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A Critical Evaluation of a Nicotine Vaccine within a Self-Administration Behavioral Model

Amira Y. Moreno,[†] Marc R. Azar,[‡] Noelle A. Warren,[‡] Tobin J. Dickerson,[†] George F. Koob,[‡] and Kim D. Janda*,[†]

Departments of Chemistry and Immunology, the Skaggs Institute for Chemical Biology, the Worm Institute of Research and Medicine, and Committee on the Neurobiology of Addictive Disorders, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

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Abstract: (*S*)-Nicotine is a psychostimulant legal drug responsible for causing addiction to tobacco smoking. Tobacco smoking has been irrevocably linked to a number of serious diseases and at present is considered the leading cause of preventable death in the United States. Despite well-documented adverse medical consequences, nicotine addiction has historically been one of the hardest to break. Current therapies have offered limited success and show high rates of relapse, emphasizing the need to engineer alternative therapies to aid nicotine cessation. The current study presents a protein-based immunopharmacotherapy approach for the treatment of nicotine addiction. Immunopharmacotherapy aims to use highly specific antibodies to blunt passage of drug into the brain thus minimizing reinforcing effects on the reward pathways of the central nervous system. Generation of a successful vaccine heavily relies on appropriate optimization of hapten design, immunogenic carrier and adjuvant. Modification of a classical nicotine hapten in conjugation with three distinct carrier proteins allowed for priming of a nicotine vaccine able to elicit significant amounts of nicotine-specific antibodies. Increased self-administration with use of a high drug dose (0.03 mg/kg/infusion; ~2 cigarettes in human) was observed in the vaccinated versus control animals suggesting a compensatory pattern and possibly reduced passage of nicotine to the brain. These results support the hypothesis that proper optimization of vaccine formulations could lead to successful nicotine vaccines for human use.

Keywords: Nicotine; vaccination; immunotherapy; self-administration

Introduction

(*S*)-Nicotine is a psychostimulant legal drug responsible for causing addiction to tobacco smoking. Upon consumption, nicotine reaches the brain and binds nicotinic acetylcholine receptors leading to a surge of adrenaline and dopamine thus providing feelings of enjoyment and reinforcing the behavior. Tobacco smoking has been irrevocably linked to a number of serious diseases and at present is considered the leading cause of preventable death in the United States.[1] Despite these well-documented adverse medical consequences, roughly 20% of the US population are current cigarette smokers and nicotine addiction has historically been one of the hardest to break. Several first line therapies exist to aid nicotine cessation ranging from nicotine-replacement products to antidepressants and nico-

* To whom correspondence should be addressed. Mailing address: 10550 North Torrey Pines Road, BCC-582, La Jolla, CA 92037. Tel: (858)784-2516. Fax: (858)784-2595. E-mail: kdjanda@scripps.edu. Homepage: http://www.scripps.edu/chem/janda/.

[†] Departments of Chemistry and Immunology, the Skaggs Institute for Chemical Biology, and the Worm Institute of Research and Medicine.

[‡] Committee on the Neurobiology of Addictive Disorders.

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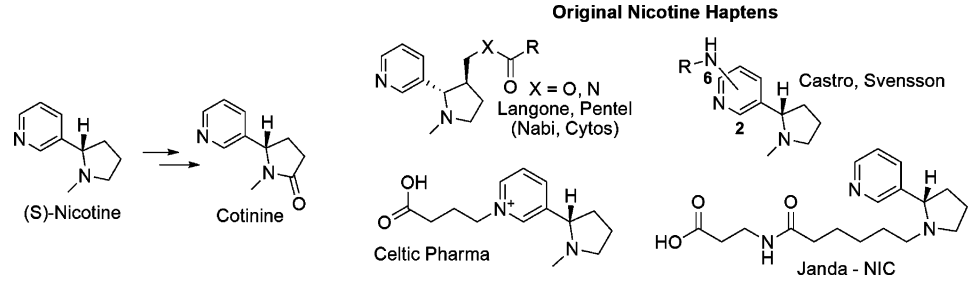
Scheme 1. Nicotine and Relevant Haptens



tinic receptor partial agonists. Current therapies have offered limited success and show high rates of relapse. Indeed, 70% of current cigarette smokers expressed a desire to quit; in 2006 40% attempted to quit and only less than 5% obtained abstinence for 3 to 12 months.[2] These estimates emphasize the need to engineer alternative therapies to aid nicotine cessation. The current study presents a protein-based immunopharmacotherapy approach for the treatment of nicotine addiction. Immunopharmacotherapy aims to use highly specific antibodies to blunt passage of drug into the brain thus minimizing reinforcing effects on the reward pathways of the central nervous system.

Formation of nicotine−protein conjugates was first reported over 30 years ago when several groups aimed to create quantitative radioimmunoassay detection methods for nicotine and its major metabolite, cotinine, in various tissue extracts and biological fluids. Nicotine and cotinine, as small molecular weight molecules, need to be appended to macromolecules in order to elicit an immune response. As both of these structures do not possess suitable functional groups for these purposes, the target scaffolds must be functionalized with an appropriate linker. Indeed, linker-nicotine regiochemical attachment has proven to be crucial for proper immune stimulation in terms of both the amount of antibody elicited and obtaining the desired antibody specificity.[3] For nicotine, several linker attachment sites have been investigated (Scheme 1) and of particular note is that of Langone et al.[4] In the original report, *trans*-3′-succinylmethylnicotine was generated and coupled to different macromolecules. Immunization of these conjugates into albino rabbits in formulation with complete Freund's adjuvant generated antibodies that allowed detection of picomolar levels of nicotine in various tissues and biological fluids even in the presence of cotinine without detectable antibody cross-reactivity. Since the original Langone report a plethora of haptens of the same general structure, i.e. functionalized at the 3′ position, have become the most widely prepared and studied in the context of nicotine haptens/vaccines.

