Prevention of Drug-Induced Memory Impairment by Immunopharmacotherapy

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One approach to treating drug abuse uses antidrug antibodies to immunize subjects against the illicit substance rather than administering therapeutics that target the specific CNS site of action. At present, passive vaccination has recognized efficacy in treating certain gross symptoms of drug misuse, namely, motor activation, self-administration, and overdose. However, the potential for antibodies to prevent drug-induced changes involving finer cognitive processes, such as benzodiazepine-associated amnesia, remains unexplored. To address this concept, a flunitrazepam hapten was synthesized and employed in the generation of a panel of high affinity monoclonal antibodies. Anti-flunitrazepam mAb RCA3A3 ($K_{d,app}$ = 200 nM) was tested in a mouse model of passive immunization and subsequent mole-equivalent challenge with flunitrazepam. Not only was flunitrazepam-induced sedation prevented but immunization also conferred protection to memory consolidation as assessed through contextual and cued fear conditioning paradigms. These results provide evidence that immunopharmacotherapeutic blockade of drug intoxication also preserves complex cognitive function.

Introduction

The challenge of drug development rests not only in identifying a novel lead compound for a given therapeutic application but also in finding a delivery method through which the druggable compound may reach its in vivo target. Small molecule therapeutics prescribed to modify mental or psychological states require a mechanism for transport across the blood−brain barrier when their site of action is within the central nervous system (CNS). For example, catecholamine and serotonin mimetics, which often contain the arylethylamine moiety common to monoamine neurotransmitters, function as agonists or antagonists of the respective neurotransmitter system. However, such direct manipulation of neural pathways by CNS-targeted therapies may incur noticeable side effects. To treat drug abuse, other routes have been explored for affecting the signaling networks altered by the illicit substance. For example, small molecule therapies that compete for the same CNS site may block drug binding. This technique either circumvents the corresponding drug high or overdose or supports user abstinence by supplying a drug substitute that endows a similar high of diminished intensity but prolonged duration. The latter instance has been used with limited success in drug-specific cases for the management of addiction (e.g., methadone, buprenorphine, and naloxone to treat heroin abuse). However, this strategy is not without drawbacks, with the most notable being the inherent addictive quality of the therapeutic agent. Further diminishing the utility of small molecule therapeutics is their incompatibility with prophylactic treatment.

In this study, we sought to demonstrate the use of immunopharmacotherapy as a prophylactic antidrug therapeutic with the capacity to stave off drug-induced cognitive impairment despite its exclusion from the CNS. Whereas immunopharmacotherapy has been validated within the scenario of attenuating the psychomotor symptoms of drug intoxication from a comparatively large drug dose that potentially overwhelms the drug-binding capacity of the administered mAb's,[a] the opposite scenario is rarely examined. As an immunotherapeutic target, benzodiazepines contrast with the traditional psychostimulants because a comparatively small number of drug molecules escaping mAb-mediated sequestration may precipitate measurable changes in cognitive processes. Protection from benzodiazepines does not probe the efficacy of a maximum mAb dose against a potential molar excess of drug, but instead it examines whether the rate of mAb-mediated drug scavenging in the periphery is sufficient to block even a minimal amount of drug from entering the CNS. To explore passive immunization against a highly potent abused small molecule, a hapten against flunitrazepam was designed and the monoclonal antibody (mAb) therapy evaluated for its ability to prevent flunitrazepam-induced memory deficits.

To date, the implementation of passive immunization strategies has been limited in scope despite a major recognized benefit over other antiaddiction therapies (methadone maintenance, nicotine patch). In particular, immunization incurs minimal physiological consequences when drug use or exposure is avoided. Currently, attempts to diminish drug dependence or attenuate drug inebriation with antidrug mAb's have been explored for morphine, PCP, cocaine, methamphetamine, mescaline, and nicotine.[1−4] These investigations into mAb-mediated pharmacokinetic antagonism have focused on the attenuation of drug self-administration, restoration or protection of basic motor skills, prevention of intoxication upon future drug intake, and reversal of lethality from drug overdose in rodent models.[5−10]

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[a] Abbreviations: mAb, monoclonal antibody; IgG, immunoglobulin; GABA, γ-aminobutyric acid; iv, intravenous; ip, intraperitoneal; VS, vehicle saline (ip); VF, vehicle flunitrazepam (ip); RS, RCA3A3-immunized saline (ip); RF, RCA3A3-immunized flunitrazepam (ip); GS, GNC92H2-immunized saline (ip); CS, conditioned stimulus (tone); US, unconditioned stimulus (foot shock); CS+ test, conditioned stimulus test session of the conditioned fear assay; NIDA, National Institute on Drug Abuse.

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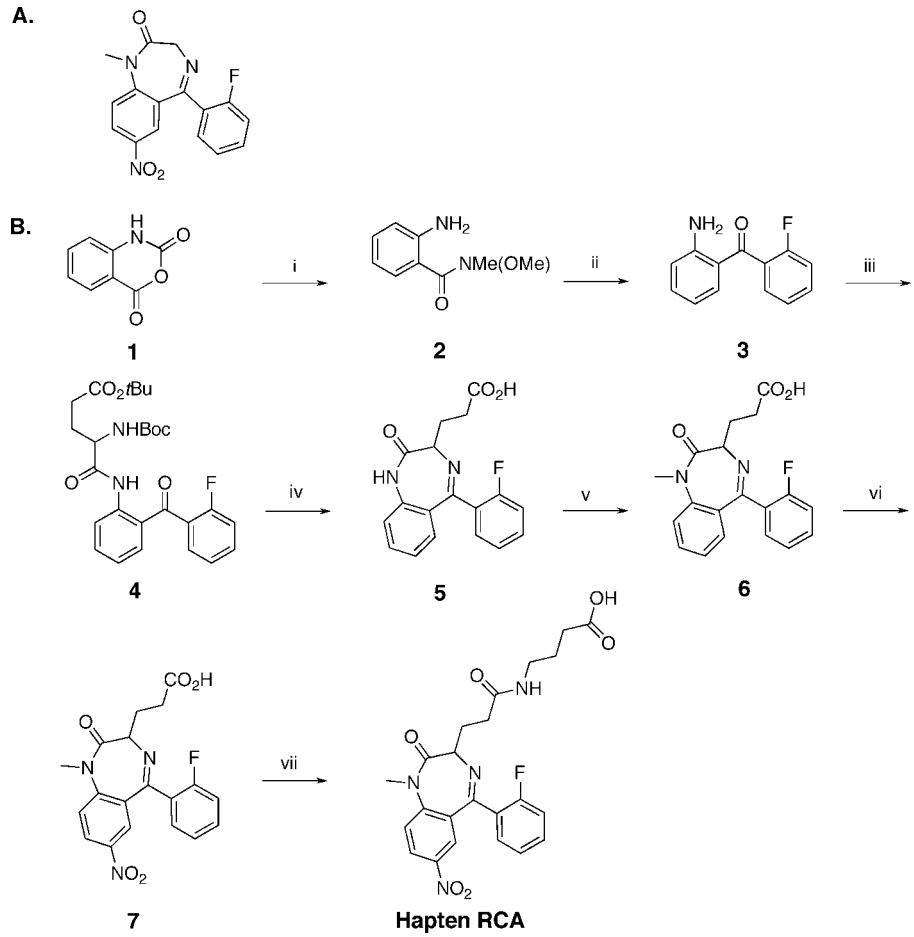

Figure 1. Design and preparation of flunitrazepam hapten RCA: (A) molecular structure of flunitrazepam; (B) synthesis of hapten RCA. Reagents and conditions are as follows: (i) *N*,*O*-dimethylhydroxylamine, EtOH; (ii) ^{n}BuLi, 2-fluorobromobenzene, −100 °C, THF, then HCl; (iii) Boc-Glu(O*t*Bu)-OH, HATU, DIEA, 75 °C, 1,2-dichloroethane; (iv) TFA/CH_2Cl_2, then pyridine, 75 °C; (v) NaH, 0 °C, THF, then CH_3I; (vi) KNO_3, fuming H_2SO_4, room temp, then ice-cold DCM/water; (vii) *tert*-butyl aminobutyrate hydrochloride, PyBOP, DIEA, DCM, then TFA/CH_2Cl_2.

