AAVrh.10-Mediated Expression of an Anti-Cocaine Antibody Mediates Persistent Passive Immunization That Suppresses Cocaine-Induced Behavior

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Abstract

Cocaine addiction is a major problem affecting all societal and economic classes for which there is no effective therapy. We hypothesized an effective anti-cocaine vaccine could be developed by using an adeno-associated virus (AAV) gene transfer vector as the delivery vehicle to persistently express an anti-cocaine monoclonal antibody *in vivo*, which would sequester cocaine in the blood, preventing access to cognate receptors in the brain. To accomplish this, we constructed AAVrh.10antiCoc.Mab, an AAVrh.10 gene transfer vector expressing the heavy and light chains of the high affinity anti-cocaine monoclonal antibody GNC92H2. Intravenous administration of AAVrh.10antiCoc.Mab to mice mediated high, persistent serum levels of high-affinity, cocaine-specific antibodies that sequestered intravenously administered cocaine in the blood. With repeated intravenous cocaine challenge, naive mice exhibited hyperactivity, while the AAVrh.10antiCoc.Mab-vaccinated mice were completely resistant to the cocaine. These observations demonstrate a novel strategy for cocaine addiction by requiring only a single administration of an AAV vector mediating persistent, systemic anti-cocaine passive immunity.

Introduction

Cocaine addiction is a worldwide problem for which there is no effective pharmacotherapy (Vocci *et al.*, 2005; Preti, 2007; Kampman, 2010; National Institute on Drug Abuse [NIDA], 2010). Chronic cocaine users have a plethora of medical problems, including a high risk of death and HIV/AIDS (Edlin *et al.*, 1994; DAWN, 2004, 2008; McCoy *et al.*, 2004; Maraj *et al.*, 2010; Ciccarone, 2011). Because addiction is a chronic relapsing illness characterized by cycles of drug abuse, abstinence, and reinstatement of abuse, vaccination against cocaine could be a lifetime therapeutic for relapse prevention (Koob and Le, 1997; Kosten *et al.*, 2002; Koob and Kreek, 2007; Orson *et al.*, 2009). One anti-cocaine vaccine strategy is to administer an anti-cocaine monoclonal antibody to prevent systemically administered cocaine from reaching its cognate receptors in the central nervous system (Kosten

and Owens, 2005; Norman *et al.*, 2009; Kinsey *et al.*, 2010). While this approach is effective in animal models, the short therapeutic half-life of monoclonal antibodies (1 to 3 weeks) implies that their clinical use in cocaine relapse prevention necessitates repeated parenteral administration, which would be expensive and problematic for long-term patient compliance (Roskos *et al.*, 2004; Haney and Kosten, 2005; Kosten and Owens, 2005; Moreno and Janda, 2009).

As an alternative, we hypothesized that an adeno-associated virus (AAV) gene transfer vector encoding for an anti-cocaine antibody would mediate persistent expression and provide an effective therapy for cocaine addiction with a single administration. To accomplish this, we designed AAVrh.10antiCoc.Mab, an AAVrh.10 nonhuman primate (Rhesus macaque)-based gene transfer vector coding for the heavy and light chains of the high affinity, anti-cocaine IgG monoclonal antibody GNC92H2 (Carrera *et al.*,

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2000; De *et al.*, 2006). If successful, AAVrh.10antiCoc.Mab should express high levels of the anti-cocaine antibody sufficient to prevent cocaine from reaching its receptors in the brain, thereby preventing the euphoric effect associated with cocaine. The data demonstrate that a single administration of the AAVrh.10antiCoc.Mab vector to mice evokes persistent levels of full-length, high-affinity, anti-cocaine antibody that blocks administered cocaine from access to the brain, preventing cocaine-induced hyper-locomotor activity.