Initial success in the generation of active cocaine vaccines strategies during the 1990s sparked interest to implement the same technology to aid nicotine cessation.[5] Furthermore, it has been hypothesized that the paradigm of nicotine addiction would make it a better candidate for immunotherapy as the maximum daily dose of drug consumed is significantly less than that of a serious cocaine addict and thus surmounting the protective effects of immunization would require considerably more effort.[6] A significant number of nicotine haptens, with five possible linker attachment sites and linker lengths, have been reported. Additionally, various carrier vehicles have been utilized such as proteins, conformationally biased peptides and viruslike particles formed from Qb bacteriophage.[7] Substitution at the 3′ position of the pyrrolidine ring has arguably become the most promising hapten structure. Pentel et al. introduced an ester−amide interchange modification to the hapten put forth

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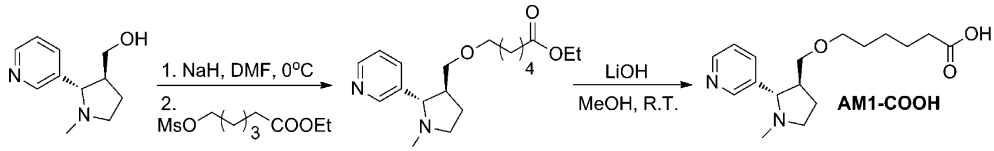
Scheme 2. Synthetic Route to AM1 Hapten



by Langone et al. and showed successful results in rat animal models.[8] The 3′-amide presenting nicotine hapten when conjugated to *Pseudomonas aeruginosa* exoprotein A in formulation with complete Freund's adjuvant is able to produce high amounts of antibody titers with nicotine binding capacities in the single-digit micromolar range even during concurrent chronic nicotine administration. Vaccination proved to reduce the brain nicotine concentration by 30−70% depending on the nicotine dosing protocol. However, while all vaccinated animals were able to surpass the 1:10 000 minimal titer threshold set forth by the authors, the range of antibody titers obtained is highly variable up to 10-fold.[8] This high variation of titer has proven to be a pitfall of this strategy as success in promoting nicotine cessation is directly related to the amount of circulating antibodies and thus high titer variability directly translates to high abstinence rate variability.

Currently, there are three companies pursuing active nicotine vaccines in human phase I and II clinical trials: NicVAX by Nabi Pharmaceuticals, NicQb by Cytos Pharmaceuticals and TA-NIC by Celtic Pharma. NicVAX and NicQb have advanced the furthest in their proof-of-concept studies. Interestingly, both vaccine formulations have used similar technologies in hapten design based on the modified Langone structure, namely, succinic acid conjugated 3′-aminomethylnicotine. Yet, each vaccine formulation has set itself apart in their use of carrier vehicle, while NicVAX employs *P. aeruginosa* exoprotein A, NicQb has employed viruslike particles as carriers. Active immunization was done using 2 to 6 doses over 2 to 4 weeks plus a later boost in the case of NicVAX. As expected, serum antibody levels steadily increased following each dose, which allowed maintenance of titers over a couple of months. Phase I results for all vaccines deemed the formulations safe and well-tolerated with only mild local and systemic reactions at the injection site that did not require further medical intervention. Results from large scale phase II trials have shown limited efficacy. A representative example is the NicVAX trial that included 201 subjects and had a total combined success rate of 11% over 6% placebo after 12 months. Importantly, most of the successful quitters were observed in the subjects that fell on the "high-titer" category which accounted for ∼30% of all participants.[9] The NicQb trial showed a similar trend where the 159 subjects had a 29.5% combined success rate over 21% placebo. Yet, up to 42% of the high titer group (53/159) participants obtained abstinence.[7d,10] In both studies, inclusion within the "high-titer" group translated into higher success rates further attesting that protective effects are directly proportional to the amount of circulating antibody elicited. The relevance of these results is the indication that an active immunization strategy can have clinical relevance in treatment of nicotine dependence and efficacy of this approach could be improved if high titers of anti-nicotine antibodies could be reproducibly elicited. In this study we aimed to optimize a nicotine vaccine formulation via modification of a 3′-substituted hapten, termed AM1, in conjugation to three distinct immunogenic carrier proteins, namely, keyhole limpet hemocyanin (KLH), tetanus toxoid (TT) and diphtheria toxin cross-reactive mutant 197 (CRM). We report that the tetanus toxoid (TT) conjugate has superior therapeutic efficacy in blunting nicotine crossing of the blood brain barrier as demonstrated by induction of a broader immune response and is able to show protective effects during high drug doses of nicotine self-administration.

Experimental Section

Materials. Unless otherwise stated, all reactions were performed under an inert atmosphere with dry reagents, solvents, and flame-dried glassware. (−)-Nicotine and (−)-cotinine were purchased from Sigma-Aldrich (St. Louis, MO). *trans*-3′-(Hydroxymethyl)nicotine was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). All other chemicals were purchased from major suppliers and used without further purification. Compounds were purified by reverse phase preparative high performance liquid chromatography (HPLC) (Grace, Vydac 218TP C_{18} 10−15 μm). All compounds were characterized using a Bruker 500 MHz NMR instrument and Agilent LC−MS (ESI) mass spectrometer. Hapten protein conjugates were analyzed using MALDI-TOF MS.

Nicotine Haptens. Racemic NIC nicotine hapten (Scheme 1) was prepared by reaction of nornicotine with the appropriate linker as previously reported.[11] AM1 nicotine hapten was synthesized according to Scheme 2. Commercially available *trans*-3′-(hydroxymethyl)nicotine (0.1 mmol) was added to a cooled stirred solution of NaH (0.3 mmol) in dry DMF (0.5 mL). After 30 min, ethyl 6-(methylsulfonyloxy)hexanoate was added neat, and the mixture