Here, immunopharmacotherapy has been proven effective, and consequently, the pursuit of mAb therapeutics for nicotine and cocaine has advanced into clinical trials.[1,2,11]

All of the drugs previously targeted by immunopharmacotherapy produce gross symptoms of intoxication that obstruct an examination of more subtle drug-induced cognitive deficits. Alternatively, the CNS depressant flunitrazepam (Figure 1A) was selected for this study because of its ability to effect higher order cognitive processes and its suitability for immunopharmacotherapy as a DEA Schedule IV abused substance.[12] Flunitrazepam binds with high affinity to benzodiazepine sites (BZ_1 and BZ_2^+) on GABA receptors to allosterically modulate GABA neurotransmission.[13] The requirement for pre-existing GABAergic tone for flunitrazepam-induced intoxication reinforces the coabuse of flunitrazepam with other drugs by potentiating the effect of GABA receptor agonists.[14] Indeed, addicts often combine flunitrazepam with other substances to temper a given drug high or to self-medicate during drug withdrawal.[15−17] In such potentially lethal cases, the available post hoc treatment is limited to administration of the benzodiazepine site antagonist flumazenil or activated charcoal.[18] The more alarming use statistic is the dramatic rise of facilitated sexual assault using "rophies", in which even mild exposure to flunitrazepam spurs behavioral disinhibition.[18,19] In response, the World Health Organization moved this benzodiazepine from a Schedule IV to Schedule III class drug, and the DEA began to track flunitrazepam use statistics more closely.[20] While administration of flunitrazepam results in the sedation and muscle-relaxation characteristic of benzodiazepines, the concurrent hypnotic and amnesic effects have also promoted its abuse. Correspondingly, the ability of flunitrazepam to cause impairment at comparatively low doses[12] make flunitrazepam intoxication amenable for study using classical rodent behavioral assays. Targeting flunitrazepam by immunopharmacotherapy presents both a worthwhile and pharmacologically interesting endeavor; the former point concerns the social hazards inherent to its abuse, and the latter point relates to the necessity of preventing the sedative as well as cognitive effects of flunitrazepam.

Results

Generation of an Anti-Flunitrazepam Monoclonal Antibody. The synthesis of hapten RCA commenced from isatoic anhydride to generate 2′-fluoro-2-aminobenzophenone **3** in two steps (Figure 1B). Slight modifications of the published methods were made in order to increase the resulting yield; specifically, the intermediate Weinreb amide **2** was pretreated with ^{n}BuLi to form a lithium amide before it was added to preformed 2-fluorophenyllithium.[21] This procedure limited the formation of the major side product 2-butylcarbonylaniline and generated **3** in 57% yield. Coupling of **3** with a stoichiometric amount of Boc-Glu(O*t*Bu)-OH in the presence of HBTU at 75 °C over 2 days proved slow and inefficient, producing only <10% conversion; however, the reaction rate and the yield were increased by switching to HATU[22] as coupling reagent and using a 6-fold excess of Boc-Glu(O*t*Bu)-OH. By use of methods previously described by Hoffmann-LaRoche, deprotection of

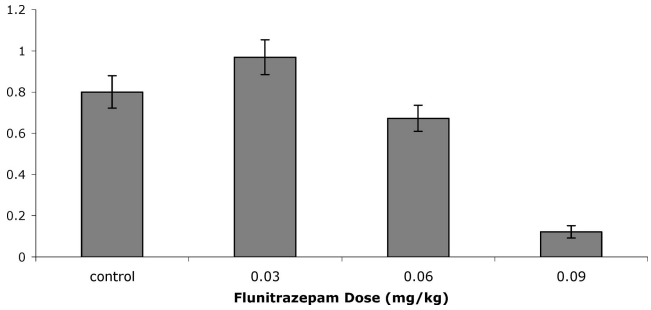

Figure 2. Flunitrazepam dose dependent impairment on rotarod performance. Approximately 20 min before testing, mouse groups received an ip injection of 0.03, 0.06, or 0.09 mg/kg flunitrazepam dissolved in 0.5% Tween-80 in isotonic saline, or an equivalent volume of 0.5% Tween-80 in isotonic saline. Values are expressed as the mean ± SEM of three balance attempts on the test day, with the speed of the rotating rod at fall for each attempt normalized to the subject's average peak performance during rotarod training.

the *tert*-butyl ester followed by heating in pyridine yielded the seven-membered ring in excellent yield.[23] Subsequent 1-*N*-methylation and *para*-nitration proceeded smoothly in the presence of the free carboxylic acid group of the linker to afford **7**.[24] The linker was extended to a total length of 10−12 Å by addition of an aminobutyrate moiety to give the hapten RCA. To generate the immunoconjugates, hapten RCA was coupled to BSA or KLH carrier proteins using EDC and sulfo-NHS activation, as previously reported.[25] MALDI-TOF analysis revealed that an average of 12 hapten molecules were conjugated to BSA.

Immunization of two 4-week old 129 GIX+ mice with RCA immunoconjugates and RIBI adjuvant generated specific antibody titers (>12800) to the RCA hapten. In this immunization procedure, four injections were delivered 3−4 days apart, and then 1 month later, a fifth injection containing 50 μg of immunoconjugate and 250 μg of alum was administered. Mice were bled 1 week after the fourth and fifth injections for monitoring of antibody titers and specificities using ELISA. Two months after the last bleed, a final booster injection of immunoconjugate (50 μg) was administered, followed by removal of the spleen 3 days later. The spleen cells were fused with a myeloma cell line to produce hybridomas according to standard techniques.[26] The hybridomas were cloned into 96-well plates and screened against the respective RCA−BSA conjugate by ELISA during the cloning process. Colonies producing antibodies that bound RCA−BSA were subcloned twice, after which 29 retained hapten binding. These were injected into mice to generate ascitic fluid, and IgG was obtained and purified using salt precipitation, anion exchange chromatography (DEAE), and affinity chromatography (protein G). In addition, the 29 members of the final panel were assessed for hapten binding and specificity to flunitrazepam using competition ELISA ($K_{d,app}$ = 24 μM to 200 nM). Of these antibodies, mAb RCA3A3 was shown to have the highest affinity for flunitrazepam ($K_{d,app}$ = 200 nM) and was selected for further in vivo study.

Optimization of in Vivo Flunitrazepam Dose. After undergoing rotarod training, drug naive mice were randomly divided into groups according to flunitrazepam dose (0.03, 0.06, and 0.09 mg/kg) and then tested on rotarod after drug injection. The normalized data for speed at fall of each subject were averaged across group, and a one-way ANOVA (group, 0.0, 0.03, 0.06, 0.09 mg/kg dose) confirmed the significant effect of drug dose on rotarod agility [$F(3,20) = 32.731$, $p < 0.0001$] (Figure 2). Mice receiving the 0.09 mg/kg drug dose were significantly impaired relative to vehicle control, 0.03 mg/kg, and 0.06 mg/kg groups ($p < 0.0001$) but were not sedated; thus, the 0.09 mg/kg dose was selected for subsequent experiments. Motor skills returned to within baseline levels in all mice 24 h after drug exposure (data not shown).

Table 1. Experimental Design for Mouse Group and Treatment Assignments[a]

	treatment	
group	0.5% Tween-80 in isotonic saline	flunitrazepam in 0.5% Tween-80 in isotonic saline
vehicle	VS	VF
RCA3A3	RS	RF
GNC92H2	GS	not included

[a] Of the three grouping categories, vehicle, RCA3A3, and GNC92H2, only vehicle and RCA3A3-immunized mice were subsequently challenged with flunitrazepam treatment.