Methods

AAVrh.10antiCoc.Mab

The AAVrh.10antiCoc.Mab vector was derived from the nonhuman primate AAVrh.10 capsid pseudotyped with AAV2 inverted terminal repeats surrounding the anti-cocaine monoclonal antibody expression cassette. The expression cassette consisted of cytomegalovirus (CMV)-enhancer chicken β -actin (CAG) promoter, the anti-cocaine IgG1 monoclonal antibody heavy chain with secretion signal and light chain sequence derived from the GNC92H2 Fab separated by a furin 2A self-cleavage site and the rabbit α -globin polyadenylation signal (Fig. 1A) (Niwa *et al.*, 1991; Carrera *et al.*, 2000; Redwan *et al.*, 2003; Fang *et al.*, 2005; De *et al.*, 2006; Mao *et al.*, 2011). The assembled full-length cDNA was sequenced by overlapping PCR to validate that the sequence was without mutations.

AAVrh.10antiCoc.Mab was produced using three plasmids: (1) pAAVGNC92H2, an expression plasmid containing (5' to 3') the AAV2 5'-inverted terminal repeat including packaging signal (ψ), the human anti-cocaine monoclonal antibody expression cassette, and the AAV2 3'-inverted terminal repeats; (2) pAAV44.2, a packaging plasmid that provides the AAV Rep proteins derived from AAV2 needed for vector replication and the viral structural (Cap) proteins VP1, 2, and 3 derived from AAVrh.10, which determine the serotype of the AAV vector; and (3) pAd Δ F6, an Ad helper plasmid that provides Ad helper functions of E2, E4, and V_A RNA (Xiao *et al.*, 1998; Zhang *et al.*, 2000; De *et al.*, 2006). For AAVrh.10 vector production, pAAVGNC92H2 (600 μ g), pAAV44.2 (600 μ g) and pAd Δ F6 (1.2 mg) were co-transfected into human embryonic kidney 293orf6 cells (American Type Culture Collection, Manassas, VA) that contain an integrated copy of the Ad E1 and E4orf6 regions, using Polyfect (Qiagen, Valencia, CA). At 72 hr after transfection, the cells were harvested and a crude viral lysate was prepared using four cycles of freeze/thaw, which was clarified by centrifugation.

AAVrh.10antiCoc.Mab was purified by iodixanol gradient and QHP anion exchange chromatography (GE Healthcare, Piscataway, NJ). The purified AAVrh.10antiCoc.Mab was concentrated using an Amicon Ultra-15 100K centrifugal filter device (Millipore, Billerica, MA) and stored in PBS, pH 7.4, -80°C . Vector genome titers were determined by quantitative TaqMan real-time PCR analysis using a CMV promoter-specific primer-probe set (Applied Biosystems, Foster City, CA).

In vitro assessment of AAVrh.10antiCoc.Mab expression

To assess AAVrh.10antiCoc.Mab-directed expression of the GNC92H2 antibody *in vitro*, 293orf6 cells were infected with AAVrh.10antiCoc.Mab at 2×10^5 genome copies (gc) per

FIG. 1. Construction and function of AAVrh.10antiCoc.Mab. **(A)** Schematic of the expression cassette. The adeno-associated virus (AAV) serotype rh.10 was used to express the anti-cocaine monoclonal antibody GNC92H2 by subcloning the cDNA for both IgG1 heavy and light chains under the control of the CAG promoter with a furin 2A cleavage site in the spacer region of the polycistronic message to allow correct post-translational processing of the two chains. **(B)** AAVrh.10antiCoc.Mab expression of the anti-cocaine monoclonal antibody *in vitro*. Human HEK-293orf6 cells were infected with AAVrh.10antiCoc.Mab at 2×10^5 genome copies (gc) per cell. After 72 hr, cell media were collected and assessed for anti-cocaine antibody expression after purification by Western analysis under reducing conditions. Lane 1, mock cell culture supernatant; lane 2, AAVrh.10antiCoc.Mab; lane 3, Bevacizumab (positive control). The Western analysis was assessed with sheep anti-human IgG heavy and light chain antibodies. Arrows indicate the IgG heavy (50 kDa) and light chains (25 kDa).

cell and harvested at 72 hr post-infection. Media from noninfected cells served as negative "mock" control. The media were evaluated for the expression of the anti-cocaine monoclonal antibody after purification by Protein G-Sepharose by Western analysis using a 1:1 mixture of separate antibody components using sheep anti-human IgG heavy chain and light chain (*k*) specific secondary antibodies conjugated to horseradish peroxidase (HRP) (Sigma, St. Louis, MO), diluted to 1:2000, and enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ). Bevacizumab, a human anti-VEGF monoclonal antibody, was used as the positive control (Ferrara *et al.*, 2005).

