Published in final edited form as:

Mol Pharm. 2010 December 6; 7(6): 2056–2068. doi:10.1021/mp900293a.

Superadditive Effects of Ethanol and Flunitrazepam: Implications of Using Immunopharmacotherapy as a Therapeutic

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Abstract

While benzodiazepine intoxication alone may elicit sedative and anti-anxiety effects, alcohol co-ingestion greatly amplifies this central nervous system depression. As a result, this drug combination gained notoriety for its role in cases of facilitated sexual assault and fatal overdose. We previously validated the ability of the novel anti-flunitrazepam monoclonal antibody (mAb) RCA3A3 to bind flunitrazepam (FLU) *in vivo* and block FLU-induced impairment of locomotion and memory. A therapeutically relevant application of this high affinity mAb ($K_{d,app}$ = 200nM), however, is to the more tenuous indication of flunitrazepam (FLU) and alcohol co-intoxication. Employing a murine behavioral model, passive immunization with mAb RCA3A3 before injection of ethanol (EtOH, low-dose: 1 g/kg, or high-dose: 1.5 g/kg), FLU (0.06 mg/kg), or a cocktail of both drugs offered partial to full restoration of motor activity levels in co-drug treated and FLU-treated mouse groups ($n = 12$), respectively. Whereas all drug treatments left contextual learning intact, auditory cued learning was severely disrupted. Prophylactic administration of mAb RCA3A3 prevented this deficit in cued learning in FLU-treated mice but not in the FLU- and EtOH-treated mice, in which co-drug exposure exacerbated the impairment in cued fear conditioning. To substantiate this finding, a dose-response study was performed, and the changes in locomotor activity incurred by different FLU (low-dose: 0.06 mg/kg, or high-dose: 0.09 mg/kg), EtOH (1.0 g/kg, 1.5 g/kg), and mAb RCA3A3 (14.5 mg/kg, 21.8 mg/kg) dose combinations illustrated the potentiation in motor effects by concomitant exposure to FLU and EtOH. Thus, motor activity and fear conditioning results demonstrated that both the amount of FLU left unbound by antibody and the pharmacological additivity between FLU and EtOH, a GABA mimetic, were limiting factors in the therapeutic efficacy of mAb RCA3A3. In sum, our study highlights the complex nature of psychomotor impairment upon co-drug versus singular drug exposure, which may pose a unique challenge to therapeutic treatment.

Keywords

Flunitrazepam; Ethanol, Alcohol; Immunopharmacotherapy; Conditioned fear; Memory; Locomotor activity; Benzodiazepine; Antibody; Passive immunization; Co-drug abuse; GABAergic system

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INTRODUCTION

Ethanol and benzodiazepines induce similar sedative and CNS depressant effects that may range from mildly diminished motor skills to substantial deficits in memory and learning. Simultaneous exposure to both drugs exacerbates these effects to the extent that otherwise non-impairing doses of either substance may become a serious medical risk.^{1,2} Mechanistically, this ability of ethanol to exacerbate benzodiazepine-induced alterations in motor and cognitive function has been attributed to several mechanisms including an ethanol-mediated attenuation of lipid metabolism, principally through inhibition of the enzyme CYP3A4 (a hepatic microsomal cytochrome P450 enzyme), and a conformational change in the benzodiazepine receptor or GABA_A receptor complex that increases its affinity to benzodiazepines.^{3–5} Much less is known regarding the neurological effect of combining these drugs. In general, benzodiazepine-dependent patients display acute impairment on a variety of neuropsychological tests of intelligence and nonverbal memory, while alcohol abuse alone or in combination with other drugs contributes to measurable losses in recall memory and memory consolidation in learning.^{10–12} Interestingly, many of the cognitive deficits associated with alcohol intoxication and with co-drug abuse are intensified in women, which resonates with the notorious use of flunitrazepam (FLU) as a 'date-rape drug'.⁹ It follows that such gaps in the scientific knowledge pertaining to benzodiazepine abuse alongside other drugs limit the application of proven overdose and addiction treatments to this scenario of co-administration.⁹

Our interest in the pharmacodynamic interactions between ethanol (EtOH) and benzodiazepines stems from our recent development of an immunopharmacotherapy for the prevention of FLU intoxication. Passive immunization with this therapeutic mAb, termed RCA3A3 ($K_{d,app}$ = 200nM) prior to drug exposure blocked the central depressant effects of FLU.¹³ While this achievement is notable because FLU represents one of the more potent benzodiazepines, we wished to further explore the therapeutic ability of RCA3A3 under a more complex model of drug abuse. Given the reports from NIDA citing the proliferation of co-drug use, and in particular, the combining of benzodiazepines with other substances, the investigation of passive vaccination against FLU in a co-drug model seemed a worthwhile endeavor.^{6–8}

Central to the pharmacological considerations of this co-drug model is the aforementioned potentiation of the effects of FLU in the presence of alcohol (ethanol, EtOH). FLU allosterically modulates GABA neurotransmission via binding to the benzodiazepine sites (BZ₁ and BZ₂⁺) on GABA receptors, and thus the exposure to additional GABA mimetics greatly enhances GABA_A receptor-mediated transmission.¹³ Indeed, previous studies depict the propensity for sub-effective doses of EtOH to yield substantial loss in motor coordination and cognition when combined with benzodiazepines.^{14, 15} To this end, the therapeutic efficacy of mAb RCA3A3 in the prevention of FLU-induced impairment may be diminished under a co-drug scenario given the potential for EtOH and unbound drug to exert additive behavioral effects. With respect to the availability of unbound FLU, pharmacokinetic examination of drug biodistribution upon immunization with an equimolar mAb dose has elucidated the propensity for drug concentrations to remain high in serum and detectable in the brain despite mAb-mediated drug sequestration and drug redistribution.^{16–19} Under these immunopharmacotherapeutic studies, the free drug that remained in circulation was often sufficient to mediate behavioral symptoms of intoxication, albeit at markedly reduced levels relative to un-immunized subjects. Thus, further study of the acute cognitive and locomotor effects of FLU within the scenario of drug co-administration and with mAb-based prophylaxis seemed both neuropharmacologically interesting and medically relevant.

