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Vaccination Protects Rats from Methamphetamine-induced Impairment of Behavioral Responding for Food

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Abstract

(+)-Methamphetamine (METH) addiction is a chronic disease that interferes with fundamental brain-mediated behaviors and biological functions like eating. These studies present preclinical efficacy and safety profiles for a METH conjugate vaccine (IC_{KLH}-SMO9) designed to treat METH abuse. IC_{KLH}-SMO9 efficacy and safety were assessed over a 16-week period by monitoring general health and stability of responding in a food maintained behavioral paradigm. Male Sprague-Dawley rats were trained to lever press for food reinforcers until stable behavior was established. Rats (n=9/group) were then immunized with 100 µg of a control antigenic carrier protein (IC_{KLH}-Cys) or IC_{KLH}-SMO9 in Alhydrogel® adjuvant, with booster immunizations at 4, 8 and 12 weeks. Health, immunization site and behavior were assessed daily. No adverse effects were found. During weeks 14–16, when antibody titers and METH affinity ($K_d = 13.9 \pm 1.7$ nM) were maximal, all rats received progressively higher METH doses (0.3–3.0 mg/kg) every 3–4 days, followed by behavioral testing. Even though the lower METH doses from 0.3–1.0 mg/kg produced no impairment in food maintained behavior, 3.0-mg/kg in control rats showed significantly ($p < 0.05$) reduced response rates and number of reinforcers earned, as well as reduced food intake. In sharp contrast, the IC_{KLH}-SMO9 group showed no changes in food maintained behavior at any METH dose, even though METH serum concentrations showed profound increases due to anti-METH antibody binding. These findings suggest the IC_{KLH}-SMO9 vaccine is effective and safe at reducing adverse METH-induced effects, even at high METH doses.

Keywords

methamphetamine; vaccine; hapten; drug abuse; preclinical studies; rats

Introduction

(+)-Methamphetamine (METH) abuse is a worldwide problem, causing deleterious effects in users and enormous costs to communities [1,2]. While METH users report positive effects

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like increased energy, euphoria and appetite suppression [3,4]; chronic use results in addiction, organ system dysfunction, weight loss and neurotoxicity [4–6]. Rather than affecting a single site of action or organ system, METH use interferes with a range of medically important functions [7,8].

Because METH addiction is a root cause of most METH-associated health problems, a variety of addiction pharmacotherapies have been tested. Unfortunately no drug(s) have proven effective by United States FDA standards, but a few drugs are useful for acute supportive and symptomatic treatment [8]. The most successful addiction therapy is cognitive behavioral intervention, but even when patients successfully complete behavioral treatment, 36% use METH within 6 months and by 13 months 51% are back to METH use [9].

Combining behavioral treatments with medications to lessen the medical impact of METH use could potentially improve patient health and increase the chance of successful treatment [3,10]. An antibody-based medication could be either anti-METH monoclonal antibody (mAb) [2,11–13] or antibodies generated by active immunization with a METH conjugate vaccine [11,13–16].

Preclinical rodent studies show that active immunization with haptens (derived from drugs of abuse) conjugated to antigenic proteins can favorably alter behavioral and pharmacokinetic properties of addictive drugs like cocaine, heroin, METH, morphine, nicotine, and oxycodone [11,17–20]. Under optimal conditions active immunization can lead to high titer and high affinity immune responses [21–24], decrease drug-induced locomotor activity [14,15,25–28], prevent drug-induced reinstatement [11,28] and alter (*i.e.*, increase or decrease) drug self-administration in animal studies [11,16,29–31].

Importantly, studies in rats show METH use during the immunization period does not affect the affinity or titer of the immune response to a METH-conjugate vaccine (MCV) [10]. This is clinically important since many patients are likely to use drugs of abuse during the development of an immune response. While vaccination is a promising treatment, two clinical trials of a nicotine vaccine and one trial of a cocaine vaccine failed to meet their Phase II clinical endpoints [21,23,24,32]. Two shared problems among all three vaccines were a large variation in response and less than optimal affinity for the drug of abuse.

The purpose of the present studies was to evaluate the efficacy and safety of a MCV [11,13] in rats, and to better understand the potential for clinically approved aluminum-based adjuvants like Alhydrogel® to augment the immune response. Behavioral studies showed that a high METH dose substantially impaired the control rats' ability to continue stable food maintained behavior, but did not affect the ability of MCV immunized rats to respond for food, or eat the earned food reinforcers, which demonstrated an effective health benefit of the MCV.