Passive immunization with AAVrh.10antiCoc.Mab

All animal studies were conducted under protocols reviewed and approved by the Weill Cornell Institutional Animal Care and Use Committee. Male BALB/c mice (Taconic, Germantown, NY) were housed under pathogen-free conditions, and at 7 to 9 weeks of age were immunized with 10^{11} gc of AAVrh.10antiCoc.Mab by intravenous injection via the tail vein in 100 μ l volume. Blood was collected at 3 to 24 weeks from transected tail veins, allowed to clot, centrifuged at $10,000 \times g$ for 10 min, and the isolated serum was collected and stored at -20°C .

To assess anti-cocaine antibody levels, sera were assessed by enzyme-linked immunosorbent assay (ELISA) using flat-bottomed 96-well EIA/RIA plates (Corning, New York, NY) coated with the cocaine hapten termed GNC (Carrera *et al.*, 1995), which was conjugated to bovine serum albumin (GNC-BSA; 1 mg/ml, 100 μ l) (Hicks *et al.*, 2011). Anti-cocaine serum antibodies were detected using a 1:2000 dilution of HRP-conjugated sheep anti-human IgG (100 μ l, Santa Cruz Biotechnology, Santa Cruz, CA) in 1% dry milk in PBS, which was incubated for 90 min at 23°C . Anti-cocaine antibody titers were calculated as previously described (Hicks *et al.*, 2011).

To demonstrate that AAVrh.10antiCoc.Mab expressed monoclonal antibodies were cocaine-specific, inhibition of binding of sera from AAVrh.10antiCoc.Mab immunized mice to GNC-BSA by ELISA were performed in the presence of increasing concentrations of cocaine, benzoylecgonine, norcocaine, ecgonine methyl ester, and cocaine ethylene fumarate (cocaethylene) from 0.1 nmol/l to 0.1 mmol/l (all from the drug supply program, National Institute of Drug Abuse, NIH Bethesda, MD). Serum from AAVrh.10antiCoc.Mab-immunized mice was used to determine affinity via a radioimmunoassay (Muller, 1983).

Cocaine pharmacokinetics of AAVrh.10antiCoc.Mab-immunized mice

To assess the effect of AAVrh.10antiCoc.Mab immunization on cocaine blood brain distribution following parenteral cocaine challenge, naive or AAVrh.10antiCoc.Mab-administered mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) 3 min prior to tail vein administration of 250 ng cocaine (0.01 mg/kg) containing 1.0 μ Ci [^3H]-cocaine. One minute later, mice were sacrificed and brain and trunk blood were collected separately. Brain tissue was homogenized in PBS, and the blood was allowed to clot for serum isolation. Separately, 300 μ l of brain homogenate and 50 μ l of serum were added to 5 ml of liquid scintillation cocktail (Ultima GoldTM, PerkinElmer, Waltham, MA) assayed in triplicate, and normalized with a standard quenching curve. For the blood, cocaine was normalized to serum volume; for brain, cocaine was normalized to brain wet weight protein, determined by the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL).

Suppression of cocaine-induced hyperlocomotor behavior in AAVrh.10antiCoc.Mab-immunized mice

Mouse locomotor behavior was measured using infrared beam-equipped activity open-field chambers (20 cm by 20 cm chamber, AccuScan Instruments, Columbus, OH) to

track their motions in both horizontal (X, Y) and vertical (Z) directions. Mice were allowed to habituate to the room for 1 hr prior to each test. Mice were placed in the chamber for 15 min to record prechallenge behavior, then removed, injected intravenously with PBS or cocaine (12.5 μ g, approximately 0.5 mg/kg), and returned to the chamber for 15 min to record the post-challenge behavior. Locomotor activity was collected as horizontal (ambulatory) activity (by number of beam breaks) and time spent in vertical movement (sec).

