Biological Psychiatry

Archival Report

Fluoroethylnormemantine, a Novel NMDA Receptor Antagonist, for the Prevention and Treatment of Stress-Induced Maladaptive Behavior

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ABSTRACT

BACKGROUND: Major depressive disorder is a common, recurrent illness. Recent studies have implicated the NMDA receptor in the pathophysiology of major depressive disorder. (*R,S*)-ketamine, an NMDA receptor antagonist, is an effective antidepressant but has numerous side effects. Here, we characterized a novel NMDA receptor antagonist, fluoroethylnormemantine (FENM), to determine its effectiveness as a prophylactic and/or antidepressant against stress-induced maladaptive behavior.

METHODS: Saline, memantine (10 mg/kg), (*R,S*)-ketamine (30 mg/kg), or FENM (10, 20, or 30 mg/kg) was administered before or after contextual fear conditioning in 129S6/SvEv mice. Drug efficacy was assayed using various behavioral tests. Protein expression in the hippocampus was quantified with immunohistochemistry or Western blotting. In vitro radioligand binding was used to assay drug binding affinity. Patch clamp electrophysiology was used to determine the effect of drug administration on glutamatergic activity in ventral hippocampal cornu ammonis 3 (vCA3) 1 week after injection.

RESULTS: Given after stress, FENM decreased behavioral despair and reduced perseverative behavior. When administered after re-exposure, FENM facilitated extinction learning. As a prophylactic, FENM attenuated learned fear and decreased stress-induced behavioral despair. FENM was behaviorally effective in both male and female mice. (*R,S*)-ketamine, but not FENM, increased expression of c-fos in vCA3. Both (*R,S*)-ketamine and FENM attenuated large-amplitude AMPA receptor–mediated bursts in vCA3, indicating a common neurobiological mechanism for further study.

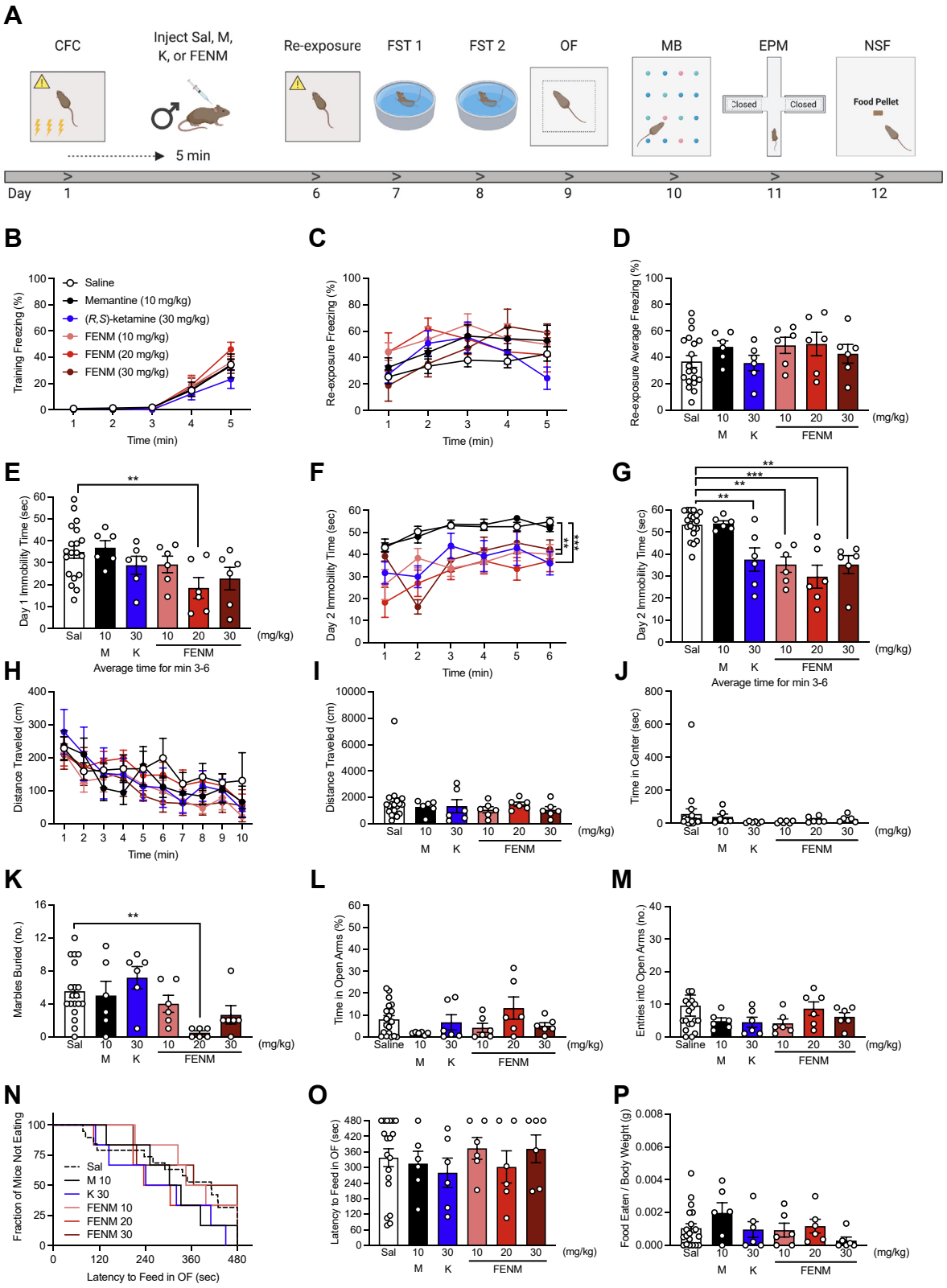
CONCLUSIONS: Our results indicate that FENM is a novel drug that is efficacious when administered at various times before or after stress. Future work will further characterize FENM's mechanism of action with the goal of clinical development.