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was stirred at room temperature for 10 h. The mixture was then cooled to 0 °C and quenched with the addition of 1 M HCl. The aqueous layer was extracted twice with diethyl ether and subsequently filtered before HPLC purification [A (aqueous phase) = 0.1% TFA H_2O, B (organic phase) = 0.1% TFA acetonitrile; $\lambda = 254$ nm; solvent gradient 1% B to 15% B in 15 min, 15% B to 95% B in 25 min]. Two peaks of interest were obtained; one main peak corresponded to the final product AM1-COOH while a second, smaller one corresponded to the protected ester. The protected ester was saponified to the desired carboxylic acid via addition of 4 equiv of 4 M LiOH in methanol and stirring at room temperature overnight. After removal of acetonitrile under reduced pressure, the pure fractions were freeze-dried to yield AM1-COOH as a pale yellow oil (17.42 mg, 54.7% yield). ^{1}H NMR (500 MHz, CD_3OD): δ 9.18 (s, 1H), 8.94 (d, J = 5.5, 1H), 8.83 (d, J = 7.3, 1H), 8.11 (m, 1H), 4.61 (d, J = 9.7, 1H), 3.96 (d, J = 18.4, 1H), 3.53 (m, 2H), 3.42 (m, 1H), 3.35 (m, 3H), 3.07 (s, 1H), 2.85 (s, 2H), 2.47 (m, 1H), 2.27 (dt, J = 15.8, 7.3, 2H), 2.13 (m, 1H), 1.52 (dd, J = 15.3, 7.6, 2H), 1.40 (m, 2H), 1.19 (dd, J = 14.7, 7.1, 2H). ^{13}C NMR (500 MHz, CD_3OD): δ 177.77, 147.43, 146.33, 136.84, 134.37, 128.92, 101.35, 72.42, 71.68, 57.22, 39.60, 35.11, 34.98, 30.58, 27.12, 26.39, 26.09. LC−MS $(M + H)^+$: calcd for $C_{17}H_{26}N_2O_3$ = 307.19; found 307.20.

Hapten−Protein Immunoconjugates. Racemic NIC was conjugated to BSA for ELISA microtiter plate coating only. For AM1 hapten; KLH, TT and CRM conjugates were prepared for immunization. AM1 was activated at room temperature for 6 h using standard EDC/sulfo-NHS (1.3 equiv each) coupling procedure in DMF. After DMF removal under reduced pressure, the residue was dissolved in 0.1 M MOPS saline pH = 7.2, and the corresponding amount of protein (1 mg of hapten:1 mg of protein) was added and allowed to stand for 12 h at 4 °C. We found MOPS buffer prevented protein unfolding better than PBS. Coupling efficiencies were monitored using MALDI-TOF MS, save for KLH which cannot be directly analyzed. As the number of lysine residues directly affects coupling, TT generally afforded a greater number of hapten copies in line with its higher molecular weight.

Active Immunization Protocols for Mouse Studies. Groups of $n = 4$ 129GIX$^+$ mice (6−8 weeks, 23−28 g) were immunized ip on days 0, 7, and 133 with a suspension of AM1−TT, AM1−KLH or AM1−CRM (0.1 mg) in phosphate buffered saline (PBS) in formulation with AS-03 adjuvant (GlaxoSmithKline). AS-03 is a GSK proprietary α-tocopherol oil-in-water emulsion based adjuvant system currently approved for use in pandemic influenza vaccines in the European Union and some countries in Asia. Our choice of adjuvant was based on its ability to produce a robust immune response combined with its approval for human use. Following vaccine administration, serum (0.1 mL) was collected on days 7, 14, and 140 via retroorbital puncture. All biological samples collected were stored at −80 °C until use to preserve integrity.

Vaccination of Rats for Self-Administration. Based on its performance in murine experiments, AM1−TT was advanced onto rat behavioral studies. Wistar-derived male rats (n = 5−6, 250−300 g) were purchased from Harlan (Indianapolis, IN) and assigned either to AM1−TT vaccine or TT-only control group. Rats were immunized with 0.1 mg of immunoconjugate in formulation with AS-03 adjuvant administered into 3 sites (2 sc; 1 ip). Four total immunizations were performed during the course of the study at days 0, 14, 28, and 53. On days 14, 27, 41, and 72 roughly 0.2 mL of serum was collected onto heparinized microcentrifuge tubes, and their immune response to date was measured by ELISA.

Immunologic Assays. Production of nicotine-specific IgG was monitored by ELISA using a NIC−BSA conjugate as the coating antigen. Titers were calculated from the plot of absorbance versus log dilution, as the dilution corresponding to an absorbance reading 50% of the maximal value. NIC−BSA was the hapten−protein conjugate of choice in order to prevent biasing of the titer measurements toward the immunized hapten. We have previously demonstrated the general suitability of using NIC−BSA for titer measument and determination of nicotine binding constants.[12] NIC−BSA and protein only controls were added to COSTAR 3690 microtiter plates and allowed to dry at 37 °C overnight. Following methanol fixation, nonspecific binding was blocked with a solution of 5% nonfat powdered milk in PBS for 0.5 h at 37 °C. Next, mouse serum was serially diluted in a 1% BSA solution across the plate and allowed to incubate for 1−2 h at 37 °C in a moist chamber. Plates were then washed with DI H_2O and treated with goat anti-mouse-HRP antibody for 0.5 h at 37 °C. Following another wash cycle, plates were developed with the TMB 2-step kit (Pierce; Rockford, IL). In the case of the rat self-administration serum, the absolute titer value obtained is deemed to be "masked" due to concurrent administration of nicotine.[13] Antibody affinity for nicotine and cotinine, a nicotine major metabolite, was approximated for comparison across groups using competition ELISA. The same procedure as above was followed except, the desired competitor (nicotine or cotinine) was added concurrently with the mouse serum previous to plate incubation.

Additionally, refined values of antibody affinity and nicotine binding capacity were determined for our rat behavioral study samples via a soluble radioimmunoassay

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(RIA). A modified version of Muller's method[14] was followed as it allows for determination of both affinity constant and concentration of specific antibody in serum. The RIA was carried out in a 96-well equilibrium dialyzer MWCO 5000 Da (Harvard Apparatus, Holliston, MA) to allow easy separation of bound and free L-[*N*-methyl-^{3}H]-nicotine tracer; specific activity = 81.7 Ci/mmol (PerkinElmer, Boston, MA). Briefly, rat serum was diluted in RIA buffer (sterile filtered 2% BSA in 1X PBS pH = 7.4) to a concentration that would bind 40% of ~24 000 decays/min of ^{3}H-nicotine tracer. A 50 μL aliquot of serum was combined with 10 μL of radiolabeled tracer (~24 000 decays/min) and 50 μL of unlabeled (−)-nicotine at varying concentrations in RIA buffer; 110 μL of PBS pH = 7.4 was added to the solvent chamber and the samples were allowed to reach equilibrium on a plate rotator (Harvard Apparatus, Holliston, MA) at room temperature for at least 22 h. A 70 μL aliquot from each sample/solvent chamber was slowly aspirated and suspended in 5 mL of scintillation fluid (Ecolite, ICN, Irvine, CA), and the radioactivity of each sample was determined by liquid scintillation spectrometry. These samples were concurrently used to construct a standard curve for use in quantitative ELISA of rat serum samples.