Statistics

All data are expressed as either geometric means (for serum titers) or group means (behavior parameter) $\pm$ standard error of the mean. Comparisons for the blood-brain distribution experimental groups were performed with two-tailed Student's *t*-test and ANOVA for multiple paired datasets. Behavioral data statistical comparisons between experimental and control groups were performed by two-way repeated-measures ANOVA, using the individual mice as the repeated measure (Genomic Suite v6.6, Partek, St. Louis, Missouri, and R [www.R-project.org]).

Results

Characterization of AAVrh.10antiCoc.Mab

To assess AAVrh.10antiCoc.Mab-directed expression of the anti-cocaine monoclonal antibody, cultured 293orf6 cells were infected with AAVrh.10antiCoc.Mab and, at 72 hr, cell media were collected and antibody expression assessed by Western analysis (Fig. 1B). Media collected from the AAVrh.10antiCoc.Mab-infected cells demonstrated that the vector expressed the heavy and light chains of the monoclonal anti-cocaine antibody.

To determine whether the AAVrh.10antiCoc.Mab vector had the capacity to express and maintain a high systemic level of the anti-cocaine antibody *in vivo*, BALB/c male mice were administered AAVrh.10antiCoc.Mab at the dosage of 10^{11} gc by intravenous administration via the portal tail vein. AAVrh.10antiCoc.Mab-derived anti-cocaine antibodies plateaued at 5 weeks and persisted for 24 weeks, the last time point evaluated (Fig. 2A). To determine the specificity and affinity of the *in vivo* expressed anti-cocaine monoclonal antibody, serum at 12 weeks post-vaccination was assessed in a competitive ELISA and radioimmunoassay. The experiment, performed three times, established the K_d for cocaine at 6.7 ± 1.5 nmol/l (Fig. 2B). The serum-derived antibody had significantly higher specificity for cocaine than the major cocaine metabolites, norcocaine (170-fold), ecgonine methyl ester (2000-fold), and benzoylecgonine (5000-fold; Fig. 2C). The active metabolite of cocaine and ethanol, cocaethylene, had similar affinity as cocaine to the anti-cocaine monoclonal antibody, suggesting that the antibody recognizes the functionally active moiety on cocaine and active cocaine analogs (Danger *et al.*, 2004).

AAVrh.10antiCoc.Mab-derived monoclonal antibody sequesters cocaine in the blood and shields the brain from cocaine

To demonstrate that AAVrh.10antiCoc.Mab-mediated anti-cocaine antibody passive immunization protects the brain from cocaine, AAVrh.10antiCoc.Mab-treated mice

FIG. 2. AAVrh.10antiCoc.Mab directed expression of an anti-cocaine antibody in mice. **(A)** Persistence of expression of the anti-cocaine antibody over 24 weeks following a single administration of AAVrh.10antiCoc.Mab. BALB/c male mice ($n=10$) were administered AAVrh.10antiCoc.Mab intravenously with 10^{11} gc, and antibody titers were measured at 0 to 24 weeks. Antibody titers in the mouse serum were assessed by ELISA against the cocaine GNC hapten conjugated to bovine serum albumin. **(B)** Antibody affinity (K_d) generated by AAVrh.10antiCoc.Mab. Serum from panel A at 12 weeks was used to determine K_d of antibody affinity for cocaine by inhibition of tracer ($[^3H]$ -cocaine) binding via cold competitor radioimmunoassay. $[^3H]$ -cocaine (1.25 nmol/l) was mixed with various concentrations of non-radiolabeled cocaine (0.1 to 1290 nmol/l), incubated with serum containing the AAVrh.10antiCoc.Mab expressed anti-cocaine antibody and precipitated with ammonium sulfate. The K_d is a mean of the three tests and was determined from the concentration of cold competitor at the point of 50% inhibition, as interpolated from the best fit line of the linear region of the curve and the concentration of bound $[^3H]$ -cocaine (Muller, 1983). Data are representative of three experiments. **(C)** Specificity of the anti-cocaine antibody generated by AAVrh.10antiCoc.Mab *in vivo*. Sera at 12 weeks from the mice in panel A were assessed for cocaine specificity by inhibition of binding of immune sera to bovine serum albumin-GNC by ELISA in the presence of increasing concentrations of cocaine, benzoylecgonine, norcocaine, ecgonine methyl ester, and cocaethylene.