The classic approach to assessing learning and memory in animal models is through the conditioned fear paradigm. Possessing an overall design that is easily manipulated based on the experimental goals, the conditioned fear test allows all aspects of memory formation, recall, and extinction to be examined in order to analyze the exact effect of a particular treatment on cognitive function. Here, the fear response, which reflects the expectation of a noxious, unconditioned stimulus (US), serves as a read-out for the learnt association between the US and the predictive contextual or auditory cues. Through various permutations of this assay, it has been shown that ethanol induces bidirectional modulation of cognitive processes in a dose-dependent manner. A moderately high dose of ethanol negatively influences cued learning, a hippocampal-independent process, and markedly disrupts the hippocampal-dependent learning of associations between contextual features and the fearful stimulus.²⁰ By contrast, administration of a comparatively low dose of EtOH enhances memory of contextual and cued conditioning.²¹ Benzodiazepines impart parallel effects on fear conditioning as EtOH, and in addition, benzodiazepines severely impair both the acquisition and retrieval of spatial memories. Predictably, the impact of benzodiazepine and EtOH co-administration is additive or superadditive, and numerous behavioral studies have elucidated that the resulting severe intoxication attenuates the fear response toward the contextual stimulus and impairs cued recall.^{14, 22–25}

Even though mAb RCA3A3 was previously demonstrated to prevent a high dose of FLU from eliciting psychomotor as well as fear memory impairment, it remains to be explored whether this immunization strategy grants similar protection of motor and cognitive skills in cases involving concurrent EtOH exposure. In the previous study, a small portion of the FLU dose was hypothesized to avoid mAb binding despite its failure to produce detectable changes in mouse behavior. However, their potentiation by EtOH co-administration may cause this negligible amount of drug to acquire pharmacologically relevant effects, which could diminish the viability of mAb-mediated therapy for this scenario of co-drug intoxication. Thus, the current study was undertaken to explore whether the co-administration of sub-effective EtOH and FLU doses would yield additive or super-additive impairment to memory and, correspondingly, whether passive immunization retained its therapeutic value in blocking the contribution of FLU to the magnified psychomotor impairment.

EXPERIMENTAL SECTION

In vivo testing of mAb RCA3A3

Subjects—Female Crl:CD-1 (ICR) mice weighing ~30–35 g during testing (mAb RCA3A3 dose-response study: N = 60, conditioned fear testing in co-administration study: N = 140 mice, locomotor dose-response in co-administration study: N = 140) were purchased from Charles River (~18–21 g) and all experiments were conducted when mice reached 6–8 weeks of age. Animals were housed four per cage prior to surgery and singly post-operatively in a temperature-controlled vivarium under a reversed 12 h:12 h light/dark cycle (lights off at 06:00 h). Following surgery, mouse groups (therapy: vehicle and mAb RCA3A3) were assigned to receive a specific drug treatment (mAb dose-response study: FLU or Saline; co-administration study: low-dose and high-dose FLU (F), low-dose and high-dose ethanol (E1 and E2), FLU and EtOH (FE1/FE2), or saline (S)). 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 (FLU) [5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one] (Sigma) was prepared in 0.5% tween-80 in sterile 0.9% saline and administered by intraperitoneal (i.p.) injection at a concentration appropriate for the i.p. injection volume, about 0.01 mg/mL. Only low-dose FLU (0.06 mg/kg) was examined in the conditioned fear testing of the FLU and EtOH co-administration study (FLU treatment groups: VF, VFE1, VFE2, RF, RFE1, RFE2). A higher FLU dose (0.09 mg/kg) was selected for the mAb dose-response study (FLU treatment groups: vehicle-FLU group and RCA3A3-immunized-FLU groups, in which the mAb was administered at a dose of 0.125, 0.25, and 0.50 times the molar equivalent (mol Eq) FLU dose), while two FLU dosing regimens were included in the locomotor dose-response testing within the co-administration study (treatment groups: VF, VFE1, VFE2, RF, RFE1, RFE2; repeated for high-dose and low-dose FLU variations).

Ethanol—Ethanol (EtOH, 200 proof) was diluted in 0.9% sterile saline to produce a 20% w/v solution, and this solution was injected i.p. at a dose of 1.0 g/kg (E1, low-dose) or 1.5 g/kg (E2, high-dose).

Immunization—One week after arrival, mice were labeled, weighed, and subjected to intravenous (i.v.) catheterization. Detailed methods on surgery and apparatus are described elsewhere.²⁴ The schedule employed for passive immunization, drug administration, and behavioral testing was based on previous studies with mAb RCA3A3 reported by our laboratory.¹³ Briefly, all animals were treated and tested in a randomized fashion during their active (dark) cycle.

Passive immunization, which was conducted 30 hours before i.p. injections of drug, involved attaching a 4-inch polyethylene tube to the indwelling catheter on the animal's back, and then slowly delivering the bolus infusion of mAb RCA3A3 or saline through the tube over the course of ~ 1 minute. Catheters were flushed with an additional 0.05 mL 0.9% saline to ensure that the entire mAb dose reached the jugular vein before returning mice to their home cages. mAb RCA3A3 was administered at the following doses (i.v. in a volume of 1 mL/kg): mAb RCA3A3 dose-response study: 2.55 mg/kg (0.125 mol Eq), 5.1 mg/kg (0.25 mol Eq), or 10.2 mg/kg (0.50 mol Eq); conditioned fear testing within the co-administration study: 10.2 mg/kg (~ 0.75 mol Eq to FLU dose); locomotor dose-response co-administration study: 14.5 mg/kg or 21.8 mg/kg (1.0 mol Eq to low-dose FLU or high-dose FLU, respectively). These mAb doses were calculated based on the molecular mass of 150 kDa and the presence of two FLU binding sites for each mAb molecule. Sterile 0.9% saline (1 mL/kg, i.v.) was substituted for mAb in the mock immunization of vehicle groups (mAb dose-response study: vehicle-saline and vehicle-FLU treatment groups; co-administration study: vehicle groups for all high-dose and low-dose drug treatments, e.g. VS, VF, VE, and VFE).

