitoneal (ip) injections of a 400 μL bolus of a RIBI adjuvant (MPL-TDM) (RIBI Immunochem Research) containing 250 μg **NIC**-KLH or KLH in 100 mM phosphate buffer, pH 7.4.[32] This initial inoculation was followed by boosts at 21 and 35 days. The last boost was administered without adjuvant. Animals were tail-bled seven days after each immunization and serum samples analyzed by enzyme-linked immunsorbent assay (ELISA).[32] For the passive immunization study, animals received a bolus iv infusion of 5, 25 or 50 mg/kg of NIC9D9 in phosphate buffered saline through the prepared intrajugular indwelling catheter in a time period of 5 s at 30 min before the testing session.

4.5. Intravenous catheterization surgery

Rats were deeply anesthetized under chronic vapor isofluorane (1.0–1.5%) and implanted with chronic silastic catheters in the jugular vein.[71] The catheter was passed subcutaneously to a polyethylene assembly mounted on the rat's back. This assembly consists of a guide cannula (C313G) (Plastic Products) attached into a one square inch piece of marlex mesh with epoxy. The marlex mesh is sutured into the rat's back. The guide cannula that protrudes from the rat's back is tightly covered with a sealed piece of tygon tubing. The rats received a seven-day post-surgical recovery period during which they were treated with a prophylactic course of Timentin antibiotic.

4.6. Nicotine levels in the brain

Rats ($n=16$) were prepared with indwelling iv catheters (see above). After a week-long recovery period, rats were infused with a bolus infusion of 30 mg/kg of NIC9D9 ($n=8$) or saline ($n=8$). Thirty minutes later, NIC9D9- and saline-treated rats received a sc nicotine injection of either 0.28 mg/kg ($n=4$/per group) or 0.56 mg/kg ($n=4$/per group). The resulting groups, NIC9D9/0.28, NIC9D9/0.56, saline/0.28, saline/0.56, all contained four rats each. Three min post-nicotine injection, rats were deeply anesthetized with isofluorane in an inverted glass bell, decapitated and their brains (brain stem to olfactory bulb regions) immediately extracted and placed on wet ice for a maximum of 30 min before HPLC determination of nicotine.

4.7. Determination of nicotine in brain samples

4.7.1. Reagents. (*S*)-(−)-nicotine and (−)-cotinine were from Aldrich Chemical Co. and (2′*S*)-nicotine-1-*N* oxide, (1′*S*, 2′*S*)-nicotine-1′-oxide, and nicotine-1,1-di-*N*-oxide were from Toronto Research Chemicals, Inc., North York, ON, Canada. Cotinine and the other metabolites were used in development of the HPLC assay to assure that their peaks would not overlap that of (*S*)-(−)-nicotine. A.C.S. grade KOH and HCl, 2-propanol (IPA) (HPLC grade), ethanol, dichloromethane (DCM), and Alconox detergent were from Fisher Scientific, Pittsburgh, PA. The dichloromethane was distilled over calcium hydride.

4.7.2. Apparatus. The vortex mixer was a Genie-2 from Fisher Scientific, the small orbital platform shaker was an IKA-VBRAX-VXR from IKA-Works, Inc., Cincinnati, OH, and the centrifuge was a clinical model from International Equipment Company, Needham Heights, MA. The rotary evaporation system consisted of a Buchi RE 111 + model 461 waterbath attached to a Welch Gem 1.0 vacuum pump all from Fisher Scientific. The HPLC system was the 7000 series by Hitachi Instruments, Inc., San Jose, CA. The system included a L-7100 pump, L-7400 ultraviolet detector set to monitor the absorbance at 254 nm, and the D-7500 integrator. The Rheodyne 7725i injector was fitted with a 20 μL loop. The analytical HPLC column was a 250 mm×4.6 mm (ID) Whatman Partisil 10 PAC (cat. # 4225-001) from Alltech Associates, Inc., Deerfield, IL. A guard column was used packed with silica gel. The chromatographic mobile phase was an isocratic solution of 97% IPA/3% water flowing at 0.75 mL/min (∼1850 psi). The IPA was degassed before use and the water was from a Barnstead NANOpure ultrapure water system.