Methods

Drugs and Chemicals

Reagents were purchased from Sigma Aldrich (St. Louis, MO) unless noted otherwise. (+)-Methamphetamine hydrochloride, (+)-amphetamine sulfate (AMP), and ³H-METH (23.5 Ci/mmol) were obtained from the NIDA Drug Supply Program. Doses were calculated as the free base.

Vaccines

The METH-like hapten HSMO9 (Figure 1) was covalently bound to immunocyanin to produce the MCV (IC_{KLH}-SMO9) by the method of Carroll et al. [11]. Immunocyanin (IC_{KLH}; Biosyn Corp., Carlsbad, CA) is derived from native keyhole limpet hemocyanin (KLH) and consisted of two stable subunit monomers (~360 and ~390 kDa). Both native KLH (8–32 million Da [33]) and IC_{KLH} [25] are used in human vaccines. The control vaccine (IC_{KLH}-Cys) was synthesized by conjugating the activated IC_{KLH} to L-cysteine (Cys), instead of HSMO9.

Gel electrophoresis and Western blot analysis

The proteins were separated using the Fluorescent LP-Next Gel system (Amresco LLC, Solon, OH) and were then transferred to an Immobilon-NC Millipore membrane (Thermo Fisher Scientific, Waltham, MA) for Western blot analysis. Detection of the METH-like hapten was with anti-METH mAb4G9 [34] and an anti-mouse IgG-conjugated peroxidase secondary antibody.

Animal Subjects

All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences. Adult male Sprague-Dawley rats (n=18; Charles River Laboratories, Raleigh, NC) were housed two per cage. Water was provided *ad libitum* and sufficient food was provided after behavioral sessions to maintain rat body weights at approximately 320 g.

Behavioral Apparatus

Experimental sessions were conducted in operant chambers (Med Associates, Georgia, VT) equipped with two levers, a white stimulus light above each lever with a house light on the opposite wall, a sound generator, and a food hopper connected to a pellet dispenser. Recording of responses and activation of lights and sound was computer controlled (MED-PC IV, Med Associates, Georgia, VT).

Food maintained behavior

Behavioral sessions were run Monday-Friday. Rats were trained to lever press for food reinforcers (45 mg grain-based Dustless Precision pellets; Bio-Serv, Frenchtown, NJ) under a second order fixed-interval (FI), fixed-ratio (FR) schedule of reinforcement. In the presence of the house and a white light above the reinforced lever, completion of every 5th response (FR5) during a 30s FI resulted in the concurrent flashing of the stimulus lights above both levers and sounding of a tone for 4s. Completion of the first FR after expiration of the FI resulted in the delivery of two food pellets (defined as one reinforcer), simultaneous with the flashing lights and sound activation. Daily sessions ended after 25 reinforcers earned, or two hrs, whichever occurred first. After responding stabilized, a 10-day baseline response measurement was collected. Responding was then extinguished by omitting the food reinforcer. Extinction sessions were run for a minimum of 10 days to achieve stable low responding. Following extinction, food reinforcers were made available and reacquisition of food maintained behavior was assessed for 10 sessions, at which point rats were back to baseline responding.

Immunization

Once trained, rats were assigned to immunization groups based on pre-established statistical criteria (as described in the statistics section) to prevent behavioral differences between groups. Rats were immunized subcutaneously with 100 µg of IC_{KLH}-Cys or IC_{KLH}-SMO9

in Alhydrogel® 85 adjuvant (Brenntag Biosector; Frederikssund, Denmark). Booster injections were at 4, 8 and 12 weeks.

Quantitation of anti-METH polyclonal antibody binding

Blood samples were collected two days before and 12 days after immunizations. Rapid equilibrium dialysis (RED; Thermo Scientific, Rockville, IL) and a [^3H]-METH tracer were used to measure antibody functional titers. Each rat serum was diluted 1:50, 1:100 and 1:300 in 0.1 M sodium phosphate with 0.15 M NaCl, pH 7.35 buffer. Then aliquots of each dilution (containing 5 nM [^3H]-METH) were added to one chamber of the RED device and buffer to the other chamber. Plates were incubated overnight at 4°C to reach equilibrium. The [^3H]-METH in each chamber was quantitated by liquid scintillation spectrophotometry to determine the percentage of bound [^3H]-METH in serum.