Intravenous Nicotine Self Administration (IVSA). As stated, the most promising immunoconjugate was moved forward into a rat behavioral model. At the start of the experiment, Wistar-derived male rats (n = 5−6, 250−300 g) were purchased from Harlan (Indianapolis, IN) and were housed in groups of two and maintained in a temperature controlled environment on a 12 h:12 h light cycle (0600 h on−1800 h off). Upon arrival at the laboratory, animals were given free access to food and water during a one-week habituation period. All animal care and use was performed according to NIH guidelines and in compliance with protocols approved by the Institutional Animal Care and Use Committee. Food training and nicotine self-administration took place in 8 standard Coulbourn operant chambers. Each chamber was housed in a sound-attenuated box. Operant chambers were equipped with two levers, mounted 2 cm above the floor, and a cue light mounted 2 cm above the lever on the back wall to the right of the food hopper. The right lever was the "active" lever, and the left lever was the "inactive" lever. For food training, a food hopper is located 2 cm to the left of the "active" lever, in the middle of the back wall. Intravenous infusions were delivered in a volume of 0.1 mL over a 1 s interval when the active lever was pressed, via an infusion pump (Razel, CT). Lever pressing was established as demonstrated by the method of Hyytia et al.[15] Initially, rats were restricted to 15 g of food daily (approximately 85% of their free-feeding body weight). After the second day of food restriction, rats were trained to respond for food under a fixed-ratio 1 (FR1) schedule of reinforcement (1 food pellet for each lever press) with a 1 s timeout (TO-1s) after each reinforcement. Training sessions lasted for 30 min daily, and the FR1 and 1 s TO were gradually increased to a FR1 and a 20 s TO. Once rats obtained steady baseline responding at a FR1-TO-20s schedule of reinforcement for food, they were returned to *ad libitum* food prior to preparation for intravenous jugular catheter implant surgery. For surgery, rats were anesthetized with an isoflurane−oxygen mixture (1−3% isoflurane) and chronic Silastic jugular catheters were inserted into the external jugular and passed sc to a polyethylene assembly mounted on the animal's back. The catheter assembly consists of a 13 cm length of Silastic tubing (inside diameter 0.31 mm; outside diameter 0.64 mm), attached to a guide cannula that is bent at a right angle. The cannula is embedded into a dental cement base and anchored with a 2 × 2 cm square of durable mesh. The catheter is passed sc from the rats back to the jugular vein where it is inserted and secured with a nonabsorbable silk suture. Upon successful completion of surgery, rats were given 3−5 days to recover before self-administration sessions start. During the recovery period, rats remained on *ad libitum* food access, and had catheter lines flushed daily with 30 units/mL of heparinized saline containing 100 mg/mL of Timentin to prevent blood coagulation and infection in the catheters. Following successful recovery from catheter implant surgery, self-administration session began, where rats were trained under "acquisition" conditions to iv self-administer nicotine (0.03 mg/kg/inf) in 1 h baseline sessions, 1−3 days per week, on a FR1-TO-20s schedule of reinforcement. During self-administration sessions, rats were flushed with saline before the test session to ensure catheter patency, and again flushed after the test session with 30 units/mL of heparinized saline, containing 100 mg/mL of Timentin, to prevent blood coagulation and infection in the catheters. If catheter patency was in question, demonstrated by an unexpected shift in response rates, or inability to draw blood from the catheter, 0.1 mL of a short-acting anesthetic (Brevital, 10 mg/mL) was infused. Animals with patent catheters exhibit rapid loss of muscle tone within 3 s. Rats with catheters no longer patent according to the Brevital test were removed from the experiment.

After establishing "baseline" responding, the test vaccine was administered as described above. Acquisition training continued throughout the initial vaccination, through boost 1 and up until 2 weeks after the second boost, where we determined *a priori* that titer levels would be sufficient to examine the effects of AM1−TT on nicotine self-administration. Following the acquisition phase of training, rats were run under stable responding conditions two weeks after boost 2 and again two weeks after boost 3, to evaluate the effects of AM1−TT vaccine on nicotine intake under FR1 and progressive ratio (PR) schedules of responding. Stable responding is defined as less than 20% variability across 2 consecutive sessions.

Following the FR1 testing (i.e., two weeks after boost 2 and again two weeks after boost 3) rats were then tested on

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a 10 h progressive-ratio schedule, with each reward resulting in a progressive increase in the number of lever presses required for the subsequent reward. The progression of lever presses was 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95 etc., where initially the first lever press delivered a nicotine reinforcer. Then the subsequent reinforcer required two presses and the subsequent reinforcer required four lever presses for the reinforcer, etc. In preliminary results, this procedure produces, under saline pretreatment conditions (no vaccine), an average intake of 6−7 rewards, with 12−15 as the last ratio completed (break-point). Break-point is arbitrarily defined as the last ratio completed with no reinforced response for 30 min.

Data Analysis. For IVSA sessions, data are collected online simultaneously from multiple operant chambers. Results of the operant procedures are reported as mean number or mean cumulative number of bar presses for nicotine. Where appropriate, data were analyzed using Student's *t* test, analysis of variance (ANOVA), or for data that did not conform to homogeneity of variance, the Mann−Whitney *U* test was used.

Results

Titers and Affinity of Nicotine-Selective Antibodies Generated by Active Immunization in Mice. In an effort to elucidate the optimal carrier vehicle for immunization, AM1 hapten was conjugated with three different carrier proteins, namely, KLH, TT and CRM. Each protein was chosen based on its ability to elucidate a potent immune response. The efficacy of all hapten−protein immunoconjugates was assessed by vaccination into groups of $n = 4$ 129GIX$^+$ mice. Bleeds obtained after only one injection ($t = 7$ days) showed no significant NIC−BSA titer and thus were deemed inadequate for further analysis. All bleeds from the sham immunized protein only controls showed no titer as well. It is well established that multiple immunizations are required for adequate stimulation even with the most successful vaccines.[16] Additional challenges are expected to give a more robust response as it increases the interaction time between antigen and immune system. Thus, while a significant response was observed after two injections, the effect of a third injection following a 4 month "rest" period was also assessed. Results obtained are summarized in Table 1. While an increase in titer was observed with all three test groups, the most drastic improvement in immunogenicity was seen with AM1−TT where the titer measurement increased more than 3-fold after a third injection to reach titers in the ∼1:100 000 level.