Immunization with RCA3A3 Prevents Flunitrazepam-Induced Sedation. The rotarod test, which aided in the flunitrazepam dose selection, was also employed as an initial screen of the therapeutic value of RCA3A3. Administration of the flunitrazepam-based mAb prior to drug exposure attenuated the deterioration of motor skills caused by flunitrazepam intoxication. Three-way repeated measures ANOVA (group × treatment × balance trials in a session) confirmed the significance of flunitrazepam-induced impairment and RCA3A3-mediated protection (Table 2) in that there was a significant interaction between treatment and the repeated measure [$F(2,82) = 3.805$ ($p < 0.05$)]. Thus, two-way ANOVA (group × treatment) and Fisher's PLSD were implemented for comparisons within balance sessions and between groups, respectively. Intoxication significantly impacted motor ability of VF mice relative to VS, RS, and GS groups ($p < 0.005$), while immunization against flunitrazepam protected RF mice from drug effects compared to VF mice ($p < 0.0001$) (Figure 3). Importantly, the immunization procedure had little effect on mice as demonstrated by the comparison of the performance of RF mice to RS mice in the rotarod assay ($p = 0.95$).

Given the utility of the rotarod test in highlighting the potential of our antiflunitrazepam vaccine to influence agility and motor skills, we conducted a locomotor activity test in order to observe the time-sensitive progression of both flunitrazepam-induced sedation and RCA3A3-mediated protection. One-way repeated measures ANOVA revealed the significance of time interval on activity level in all locomotor activity recording sessions. Specifically, all mice demonstrated transient arousal to a novel environment, a trait inherent to the assay (Figure 4).[27] This arousal or hyperactivity decreased across time intervals and over subsequent sessions as mice became habituated to the testing cage. To confirm that locomotor activity data of VF mice portrayed the initial motor activation and then stereotyped sedative effects characteristic of benzodiazepines, interactions between interval and group or between interval and treatment were examined for the five intervals of the test session via three-way ANOVA (Table 2). Further comparative statistics using two-way ANOVA indicated that treatment had a significant main effect on locomotion in the first time interval [$F(1,42) = 6.044$, $p < 0.05$], which was attributed to the initial motor activation caused by flunitrazepam. A significant interaction between group × treatment ($p < 0.05$) persisted in the third through fifth time intervals, in which flunitrazepam caused sedation in the VF group but not in the immunized RF group.

Locomotor data analysis by three-way ANOVA with repeated measures (group × treatment × time interval) and the Student's *t* test proved that immunization with RCA3A3 prevented

Table 2. Statistical Analysis of Rotarod Agility and Locomotor Activity Test Results

Motor Activity, Rotarod		
Three-way ANOVA[a]	VS, VF, RS, RF	$F(2,82) = 3.805$ ($p < 0.05$)
Two-way ANOVA (group × treatment)	VF vs VS VF vs RF RF vs RS	$p < 0.005$ $p < 0.0001$ $p = 0.95$
Motor Activity, Locomotor		
Three-way ANOVA	VS, VF, RS, RF	$F(4,186) = 4.017$ ($p < 0.005$)[b] $F(4,186) = 10.656$ ($p < 0.0001$)[c] $F(4,186) = 4.017$ ($p < 0.05$)[d]
Three-way ANOVA[d]	VF vs VS VF vs RF RF vs RS	$F(4,92) = 8.645$ ($p < 0.0001$) $F(4,84) = 3.967$ ($p = 0.006$) $F(4,76) = 3.505$ ($p < 0.05$)
Student's t test[e]	VF vs VS VF vs RF RF vs RS	$p < 0.05, 0.05, 0.005$ $p < 0.05$, all $p \gg 0.05$, all

[a] (Group × treatment × balance trial), balance trials as repeated measure. [b] (Group × time interval × session). [c] (Treatment × time interval × session). [d] (Group × treatment × time interval), time interval as repeated measure. [e] p values shown for 3rd, 4th, and 5th intervals.

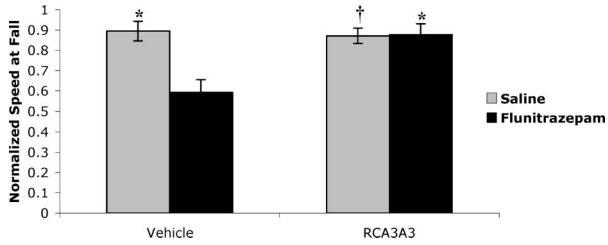

Figure 3. Effect of flunitrazepam intoxication and immunization with mAb RCA3A3 (RF, RS groups) on rotarod performance. Flunitrazepam (VF, RF; black bars) or 0.5% Tween-80 in isotonic saline (VS, RS; gray bars) ip injections were given 20 min prior to rotarod testing. Bars indicate the group mean ± SEM for all rotarod balance trials on the day of drug administration. Individual subject data were calculated by normalizing the speed of the rotating rod upon the subject's fall for all balance attempts on the test day to the average peak performance (e.g., mean speed upon fall) of that subject from the last training day. A repeated measures ANOVA was used to evaluate the statistical significance of rotarod impairment in VF mice compared VS, RS, and RF mice: (∗) $p < 0.05$; (†) $p = 0.058$.

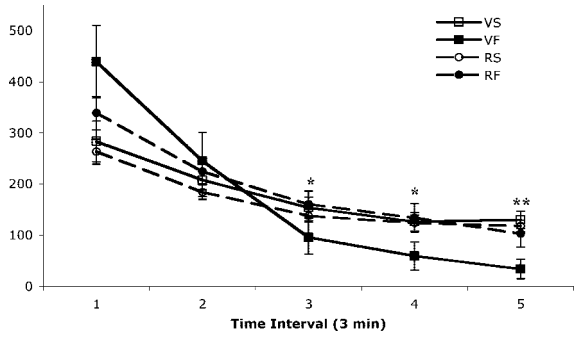

Figure 4. Flunitrazepam-induced sedation as depicted by locomotor activity response. Mice were passively immunized (iv) with mAb RCA3A3 (dashed line) or vehicle isotonic saline solution (solid line) 3 days before ip injection of flunitrazepam ((■) VF, (●) RF) or 0.5% Tween-80 in isotonic saline ((□) VS, (○) RS). Data, which reflect locomotor activity monitoring for 15 min following ip injection, are expressed as the group mean ± SEM of activity counts (photobeam breaks) summated across 3 min intervals for each mouse. Statistical significance is shown for the third through fifth time intervals: (∗) $p < 0.05$ and (∗∗) $p < 0.01$ for comparison of VF and VS mice; (†) $p < 0.05$ for comparison of VF and RF mice.

flunitrazepam-induced sedation (Table 2, Figure 4). Specifically, statistical analysis confirmed the significant differences in locomotor activity between VS and VF mice, between VF and RF mice, and between RF and RS mice. This latter result that the behavior of RS and RF mice is statistically distinguishable suggests that a portion of the injected flunitrazepam may have entered the CNS in RF mice. However, upon performing the Student's t test for between group comparisons at individual time intervals, the repeated measures ANOVA result for the RS versus RF comparison is refuted (Table 2). Analysis of shorter observation intervals allows for more sensitive detection of dynamic changes in activity level specific to group or treatment effects as opposed to overall behavioral trends. No statistically significant difference was found in locomotor activity for comparisons between VS and RF groups, RS and RF groups, and RS and VS groups when intervals were analyzed separately. This substantiated the conclusion that passive immunization alone imparted no significant change in locomotor activity (vide supra). The Student's t test was also employed to verify that immunization prevented flunitrazepam intoxication and the corresponding activity changes. Gratifyingly, the locomotor activity of VF mice was significantly altered in comparison to VS and RF mice in the third, fourth, and fifth time intervals (Table 2). In sum, when flunitrazepam was given to RCA3A3-immunized mice, drug-induced changes in locomotor activity were prevented (Figure 4).