Effect of passive immunization against flunitrazepam—After their recovery from the catheterization surgery, mice were randomly assigned to treatment groups. In the mAb RCA3A3 dose-response study, the mAb RCA3A3 dose was progressively lowered in order to examine the reduction in its efficacy at doses greatly submolar to drug. The following groups were used: 0.125-FLU group, 0.25-FLU group, or 0.50-FLU group, which represent 0.125, 0.25, or 0.5 times the molar equivalent FLU dose (0.09 mg/kg) delivered by i.p. injection on the test day. Three control groups were employed: vehicle-saline: vehicle (i.v.), saline (i.p.); vehicle-FLU: vehicle (i.v.), FLU (i.p.); 0.50-saline: mAb RCA3A3 at 0.50 mol Eq dose (i.v.), saline (i.p.). Upon confirming through statistical analysis that immunization with mAb RCA3A3 imparted no effect on locomotor or cognitive behavior, the 0.50-saline control group data were excluded from presentation in the results.

For the co-administration study (see Table 1), the following groups were included: VS: vehicle saline (i.v.), saline (i.p.); VF: vehicle (i.v.), FLU (i.p.); VE1: vehicle (i.v.), low-dose EtOH (i.p.); VE2: vehicle (i.v.), high-dose EtOH (i.p.); VFE1: vehicle (i.v.), low-dose EtOH (i.p.) and FLU (i.p.); VFE2: vehicle (i.v.), high-dose EtOH (i.p.) and FLU (i.p.); RS: RCA3A3 (i.v.), saline (i.p.); RF: RCA3A3 (i.v.), FLU (i.p.); RE2: RCA3A3 (i.v.), high-dose EtOH (i.p.); RFE1: RCA3A3 (i.v.), low-dose EtOH (i.p.) and FLU (i.p.); and RFE2: RCA3A3 (i.v.), high-dose EtOH (i.p.) and FLU (i.p.). Group size ($n = 12$) was increased for the conditioned fear testing in order to detect significant differences in fear memory deficits, and only the lower dose of FLU was examined. In the locomotor activity monitoring portion of the co-administration study, two sets of the aforementioned groups ($n = 8$) were prepared so as to test both low and high FLU dosing regimens.

In the 30-hour interim between passive immunization and drug treatment, mice were habituated to the locomotor activity cages (two sessions) and to the conditioned fear apparatus (one session). After administration (i.p.) of drug treatments (FLU: VF and RF groups, the 0.125, 0.25, and 0.50 mol Eq groups, and the vehicle-FLU group; EtOH: VE1, VE2; 0.5% tween-80 in isotonic saline: VS and RS, the vehicle-saline and immunized 0.50-saline groups; FLU and EtOH in single i.p. injection: VFE1, VFE2, RFE), mice were monitored for drug-induced motor changes in the locomotor activity test session (see Supporting Information) and then transferred to the CF apparatus for fear conditioning within 20 minutes of the drug injections so as to complete behavioral testing within the timeline of acute intoxication by FLU and/or EtOH exposure. The latter sequence of behavioral tests, which included the locomotor activity retest session as well as the two CF test sessions for contextual and cued memory, did not commence until 24 hrs after drug injection.

Locomotor activity testing—Locomotor activity was measured in Plexiglas cages ($42 \times 22 \times 20$ cm) placed into frames (25.5×47 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 USA). 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 two days preceding drug treatments (i.p.), daily locomotor box habituation sessions were conducted to minimize transient arousal during the test session, an experimental artifact caused by exposure to the novel environment. Mice were placed singly in the activity boxes for a recording session directly following injection (i.p.) of a drug treatment, and then subjects were either returned to their home cages or transferred to the conditioned fear apparatus. All mice were administered sterile 0.9% saline (10 mL/kg, i.p.) immediately prior to the two locomotor activity habituation sessions as well as before the locomotor activity retest session one day after drug treatments in order to reproduce the environment of drug administration. Recording sessions lasted for 10 minutes in the coabuse study and in the mAb RCA3A3 dose-response study, and activity counts were summated across 2-minute intervals for analysis. For the locomotor activity recording within the co-administration study, sessions were lengthened to 3 hours to ensure that the motor effects of drug treatments had disappeared. The second habituation session was used to create a baseline response curve of the activity counts across the entire recording session for each subject. This procedure was repeated with the activity counts collected on the day of drug administration. The area-under-the-curve (AUC) was calculated for both the baseline activity curve and the drug-day activity curve for each individual mouse, and the difference between these calculations was used to estimate the total change in activity imparted by a given drug treatment. Data were normalized to vehicle control group activity for presentation in Figure 6.

Cued and contextual conditioned fear testing—The conditioning system consisted of a soundproofed box of white interior housing a Freeze Monitor chamber (San Diego Instruments). The Plexiglas conditioning chamber (26 × 26 × 17 cm) was equipped with a speaker, a light, and a shockable grid floor under which eugenol-scented cedar chips were scattered. The stainless steel rod grid was connected to a shocker-scrambler unit delivering shocks of defined duration and intensity (0.8 mA). Based on previous reports in the literature, all treatments were expected to leave proprioception intact in intoxicated mice.^{14, 26} However, all mice were observed by the experimenter during conditioning sessions for the flinch response and vocalization to verify that none had been rendered insensitive to the aversive foot shock.

On day 1 of the conditioned fear assay, mice were individually acclimated to the chamber via a 5-min habituation session immediately after completing the second locomotor activity habituation session. Mice were returned to their home cages for a minimum of 5 hours before undergoing fear conditioning. Upon completion of the locomotor activity test session, mice were exposed to aversive foot shocks alongside auditory cues (CS, conditioned stimulus) in the conditioning chamber (context) over a 5.5-min conditioning session. The predictive, 30-second tones (3000 Hz, 80 dB), which were emitted 120 s and 270 s into the session, each terminated with the delivery of a 2-second, 0.8 mA scrambled foot shock (US, unconditioned stimulus). After a 24-hr delay and locomotor activity retesting to verify the absence of residual motor effects from intoxication, the contextual memory test was carried out by placing the subject in the eugenol-scented conditioning chamber for a 5-min test session and monitoring its 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 a minimum of 5 hrs after contextual testing 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 eugenol-scented cedar chips with a peppermint oil-soaked sterile gauze pad placed in the bedding tray beneath the flooring. After exposure to the novel context for the first 3 min, mice were tested for their association between the foot shock and the tonal cue through tone presentation for the latter 3-min period of the test session. Mice were returned to their home cages both between test sessions and at the conclusion of each testing day.

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. An initial evaluation of the total time spent freezing for each of the test sessions (habituation, conditioning, context, CS + test: before and after tone) was performed to verify that all subjects displayed similar activity during the habituation session and during the first period of the CS+ test session prior to cue presentation. Graphical figures that depict the results of the conditioned fear assay have been labeled by test session: “Context Test” and “CS+ Test: after tone”.