were challenged intravenously with $[^3H]$ -cocaine and cocaine levels assessed in serum and brain (Fig. 3). Assessed 1 min post-administration of cocaine, cocaine levels in the brain of AAVrh.10antiCoc.Mab-immunized mice showed a reduction of 68% compared to nonimmunized mice ($p < 0.00001$, $df=22$, $t=21.6$; Fig. 3A). In contrast, serum cocaine levels in AAVrh.10antiCoc.Mab-treated mice were 10-fold greater than nonimmunized mice, with 89% of the serum cocaine IgG bound ($p < 0.00001$, $df=6$, $t=18.5$; Fig. 3B). Overall, there was a 31-fold reduction in the ratio of brain to blood cocaine level in the AAVrh.10antiCoc.Mab-treated mice compared to controls.

Passive immunity with AAVrh.10antiCoc.Mab prevents cocaine-induced hyperactivity

To demonstrate that anti-cocaine monoclonal antibodies elicited by passive immunization with AAVrh.10antiCoc.Mab could prevent cocaine-induced hyperactivity, AAVrh.10antiCoc.Mab-treated mice were challenged on a weekly basis (12 to 17 weeks post-vaccination) with intravenously administered cocaine (12.5 μ g, 0.5 mg/kg); activity was monitored by infrared-beam open-field activity chambers. Quantitative measurement of ambulatory activity was assessed by number of beam breaks for each mouse (treated and control) for 15 min (post-cocaine administration). In successive weekly cocaine challenges over 4 weeks, the

AAVrh.10antiCoc.Mab mice consistently demonstrated suppression of cocaine-induced hyperactivity, as measured by a reduction in ambulatory activity (beam breaks) and were indistinguishable from the nonimmunized mice with PBS controls, with a 20% to 27% reduction in ambulatory activity (beam breaks) compared to nonimmunized mice with administered cocaine (Fig. 4A; p values between all groups, $p < 0.002$, $F_{2,22}=8.0$; between immunized+cocaine and naive+cocaine groups, $p < 0.008$, $df=1$, $F=11.6$; immunized+cocaine and naive+PBS groups, $p > 0.9$, $df=1$, $F=0.02$; naive+cocaine and naive+PBS groups, $p < 0.04$, $df=1$, $F=8.6$; two-way ANOVA with repeated measures). Cocaine-induced hyperactivity increases with repeated cocaine challenge: $F_{3,66}=10.4$, $p < 0.0001$. The interaction between group and day was not significant ($p > 0.95$, $F_{6,66}=0.218$). *Post hoc* analysis using the Dunnett's t -test showed significant difference ($p < 0.04$, $Z=2.3$) between control (naive+PBS) and unvaccinated mice (naive+cocaine); whereas there was no significant difference ($p=1.0$, $Z=-0.05$) between control (naive+PBS) and vaccinated mice (AAV-anticocaine+cocaine). After the fourth challenge (17 weeks post-vector administration), the AAVrh.10antiCoc.Mab mice challenged with cocaine still displayed activity levels indistinguishable from nonimmunized control mice injected with PBS, whereas naive nonimmunized mice challenged with cocaine showed a rapid rise in ambulatory activity over the 15-min test (p values between all groups,

FIG. 3. Levels of cocaine in brain and serum of BALB/c mice challenged with cocaine. Shown are cocaine levels in the serum (ng/ml serum) and brain (ng/g brain) of nonimmunized and AAVrh.10antiCoc.Mab-treated mice following [3 H]-cocaine challenge at 12 weeks post-administration with AAVrh.10antiCoc.Mab. [3 H]-cocaine (0.25 μ g, 0.01 mg/kg) was administered intravenously to BALB/c mice ($n=4$). Evaluations were at 1 min following [3 H]-cocaine challenge. **(A)** Brain cocaine levels. Shown are the amounts of [3 H]-cocaine detected in the mouse brains for nonimmunized and AAVrh.10antiCoc.Mab-treated mice ($p < 0.00001$, $df=22$, $t=21.6$). **(B)** Blood cocaine levels. Shown are amounts of IgG bound cocaine, total cocaine, and unbound cocaine in the serum. After 1 min, serum was obtained, mixed with protein G-sepharose, and assessed for bound [3 H]-cocaine (i.e., IgG bound) relative to the [3 H]-cocaine in the supernatant. Comparisons between groups were conducted by two-tailed, unpaired Student t -test. Unbound (nonimmunized vs. AAVrh.10antiCoc.Mab) at $p > 0.45$, $df=6$, $t=0.7$; IgG-bound (nonimmunized vs. AAVrh.10antiCoc.Mab, $p < 0.00001$, $df=6$, $t=34.9$); total serum (nonimmunized vs. AAVrh.10antiCoc.Mab, $p < 0.00001$, $df=6$, $t=18.5$).