Acquisition of Fear Memory in Flunitrazepam-Intoxicated and RCA3A3-Immunized Mice. Of the four testing days (habituation, conditioning, context test, and CS+ test), data were analyzed for total time spent freezing within each test session. There were no differences between the groups in freezing behavior during the habituation trials. In addition, all mice were examined for freezing and/or hyperactivity in response to the foot shocks during the conditioning session. The presence of altered activity in all mice reflected that flunitrazepam did not severely interrupt either proprioception or motor response to the aversive foot shock despite heavy sedation. It follows that changes in freezing behavior of intoxicated mice were directly attributable to disruption of associative fear memory, and the potential antinociceptive effect of flunitrazepam was not sufficient to minimize the perception of the shock as aversive. Lastly, to ensure that no negative effects resulted from the immunization procedure, one experimental group (GS) included mice immunized with the anticocaine mAb GNC92H2 and injected with saline (ip) as the treatment condition. The freezing behavior of GS, VS, and RS mice was statistically indistinguishable in the context test session, as evaluated by one-way ANOVA [group: $F(2,30) = 1.305$, $p > 0.05$] and two-way

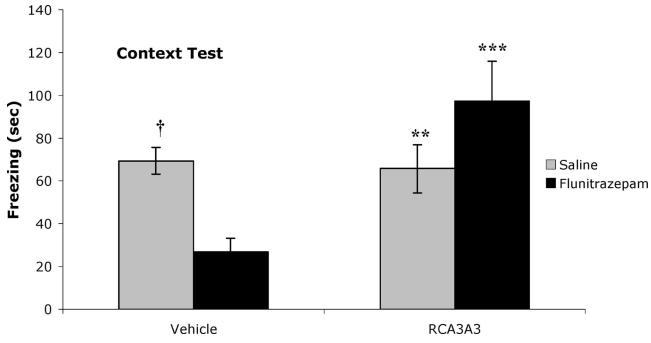

Figure 5. Fear behavior resulting from contextual conditioning in vehicle (left) and RCA3A3-immunized (right) mice. Gray bars depict ip injection of vehicle (0.5% Tween-80 in isotonic saline; VS, VF), and black bars indicate ip injection of flunitrazepam (dissolved in 0.5% Tween-80 in isotonic saline; VF, RF). Mice were fear conditioned to the cage context 20 min after ip injection and then tested for their contextual fear memory 24 h later. A decrease in freezing behavior reflects a disruption of fear memory consolidation. Data for time spent freezing over the entire context test session are presented as the mean ± SEM. Statistical significance, as calculated using the unpaired Student's *t* test, compares the freezing behavior of the VF group to that of VS, RS, and RF groups: (**) $p < 0.01$; (***) $p < 0.005$; (†) $p < 0.0001$.

Table 3. Statistical Analysis of Conditioned Fear Context Test Results

Three-Way ANOVA		
group	$F(1,38) = 8.543$ ($p = 0.0058$)[a]	
group × treatment	$F(1,38) = 10.578$ ($p < 0.005$)[a]	
Two-Way ANOVA		
	context: 1st interval	context: 2nd interval
group × time interval	$F(1,38) = 6.260$ ($p < 0.05$)	$F(1,38) = 8.760$ ($p = 0.0053$)
group × treatment	$F(1,38) = 12.026$ ($p = 0.0013$)	$F(1,38) = 7.571$ ($p < 0.01$)
Two-Way ANOVA of repeated measures, group × time interval		
RF vs VS, RS	statistically indistinguishable	
VF vs VS	$F(1,20) = 22.162$ ($p = 0.0001$)	
VF vs RF	$F(1,18) = 12.838$ ($p < 0.005$)	

[a] Group × treatment × time interval, repeated measure.

repeated measures ANOVA [group × time interval: $F(1,19) = 0.508$, $p = 0.48$]. Furthermore, GS mice displayed freezing behavior similar to VS and RS mice in the CS+ test [group × time interval: $F(1,19) = 0.185$, $p > 0.5$]. In light of this data, we concluded that mAb therapy conferred minimal effects on fear conditioning and memory, and as such, the GS group data were excluded from subsequent analyses of conditioned fear test data.

Restoration of Contextual Fear Memory Formation. Conditioned mice were examined for their contextual memory of the environment in which they received the tone-foot shock pairing 24 h prior (Figure 5). There were dramatic differences in freezing behavior between mice conditioned during flunitrazepam exposure (VF) and mice receiving the immunotherapy (RF), as confirmed by three-way ANOVA, two-way ANOVA (group × treatment) for individual time intervals, and two-way repeated measures ANOVA (group × time interval) (Table 3). Specifically, VF mice failed to freeze in response to the contextual stimulus, whereas control and immunized mice froze upon re-exposure to the conditioning box during context test sessions (Figure 5). The freezing behavior of RF mice was restored to levels statistically indistinguishable from VS and RS groups as evaluated by ANOVA and unpaired Student's *t* tests (data not shown), confirming that immunization with RCA3A3 effectively blocked flunitrazepam-induced impairment of contextual memory. Analysis by Student's *t* test for each time interval supported the above conclusions in that VF mice showed significantly less freezing relative to VS mice (first interval, $p < 0.0001$; second interval, $p = 0.016$) and RF mice (first interval, $p = 0.0017$; second interval, $p < 0.005$). Any differences in freezing behavior between RF, VS, and RS groups were statistically undetectable, implying that immunization alone incurred no changes to freezing response and that immunization with RCA3A3 before flunitrazepam exposure protected the formation of contextual fear memory.

Immunization Protects Associative Aversive Learning Cued Stimulus. The CS+ test assessed the presence of a lingering associative memory between the tone and the aversive shock despite the novel context. CS+ test session data were divided into two parts with the first 3 min representing baseline activity in the novel context (Figure 6A) and the last 3 min testing memory of the cued stimulus by exposing mice to the conditioned tone (Figure 6B). As with the context session, there was no significant difference in freezing behavior between GS and RS mice either before the tone [group × time interval, $F(1,17) = 3.350$, $p > 0.05$] or during the tone [group × time interval, $F(1,17) = 0.022$, $p > 0.5$]. All mice behaved similarly during the first 3 min of the CS+ session in that they exhibited hyperactive, exploratory activity in response to the novel environment and thus spent minimal time freezing regardless of group or treatment (Table 4, Figure 6A).

During the second 3 min of the CS+ session during which mice were exposed to the conditioned stimulus, freezing behavior reappeared in all mice but those in the VF group (Figure 6B). Results of a three-way ANOVA with group and treatment as between factors and time interval as the within factor justified further statistical analysis (Table 4). VF mice exhibited significantly less freezing behavior than mice in all other groups (H_O, VF = VS, $p < 0.01$; H_O, VF = RF, $p = 0.014$), while there was no statistically significant difference in behavior between RF, RS, and VS mice, as concluded using the Student's *t* test.

Discussion

To test the hypothesis that passive immunization offers an effective treatment against drug-induced cognitive impairment, we embarked upon the design of a hapten capable of eliciting antibodies that could bind flunitrazepam with high affinity (Figure 1A). This process followed from previous experience in the preparation of haptens for other drugs of abuse including cocaine, nicotine, and Δ^9-tetrahydrocannabinol.[25,28−32] The molecular features of flunitrazepam, specifically the rigid hydrophobic core, were expected to elicit complementary aromatic residues that are favored in antibody binding pockets.[33] In addition, the general structure of flunitrazepam-like hapten RCA incorporated a linker at the C-3 position of the 1,4-benzodiazepine-2-one ring for covalent attachment to carrier proteins (Figure 1B). An aminobutyrate moiety was incorporated into the linker given that increased linker length has been shown to afford superior hapten recognition upon immunization.[29,34]

To examine the efficacy of flunitrazepam binding and sequestration by RCA3A3, a dose−response relationship for flunitrazepam-induced locomotor effects was generated in a mouse model. While reports of flunitrazepam-induced immobilization, decreased exploratory behavior, and overall sedation were used for initial drug dose parameters,[35,36] the limited number of studies using this specific benzodiazepine necessitated