https://doi.org/10.1016/j.biopsych.2021.04.024

Major depressive disorder (MDD) is the leading cause of disability worldwide, affecting more than 17 million adults in the United States alone (1,2). Current pharmacological treatments for MDD, such as selective serotonin reuptake inhibitors and monoamine oxidase inhibitors, are based on the monoamine hypothesis of depression, which attributes psychopathology to the reduced availability of monoaminergic chemicals in the brain (3). Emerging evidence, however, increasingly supports the now prevalent glutamatergic theory of depression, which proposes that an imbalance of excitatory and inhibitory neurotransmitters, perhaps due to the abnormal activity of NMDA receptors (NMDARs), perturbs physiological homeostasis in the central nervous system, thus leading to symptoms of MDD (4,5).

Perhaps the most compelling evidence for the glutamatergic hypothesis is the discovery that (*R,S*)-ketamine, a commonly used anesthetic, is a rapid-acting antidepressant (6). (*R,S*)-ketamine acts as a noncompetitive NMDAR antagonist (7–9). Administered subanesthetically, (*R,S*)-ketamine can relieve depressive symptoms within 2 hours, last up to 3 weeks, and remain effective in patients suffering from treatment-resistant depression (10–13). A stereospecific version of (*R,S*)-ketamine, Spravato (esketamine) (Janssen Pharmaceuticals, Inc., Beerse, Belgium), recently became the first novel Food & Drug Administration–approved treatment for MDD in nearly 20 years (14,15). (*R,S*)-ketamine can also prevent the onset of learned fear and behavioral despair in mice (16–24) and may prevent postpartum depression and posttraumatic stress disorder in humans (25–28). However, despite

 ISSN: 0006-3223


A
CFC
Inject Sal, M, K, or FENM
5 min
Re-exposure
FST 1
FST 2
OF
MB
EPM
NSF
Closed
Closed
Food Pellet
Day 1 6 7 8 9 10 11 12
B
Saline
Memantine (10 mg/kg)
(R,S)-ketamine (30 mg/kg)
FENM (10 mg/kg)
FENM (20 mg/kg)
FENM (30 mg/kg)
Training Freezing (%)
Time (min)
C
Re-exposure Freezing (%)
Time (min)
D
Re-exposure Average Freezing (%)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
E
Day 1 Immobility Time (sec)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
Average time for min 3-6
F
Day 2 Immobility Time (sec)
Time (min)
G
Day 2 Immobility Time (sec)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
Average time for min 3-6
H
Distance Traveled (cm)
Time (min)
I
Distance Traveled (cm)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
J
Time in Center (sec)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
K
Marbles Buried (no.)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
L
Time in Open Arms (%)
Saline 10 30 10 20 30 (mg/kg)
M K FENM
M
Entries into Open Arms (no.)
Saline 10 30 10 20 30 (mg/kg)
M K FENM
N
Fraction of Mice Not Eating
Sal
M 10
K 30
FENM 10
FENM 20
FENM 30
Latency to Feed in OF (sec)
O
Latency to Feed in OF (sec)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
P
Food Eaten / Body Weight (g)
Sal 10 30 10 20 30 (mg/kg)
M K FENM

(*R,S*)-ketamine's remarkable actions, the compound's side effects include psychotropic effects and high abuse potential, representing a hurdle toward its development as a clinical treatment. These challenges have led researchers to search for novel NMDAR antagonists that can exhibit similar antidepressant and/or prophylactic efficacy with reduced nonspecific effects.