METH K_d (dissociation constant) determinations in IC_{KLH}-SMO9 immunized rats

After the third boost, K_d values for METH binding in serum samples were determined using a bead-based RIA that is specific for IgG binding [13]. METH K_d values from each antiserum were determined after correction for the binding of [^3H]-METH tracer in the presence of unlabeled METH [35]

METH challenge dosing

The goal was to test the ability of the IC_{KLH}-Cys (control) and IC_{KLH}-SMO9 vaccine to alter METH (and AMP metabolite) pharmacokinetics and METH-induced behavioral responses at week 14–16. Rats received subcutaneous saline followed by progressively increasing METH doses every 3–4 days (0.3–3.0 mg/kg METH). Immediately after dosing, a food maintained behavioral session was conducted. Testing of food maintained behavior continued Monday-Friday. Two hrs after saline or METH administration, a blood sample was collected to measure METH and AMP serum concentrations by LC-MS/MS [36].

Treatment safety observations

Rats were weighed and checked for signs of ill health Monday-Friday. Careful attention was given to sites of immunization for signs of inflammation, lesions or swelling. After METH challenge sessions, rats were observed in their home cage at regular intervals for unexpected changes in general appearance or behavior. The trained observer was blind to the treatments.

Statistical Analysis

Average number of responses, session time and response rate during acquisition, extinction and reacquisition of food maintained behavior was used to sort the rats into performance-matched immunization groups, verified by t-tests. Behavioral response data were analyzed using two-way repeated measures (RM) analysis of variance (ANOVA) (day $\times$ immunization group), followed by Bonferroni Multiple Comparison tests.

To assess food maintained behavior and body weight data during the immunization period, a two-way RM ANOVA (time point $\times$ immunization group) was conducted, followed by Bonferroni Multiple Comparison tests. To determine immunization effects on food maintained behavior after METH challenges, a two-way RM ANOVA was performed (METH dose $\times$ immunization group), followed by Bonferroni Multiple Comparisons.

Serum METH and AMP concentrations after METH challenges were analyzed using a two-way RM ANOVA (METH dose $\times$ immunization group), followed by Bonferroni Multiple Comparison tests. Statistical analyses were conducted using SigmaStat (Aspire Software International, Ashburn, VA). Comparisons were considered statistically different at $p < 0.05$.

Results

Gel and Western blot analysis

While the initial electrophoresis step after maleimide activation of IC_{KLH} did not separate the ~360 and ~390 kDa proteins (Figure 2), the final IC_{KLH}-Cys and IC_{KLH}-SMO9 antigens showed two bands, with a significant and equal amount of METH hapten conjugated to both immunocyanin monomers.

Food maintained behavior performance testing and METH binding over time

Table 1 summarizes the food maintained behavior results before the assignment to immunization groups. Daily testing during the immunization period showed no differences between immunization groups for any measures ($p > 0.23$). Figure 3 shows the serum METH binding after each immunization, as measured with RED and [³H]-METH binding.

Both immunization groups showed similar behavioral responses by all measures following METH doses from 0.3 to 1.0 mg/kg (Figure 4). The major changes occurred after the 3.0 mg/kg METH challenge dose, when the IC_{KLH}-Cys immunized rats showed decreased responding that was not statistically different ($p < 0.07$), decreased response rates ($p < 0.001$), decreased reinforcers earned ($p < 0.001$), and increased session time ($p < 0.001$) relative to IC_{KLH}-SMO9 treated rats.

METH and AMP concentrations and K_d values in serum after immunization

Rats from both treatment groups showed METH-dose dependent increases in METH serum concentrations (Figure 5). However, IC_{KLH}-SMO9 immunized rats showed significantly and substantively higher METH serum levels than control rats at all METH doses, except the lowest dose ($p < 0.06$, Fig 5). AMP concentrations also increased in a dose-dependent manner, but only at 3.0 mg/kg did the METH IC_{KLH}-SMO9 immunized rats show significantly higher AMP levels. The K_d value for METH binding at week 14 was 13.7 ± 1.7 nM ($n = 7$).