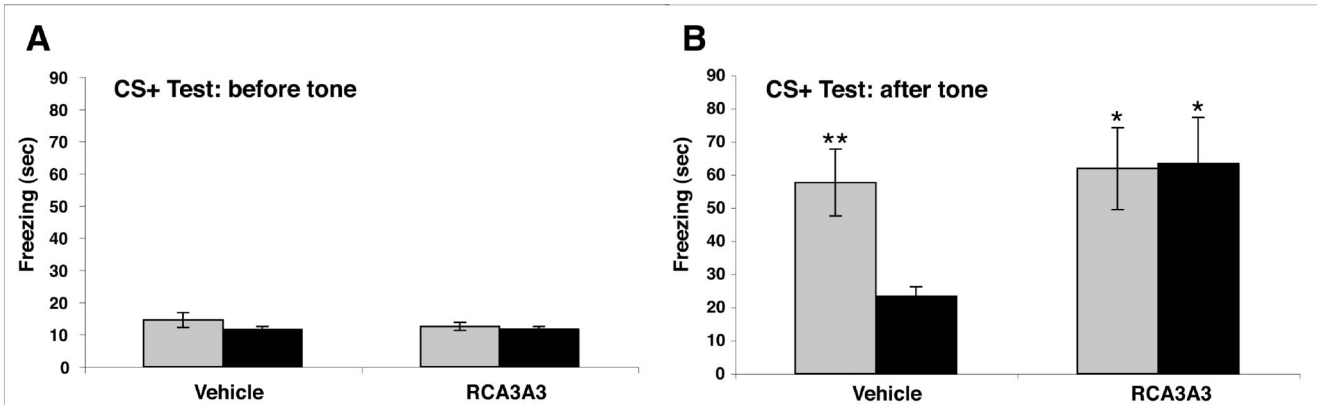

Figure 6. Freezing response to the cued stimulus in the CS+ test. Vehicle (VS, VF) and immunized (RS, RF) mice were evaluated for their cued fear memory 48 h after ip injection (VF, RF, flunitrazepam, black bars; VS, RS, vehicle, gray bars) and fear conditioning. All mice spent minimal time freezing before presentation of the tone (A), which indicates that the cage was adequately disguised to mask any contextual cues. Upon exposure to the cued stimulus (tone) during the second time interval (B), VS, RS, and RF mice showed significant increases in freezing behavior while VF mice display less freezing compared to control levels. Data are expressed as the mean ± SEM of time spent freezing within either interval (A or B) of the CS+ test, and statistical significance represents results from the unpaired Student's *t* test comparing VF group data to VS, RS, and RF group data: (*) $p < 0.05$; (**) $p < 0.01$.

Table 4. Statistical Analysis of Conditioned Fear CS+ Test Results

Conditioned Fear: CS+ Test, Before Tone	
Three-Way ANOVA[a]	
group × time interval	$F(1,37) = 0.358$, ($p > 0.5$)
group × treatment	$F(1,37) = 0.474$, ($p = 0.495$)
treatment × time interval	$F(1,37) = 1.621$, ($p = 0.211$)
Conditioned Fear: CS+ Test, After Tone	
Three-Way ANOVA[a]	
group × time interval	$F(1,38) = 4.168$ ($p < 0.05$)
Student's *t* Test	
H_0: VF = VS	$p < 0.01$
H_0: VF = RF	$p = 0.014$
H_0: RF = VS, VS = RS, RF = RS	statistically indistinguishable

[a] Group × treatment × time interval.

further pharmacodynamic characterization of drug effects in mice. The rotarod assay for motor coordination was used to create a qualitative dose-motor response curve and thereby confirms reports in the literature of the appropriate dose of flunitrazepam conferring substantial locomotor impairment. The dose range tested here illustrated the biphasic dose response curve common to benzodiazepines, ethanol, and other abused substances in that agility and coordination improved with a low flunitrazepam dose (0.03 mg/kg), while motor impairment and sedation resulted at higher doses (Figure 2).[37] Doses exceeding 0.09 mg/kg were not explored, as it was critical that sedated mice maintain consciousness for assessment of the drug-induced memory deficits in behavioral tests. The rotarod assay thus served as an appropriate behavioral screen for measuring motor deficits upon intoxication and permitted selection of the 0.09 mg/kg drug dose for subsequent psychomotor testing.

The primary behavioral paradigms employed to investigate mAb efficacy in vivo have included tests of locomotor activity and drug self-administration. Analogous to these studies, locomotor activity and agility were measured to assess flunitrazepam-induced sedation and the corresponding benefit derived from passive immunization with mAb RCA3A3. Intoxicated mice (VF) exhibited some hyperlocomotion during the first time interval of the locomotor assay, reflecting both the initial motor activating effect of the drug, which precedes sedation, and the anxiolytic effect of benzodiazepines, with heightened "fearlessness" translating to increased exploratory behavior. When flunitrazepam was administered to RCA3A3-treated mice (RF), these drug-induced changes in locomotor activity were prevented (Figure 4). Similarly, RCA3A3 also protected mice from the acute deterioration in motor coordination following drug exposure, as assessed by the rotarod test (Figure 3). Given the severe memory deficits produced by flunitrazepam alongside this demonstrated motor impairment, testing of the in vivo efficacy of RCA3A3 also presented an ideal scenario for extending immunopharmacotherapeutic strategies to the prevention of drug-mediated cognitive impairment. Thus, following rotarod and locomotor activity testing, the conditioned fear assay was implemented to then examine the potential of mAb RCA3A3 to protect normal memory consolidation in drug-exposed mice.

Conditioned fear is commonly used to probe the neural mechanisms of memory. Not only is fear memory comparatively robust, facilitating comparison between initial memory consolidation versus long-term memory, but also fear-potentiated startle experiments are easily adapted to rodent models for studies of learning and memory processes. More importantly, the conditioned fear assay has previously been implemented to show the propensity of benzodiazepines or benzodiazepine site ligands to elicit anterograde amnesia as assessed by weakened associations between an environmental context and cue to an aversive foot shock in mice and in other animal models.[38−41] In this study, we determined the ability of an antiflunitrazepam mAb that is inherently localized to the periphery to protect fear memory of such associations despite flunitrazepam administration during the conditioning phase of the experiment. Subjects administered drug or saline (control) injections were twice exposed to a 30 s tone and white light, both co-terminating with a 1.0 mA foot shock during the conditioning session. Upon re-exposure to the foot shock chamber 24 h after conditioning, mice that spend a substantial amount of time freezing possess intact memory of the aversive foot shock delivered in the conditioning cage. However, any flunitrazepam-induced deficit in fear learning translates to a blunted freezing response in mice. Our results were in agreement with the initial hypothesis because drug-naive mice (VS) spent more time freezing to the contextual stimuli than intoxicated mice (VF). This flunitrazepam-induced deficit in the retrieval of fear memory seen in VF mice was not observed in RF mice. Passive immunization with RCA3A3

enabled the formation and retention of memory for the shock-associated context as shown by their normal freezing response (Figure 5).

Because the conditioned fear assay discriminates between contextual hippocampal-dependent and cued hippocampal-independent learning,[42] it has been used previously to show that the effect of benzodiazepines on fear conditioning may be specific to the environmental context,[43,44] a conclusion supported by our results. However, we obtained similar results for the CS+ test as in the context test. Analysis of freezing behavior during tone presentation in CS+ sessions confirmed the significant detrimental effect of flunitrazepam on fear memory, which was blocked by pretreatment with RCA3A3 (Figure 6B). This finding challenges previous studies that found benzodiazepine-mediated disruption of associations between the conditioned stimulus (cue) and unconditioned stimulus (foot shock) to be under contextual control.[43,44] As evidenced by the absence in freezing of VF mice to the tone, flunitrazepam induced inhibition of fear memory of the cued stimulus-foot shock association. VS, RS, and RF mice all exhibited freezing upon tone presentation, or retrieval of intact associative memory between the tone and the aversive shock, regardless of the novel context. There was no significant difference in freezing behavior between RF, RS, and VS groups, suggesting that treatment with RCA3A3 protected memory of the aversive stimulus and the associated auditory cue. In addition, passive immunization had no detectable effect on general anxiety-like behavior given the similar fear responses shown by VS, RS, and GS mice in both the context and CS+ tests. The first interval of the CS+ test, which entailed monitoring the exploratory behavior of all mice in response to the disguised cage, served as an internal assay control (Figure 6A). All groups displayed similar behavior before exposure to the conditioned tone.