4.7.3. Procedures. The nicotine extractions from brain tissue were carried out in translucent 10 mL screw-capped Teflon centrifuge tubes (Nalgene FEP Oak Ridge type) from Fisher Scientific. Before use, each tube and cap was rinsed with an aqueous 5 M KOH solution, soaked in and thoroughly washed with an aqueous Alconox detergent solution, and then rinsed with warm tap water, followed by deionized water, and finally ethanol. The tubes and caps were blown dry with nitrogen and allowed to further air dry in a dust-free environment. Immediately before every extraction, each tube with cap was weighed. Brain samples, temporarily stored cold in aluminum foil on ice, were processed through the following first step within min of their removal from the rat. Each whole brain was quickly diced with a clean razor blade and placed into separate tubes that were reweighed to obtain the initial brain mass. To each tube was added 2 mL of water and 1 mL of 5 M KOH to create a strongly basic (pH > 9) solution, in which the tissue would be digested. Each tube was vigorously shaken by hand and mixed on a vortexer (set on high speed 6–8) for several min to break up the tissue as much as possible. The tubes were then placed horizontally on a small orbital platform shaker (set on a speed of 1000–1200) and the tissue was homogenized overnight (15–18 h).

The brain tissue solutions were acidified (pH < 3) by adding 25–30 drops (∼800 μL) of 6 M HCl from a glass Pasteur pipet. After vortex-mixing for 30 seconds, 3 mL of DCM was added. Nicotine remains in the aqueous phase at this pH. The tubes were vortexed and then shaken horizontally for 20 min. Centrifugation at high speed for 20 min separated the resulting emulsion into two phases. The lower DCM organic phase was removed with a glass pipet and discarded, and the DCM extraction step repeated.

The remaining aqueous solution was made basic (pH > 9) by the addition of 15–20 drops (∼500 μL) of 5 M KOH. The tubes were vortexed 30 s and placed on

the shaker for 20 min. Nicotine (free base) was removed from the aqueous solution by extracting four times with 2.5–3.0 mL of DCM. Each time the tubes were vortexed 30 s, shaken horizontally for 20 min, followed by another 20 min of centrifugation for phase separation. The lower organic phase was removed with a glass pipet and then dried by pushing it through another glass pipet equipped with a small cotton plug topped with a short pad of anhydrous $MgSO_4$. The DCM extractions for each sample were combined in individual Teflon tubes. After the second DCM extraction, the organic phase was concentrated ($\sim$90% removed) with a rotary evaporator (adapter for the threaded Teflon tube was from CHEMGLASS Scientific Apparatus, Vineland, NJ) to allow for the remaining two DCM extractions. After all the organic extractions were combined for individual samples, the DCM in each tube was completely removed using the rotary evaporator over a warm ($\sim$35–40 °C) water bath.

The dried extraction sample (nicotine free base) in each tube was then reconstituted with the addition of 100 μL of DCM measured with a Hamilton syringe. The tube was capped and vortexed for 30 s with additional manual manipulation of the tube to insure that all inner surfaces of the tube were rinsed. A final centrifugation for 5 min recombined all the organic solvent together at the bottom of the tube. From each reconstituted extraction sample, 30 μL was injected for HPLC. Nicotine present in the sample eluted at a retention time of 11.1–11.5 min. There was enough solution in the bottom of the tube to inject each sample at least twice. The concentration of nicotine was determined by comparing the peak area and height to calibrated nicotine standards. The total mass of nicotine (ng) present in the tube, which was that extracted from each whole brain sample, was calculated based on the reconstitution volume of 100 μL, and divided by the initial brain mass (g) to give the reported ng nicotine/g brain. No attempt was made to correct the values for estimated nicotine extraction efficiencies.

5. Statistical analyses

Tests for homogeneity of variance were performed on test scores. Upon compliance with the assumption of homogeneity of variance, appropriate analyses of variance (ANOVAs) were performed. Scores violating homogeneity of variance were transformed accordingly or analyzed using appropriate nonparametric tests. Following ANOVAs, individual means were compared using the Newman–Keuls a posteriori test.

Acknowledgements

This work was supported by funding from the Skaggs Institute for Chemical Biology, and the California Tobacco-Related Disease Research Program (TRDRP) 11RT-0174 (K.D.J.).

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