General safety assessment

Rat body weights showed a small increase from an initial weight of 327 ± 5.4 g to 349 ± 4.7 g (mean $\pm$ S.E.M.) after 16 weeks, but there were no differences between the IC_{KLH}-Cys and IC_{KLH}-SMO9 immunization groups ($p > 0.41$). Daily health and behavioral monitoring did not reveal any signs of ill health or adverse effects attributable to the immunization, METH dosing, or the combination of immunization and dosing.

Discussion

These studies provide a preclinical efficacy and safety profile for a MCV designed to treat human METH addiction. We chose to use the ability of the animals to maintain stable food maintained behavior as an indication of efficacy, as well as a general measure of animal health. While this is not a standard test of vaccine protection from addiction behaviors, we hypothesized it could be an important measure since METH has significant negative health effects on eating behavior and body weight in humans [4]. Indeed, METH is approved in the US to treat obesity where other diets or medications have failed to produce weight loss.

At the highest METH challenge dose, control rats showed significant impairment (Figure 4) in their ability to continue normal behaviors. In contrast, the IC_{KLH}-SMO9 treated rats appeared unaffected. An important observation is that even though some of the control rats did not seem overtly impaired in their performance of food maintained behavior after 3.0 mg/kg METH, five out of nine rats did not eat the food earned during the session. By

comparison eight out of nine of the IC_{KLH}-SMO9 immunized rats responded normally for the food, and all nine ate all earned food pellets. The significant adverse effect on control rats at this dose could be explained by METH-induced disruptions in a chain of behaviors, suggesting rats were able to respond on the lever, but unable to complete the process of eating the food. It is also possible the animals were too profoundly affected to eat the food reward because of METH-induced behaviors like sniffing, rearing, repetitive head and limb movement [37].

One of the most impressive behavioral findings was that the IC_{KLH}-SMO9 immunized rats had sufficient antibody binding capacity to protect against the pharmacological effects of the highest dose of METH. Especially since it was administered after a 0.3, 0.56, and 1.0 mg/kg dose of METH. These progressively increasing doses, followed by the final 3.0 mg/kg METH dose should have substantially exceeded the stoichiometric binding capacity of the antibodies generated by active immunization. Thus the mechanism of action cannot be solely dependent on antibody binding capacity, as previously demonstrated [38,39].

In contrast to control rats, eight of the nine the IC_{KLH}-SMO9 immunized rats were able to complete the behavioral task at the 3.0 mg/kg METH dose. The one adversely affected rat showed increased session time and failed to obtain all reinforcers within the 2 hr session. Although a Grubb's outlier test showed this rat's data were a statistical outlier in the IC_{KLH}-SMO9 treatment group, we did not remove the data set. This rat's immune response was the third lowest anti-METH titer on week 14 (Figure 3), and the only rat in this group to not respond normally for the food.

The finding that a low-titer rat was not fully protected from the METH-induced behavioral effects appears to partially mimic recent findings in clinical trials for cocaine and nicotine vaccines, where only a portion of the human populations achieved even a modest immune response [21,23,32]. In our study all IC_{KLH}-SMO9 immunized rats had significantly higher METH serum concentrations compared to METH serum concentrations in controls (Figure 5), and even the rat with the lowest measured titer had elevated METH concentrations compared to controls. Thus, we think improvements in the choice of adjuvant, immunization schedule, and METH hapten epitope density could improve the uniformity and magnitude of the immune response. These studies are ongoing.

Based on our experience with METH-induced stimulant and stereotypic behaviors (*e.g.*, [40], we expected the highest doses of METH (1.0 and 3.0 mg/kg) to produce significant and long lasting (*e.g.*, up to 4–6 hrs) stereotypic and locomotor behaviors that would interfere with accurate and timely behavioral responses (Figure 4). A possible reason for not observing disruptions in their behavioral task may lie in the timing of the testing. We started the 2 hr testing period immediately after the METH challenge, and on average the rats completed the task within 15 min, up to the 1 mg/kg METH dose. However, the peak for potential disruptive locomotor activity and stereotype behaviors occurs at about 20 min after a 1 mg/kg METH dose [40,41] and even later after a 3.0 mg/kg dose.