The value of applying immunopharmacotherapy to the treatment of cognitive dysfunction has been highlighted by the procurement of an anti-flunitrazepam mAb capable of attenuating both the sedative and amnesic central effects of intoxication. In terms of therapeutic value, mAb RCA3A3 blocked the appearance of contextual learning impairments caused by exposure to a benzodiazepine and it preserved the subsequent retrieval of fear memory for the conditioned cue, or tone, when it was presented outside the conditioning context. This result, a finding of pharmacological relevance in addition to validation of our original hypothesis, refutes the assumption that the conditioned-unconditioned stimuli association was solely under contextual control. Because mAb RCA3A3 abolished deficits in both motor activity and memory, this study illustrates that immunopharmacotherapeutic strategies possess applications beyond the treatment of addictive behavior and the prevention of gross symptoms of intoxication, such as overdose, hyperlocomotion, and seizures. Instead, immunopharmacotherapy represents a valid approach for addressing even the more subtle cognitive effects incurred by drug exposure.

Experimental Section

Hapten Synthesis. ^{1}H and APT ^{13}C NMR spectra were recorded on a Bruker DRX-500 spectrometer, and chemical shifts (ppm) were referenced to internal $CDCl_3$ standard (^{1}H, 7.26 ppm; ^{13}C, 77.0 ppm). Carbon signals marked with an asterisk represent methyl and methyne carbons as determined by APT experiments. High-resolution mass spectra were recorded using electrospray ionization (ESI) or MALDI-TOF techniques. All chemical reagents and solvents were from Aldrich Chemical Co., unless otherwise noted, and used without further purification. Glassware and solvents were dried by standard methods. Flash chromatography was performed on silica gel 60 (230−400 mesh) and thin layer chromatography on glass plates coated with a 0.02, 0.5, or 1.0 mm layer of silica gel 60 F-254. Analytical RP-HPLC was performed on a Hitachi Elite LaChrom L-2000 series HPLC chromatograph using a Vydac 218TP5415 column at a flow rate of 1 mL/min, with detection at 254 nm during a linear gradient of 10−95% acetonitrile/0.09% TFA over 40 min at 35 °C. Preparative RP-HPLC was performed using a custom-packed, 2-in. bore C18 column at a flow rate of 10 mL/min, with detection at 254 and 280 nm; fractions were analyzed off-line using an ABI/Sciex 150EX single quadrupole mass spectrometer. Compounds **2** and **3** have been previously reported, and their spectral data were consistent with prior literature.[21]

2′-Fluoro-2-aminobenzophenone (3). To a stirred solution of **2** (4.90 g, 27.2 mmol) in THF at −100 °C under argon was added 1.5 M *n*BuLi in hexanes (18.1 mL, 27.2 mmol). In a separate flask, 1.5 M *n*BuLi in hexanes (18.1 mL, 27.2 mmol) was added to a solution of 2-fluorobromobenzene (4.76 g, 27.2 mmol) in THF at −100 °C under argon. After 10 min, the solution of 2-fluorophenyllithium was transferred to the first flask containing the lithium amide via cannula. After a further 10 min of stirring, 1 M HCl (aq) was added and the resulting mixture was extracted with ethyl acetate. The organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified on silica gel with EtOAc/hexanes (15:85) as the eluent to give **3** (3.33 g, 57%).

***tert*-Butyl 4-(*tert*-Butoxycarbonylamino)-5-(2-(2-fluorobenzoyl)phenylamino)-5-oxopentanoate (4).** Boc-Glu(O*t*Bu)-OH (3.38 g, 11.1 mmol) and HATU (4.24 g, 11.1 mmol) were suspended in anhydrous 1,2-dichloroethane (20 mL). Diisopropylethylamine (5.8 mL, 33.3 mmol) was added, and the mixture was stirred under an atmosphere of argon at ambient temperature until a nearly clear yellow solution was obtained (15 min). **3** (400 mg, 1.86 mmol) was added, and the mixture was heated at 75 °C for 16 h, after which the mixture was cooled, diluted with ethyl acetate (40 mL), and transferred into a separatory funnel. The organic phase was washed consecutively with water, saturated aqueous sodium bicarbonate (3×), 10% aqueous citric acid (2×) and dried over anhydrous sodium sulfate. The organic solvents were evaporated under reduced pressure and the dark syrupy residue was purified by column chromatography on silica gel using hexane−ethyl acetate (5:1 → 3:1 → 2:1, v/v) as eluent, yielding 0.74 g (80%) of **4** as yellow syrup. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 11.85 (s, 1H), 8.76 (d, 1H, J = 8.4 Hz), 7.58 (t, 1H, J = 7.6 Hz), 7.55−7.48 (m, 2H), 7.44 (dt, 1H, J = 1.6, 7.5 Hz), 7.26 (dt, 1H, J = 0.7, 7.6 Hz), 7.16 (t, 1H, J = 9.1 Hz), 7.07 (t, 1H, J = 7.5 Hz), 5.45 (d, 1H, J = 6.1 Hz), 4.32 (m, 1H), 2.42 (m, 2H), 2.28 (m, 1H), 2.05 (ddd, 1H, J = 7.4, 14.8, 15.0 Hz), 1.43 (s, 18H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 196.5, 172.3, 171.3, 160.4, 158.4, 155.5, 140.6, 135.3*, 134.0*, 132.9*, 132.9*, 130.2*, 127.6, 127.5, 124.2*, 122.8, 122.6*, 120.8*, 116.3*, 116.1*, 80.8, 80.2, 56.0*, 32.0, 28.2*, 28.0*, 27.4. FTMS calcd for $C_{27}H_{33}N_2O_6F$ $[M + Na]^+$: 523.2220, found 523.2230.

3-(5-(2-Fluorophenyl)-2-oxo-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)propanoic Acid (5). **4** (345 mg) was dissolved in dichloromethane (10 mL), and trifluoroacetic acid (10 mL) was added while stirring. Stirring was continued for 1 h, after which the volatile components where evaporated under reduced pressure. The dark syrupy residue was dissolved in pyridine (10 mL) and heated at 75 °C for 14 h. The reaction was monitored by TLC (10:1 CH_2Cl_2/MeOH) and ESI-Q MS. Pyridine was evaporated under vacuum and the dark syrupy residue was purified by column chromatography on silica gel using dichloromethane−methanol (20:1, v/v) as eluent, yielding **5** (221 mg, 98%) as an amber syrup. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.90 (d, 1H, J = 4.2 Hz), 7.53 (dt, 1H, J = 1.4, 7.5 Hz), 7.49 (m, 1H), 7.41 (m, 1H), 7.20 (m, 3H), 7.12 (t, 1H, J = 7.6 Hz), 7.03 (t, 1H, J = 9.2 Hz), 3.73 (dd, 1H, J = 5.2, 8.1 Hz), 2.74 (m, 2H), 2.61 (ddd, 1H, J = 6.6, 14.5 Hz), 2.46 (ddd, 1H, J = 7.1, 14.0 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.8, 171.8, 167.0, 161.4, 159.4, 137.2*, 132.2*, 132.1*, 131.6*, 130.1*, 128.1, 127.3, 127.2, 125.3*, 124.3*, 124.3*, 124.0*, 121.3*, 116.2*, 116.1*, 61.8*, 53.4, 30.6, 25.7. FTMS calcd for $C_{18}H_{15}N_2O_3F$ $[M + H]^+$: 327.1145, found 327.1125.