Statistical analyses—All values were expressed as mean ± SEM. For mean freezing times within the conditioned fear assay sessions and mean activity counts within the locomotor activity monitoring, values differing by more than two standard deviations from the mean were excluded (approximately one mouse per test group). Test scores of locomotor and fear conditioning behavioral assays were analyzed for homogeneity of variance. Assuming scores met this criterion, analyses of variance (ANOVA) were performed with mouse groups as the between subject factor and, where appropriate, time interval within a session as the within subject factor or repeated measure. ANOVAs were also conducted using therapy (vehicle or immunized) and/or treatment (i.p. injection of saline, low-dose and

high-dose FLU, low-dose and high-dose EtOH, FLU and EtOH co-administration treatments for various dose combinations) as the between subject factors. 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.

RESULTS

Dose Dependence of Immunopharmacotherapeutic Protection

Prior to testing RCA3A3 efficacy with FLU and EtOH co-administration, an mAb dose-response study was performed to assess the level of protection granted by mAb doses at one eighth (0.125-FLU), one fourth (0.25-FLU), or one half (0.50-FLU) of the molar equivalent (mol Eq) FLU dose (0.09 mg/kg, i.p.). Motor and cognitive impairment from FLU was measured via both locomotor and conditioned fear assays. All mAb doses significantly attenuated the FLU-induced change in motor activity over the entire locomotor testing period, as confirmed by a repeated measures ANOVA (time interval) with group as the main factor (data not shown). An ANOVA comparing the activity data of each group within individual time intervals of the locomotor test depicted the significant differences in activity within the first and last intervals, time points at which the motor-impairing effects of FLU observed in un-immunized mice were greatest [$F(5,58) = 2.601$, $p < 0.05$; $F(5,58) = 2.812$, $p < 0.05$]. Specifically, passive immunization with higher mAb doses (0.25-FLU, 0.50-FLU groups) moderated both the initial FLU-induced hyperactivity and the subsequent locomotor depression (Fig. 1).

Even though passive immunization with lower mAb RCA3A3 doses attenuated the alterations in locomotor activity incurred by FLU (Fig. 1), neither the 0.125-FLU nor the 0.25-FLU groups exhibited normal levels of contextual or cued fear memory (Fig. 2). A one-way ANOVA of all vehicle and RCA3A3-immunized groups confirmed the main effect of group on freezing to the context [$F(4,50) = 5.754$, $p < 0.001$] and on freezing during cue presentation [$F(4,50) = 3.839$, $p < 0.01$]. Subsequent group comparisons by the unpaired Student's t test illustrated that, contrary to the protection of fear memory conferred by a 0.50 mol Eq mAb dose, 0.125 and 0.25 mol Eq mAb doses failed to prevent the contextual and cued memory impairment mediated by FLU (Fig. 2). In the context test, the diminution in freezing times of 0.125-FLU and 0.25-FLU groups in comparison to the intact freezing behavior of the 0.05-FLU group was statistically significant ($p < 0.05$).

Impairment of Contextual and Cued Learning by Flunitrazepam and Ethanol

The conditioned fear paradigm not only measures the presence of memory impairment but it also differentiates between specific forms of cognitive deficit. Thus, this test may detect any disparate effects on memory consolidation incurred by varying states of intoxication: FLU alone, EtOH alone at high or low doses, or drug co-administration. Simultaneous exposure to FLU (0.09 mg/kg) and EtOH occasionally produced full sedation and disrupted footshock proprioception, and so a lower FLU dose (0.06 mg/kg) was implemented for conditioned fear testing. Locomotor activity was recorded for all mice during the time period between treatment injection and peak motor effects in order to complete fear conditioning when drug exposure would disrupt contextual and cued learning (see Supporting Information for accompanying locomotor analysis).

To examine the effect of drug treatment on spatial memory acquisition, conditioned mice were re-exposed to the context where they had received the aversive foot shocks 24 hours prior, and their activity in the chamber was recorded. All treatments, including co-administration of the EtOH (1.5 g/kg) with FLU, failed to cause statistically significant

differences in suppression of motility relative to VS mice re-exposed to the context (Fig. 3). A statistical comparison of all vehicle groups by one-way ANOVA confirmed the absence of a main effect of treatment on contextual fear memory [$F(5,72) = 0.982, p > 0.05$]. Even though a comparison of freezing times between all groups by one-way ANOVA suggested the significance main effect of group [$F(10,127) = 2.723, p < 0.005$], there were no significant alterations in fear behavior due to the different drug treatments or mAb when the relevant group comparisons were analyzed individually via ANOVA and the Student's *t* test. Whereas the high dose of EtOH was linked to a decrease in freezing behavior, this reduction disappeared upon the coadministration of FLU (see Fig. 3A, (*)-labeled bars).

FLU and EtOH were evaluated for their ability to directly hinder the formation of non-spatial memory in mice using the CS+ test of the conditioned fear paradigm. The first three-minute period of the CS+ test habituated groups to the novel, disguised context, and served as an internal assay control. Namely, all mice are expected to exhibit minimal freezing behavior, and thus their behavior should be statistically indistinguishable up until CS presentation. The freezing data met this criterion as the inter-group variability was found to be insignificant by one-way ANOVA [group: $F(10,137) = 1.779, p > 0.05$]. The second three-minute period challenged fear memory of mice for the CS (tone), which was first presented in the conditioning session. The freezing behavior of mice varied significantly between groups [$F(10,137) = 5.635, p < 0.0001$]. A two-way repeated measures ANOVA, in which groups of all therapy and treatment combinations were compared across the two CS+ test periods confirmed the statistical significance of the main effect of group on freezing behavior [group $\times$ time interval, $F(10,37) = 4.769, p < 0.0001$]. There was also a significant interaction between group and time interval on freezing level [$F(10,137) = 5.970, p < 0.0001$].

The presence of intact associative memory between the cue and aversive stimulus was examined under the scenario of FLU administration alone. Even though the effect of immunopharmacotherapy on the disruption of contextual memory consolidation was ambiguous, its capacity to protect cued memory consolidation was realized in the CS+ test. FLU administration had a significant main effect on freezing behavior, and this effect was prevented by immunization [VS vs. VF, treatment $\times$ time interval: $F(1,26) = 16.850, p < 0.0005$; VF vs. RF, therapy $\times$ time interval: $F(1,20) = 6.641, p < 0.05$]. A comparison of immunized groups receiving saline or FLU confirmed the protection offered by mAb RCA3A3 as there was no main effect of treatment on freezing times [RS vs. RF, treatment $\times$ time interval: $F(1,19) < 1.0$].