The polyclonal antibodies generated by IC_{KLH}-SMO9 immunization showed a high affinity for METH ($K_d = 13.7$ nM, see Figure 3 legend), which was comparable to three of the highest affinity anti-METH mAb produced in our laboratory (*i.e.*, 6.8, 9, and 16 nM for METH) [13]. Studies of another anti-METH KLH-based vaccine report polyclonal anti-METH antibody K_d values of 130 nM in mice [42] and 30.4 nM in male Sprague-Dawley rats [14]. While the rats were the same outbred strain as in the current studies, most other study aspects differed including use of intact native KLH instead of pharmaceutical grade IC_{KLH} monomers, a different hapten design (named MH6), a different immunization schedule, and the use of Sigma Adjuvant System instead of Alhydrogel®.

Antibody titers in IC_{KLH}-SMO9 immunized rats rose steadily across the immunization period, resulting in an approximate 2-fold increase in titers from the first to third boost. However, the individual week 14 rat titers (and earlier) were variable (Figure 3), ranging from 40–88% (n=9) with a percent coefficient of variation of 23%. In our experience with rodents (unpublished observation), Alhydrogel® usually elicits lower antibody titers than other more potent preclinical adjuvants like Freund's Complete adjuvant [43] or Sigma Adjuvant System. However, Alhydrogel® is one of only a few adjuvants approved for human use by the United States FDA. We think that the poor and variable immune responses in previous clinical trials of anti-cocaine and anti-nicotine vaccines was in part due to the poor ability of aluminum-based adjuvants like Alhydrogel® to stimulate robust immune responses [21], [23], [32]. This will need to be addressed in future anti-addiction vaccine trials.

Another essential aspect of vaccine development is safety of the vaccine protocol, and potential for adverse interactions with the antibody target (*i.e.*, METH). Our MCV produced no reactions at the injection site or observable adverse health effects. Food maintained behavior (prior to METH) and home cage behavior were not different between control and IC_{KLH}-SMO9 immunized rats, indicating no apparent negative effects. When METH was administered there were no observable additive or synergistic effects of the MCV on METH-induced effects.

Conclusions

In conclusion, repeated immunization with a high dose of IC_{KLH}-SMO9 produced no adverse effects on health, body weight or performance during food maintained behavioral testing. Even when rats were challenged with increasing METH doses over a two-week period, there were no apparent additive or synergistic interactions between the immunization and METH. Administration of 3.0 mg/kg METH dramatically impaired food maintained behavior in control, but not IC_{KLH}-SMO9 immunized rats. Even though low METH challenge doses did not seem to affect behavior differently in the treatment groups, IC_{KLH}-SMO9 immunized rats still had higher METH serum concentrations for all doses, indicating that the antibodies generated by the IC_{KLH}-SMO9 vaccine were effective at sequestering METH in the bloodstream. In a broader perspective these data suggest anti-METH vaccines could provide improvements in general health even when patients use METH at exceedingly high doses.

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Abbreviations

AMP	(+)-amphetamine
Cys	L-cysteine
FI	fixed-interval schedule of behavioral reinforcement
FR	fixed-ratio schedule of behavioral reinforcement
[³H]-METH	(+)-[2',6' - ³ H(n)]-methamphetamine
HSMO9	the METH-like hapten (S)-N-(2-(mercaptoethyl)-6-(3-(2-(methylamino)propyl)phenoxy)hexanamide

IC_{KLH}	immunocyanin monomers derived from native KLH
IC_{KLH}-Cys	maleimide activated immunocyanin end capped with cysteine
IC_{KLH}-SMO9	the METH-conjugate vaccine
K_d	equilibrium dissociation constant
KLH	keyhole limpet hemocyanin
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
mAb	monoclonal antibody
METH	(+)-methamphetamine
MCV	METH-conjugate vaccine
RED	rapid equilibrium dialysis

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Highlights

- A vaccine (IC_{KLH}-SMO9) for treatment of methamphetamine abuse was tested in rats
- Repeated immunization with IC_{KLH}-SMO9 caused no apparent adverse effect
- IC_{KLH}-SMO9 protects from methamphetamine-induced reductions in responding for food
- METH serum concentrations substantially increased due to anti-METH antibody binding
- These studies suggest the vaccine is effective and safe

Figure 1.