While antibody titer data can be extremely promising and reveal the overall immunogenicity of a vaccine candidate, an equally important parameter in predicting efficacy is the ability of the polyclonal antibody response to bind its desired antigen, in this case, nicotine. It is important to note that the binding constants measured via competition ELISA are inherently higher than what would be observed with other more precise methods such as equilibrium dialysis with radioactive labeled drug. Due to experimental ease, we used competition ELISA as a first line of analysis for comparison of the immune response elicited by each vaccine group. We expect a viable active vaccine candidate to show binding constants in the order of 10 μM on the ELISA scale, which on the soluble immunoassay scale corresponds to low nanomolar binding (vide supra). For our analysis, a measurable NIC−BSA titer must be present in order to measure the nicotine binding constant as this is the relevant competition we are attempting to measure (i.e., NIC−BSA versus free nicotine in solution). As stated, no competition data is available for the first bleed as well as the control groups. The second bleed data shows some affinity toward nicotine, but acceptable levels were not achieved until the third injection. In particular, the TT and CRM groups appear to greatly benefit from an additional challenge as the affinity toward nicotine is increased by a full order of magnitude between the two data points. Importantly, this was accomplished without losing specificity as binding constants against cotinine, nicotine's main metabolite, remained negligible, in line with what has been previously reported with similar haptens.[8]

Table 1. Average ELISA Titer and Nicotine Binding Constant Measurements Obtained from $n = 4$ Immunized 129GIX$^+$ Mice on NIC−BSA Coated Plates[a]

	AS-03 adjuvant			
	second bleed		third bleed	
immunoconjugate	titer	K_d (μM)	titer	K_d (μM)
AM1−KLH	18 400	44.16 ± 4.61	21 333	27.39 ± 8.69
AM1−TT	28 000	102.94 ± 11.40	94 400	14.63 ± 2.19
AM1−CRM	22 400	138.16 ± 17.72	64 000	13.22 ± 1.54

[a] These results are for comparative purposes only and are not representative of precise binding constants.

Generation of Nicotine Specific Antibodies by Active Immunization during Concurrent Nicotine Self-Administration in a Rat Model. Once the optimal immunogenic carrier protein was elucidated, the most promising vaccine candidate was advanced into rat-behavioral studies. Rats have a well-characterized central nervous system whose neurochemical pathways, particularly in the limbic and motivational parts of the brain, correspond qualitatively to those of humans. Their behavioral repertoire is well-characterized and shows a characteristic dependence syndrome during chronic administration. The objective of the self-administration experiment was to assess any behavioral changes induced by vaccination on rats trained to intravenously self-administer nicotine, at a dose of 0.03 mg/kg/infusion during 1 h sessions on a FR1 schedule. This dose was used to mimic the intake of a heavy smoker as it is roughly equal to the nicotine infusion of 2 cigarettes in a human.[8] Two groups were included, a TT-only protein control and an AM1−TT vaccine group. Initial cell sizes of $n = 5$−6 were considered enough to provide reliable estimates of drug effects; the immune data

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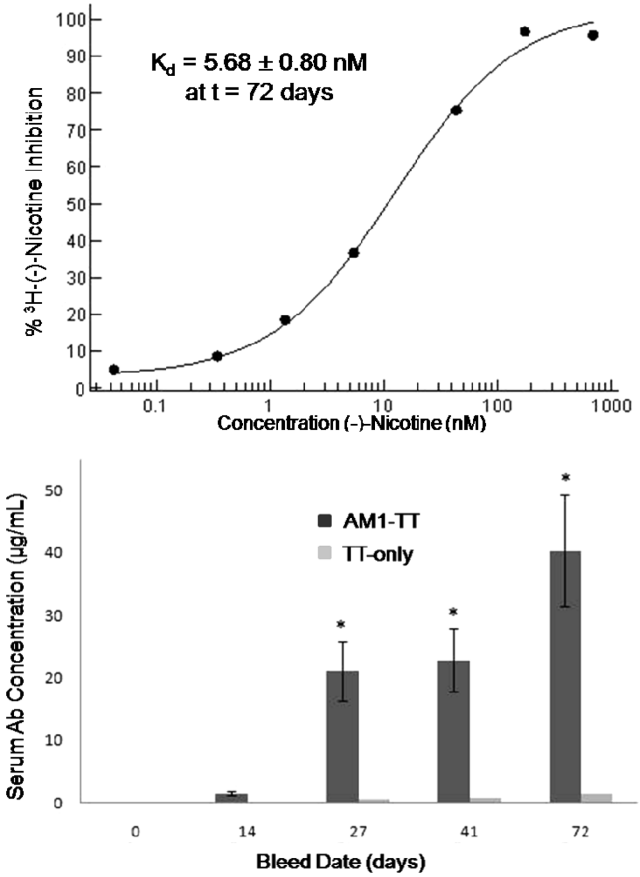

Figure 1. Nicotine specific antibody affinity and concentration elicited in rats during course of self-administration. *$p < 0.05$ (by Student's t test).

presented here is representative of those subjects that maintained catheter patency throughout the study ($n = 3-4$/group).

Serum concentrations of nicotine specific antibodies were measured by quantitative ELISA using NIC−BSA coated microtiter plates. A standard curve was constructed using sera in which anti-nicotine antibody concentrations had been independently determined by radioimmunoassay. Importantly, and as expected, all samples from the TT-only immunized controls showed no titers on NIC−BSA. Samples collected from the AM1−TT vaccinated groups demonstrated a steady increase in titer over time after each boost. At the time of the last bleed, K_d for nicotine was 5.68 ± 0.80 nM as calculated by soluble RIA (Figure 1). Nicotine binding capacity calculated from these data was $5.36 \pm 1.20 \times 10^{-7}$ M, which is equivalent to 40.26 ± 8.97 µg/mL of nicotine-specific IgG. IgG was assumed to have a molecular weight of 150 kDa and two nicotine-binding sites per molecule. This nicotine specific IgG concentration in serum corresponds to a nicotine binding capacity in serum of 87.10 ± 19.40 ng/mL.