Each drug treatment (F, E1, E2, FE1, FE2) resulted in measurable deficits in cued fear memory (Tables 2–3). Whereas saline-treated mice froze for approximately 30% of the time interval of CS presentation, mice that were conditioned while intoxicated exhibited freezing behavior for approximately 10% of the same time period (Fig. 3B). The deficits in cued memory resulting from low-drug co-administration were statistically indistinguishable between EtOH doses (VFE1 vs. VFE2, $p > 0.05$). The VE1 mice displayed moderate levels of freezing to the tonal cue, and a Fisher's PLSD post-hoc analyses of the one-way ANOVA between all groups suggested that their level of motility was significantly different from that of VS and VFE1 groups (VE1 vs. VS, VE1 vs. VFE1: $p < 0.05$). Subsequent statistical analyses of VE1 freezing relative to VS or VFE1 group data by Student's *t* test confirmed the significant difference in freezing between VE1 and VFE1 groups, but not between VS and VE1 groups (Table 3). The amnesiac effects of other treatments (VF, E2, FE2) were statistically indistinguishable according to CS+ session freezing data.

Immunization was not able to rescue the disruption of CS-US associations mediated by drug co-administration. Whereas the pairing of FLU with low-dose EtOH (VFE1) caused greater

deficits in cued memory than EtOH alone (VE1), mAb RCA3A3 granted some restoration of cued learning (H_0 : VE1 = RFE1, $p = 0.054$). The effect of immunization was slight, however, and the freezing behavior of VFE1 and VFE2 groups was undistinguishable from that of the immunized counterparts, (H_0 : VFE1 = RFE1, $p > 0.1$; VFE2 = RFE2, $p > 0.1$).

Dose–response Relationship for Flunitrazepam and Ethanol Co-administration

The modulation of locomotor activity by FLU and EtOH was profiled using two FLU doses, two EtOH doses to elucidate their dose-response relationship. The mAb RCA3A3 dose (15 mg/kg, 22 mg/kg) was administered at an approximate 1:1 stoichiometric ratio to the FLU dose (0.06 mg/kg, 0.09 mg/kg) to minimize the amount of drug that escaped mAb binding. Passive immunization with this higher mAb dose eliminated the locomotor alterations that were observed in the FLU-injected vehicle mice (see Fig. 4A, (**)-labeled markers) as highlighted by the significant interaction between group and time interval in the repeated measures ANOVA [RF vs. VF, 0.09 mg/kg FLU: $F(7,105) = 4.430$, ($p < 0.001$)].

It was hypothesized that the motor effects of trace amounts of unbound FLU would be magnified based on the synergistic interaction between benzodiazepines and EtOH. Whereas the lower FLU dose (0.06 mg/kg) produced significantly less hypoactivity than the higher FLU dose (Fig. 4B), its co-administration with either EtOH dose magnified the reduction in motor activity, as confirmed with a repeated measures ANOVA (time interval) comparing the main effect of low-dose FLU administration in vehicle and EtOH-coadministered groups [VF1 vs. VF1E: 1.0 g/kg EtOH, $F(1,17) = 10.462$, $p < 0.005$; 1.5 g/kg EtOH, $F(1,15) = 11.787$, $p < 0.005$]. Administration of high-dose FLU (0.09 mg/kg) precipitated substantial changes in locomotor activity alone, while EtOH co-administration further aggravated the motor deficits within later time intervals (Fig. 4B, 5). A main effect of high-dose FLU administration was found in group comparisons by repeated measures ANOVA (time interval) between vehicle and EtOH-administered groups receiving high-dose FLU [VF vs. VFE: 1.0 g/kg EtOH, $F(1,14) = 10.640$, $p < 0.01$; 1.5 g/kg EtOH, $F(1,14) = 5.359$, $p < 0.05$].

Thus, mice co-administered FLU and EtOH displayed progressive motor depression across each 3-min time interval, whereas in the absence of FLU, these changes in activity level were attenuated. Passive immunization significantly blunted the contribution of FLU to the net hypoactivity from FLU and EtOH co-administration (see Fig. 5, §-labeled time intervals), as confirmed via a repeated measures ANOVA (time interval) comparing immunized and vehicle groups co-administered high-dose FLU and EtOH [RFE vs. VFE: 1.0 g/kg EtOH, $F(1,12) = 10.951$, $p < 0.01$; 1.5 g/kg EtOH, $F(1,12) = 5.967$, $p < 0.05$]. Further comparison of group locomotor activity by the Student's t test demonstrated the statistically significant differences in activity between groups administered EtOH alone versus with FLU during the 2nd, 3rd and 5th intervals, between immunized and vehicle groups co-administered FLU and EtOH during the 2rd–5th intervals (see Fig. 5B, §-labeled time intervals), and between mice administered FLU alone or with EtOH during the 1st–3rd, 5th intervals. Moreover, the activity behavior of immunized mice co-administered FLU and EtOH (1.0 g/kg) was statistically indistinguishable from immunized mice receiving only low-dose EtOH by a repeated measures ANOVA [RFE vs. RE, 1.0 g/kg EtOH: main effect of group, $F(1,13) = 1.860$, $p > 0.05$; group $\times$ time interval, $F(5,65) = .698$, $p > 0.05$].

Immunization attenuated the onset of sedation in mice co-administered EtOH (1.5 g/kg) and FLU (Fig. 5A, §-labeled time intervals). However, by the 6th time interval, the higher EtOH dose in combination with the fraction of FLU avoiding mAb capture were sufficient to cause locomotor deficits statistically equivalent to vehicle mice co-administered FLU and EtOH. Nevertheless, a repeated measures ANOVA (time interval) comparing these high-dose co-administration groups confirmed the significant main effect of immunization on activity

levels [RFE vs. VFE: 1.5 g/kg EtOH, $F(1,12) = 5.967$, $p < 0.05$], which implied that immunization offered a net protective effect against FLU-related motor deficits.