Chemical structures of METH, METH-like hapten (HSMO9; (S)-N-(2-(mercaptoethyl)-6-(3-(2-(methylamino)propyl)phenoxy)hexanamide), maleimide activated immunocyanin, IC_{KLH}-Cys, and the MCV (IC_{KLH}-SMO9). The large molecular weight immunocyanin (IC_{KLH}) antigenic carrier protein is symbolically represented as a large circle. When the synthesis reactions for IC_{KLH}-Cys and IC_{KLH}-SMO9 were complete, a large excess of L-cysteine was added to the reaction mixture to block any potentially unreacted maleimide sites before use in immunizations.

Figure 2.

Gel electrophoresis and Western blot analysis of IC_{KLH} , $IC_{KLH-Cys}$ and $IC_{KLH-SMO9}$. Protein concentration for all lanes ranged from 0.73–0.75 mg/ml. The upper panel shows the migration of IC_{KLH} carrier protein (Lane 1). $IC_{KLH-Cys}$ is shown in Lanes 2 and 5, and the final MCV $IC_{KLH-SMO9}$ is shown in Lanes 3 and 4. The proteins shown in Lanes 2–3 were treated with an additional large excess of L-cysteine as a final step in the synthesis to “cap” or block potentially unreacted maleimide groups in the $IC_{KLH-Cys}$ (control) and $IC_{KLH-SMO9}$ antigens. The purpose of this step was to reduce the possibility of unwanted covalent binding to protein and cellular sites *in vivo*. The proteins in Lanes 4 and 5 were not treated with an excess of Cys. The lower panel shows the same proteins analyzed by Western blot. The antibody for the Western blot analysis was anti-METH mAb4G9 [13,34]. The immune reactivity of the $IC_{KLH-SMO9}$ protein with mAb4G9 appeared unaffected by the addition of excess L-cysteine, and did not show immunoreactivity with the proteins in other lanes, which did not contain the METH hapten.

Figure 3.

Development of anti-METH polyclonal antibody binding titers in rats using $\text{IC}_{\text{KLH-SMO9}}$ (open circles indicate individual rat results) or $\text{IC}_{\text{KLH-Cys}}$ (without METH haptens, closed circle at week 14 along the x-axis) antigens with Alhydrogel® adjuvant during a 14-week period. The dashed line crosses each data set at the mean value for the group ($n = 9$ per group). Serum samples were obtained two days before and 12 days after each immunization. Arrows indicate the time of booster immunizations. The K_d value for METH binding at week 14 was 13.7 ± 1.7 nM ($n=7$). We were unable to accurately measure the K_d values in serum samples from the two rats with the lowest titers.

Figure 4.

Effects of IC_{KLH}-Cys (control) and IC_{KLH}-SMO9 immunization on food maintained behavior immediately after METH challenge doses from 0.3 to 3.0 mg/kg. The graphs show the number of lever presses on the reinforced lever (upper left), response rates (responses per second, upper right), session times (lower left) and number of food pellet reinforcers earned (lower right). Data are expressed as mean ± S.E.M. (n = 9 per group). The ** indicates a significant difference (p < 0.001) relative to the IC_{KLH}-Cys control rats.

Figure 5.

METH (upper graph) and AMP (lower graph) serum concentrations measured in rat serum samples collected 2 hrs after a METH challenge dose from 0.3 to 3.0 mg/kg in IC_{KLH}-Cys control (open circle) and IC_{KLH}-SMO9 immunized (closed circle) rats. The progressively increasing METH challenge doses were administered every 3–4 days from weeks 14–16. Each point represents an individual rat response and the horizontal line indicates the mean of the group. The * indicates a significant difference between IC_{KLH}-Cys (control) and IC_{KLH}-SMO9 immunized rats.

Table 1

Summary of food maintained behavioral testing results before the assignment to immunization groups. All rats were trained to stable performance by 13.8 ± 0.3 days.

Test stage	Responses (number)	Response Rate (responses/s)	Reinforcers (number)	Session time (min)
Baseline	414.3 ± 28.0	0.51 ± 0.04	25.0	15.4 ± 0.2
Extinction	35.2 ± 9.7	0.005 ± 0.001	5.0 ± 1.4	120 ± 6.9
Reacquisition	Within 4 days, baseline levels of performance were reestablished.			

All values are the mean $\pm$ S.E.M. (n=18 rats)