Importantly, we did not observe drastic variations in titers; the difference between the high and low responders was ~2−3-fold (versus 10-fold as reported by other groups in similar studies[8]) supporting our hypothesis of advantageous hapten design.

Table 2[a]

treatment group	prevaccine	vaccine	boost 1
TT-only	16.3 (1.6)	15.7 (1.6)	13.6 (1.5)
AM1−TT	17.3 (2.3)	18.4 (3.1)	19.0 (2.9)

[a] Data represent mean (SEM) number of reinforced responses for nicotine under a fixed-ratio 1 (FR1) schedule of responding during the last 2 sessions in each phase of acquisition for nicotine, prior to responding for nicotine under "stable" conditions, two weeks after boost 2 ($n = 3-4$/group).

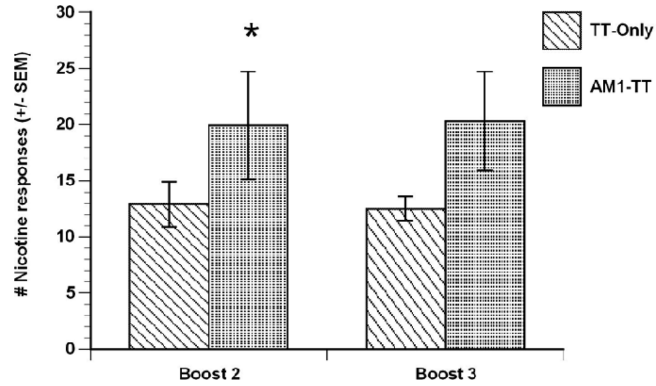

Figure 2. Data represent mean (± SEM) number of reinforced responses for nicotine under a fixed-ratio 1 (FR1) schedule during stable responding two weeks after boost 2 and boost 3. Analysis revealed a significant increase in nicotine responses in AM1−TT treated rats, and a trend toward increased responding for nicotine in AM1−TT treated rats two weeks after boost 3, when peak titer values were observed ($n = 3-4$/group). *$p < 0.05$ vs TT-only controls.

Nicotine Self-Administration in Rats. The results from nicotine self-administration during the acquisition phase of the study are presented in Table 2 and Figure 1 in the Supporting Information. During this phase of the study, rats were only run 1−3 times per week, in order to maintain lever pressing for the reinforcing properties associated with nicotine, therefore, analysis of these data were not conducted.

Following the acquisition phase of training, rats were run under stable responding conditions two weeks after boost 2, for 6 IVSA sessions, and again two weeks after boost 3, for 2 IVSA sessions, prior to PR testing, to evaluate the effects of the AM1−TT vaccine on nicotine intake under a FR1-TO-20 schedule of responding. During these testing phases, 2 rats were dropped from the TT-only group and 1 rat from the AM1−TT group, due to loss of catheter patency. Results from these IVSA sessions are presented in Figure 2. Analysis of the effects of AM1−TT vaccine, two weeks after boost 2, on nicotine intake revealed a significant increase in nicotine responses in rats vaccinated with AM1−TT, relative to TT-only controls ($p < 0.05$). Analysis of data two weeks after boost 3 produced a noticeable trend toward increased nicotine intake, but analysis only revealed a near significant effect of AM1−TT treatment on increased nicotine intake ($p = 0.09$).

Cumulative response data across the 60 min IVSA sessions, two weeks after boost 3, better illustrate the effects

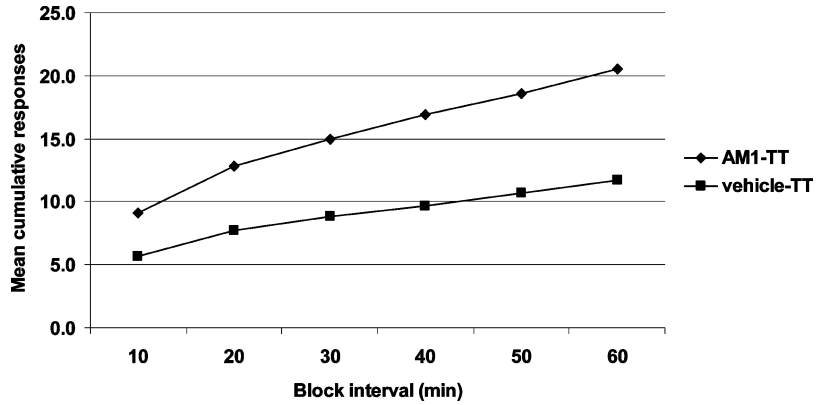

Figure 3. Data represent mean cumulative reinforced responses for nicotine during the last 2 IVSA sessions under a FR1 schedule of responding two weeks after the final boost (boost 3). The data demonstrate a clear increase in the loading phase (37%) during the first 10 min of responding, and an overall average increase at the end of the 1 h IVSA sessions of 43% in the AM1−TT treatment group ($n = 3-4$/group).

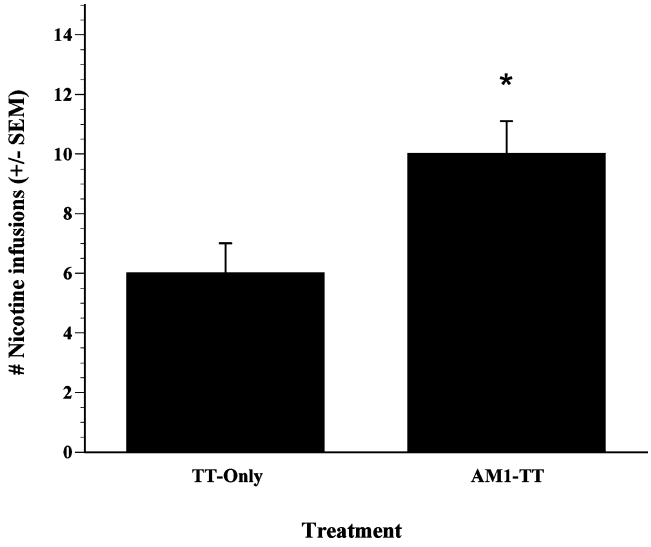

Figure 4. Data represent the average number (±SEM) of infusions for 0.03 mg/kg/infusion of nicotine under a progressive ratio schedule of responding. ANOVA revealed a significant increase in nicotine infusions in rats vaccinated with AM1−TT relative to TT-only controls ($n = 3-4$/group). *$p < 0.05$ vs vehicle controls (TT-only).