Potentiation of Locomotor Impairment by Drug Co-administration

From the 3-hour activity recording periods, a base-line activity curve was generated for each subject from the second habituation session for direct comparison to the corresponding activity profile of each subject upon drug administration. To demonstrate the superadditive motor effects imparted by FLU and EtOH co-administration, the net difference in the aforementioned activity curves for the first hour of monitoring was calculated via area-under-the-curve (AUC) analysis. The net change in activity of each subject due to drug administration was then normalized to the average change in activity between habituation and test sessions of vehicle mice administered saline. Thus, the resultant activity data for each group (Fig. 6) reflect the proportion of recorded activity counts that may be attributed to a specific drug treatment. Varying the FLU dose had minimal effect on the net change in locomotor activity within co-administration groups, and vehicle mice receiving high-dose FLU alone displayed a net change in activity of 104 ± 44 counts. The low and high EtOH doses were attributed to 96 ± 32 counts and 114 ± 50 counts, respectively, which increased to 309 ± 68 counts and 578 ± 100 counts with low-dose FLU co-administration (Fig. 6). Given that vehicle mice treated with high-dose FLU and low or high EtOH doses exhibited mean changes in locomotor activity of 348 ± 38 counts and 661 ± 139 counts, the behavioral effects of FLU and EtOH were amplified upon their co-administration.

DISCUSSION

The anti-flunitrazepam mAb RCA3A3 was previously shown to block the locomotor-sedating and memory-impairing effects of acute FLU intoxication in a mouse model.¹³ This study reflected a notable step in the field of immunopharmacotherapy as it demonstrated the successful application of an anti-drug mAb to the attenuation of the more subtle psychomotor effects of intoxication rather than of gross behavioral phenomena such as motor activity, self-administration, and lethality.^{28–33} To further characterize the preclinical utility of this immunopharmacotherapeutic treatment, mAb RCA3A3 was tested in a mouse model designed to mimic the “real-world scenario” of alcohol and benzodiazepine co-administration.³⁴ Both EtOH and benzodiazepines target the GABAergic system, and this drug combination has previously been reported to elicit additive or super-additive pharmacological effects as.^{5, 35} Thus, the efficacy of mAb RCA3A3 hinges on its ability to sequester a sufficient amount of the FLU dose so that EtOH is unable significantly magnify its behavioral effects.

To verify the therapeutic efficacy of passive immunization for the case of singular drug abuse, the restoration of normal locomotor activity by pretreatment with mAb RCA3A3 was tested in FLU-injected mice. Although minor, the motor deficit observed in VF mice was absent in RF mice (Fig. 4A), which suggested that immunization prevented both the mild hyperactivity and locomotor depression characteristic of FLU. The protection of motor function conferred by mAb RCA3A3 was realized briefly in cases of drug co-administration. The acute intoxication of vehicle mice co-administered FLU and high-dose EtOH (VFE2), which manifested itself as immediate sedation without the precursory hyperactivity, was significantly ameliorated in the corresponding immunized group (RFE2). Indeed, a repeated measures ANOVA (time interval) illustrated that there was a statistically significant main effect of mAb RCA3A3 on locomotor activity [$F(1,12) = 5.967$, $p < 0.05$]. Immunization also modified the behavior of mice administered the lower EtOH dose alongside FLU. During the latter time intervals the severity of motor depression was attenuated in immunized mice receiving FLU and low-dose EtOH (RFE1), and their activity level generalized to that expected upon sole administration of low-dose EtOH.

The results of locomotor activity testing highlighted two important traits of this co-administration model: the biphasic effects of either drug alone and the synergistic behavioral effects upon drug co-administration. The onset of intoxication was marked by increased locomotion during the 1st and occasionally the 2nd time interval of locomotor monitoring in VE1, VE2, VF, and the low dose VFE1 groups. Prior studies have linked comparatively low EtOH doses (1–1.5 g/kg) to the generation of greater initial hyperactivity, and mice within the early adolescent to periadolescent age range appeared more sensitive to this trend in altered locomotion.³⁶ Indeed, our data was consistent with this observation. This hyperactivity transitioned into a progressive locomotor depression during the latter 3rd–5th time intervals. The onset and severity these alterations in activity level were significantly accelerated in drug co-administration groups, and this precursory hyperactivity was absent in vehicle mice receiving the higher EtOH dose alongside FLU.

Previous research has elucidated the detrimental influence of both benzodiazepines and EtOH on fear-conditioned contextual memory.^{21, 37–39} In the current study, the FLU and EtOH doses were selected for their ability to cause mild to moderate impairment alone, while drug co-administration would yield severe intoxication with significant ramifications to both motor function and memory consolidation.^{14, 23} The locomotor activity results for each treatment group illustrated the graduated levels of motor changes anticipated for each drug dose. On the contrary, the correlation between drug treatment and freezing behavior was inconsistent, and the conditioned fear results belie the complexity of drug interactions during intoxication.

Both FLU and EtOH are notable in their capacity to mediate biphasic dose-response (i.e., dose-activity) effects.^{21, 26, 40} At lower doses, these drugs not only cause increased locomotion, but they may also facilitate certain cognitive processes, such as memory recall after acquisition. At higher doses, these drugs suppress psychomotor activity and impair contextual conditioning. While cued conditioning appears to be less susceptible to disruption, many studies have documented large impairments to cued or hippocampus-independent memory, which were then attributed to subtleties of the experimental design. Indeed, the cognitive effects of drug exposure have been proven to be highly dependent on time, drug dose, environment, stimulus type (e.g., contextual or cued stimulus learning), and stimulus saliency.

Even though the FLU, EtOH and co-drug treatments failed to significantly impact the learned association between a context and an aversive footshock, the treatments severely disrupted motor activity and cued memory. The potency of this co-drug combination was verified through the performance of a dose-response study in which the modulation of locomotor activity was assessed over extended time periods upon singular drug or co-drug administration. Results of this study depicted the superadditivity of FLU and EtOH *in vivo*, as their co-administration imparted a greater change in locomotor activity than that which would be expected by the sum of their individual effects.