$p < 0.00001$, $df=2$, $F=3.4$; between immunized+cocaine and naive+cocaine groups, $p < 0.00001$, $df=1$, $F=2.1$; immunized+cocaine and naive+PBS groups, $p > 0.9$, $df=1$, $F=2.1$; naive+cocaine and naive+PBS groups, $p < 0.0004$, $df=1$, $F=5.9$; three-way ANOVA with repeated measures; Fig. 4B).

Discussion

The primary challenge in developing an anti-addiction vaccine against cocaine is generating an effective immunity that prevents cocaine from reaching its receptors in the brain, thus blocking the psychotropic effects of cocaine. The present study tests the efficacy of passive immunotherapy against cocaine mediated by AAVrh.10antiCoc.Mab, an AAV gene transfer vector expressing a persistent high-affinity anti-cocaine antibody. When mice were vaccinated with AAVrh.10antiCoc.Mab, high-titer, high-affinity cocaine-specific antibodies were expressed at high levels throughout a 6-month trial. When the vaccinated mice were challenged with cocaine, the cocaine was partially sequestered in the blood, restricting the administered cocaine from reaching the brain. Importantly, the AAVrh.10antiCoc.Mab-vaccinated mice demonstrated reduced cocaine-induced hyperactivity on a persistent basis, despite repeated challenges with intravenous cocaine. Together, the data support the concept that AAVrh.10antiCoc.Mab is a potential candidate for clinical development as an effective anti-cocaine vaccine.

Anti-cocaine vaccines

An effective treatment of cocaine addiction is plausible only when sufficient persistent high levels of high-affinity anti-cocaine antibodies are evoked by the vaccine (Danger *et al.*, 2004; Martell *et al.*, 2009). There have been several ap-

proaches to developing active and passive anti-cocaine vaccines.

Active anti-cocaine vaccines have been developed by linking cocaine analogs to large proteins (BSA, keyhole limpet hemocyanin [KLH], and cholera toxin) (Bagasra *et al.*, 1992; Carrera *et al.*, 1995, 2000; Fox *et al.*, 1996; Johnson and Ettinger, 2000; Kantak *et al.*, 2000; Kosten *et al.*, 2002). In rodent models, all of these approaches have shown efficacy in evoking anti-cocaine antibodies and suppressing cocaine-induced psychotropic behavior (Carrera *et al.*, 1995, 2000; Fox *et al.*, 1996; Johnson and Ettinger, 2000; Kantak *et al.*, 2000, 2001). We recently extended this concept with the development of a vaccine consisting of a cocaine analog covalently linked to a disrupted serotype 5 human adenovirus (Hicks *et al.*, 2011). Studies in mice demonstrated the vaccine evoked persistent high levels of high affinity serum anti-cocaine antibodies that protected the mice from repeated administration of cocaine. Studies in rats demonstrated parallel efficacy, including suppression of addiction-related self-administration behavior (Wee *et al.*, 2012). To date, only one cocaine vaccine, TA-CD (succinyl-norcocaine coupled to a recombinant cholera toxin B subunit), has been tested in clinical trials (phase I study [Kosten *et al.*, 2002], phase II [Haney and Kosten, 2005; Martell *et al.*, 2009; Haney *et al.*, 2010]). However, only 38% of treated subjects achieved high serum antibody titers and, of those subjects, only 53% had reduced their cocaine usage in half (Martell *et al.*, 2009).