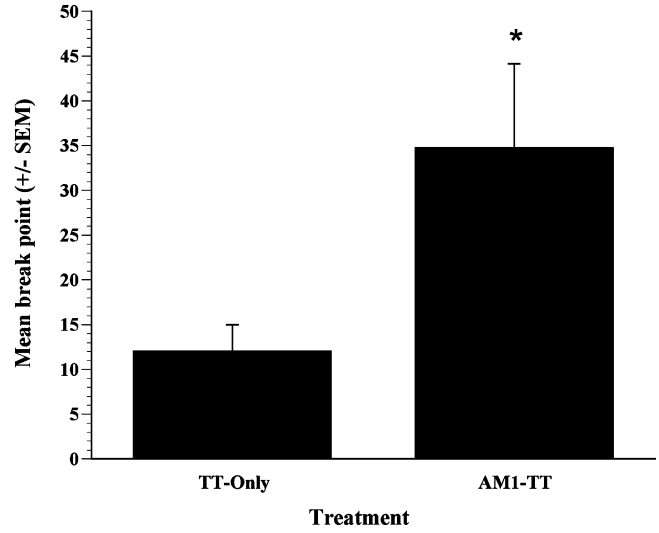

Figure 5. Data represent the average break-point (±SEM) when responding for 0.03 mg/kg/infusion of nicotine under a progressive ratio schedule. Analysis of break-points using the Mann−Whitney *U* test revealed a significant increase in the break-point in rats vaccinated with AM1−TT relative to TT-only controls ($n = 3-4$/group). *$p < 0.035$ vs vehicle controls (TT-only).

of AM1−TT vaccination on the loading phase (the first10 min of the IVSA session) and the steady increase in responding for nicotine in the AM1−TT group (Figure 3). As can be seen in Figure 3, there is a 37% average increase in responding for nicotine during the loading phase, and an overall average increase at the end of the 1 h IVSA sessions of 43% in the AM1−TT treatment group, relative to TT-only controls.

Figures 4 and 5 illustrate that, under a progressive ratio schedule, AM1−TT vaccinated rats demonstrated a significant increase in the number of nicotine infusions and in break-point. ANOVA of nicotine infusion during the PR test (Figure 4) revealed a significant increase in nicotine infusions in rats vaccinated with AM1−TT relative to TT-only controls ($F = 6.85$, df 1, 5, $p < 0.05$).

Progressive ratio break-point data were analyzed using the nonparametric Mann−Whitney *U* test, and results revealed a significant increase ($p < 0.035$) in the break-point in AM1−TT rats relative to TT-only controls (Figure 5). Additionally, to better illustrate and support the hypothesis that AM1−TT vaccination increased motivation to work for nicotine in the PR test, the mean number of lever presses on the active lever and inactive lever are presented in Table 3.

Finally, while this study did not attempt to elicit the tolerability of the hapten−protein conjugates, we note that all animals survived immunization and none presented adverse side effects stemming from vaccination. In order to assess the overall health of the test subject undergoing intravenous self-administration, their body weight was monitored for the length of the study. We found that AM1−TT immunized animals presented slightly lower

Table 3[a]

Treatment Group	Mean PR Active Lever Presses	Mean PR Inactive Lever Presses
TT-Only	50 (15)	5 (4)
AM1−TT	171 (59)*	22 (11)

[a] Data represent mean (SEM) number of PR active lever presses and PR inactive lever presses for nicotine under a progressive ratio schedule. Progressive ratio testing was conducted two weeks after boost 3. Analysis of mean lever presses using the Mann−Whitney *U* test revealed a significant increase in overall active lever pressing in rats vaccinated with AM1−TT relative to TT-only controls ($n = 3-4$/group). *$p < 0.035$ vs vehicle controls (TT-only).

weights than the TT-only control group. At $t = 72$ days, the AM1−TT immunized group weighed an average of 10% less than those of control (data not shown).

Discussion

While we are aware of the three ongoing clinical nicotine vaccine trials, lack of consistent and robust immune stimulation could prevent this technology from reaching its full potential. Several approaches aiming to improve the immunogenicity of nicotine vaccines in rodent models have been developed. Our laboratory has an active immunopharmacotherapy program which has focused on ways to maximize the immunogenicity of vaccines against drugs of abuse, primarily cocaine and nicotine, via optimization of hapten design. Several publications have emerged from our efforts including generation of a stereochemically defined hapten, termed NIC,[11] and spatially constrained designs, termed CNA and CNI,[12] which targeted the two most stable conformers of nicotine in solution as established by computational studies.[17] The current study incorporates an additional structure to our arsenal of nicotine haptens termed AM1. AM1 is an unconstrained hapten that follows the general 3′-pyrrolidine substitution pattern.

We hypothesized AM1 would possess a hapten design advantageous over those set forth by Langone and Pentel based on the following assertions. First, we present a stability argument where the presence of liable ester linkage in the Langone design translates into spontaneous detachment of antigen from the carrier protein and thus loss of anti-nicotine immunogenicity; while such a design would not preclude monoclonal antibody production it could severely impinge on an active vaccine's efficacy. Spontaneous ester hydrolysis under physiological conditions is a known phenomenon, and increasing hapten stability via an ester−amide interchange was previously investigated by our laboratory during our cocaine immunopharmacotherapeutical efforts.[18] We suspect this reasoning was set forth when Pentel made said modification to the Langone design.[8] Amide bonds are resistant to hydrolysis unless specific proteases are present which significantly minimizes the possibility of loss of hapten cargo during immunization. However, while a peptide bond is able to confer hapten stability, it also introduces an additional immunogenic moiety to the target structure. Indeed, in the case of cocaine catalytic antibody haptens, the presence of this additional antigenic moiety was required to obtain clones of desired activity.[19] In the case of the 3′-substituted nicotine scaffold, however, we posit the proximity of this antigenic "switch" to the target structure could have detrimental effects and lead to imprecise stimulation where the immune focus is shifted toward a non-native amide bond rather than the innate antigenic moieties of the nicotine structure. Previous uses of β-alanine linkers usually present the amide bond moiety at least 6 carbon atoms away from the target scaffold, where it serves two roles, as it not only increases immune recognition but also elongates the linker distance between the carrier and cargo, thus ensuring better presentation of the target structure to the immune cells.[3,19] In the case of the Pentel hapten, only a single methylene carbon separates the target pyrrolidine ring from the antigenic amide bond, which could result in overlapped immune presentation and consequently decreased nicotine specificity as additional H-bonding pairs are introduced into the antibody−antigen complex of the hapten versus naive drug. In the case of AM1, we have substituted the amide moiety with a simple ether appendage which not only provides hapten stability but also allows for a "masked" appendage site which focuses the immune response onto the desired nicotinic target. The proposed ether linker effectively mimics a lipid structure known in the gene-delivery world to have distinct nonimmunogenic character and low cytotoxicity thus allowing for a muted linker attachment site from an immunological standpoint.[20]