Though locomotor activity monitoring was capable of distinguishing between enhancement and depression of motor function by FLU and EtOH, the biphasic dose-response profiles of both drugs obscured the interpretation of the conditioned fear data. For example, the apparent absence of changes in freezing level relative to VS mice may indicate that the freezing response of intoxicated mice neared the inflection point on the drug dose-response curve. Specifically, the level of inebriation of drug-treated groups exceeded that which might convey attention or memory-enhancing effects but fell short disrupting contextual memory consolidation. Even though this explanation is refuted by the overall lack of differences between treatments, the extreme locomotor sedation observed drug co-administration suggested that the dose had surpassed the estimated inflection point, and it

thwarted any exploration of higher drug doses within the conditioned fear assay. Other factors that may have biased the experimental results are the between-subject variation inherent to the nature of the conditioned fear assay and the use of a genetically heterogeneous, outbred strain of mice.⁴¹

Lastly, the salience and thereby the associative fear memory of the context relative to the cued and unconditioned stimuli is subject to modulation by numerous variables specific to the conditioned fear testing paradigm.⁴² Extended pre-exposure to the training context has been shown to cause slight latent inhibition of the contextual association to the US (foot shock) in less-intoxicated mice. Even though context pre-exposure generally attenuates benzodiazepine-induced deficits in fear memory, the notable salience of the context in this study, which was accomplished through the presence of distinctive eugenol odors and grid flooring during habituation, conditioning, and contextual testing sessions, provided associative strength to the overall context and thus reinforced the consolidation of context-US associations during conditioning.³⁸

The corollary to this strong effective context-US association is the subsequent overshadowing of the CS-US association.⁴² The CS+ test results aligned with this proposed susceptibility of cued memory to disruption by intoxication, as illustrated by the equivalent suppression of motility with singular drug and co-drug intoxication. Immunization prevented such deficits in cued memory deficit, and the protection afforded by RCA3A3 was dose-dependent (RF vs. VF, 0.25-FLU, 0.50-FLU: $p < 0.05$). This pattern of fear memory deficits, which has been studied previously and termed the comparator hypothesis⁴³, provides a plausible explanation for the minimal effect FLU imparted on contextual memory in this study, and it supports rather than contradicts the FLU-induced suppression of cued memory in the disguised context.

CONCLUSION

To this end, our study depicts the therapeutic utility of an anti-flunitrazepam passive vaccine in the prevention of severe psychomotor impairment under a complex scenario of drug co-administration. Immunization with murine mAb RCA3A3 prior to drug exposure blunted the FLU-induced alterations in motor activity, countering both the initial hyperactivity and the subsequent locomotor depression despite EtOH-mediated potentiation of motor changes. We previously reported on the mAb RCA3A3-mediated prevention of contextual and cued memory impairment upon exposure to a high dose of FLU, and this protection of cued memory was recaptured here under a lower FLU dose. The new dosing paradigm left contextual memory fully intact even when FLU was co-administered with EtOH, and this dichotomy between contextual memory formation and cued memory ablation poses a unique phenomenon in terms of the cognitive effects that arise from drug co-administration. Even though this immunopharmacotherapeutic mAb was unable to attenuate the disruption of cued memory acquisition, our investigation into the motor and cognitive impairment caused by simultaneous alcohol (EtOH) and benzodiazepine exposure directly illustrates the superadditive pharmacological interaction of this drug combination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge the financial support of the Skaggs Institute for Chemical Biology, the National Institute on Drug Abuse (DA 21939 to J.B.T., DA 08590 to K.D.J.), and NIH Blueprint for Neuroscience Research (NS057096 to A.J.R.).

NONSTANDARD ABBREVIATIONS FOOTNOTE

mAb	monoclonal antibody
IgG	immunoglobulin
GABA	γ -aminobutyric acid
i.v.	intravenous
i.p.	intraperitoneal
FLU	flunitrazepam
EtOH	ethanol
VS	vehicle (i.v.) saline (i.p.)
VF	vehicle (i.v.) flunitrazepam (i.p.)
VE1 and VE2	vehicle (i.v.) ethanol (i.p.) at low (E1) or high (E2) dose
VFE1 and VFE2	vehicle (i.v.) flunitrazepam plus low/high-dose ethanol (i.p.)
RS	RCA3A3 (i.v.) saline (i.p.)
RF	RCA3A3 (i.v.) flunitrazepam (i.p.)
RE2	RCA3A3 (i.v.) high-dose ethanol (i.p.)
RFE1 and RFE2	RCA3A3 (i.v.) flunitrazepam plus low/high-dose ethanol (i.p.)
mol Eq	molar equivalent
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.

Dose-dependence of RCA3A3-mediated protection of motor function. Mice were passively immunized either with mAb RCA3A3 at 0.125, 0.25 or 0.50 times the mol Eq dose FLU, or with a vehicle solution (sterile 0.9% saline) before the start of behavioral testing. Locomotor activity levels in vehicle-immunized and mAb-immunized mice were recorded for a 15-minute period following administration (i.p.) of FLU (0.09 mg/kg, vehicle-FLU group and all mol Eq dose groups) or 0.5% tween-80 in isotonic saline (10 ml/kg, vehicle-saline group). Data are expressed as the mean $\pm$ S.E.M. of total activity counts for each mouse within 3-min intervals. Statistical significance, as calculated using an unpaired Student's *t* test, indicates the difference in locomotor activity between vehicle-FLU and 0.25-FLU (*), vehicle-FLU and 0.50-FLU (**), or vehicle-FLU and vehicle-saline (\$) groups; $p < 0.05$.

Figure 2.

Fear behavior resulting from contextual and cued conditioning in mice passively immunized with mAb RCA3A3 at 0.125, 0.25, 0.50 times the mol Eq dose FLU. Vehicle-saline mice were i.p.-injected with 0.5% tween-80 in isotonic saline, while immunized (bars at 0.125, 0.25, 0.50) and vehicle-FLU (bar at 0) mice were i.p.-injected with FLU (0.09 mg/kg). **A**, In the context test, time spent freezing is summed over the entire test session, and data represent group means $\pm$ SEM. **B**, In the CS+ test, all mice spent minimal time freezing before presentation of the tone. Upon exposure to the cued stimulus, or tone, vehicle-saline and 0.50-FLU mice showed significant increases in freezing behavior while 0.125-FLU, 0.25-FLU, and vehicle-FLU mice displayed less freezing compared to control levels. All data are expressed as group means $\pm$ SEM of time spent freezing within each test session or interval (context, CS+ before tone, CS+ after tone). Statistical significance, as calculated using the unpaired Student's t-test, compares the freezing behavior of the designated group to that of the vehicle-FLU group; * $p < 0.05$.

Figure 3.