Passive anti-cocaine vaccines comprised of exogenously produced cocaine-binding antibodies confer immediate immunity against cocaine. Several anti-cocaine monoclonal antibodies with high affinities for cocaine have been developed, all of which prevented cocaine-induced central

FIG. 4. Prevention of cocaine-induced hyperlocomotor activity by prior treatment with AAVrh.10antiCoc.Mab. Vaccinated mice were tested weekly for cocaine-induced increased hyperactivity and measured in infrared-beam open-field chamber boxes. Three groups of mice ($n=10$ in each group) were assessed: nonimmunized + cocaine (▲ 12.5 μ g intravenous); non-immunized administered PBS intravenously (●); and AAVrh.10antiCoc.Mab + cocaine (○, 12.5 μ g intravenous). **(A)** Total ambulatory activity of nonimmunized compared to AAVrh.10antiCoc.Mab-immunized mice following weekly challenges of PBS or cocaine (12.5 μ g, 0.5 mg/kg), 12 to 16 weeks post-administration. All mice received a baseline PBS injection at week 12 (indicated by the nonconnected symbols). The first weekly cocaine challenge corresponds to week 13 post-administration of AAVrh.10antiCoc.Mab or PBS. **(B)** Cumulative ambulatory counts assessed as a function of time post-administration of PBS or cocaine. The locomotor activity was assessed by number of beam breaks by the mice in the chambers. Shown is the cumulative ambulatory activity over the first 15 min post-challenge from the last challenge, 16 week post-administration of AAVrh.10antiCoc.Mab.

nervous system stimulation in rodent models (Carrera *et al.*, 2000, 2001; Kantak *et al.*, 2000; Paula *et al.*, 2004; Norman *et al.*, 2009). The anti-cocaine antibody gene sequence of our current study was derived from GNC92H2, a murine monoclonal antibody with exquisite cocaine binding specificity and affinity (K_d 13 to 240 nM) that was elicited using the cocaine analog (GNC) conjugated to KLH (Carrera *et al.*, 2000; Treweek *et al.*, 2011). This monoclonal has demonstrated therapeutic efficacy in rat and mouse models of cocaine reinstatement and cocaine overdose prevention (Carrera *et al.*, 2005; Treweek *et al.*, 2011). However, like all of the anti-cocaine monoclonal antibodies, the GNC92H2 monoclonal has a short half-life, requiring repetitive systemic administration to maintain protective immunity (Fox *et al.*, 1996; Kantak *et al.*, 2000; Carrera *et al.*, 2001; Roskos *et al.*, 2004; Norman *et al.*, 2007). Thus, its primary clinical application is for the indication of acute cocaine toxicity, given the prohibitive cost of monthly prophylactic immunizations and the anticipated poor patient compliance among drug abusers in ongoing treatment schedules (Stitzer *et al.*, 2010).

AAVrh.10antiCoc.Mab vaccine

To circumvent the need for frequent administration of an anti-cocaine monoclonal, we developed a gene transfer approach to deliver the genetic code for an anti-cocaine monoclonal antibody using an AAV vector. AAVs mediate long-term expression, enabling a persistent passive immunity against cocaine. Based on AAVrh.10, a nonhuman primate-derived gene transfer vector known from prior studies to be effective in expressing robust levels of antibodies *in vivo* (De *et al.*, 2006; Sondhi *et al.*, 2008b; Watanabe

et al., 2010; Mao *et al.*, 2011), and having minimal preexisting immunity in humans, we designed a vector coding for the heavy and light chains of the anti-cocaine IgG monoclonal antibody GNC92H2. While the development of high antibody titers mediated by AAVrh.10antiCoc.Mab would take 2 to 3 weeks, instead of minutes with acute administration of the monoclonal *per se*, the long-term benefits to the individual abusing cocaine would be long-term efficacy with AAVrh.10antiCoc.Mab with a single administration and no additional compliance requirements. In this regard, AAVrh.10antiCoc.Mab generated high-titer, high-affinity cocaine-specific antibodies in mice that were persistent for more than 24 weeks, and demonstrated a strong affinity and specificity for cocaine. The cross-reactivity to the more potent cocaethylene is an important result in that cocaethylene is a product of cocaine and alcohol, thus addressing an important problem for cocaine-abusing individuals who mix cocaine and alcohol (Danger *et al.*, 2004).