The major findings of this study are that vaccination with a *trans*-3′-(hydroxymethyl)nicotine derived hapten (AM1) results in a potent immunogenic cocktail able to elicit elevated levels of anti-nicotine antibodies in both mice and rat rodent models. The native antigenicity of the hapten is highlighted by the fact that high antibody titer levels were obtained regardless of carrier protein when checked for cross reactivity with a nonimmunized nicotine analogue (NIC). Importantly, tetanus toxoid (TT) gave antibody titer levels 4-fold higher than that of the traditional KLH formulation although this difference was only obtained after two booster injections several months after vaccination dosing began. Furthermore, the immune response observed in rats had noticeably less variability than what has been reported with similar haptens.

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Our AM1−TT vaccine formulation allowed for generation of nicotine specific antibodies even with concurrent self-administration of high doses of naive drug in Wistar derived male rats. This result is important and supports the hypothesis that vaccination can be initiated before smoking cessation begins, even in heavy smokers, without affecting vaccine immunogenicity.[8] Furthermore, the presence of these anti-nicotine antibodies actively altered the intravenous self-administration pattern in immunized subjects, where protective effects of vaccination are mirrored in an increased drug intake and an increased motivation to take the drug. The results reached statistical significance with subsequent boosts suggesting that a longer vaccination schedule may ultimately prove optimal. It was seen from our mouse model that AM1−TT saw a drastic increase in titer generation only after a given "rest" period before boosting. Thus it is likely a similar effect would have been seen in rats and higher titers have long been established as a direct indicator for induction of changes in self-administration behavior.

The data presented here shows what appears to be a paradoxical effect of an increased nicotine intake and increased motivation to take the drug as measured by a progressive ratio schedule. These results became evident in animals after the initial acquisition of nicotine self-administration, establishment of stable responding and after the second and third boosts with vaccine. While there was some decrease in nicotine self-administration in the control group during this acquisition phase, this observation often occurs as animals are tested every day and the increase in intake following deprivation disappears.[21] By the second boost, the self-administration of the control rats had stabilized and the increased self-administration produced by the treatment became manifest. It is clear that the animals had a motivation to obtain nicotine in the dose range studied here as both the controls and treatment animals showed breakpoints above 10 and the treatment animals pressed on average of 35 times for one infusion.

To our knowledge there are only two reports in the literature that probe the effects of vaccination in nicotine self-administration. In the first report, from Svensson,[22] active immunization prevented reinstatement of nicotine-seeking behavior in a low nicotine dose group (0.001 mg/kg) as compared to control. Use of a second dose, equivalent to what was used in the current work (0.03 mg/kg), failed to significantly reinstate nicotine-seeking behavior in both vaccinated and vehicle animals. The second report by Le Sage et al.[23] surveyed the effects of vaccination on acquisition and reinstatement of nicotine self-administration at a dose of 0.01 mg/kg. Their findings provided evidence that nicotine self-administration was significantly lower than controls in both behavioral paradigms. Our own results are in contrast to these findings, hence, we posit the differences seen, i.e. compensation versus decrease of drug intake, can in part be explained based on two parameters: drug and antibody concentrations. First, both previous studies used lower doses of nicotine to observe the effects seen (3× lower for Pentel and 30× lower for Svensson). Second, while ELISA titer values are presented in both studies; a later report by Pentel[24] with a similar immunoconjugate as reported in the current study allows for a direct comparison of serum nicotine antibody concentrations. In this later study, titers elicited were 3−4-fold higher than what we observed. In sum, the presence of increased amounts of circulating drug coupled with potentially reduced antibody concentrations in essence would translate into saturation of antibody binding sites. Insufficient sequestering of drug would equate to a "spillover" of nicotine, thus, paralleling an inability of vaccinated subjects to fully escape the reinforcing cycle.

One likely interpretation of the results seen from our self-administration experiment is that the rats responded to the presence of anti-nicotine antibodies by attempting to surmount these protective effects via higher drug intake. AM1−TT immunized animals were effectively "working harder" to get the nicotine induced rewarding effects. Similar results were obtained during cocaine self-administration experiments that uncovered an inverted U-shaped function shift during competitive drug antagonism.[25] That is, lower doses of cocaine will be self-administered similar to the level of saline (non-reinforcer) and higher doses will be self-administered more readily with a shorter interval between injections. It is common to observe that animals increase the rate of responding under an FR schedule when the unit dose of cocaine for self-administration decreases, which suggests that the animals compensate the decreased unit amount of cocaine per injection by increasing the rate of injection. Therefore, increased responding for drug under an FR schedule by immunization may suggest that rats compensate the partial blockage of drug delivery to the central nervous system in the presence of antibodies by increasing the rate of self-administration. Translated to the human nicotine addiction, immunized subjects would find the cost of smoking greatly increased as if the cost of the pleasure derived from a pack of cigarettes had been doubled. Together

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the data presented here show a significant increase in nicotine self-administration with vaccine treatment that needs to be incorporated into the conceptual framework of a broad vaccine approach to addiction treatment.

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Supporting Information Available: An additional figure showing the self-administration patterns observed during the course of the study. This material is available free of charge via the Internet at http://pubs.acs.org.

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