3-(5-(2-Fluorophenyl)-1-methyl-2-oxo-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)propanoic Acid (6). A 60% suspension of NaH in mineral oil (84 mg, 2.1 mmol) was washed thrice with hexane, suspended in dry THF, and added to a stirred solution of the **5** (229 mg, 0.70 mmol) in dry THF (1 mL) at 0 °C. The mixture was stirred for 10 min at 0 °C, and methyl iodide (175 μL, 2.8 mmol) was added via syringe. The mixture was stirred at 0 °C for 1 h, then allowed to warm to ambient temperature and stirred for 1 h. The excess sodium hydride was quenched by addition of glacial acetic acid (500 μL), and the volatile components were evaporated under vacuum. The dark gummy residue was triturated with dichloromethane−methanol (10:1, v/v), and the resulting suspension was filtered through a pad of silica gel. The yellow filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography on silica gel using dichloromethane−methanol (20:1 → 10:1, v/v) as eluent, yielding **6** (153 mg, 64%) as light-yellow syrup, which solidifies upon standing. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 12.08−10.36 (br s, 1H), 7.61 (dt, 1H, J = 1.7, 7.5 Hz), 7.55 (m, 1H), 7.43 (m, 1H), 7.35 (d, 1H, J = 8.2 Hz), 7.22 (m, 2H), 7.16 (t, 1H, J = 7.5 Hz), 7.03 (m, 1H), 3.69 (dd, 1H, J = 5.5, 7.6 Hz), 3.45 (s, 3H), 2.81 (m, 1H), 2.70 (m, 2H), 2.60 (ddd, 1H, J = 6.6, 14.0 Hz), 2.41 (m, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 178.1, 169.9, 166.3, 161.5, 159.5, 142.4, 132.2*, 132.2*, 131.8*, 131.4*, 131.4*, 129.9, 129.0*, 126.9, 126.8, 124.4*, 124.3*, 124.3*, 121.4*, 116.2*, 116.0*, 61.9*, 35.3*, 30.7, 26.1. FTMS calcd for $C_{19}H_{18}N_2O_3F$ [M + H]$^+$: 341.1301, found 341.1311.

3-(5-(2-Fluorophenyl)-1-methyl-7-nitro-2-oxo-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)propanoic Acid (7). KNO_3 (59 mg, 0.58 mmol) was added to a stirred solution of **6** (99 mg, 0.29 mmol) in fuming H_2SO_4 (2 mL), and the mixture was allowed to stir at ambient temperature (4 h). Small (∼20 μL) aliquots were taken after 2 h of stirring and partitioned between dichloromethane and water (∼200 μL each). The organic layer was carefully separated off with a pipet, dried over Na_2SO_4, passed through a small pad of silica gel, and evaporated. The residue was dissolved in 100 μL of methanol and analyzed by ESI-Q MS. When the reaction was judged as complete, the mixture was transferred to an ice-cold mixture of dichloromethane and water. The organic layer was dried over Na_2SO_4, concentrated, and used toward the next step without further purification.

4-(3-(5-(2-Fluorophenyl)-1-methyl-7-nitro-2-oxo-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)propanamido)butanoic Acid (Hapten RCA). A solution of the crude **7**, PyBOP (170 mg, 0.33 mmol), and diisopropylethylamine (130 μL, 0.75 mmol) in dichloromethane (3 mL) was stirred under an atmosphere of argon for 15 min. *tert*-Butyl aminobutyrate hydrochloride (65 mg, 0.33 mmol) was added, and the reaction mixture was allowed to stir for 3 h. The mixture was partitioned between EtOAc and water, and the organic layer was washed consecutively with saturated aqueous sodium bicarbonate (3×) and 10% aqueous citric acid (2×), then dried over anhydrous sodium sulfate and evaporated. The residue was purified by flash chromatography on silica gel with dichloromethane−acetone (3:1) as the eluent to give the *tert*-butyl protected intermediate, which was treated with 50% TFA/CH_2Cl_2 (2 mL) for 1 h. The volatile components were evaporated, and the syrupy residue was purified by preparative reverse-phase HPLC using a linear gradient of 15% acetonitrile/0.09% TFA to 40% acetonitrile/0.09% TFA in 0.1% TFA/water over 30 min while monitoring at 254 and 280 nm. The fractions containing the target material were pooled and lyophilized to give RCA hapten (46 mg, 30% over three steps from **6**). The purity of the product was checked by analytical HPLC and found to be greater than 95%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 10.12 (br s, 1H), 8.50 (dd, 1H, J = 2.6, 5.8 Hz), 8.36 (m, 1H), 7.64 (t, 1H, J = 7.5 Hz), 7.43 (d, 1H, J = 8.3 Hz), 7.16−7.29 (m, 3H), 6.97 (m, 1H), 3.74 (m, 1H), 3.48 (s, 3H), 3.32 (m, 2H), 2.55 (m, 4H), 2.35 (t, 2H, J = 6.8 Hz), 1.82 (p, 2H, J = 6.7 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 176.7, 175.1, 169.5, 164.6, 162.8, 157.2, 144.3, 142.7, 132.9*, 128.8*, 128.4, 127.9*, 127.8*, 127.6*, 127.6*, 127.4, 125.0*, 122.0*, 117.7*, 117.5*, 62.7*, 39.3, 35.7*, 32.5, 31.2, 27.3, 24.2. FTMS calcd for $C_{23}H_{23}N_4O_6F$ [M + H]$^+$: 471.1680, found 471.1685.

Preparation of Immunoconjugates. The RCA hapten (5 mg) was treated with 1.3 equiv of 3-sulfo-*N*-hydroxysuccinimide and 1.2 equiv of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride for 24 h in DMF. This mixture was then added to solutions (5 mg/mL) of keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) in PBS (50 mM, pH 7.4) and allowed to react for 24 h at 4 °C. The resulting conjugate was used without further purification.

Hydridoma Production. Hybridoma production followed standard protocols.[26]

Flunitrazepam Competition ELISA. An ELISA plate (Costar, 96-well) was coated uncovered overnight at 37 °C with 25 μL of the RCA−KLH conjugate. To this, methanol (50 μL/well) was added and allowed to sit for 5 min at room temperature. The methanol was then removed from the wells, and the plates were allowed to dry. Wells were then blocked with 50 μL of Blotto (5% skim milk powder in PBS) for 5 min at 37 °C. Typically, 25 μL of a solution containing a 1:100 dilution of mAb in Blotto along with varied serial dilutions of flunitrazepam (24 μM to 24 nM) was then added and incubated for 1 h at 37 °C. After the mixture was washed, 25 μL of a 1:1000 dilution of a goat anti-mouse/horseradish peroxidase conjugate in Blotto was added and incubated for 30 min. The plate was developed with the colorimetric reagent 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) for 30 min and the absorbance measured on an ELISA plate reader at λ = 405 nm. Binding constants were determined by analysis of plots of A_{405} versus flunitrazepam concentration. The $K_{d,app}$ was defined as the concentration at which A_{405} was half-maximal.

In Vivo Testing of RCA3A3. Subjects. Female CD-1 mice (20−40 g; dose determination, N = 24 mice; passive immunization, N = 110 mice) were purchased from a breeding colony at The Scripps Research Institute at 6 weeks of age. Animals were housed four per cage in a temperature-controlled vivarium under a reversed 12 h/12 h light/dark cycle (lights off at 06:00 h). Food and water were available ad libitum throughout the study. All experiments were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute and conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Every effort was made to reduce the number of animals used.

Flunitrazepam. In all cases, flunitrazepam [5-(2-fluorophenyl)-1,3,dihydro-1-methyl-7-nitro-2*H*-1,4-benzodiazepin-2-one)] (Sigma) was prepared in 0.5% Tween-80 in sterile 0.9% saline and administered by intraperitoneal injection.

Determination of Flunitrazepam Dose. One week after arrival, animals were weighed and randomly divided into four experimental groups (n = 6) based on drug dose, administered by intraperitoneal injection: vehicle with 0.5% Tween-80 in isotonic saline, 0.03 mg/kg flunitrazepam in 0.5% Tween-80 in isotonic saline, 0.06 mg/kg flunitrazepam in 0.5% Tween-80 in isotonic saline, and 0.09 mg/kg flunitrazepam in 0.5% Tween-80 in isotonic saline. The motor impairment conveyed by a particular drug dose was assessed via the rotarod test. Mice were trained on the rotarod 3 consecutive days prior to drug administration. On the fourth test day, subjects were injected with drug (or saline) and then returned to their home cages for 20 min before rotarod testing. Rotarod testing was repeated 24 h after drug administration to ensure motor activity had returned to baseline ability.