Freezing response to contextual (**A**) and cued (**B**) stimuli in vehicle (light grey bars) and mAb RCA3A3-immunized (dark grey bars) mice i.p.-injected with one of the following treatments: Saline (S); FLU (F, 0.06 mg/kg); FLU, EtOH (FE, co-injection of 1.5 g/kg EtOH and 0.06 mg/kg FLU); EtOH (E, 1.5 g/kg). The time spent freezing during the context or CS + test session was averaged for all mice within a treatment group and presented as mean $\pm$ SEM. **A**, Drug exposure did not have a statistically significant impact on contextual memory. **B**, The CS+ test results only depict freezing behavior upon tone presentation. VS, RS, and RF mice exhibited significant suppression in motility after the tone, while VF and VE mice exhibit diminished levels of freezing. * $p < 0.05$, denotes statistical significance of the unpaired Student's *t* test for designated group comparisons.

Figure 4.

Modulation of locomotor activity by FLU and EtOH. **A**, mice were passively immunized (i.v.) with mAb RCA3A3 (open marker) or isotonic saline solution (solid marker) 30 hours before i.p. injection of FLU (● FLU, ○ FLU, mAb; solid line) or 0.5% tween-80 in isotonic saline (◇ Saline, ◆ mAb; dashed line). **B**, the motor effects of FLU (● 0.09 mg/kg, ○ 0.06 mg/kg) and EtOH (◇ 1.5 g/kg, ◆ 1.0 g/kg) administration were recorded for two doses of either drug. Data, which reflect locomotor activity monitoring for 18 minutes following i.p. injection, are expressed as group means $\pm$ SEM of activity counts (photobeam breaks) summated across 3 min intervals for each mouse. Statistical significance is shown for group comparisons by Student's *t* test for designated time intervals; **A**, Saline vs. FLU: * $p < 0.05$, FLU vs. FLU, mAb: ** $p < 0.05$; **B**, 0.06 mg/kg FLU vs. 0.09 mg/kg FLU: § $p < 0.05$, Saline vs. 1.5 g/kg EtOH: * $p < 0.05$, Saline vs. 1.0 g/kg EtOH: ** $p < 0.05$.

Figure 5.

Fluctuation in locomotor activity caused by drug co-administration. Mice were passively immunized (i.v.) with mAb RCA3A3 or vehicle isotonic saline solution 30 hours before i.p. injection of drug treatment: 0.5% tween-80 in isotonic saline (◆ Saline, ◇ FLU (**A**) or EtOH (**B**); dashed lines), or co-administration of FLU and EtOH (● EtOH, FLU; ○ EtOH, FLU mAb; solid line). The contribution of EtOH to changes in locomotor activity was examined for two EtOH doses: **A**, 1.0 g/kg low-dose; **B**, 1.5 g/kg high-dose. Data, which reflect locomotor activity monitoring for 18 minutes following i.p. injection, are expressed as group means $\pm$ SEM of activity counts (photobeam breaks) summated across 3-minute intervals for each mouse; statistical significance is shown for Student's *t* test comparisons between groups within designated time intervals: Saline vs. FLU (**A**) or EtOH (**B**): * $p < 0.05$; Saline vs. EtOH, FLU: ** $p < 0.05$; FLU, EtOH vs. FLU, EtOH, mAb: § $p < 0.05$.

Figure 6.

Dose-effects of drug co-administration on locomotor activity. Mice were monitored for their locomotor activity in a 3-hour recording session to create a baseline activity curve. On the day of drug administration, vehicle and mAb RCA3A3-immunized mice were i.p. injected with EtOH (1.0, 1.5 g/kg) and either vehicle (sterile, 0.9% saline) or FLU (0.06, 0.09 mg/kg) directly before the start of a 3-hour activity monitoring session, with activity counts reported in 20-sec intervals. The locomotor activity curve of each subject was corrected for its baseline activity, and this change in activity counts was tallied across a 30-min period before normalization to the vehicle control group activity. Data are presented as group mean $\pm$ SEM.

Group assignments.

Table 1

mAb RCA3A3 Dose-Response		Co-Administration Study ^a			
	Saline	FLU	FLU	EtOH	FLU + EtOH
<i>vehicle (V)</i>	VS	vehicle-FLU	VF	VE1	VFE1
		0.125-FLU		VE2	VFE2
		0.25-FLU		RE1	RFE1
<i>immunized (R)</i>	0.50-saline (RS)		RF
		0.50-FLU			

^aGroups performed in duplicate for locomotor activity testing of the co-administration study in order to examine the dose-response to both low-dose and high-dose FLU.

Table 2

Statistical analysis of contextual and cued fear memory.

One-Way ANOVA (group) or Two-Way ANOVA (treatment × therapy) or Two-Way repeated measures ANOVA (group × time interval) ^a	
<i>Context Test</i>	
therapy: vehicle (V), mAb (R)	therapy: $F(2,78) = 4.593, p < 0.05$
group: all	group: $F(10,127) = 2.723, p < 0.005$
<i>CS+ Test</i>	
treatment: saline (S), FLU (F)	treatment: $F(1,45) = 4.055, (p = 0.0501)$
therapy: vehicle (V) or mAb (R)	therapy: $F(1,45) = 4.496, (p < 0.05)$
group: saline (S), low EtOH (VE1), low EtOH + FLU (FE1) treatments; all therapies (V, R)	group: $F(3,50) = 8.132, (p < 0.0005)$ main effect: $F(5,75) = 5.778, (p < 0.0001)$ interaction: $F(5,75) = 7.541, (p < 0.0001)$
treatment: saline (S), high EtOH + FLU (FE2)	treatment: $F(1,55) = 22.541, (p < 0.0001)$
group: all groups	group: $F(10,137) = 5.635, (p < 0.0001)$

^aRepeated measures ANOVA (time interval): main effect of group, or interaction of group × time interval

Table 3

Between-group differences^a in freezing behavior to the cued stimulus.

Low-dose EtOH		High-dose EtOH	
VS = VE1	<i>ns</i>	VS = VE2	$p < 0.005$
VS = VFE1	$p < 0.0001$	VS = VFE2	$p < 0.005$
VE1 = VFE1	$p < 0.05$	VE2 = VFE2	<i>ns</i>
VFE1 = RFE1	<i>ns</i>	VFE2 = RFE2	<i>ns</i>

^a Student's *t* test; *p* values for group comparisons, H₀