The use of an AAV-mediated gene transfer for passive immunity against cocaine is based on several factors: (1) AAV vectors can express the transgene at high levels on a persistent basis; (2) unlike an active vaccine, the genetic delivery of a monoclonal antibody assures that, by design, the antibody is high affinity and equally effective in all genetic backgrounds; (3) small addictive drugs are typically not immunogenic and thus poor targets for an active vaccine, which may suffer in potency or nonpersistent responses; and (4) the persistent expression of the antibody by a single administration of AAV is compatible with a therapeutic approach for addicts who may be unlikely to return for the required boosters of an active vaccine.

AAV has been the viral vector of choice for therapeutic applications that require persistent gene expression for

long-term correction of genetic defects and as a therapeutic vector for the delivery of antibodies for genetic and infectious disease (De *et al.*, 2006; Johnson *et al.*, 2009; Watanabe *et al.*, 2010; Mao *et al.*, 2011). The present study demonstrates the extension of using this vector class as a therapeutic for drug addiction. AAV has an excellent safety profile that has been established in numerous clinical trials (Li *et al.*, 2011). The use of AAVrh.10 has been shown to be safe through the rigors of a formal safety and toxicology study that we used to support our ongoing clinical trial for Batten disease (Sondhi *et al.*, 2008a). Finally, we have carried out a number of experimental animal studies using an AAVrh.10 vector to deliver therapeutic antibodies (Watanabe *et al.*, 2010; Wang *et al.*, 2010; Mao *et al.*, 2011). In all these studies, as in the present study, the serum levels of the antibody expressed by the vector were stable over time, suggesting that functional anti-antibody responses are not an issue in using this vector system to express monoclonal antibodies *in vivo*.

Since cocaine is rapidly absorbed by the nasal and pulmonary epithelium, an effective anti-cocaine vaccine must be capable of evoking immunity that rapidly reduces unbound cocaine blood levels following drug intake. Analysis of *in vivo* pharmacokinetics, following an intravenously administered cocaine bolus with radioactive tracers, demonstrated the efficiency of AAVrh10antiCoc.Mab. The rapid binding of the monoclonal anti-cocaine antibody to cocaine *in vivo* successfully sequestered cocaine, partially preventing it from reaching its receptors in the brain. When the immunized mice were repeatedly challenged with cocaine at doses that yield serum levels comparable to those observed in humans after cocaine administration (Benuck *et al.*, 1987; Evans *et al.*, 1996), they continued to display an overall reduction in typical cocaine-induced hyperactivity, showing a locomotor response nearly identical to control animals receiving only saline. This effect remained consistent over 4 weeks of cocaine challenges, showing the persistence of the AAVrh.10antiCoc.Mab-derived antibodies to suppress cocaine-induced hyperactivity in the rodent model.

A therapy based on AAVrh.10antiCoc.Mab is not aimed at "curing" cocaine addiction *per se*, but as a therapeutic aimed at helping the addict avoid recidivism by diminishing the reward in reinstatement of drug use. We envision the use of this vaccine as an adjunct with on-going psychotherapy, such as cognitive behavioral relapse prevention therapy, to help minimize the withdrawal cravings and provide a bridge for the post AAVrh.10antiCoc.Mab administration delay for expression of anti-cocaine antibodies. There remains the important issue of "override" for this vaccine as with other cocaine vaccines. If the AAVrh.10anticoc.Mab can generate high antibody titers in humans, then perhaps the desire for cocaine can be lessened and the user will not seek to double his or her normal intake of cocaine.

In summary, the present study presents a passive immunization strategy in which a high-affinity anti-cocaine antibody is expressed *in vivo* using an AAV-based vector. With a single administration vaccine that persistently produces high-affinity anti-cocaine antibodies, the requirement for proactive engagement by an individual abusing cocaine would be dramatically reduced. This approach represents an important addition to the future toolbox for therapeutic in-

tervention for cocaine addiction for which the current alternatives remain only behavioral therapies.

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No competing financial interests exist.

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