Effect of Passive Immunization against Flunitrazepam. One week after arrival, animals were labeled, weighed, and subjected to intravenous catheterization. Detailed methods on surgery and apparatus are described elsewhere.[27] After a 5 day recovery period, mice were randomly assigned to the following experimental groups (n = 22, Table 1): VS, vehicle control, saline (ip); VF, vehicle control, saline (iv), flunitrazepam (i.p.); GS, GNC92H2 (iv), saline (ip); RS, RCA3A3 (iv), saline (ip); and RF, RCA3A3 (iv), flunitrazepam (ip). The GS test group, in which mice were immunized with the anti-cocaine mAb GNC92H2, was included as a control for effects of antibody administration. The animals

were treated and tested in Latin square fashion. Flunitrazepam solution, prepared in 0.5% Tween-80 in isotonic saline (at a dose appropriate for ip administration, about 0.01 mg/mL) was injected 3 days after passive immunization or saline iv infusion. Passive immunization involved attaching a 4-in. polyethylene tube to the in-dwelling catheter on the animal's back and then slowly delivering the bolus infusion of antibody or saline through the tube over the course of ~1 min.

Of the original test groups (VS, VF, RF, RS, GS; $n = 22$ per test group) half of the mice were trained on the rotarod assay before receiving drug or saline injection and subsequent rotarod testing. The remaining mice were habituated to the locomotor activity cages and conditioned fear setup. Upon drug or saline injection, these mice were then tested using the locomotor activity and conditioned fear behavioral assays to examine the ability of RCA3A3 to prevent flunitrazepam-induced cognitive impairment.

Immunization. Passive immunization was conducted by a bolus infusion of mAb (10.2 mg/kg, iv in a volume of 1 mL/kg) before onset of behavioral testing or 3 days before flunitrazepam treatment or control saline injection. Animals in test groups receiving the nonspecific antibody (iv) were infused with the anti-cocaine mAb GNC92H2[25] at the same dose and volume. Control groups were infused with isotonic saline (1 mL/kg, iv). The infusion was delivered at a slow flow over 1 min, and antibody infusions were followed by a small amount of saline to clear the catheter of any antibody and to ensure that the entire amount reached the jugular vein. Animals were then returned to their home cages.

Rotarod Testing. The rotarod (IITC, Inc., Life Sciences, Woodland Hills, CA) consisted of a rubber-matting-covered 3.8 cm diameter rod suspended in a test box and divided into five lanes for simultaneous testing of mice. The rotarod treadmill was programmed to reach a top speed of 30 rpm via a constant acceleration of 10 rpm per minute. The chamber and rotarod were cleaned between mice. All test groups of mice were randomly subdivided into five and sequentially placed on the rotarod (one mouse per lane). Once all five mice in a subgroup were situated on and acclimated to balancing on the rod, the rod was set to accelerate at a constant rate of 10 rotations/min^2 over the course of 3 min to a maximum speed of 30 rpm. Upon falling from the rod, mice crossed through a light beam sensor located just below the rod, and the resulting beam break was recorded by the computer. After all five mice had fallen, they were immediately placed back on the rod for another attempt. One session consisted of three consecutive balance attempts before proceeding to the next set of five mice. Recordings were measured in terms of rod speed at fall, and a speed of 30 rpm was recorded for mice remaining on the rotarod for the entire 3 min. The training process was repeated for a total of three sessions per day over 3 consecutive days. The data from the last day (three sessions of three balance attempts per session) were averaged for each mouse to calculate individual peak baseline performance. On the fourth day, mice underwent one session of rotarod testing 15−20 min after administration of flunitrazepam or saline. The rotarod balance test was repeated in the same manner (i.e., one session, with saline injections substituting for drug injections) 24 h after drug administration. This last session on the fifth day was implemented to verify that rotarod performance returned to baseline levels. For data analysis, the speed of the rotarod at fall for each balance attempt of the test day was divided by the average peak baseline performance of the individual mouse (calculated from the last day of rotarod training). These normalized data for each mouse were then averaged across test group (Figures 2 and 3).

Locomotor Activity Testing. Locomotor activity was measured in Plexiglas cages (42 cm × 22 cm × 20 cm) placed into frames (25.5 cm × 47 cm) mounted with two levels of photocell beams at 2 and 7 cm above the bottom of the cage (San Diego Instruments, San Diego, CA). These two sets of beams allowed for the recording of both horizontal (locomotion) and vertical (rearing) behavior. A thin layer of bedding material was scattered across the bottom of the cage. On the 2 days preceding drug administration, mice received daily locomotor box habituation sessions to minimize transient arousal during the test session produced by exposure to the novel environment. Mice were placed singly in the activity boxes for 15 min of monitoring directly following saline or drug injection, and then subjects were either returned to their home cages (locomotor activity habituation days) or transferred to the conditioned fear apparatus (test days). All mice received ip injections of isotonic saline (10 mL/kg) immediately prior to all habituation sessions as well as before the locomotor activity retest session in order to reproduce the environment of drug administration.

Cued and Contextual Conditioned Fear Testing. The conditioning system consisted of a sound-proofed box of white interior housing a Freeze Monitor chamber (San Diego Instruments). The Plexiglas conditioning chamber (26 cm × 26 cm × 17 cm) was equipped with a speaker, a light, and a shockable grid floor under which orange oil-scented cedar chips were scattered. The stainless steel rod grid was connected to a shocker-scrambler unit delivering shocks of defined duration and intensity (1.0 mA). On the first day of the conditioned fear assay, mice were individually acclimated to the chamber via a 5 min habituation session. On the second day, mice were trained to associate an aversive foot shock with the conditioned stimuli, or cage context and an auditory cue, during a 5.5 min conditioning session. Specifically, a tone (3000 Hz, 80 dB) lasting 30 s was emitted 120 and 270 s into the session, and upon termination of each tone, the unconditioned stimulus (a 2 s continuous foot shock at 1.0 mA) was delivered. Testing of contextual memory was conducted on the third day by placing the subject in the conditioning chamber for a 5 min test session and monitoring freezing behavior. Neither a tone nor a shock was administered as this session examined recognition of the association between the shock and the context. The mice were then analyzed for cued conditioning on the fourth day via the 6-min CS+ test. Here, the context of the conditioning chamber was disguised by the addition of a floormat over the metal grid floor, the insertion of patterned walls, and the replacement of orange oil soaked cedar chips with vanilla oil soaked chips in the bedding tray beneath the flooring. After exposure to this novel context for the first 3 min, the association between the foot shock and the tonal cue was assessed by sounding the tone for the last 3 min of the test session. Mice were tested during their active (dark) cycle and were returned to their home cages after each day of testing. Freezing behavior, or the absence of all voluntary movements except breathing, was measured in all four sessions (habituation, conditioning, context test, and CS+ test) by a validated computer-controlled recording of photocell beam interruptions. Scoring of the freezing response entailed summing time spent freezing (the absence of laser beam breaks for ≥2 s) across 5 s intervals for the entire test session duration.

Statistical Analyses. All values were expressed as the mean ± SEM. For mean freezing times within the conditioned fear assay sessions, values differing by more than 2 standard deviations from the mean were excluded (approximately one mouse per test group). Test scores of rotarod, locomotor, and fear conditioning behavioral assays were analyzed for homogeneity of variance. Assuming scores met this criterion, appropriate analyses of variance (ANOVA) were performed with group (vehicle or immunized) and/or treatment (saline or flunitrazepam injection) as the between subject factors and, where appropriate, time interval within a session as the within subject factor. Differences were considered statistically significant at $p < 0.05$. Subsequently, individual means were compared using the Student's t test for comparison between two groups, Fisher's PLSD, or the Scheffe's test in the case of unequal group size. Data from the GS, VS, and RS groups were compared by one-way ANOVA for all behavioral tests, and given no statistically detectable difference between groups, the GS data were eliminated from further analysis. The GS group was originally included to confirm the lack of adverse effects of passive immunization, and its subsequent exclusion from analysis allowed the 2 × 2 structure of ANOVA tests on VS, VF, RS, and RF group data to be upheld (Table 1).

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra and HPLC traces of compounds **4**−**6** and the RCA hapten. This material is available free of charge via the Internet at http://pubs.acs.org.

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