Recently, a novel NMDAR antagonist, fluoroethylnormemantine (FENM), was derived from the NMDAR antagonist memantine. To determine its biodistribution and safety profile, FENM was developed into the radiolabeled compound [^{18}F]-FENM (29,30). In rats, [^{18}F]-FENM (44 ± 11 MBq) stabilized in the brain 40 minutes after injection, with 0.4% of the injected dose found in the brain. In rats anesthetized with isoflurane before [^{18}F]-FENM injection, combined ex vivo autoradiography and immunohistochemical staining demonstrated a strong colocalization of NMDARs and [^{18}F]-FENM binding, particularly in cortical regions and the hippocampus (HPC) (29). Most interestingly, if rats were anesthetized with (*R,S*)-ketamine (80 mg/kg) immediately before [^{18}F]-FENM injection, the [^{18}F]-FENM autoradiographic signal no longer correlated with NMDAR staining, indicating that binding was disabled or blocked. [^{18}F]-FENM was calculated to have a K_i of 3.5 μM, compared with (*R,S*)-ketamine, which exhibits a K_i of 0.53 μM (29,31). Furthermore, FENM was recently shown to facilitate extinction learning in male rats without altering sensorimotor behavior (32). However, it remains unknown whether FENM can be efficacious as a prophylactic or antidepressant.

Here, we sought to characterize FENM as a novel compound to reduce stress-induced maladaptive behavior in mice. A single injection of saline, memantine (10 mg/kg), (*R,S*)-ketamine (30 mg/kg), or FENM (10, 20, or 30 mg/kg) was administered before or after contextual fear conditioning (CFC) stress in 129S6/SvEv mice. Memantine and (*R,S*)-ketamine were administered in order to compare their behavioral effects with those of FENM. Drug efficacy was assayed using a variety of behavioral tests. Immunohistochemistry and Western blotting were used to determine expression of the immediate early gene c-fos, the NMDAR subunit NR2A, and AMPA receptor (AMPAR) subunit GluR1 in the HPC. We used patch clamp electrophysiology to measure glutamatergic activity 1 week after drug administration. When administered directly after stress, FENM decreased stress-induced behavioral despair and perseverative behavior. FENM facilitated fear extinction when given directly after context re-exposure. When administered 1 week before stress, FENM attenuated learned fear and decreased stress-induced behavioral despair. FENM exerted behavioral effects in both male and female mice. FENM robustly attenuated large-amplitude bursts of AMPAR-mediated glutamatergic activity in ventral hippocampal cornu ammonis 3 (vCA3) similarly to (*R,S*)-ketamine. Our results indicate that FENM is effective in reducing stress-induced maladaptive behavior when administered at various timepoints before or after stress. Furthermore, our data show that both (*R,S*)-ketamine and FENM alter hippocampal activity mediated by AMPARs, suggesting a common neurobiological mechanism for further study.

METHODS AND MATERIALS

For a full description of Methods and Materials, refer to the Supplemental Methods in Supplement 1.

Drugs

All drugs were resuspended in saline and made fresh for each experiment.

Memantine. Memantine (memantine hydrochloride; Tocris Bioscience, Minneapolis, MN) was administered in a single dose at 10 mg/kg of body weight. This dose was chosen based on previous studies (33).

(*R,S*)-Ketamine. (*R,S*)-ketamine (Ketaset III, ketamine HCl injection; Fort Dodge Animal Health, Fort Dodge, IA) was administered in a single dose at 30 mg/kg of body weight. This dose was chosen based on previous studies (16–20).

Fluoroethylnormemantine. FENM (M2i, Saint-Cloud, Paris, France) was administered in a single dose at 10, 20, or 30 mg/kg of body weight.

Statistical Analysis

Data were analyzed using Prism (version 8.0; GraphPad Software, La Jolla, CA). α was set to 0.05 for all analyses. Generally, the effect of drug or group was analyzed using an analysis of variance, using repeated measures where appropriate. Post hoc Dunnett's or Tukey's tests were used where appropriate. All statistical tests and *p* values are listed in Table S1 in Supplement 2. Key resources are provided in Table S2 in Supplement 2. A summary of behavioral results is provided in Table S3 in Supplement 1.

Figure 1. FENM is a novel antidepressant when administered after stress exposure. **(A)** Experimental design. **(B)** Freezing was comparable across all groups during CFC training. **(C, D)** During CFC re-exposure, freezing was comparable across all groups. **(E)** On day 1 of the FST, FENM (20 mg/kg), but no other drugs or doses tested, reduced immobility time compared with controls. **(F, G)** On day 2 of the FST, (*R,S*)-ketamine and FENM at all doses, but not memantine, significantly reduced immobility time when compared with saline mice. **(H–J)** In the OF, all groups of mice traveled a comparable distance traveled and spent a comparable amount of time in the center of the arena. **(K)** In the MB task, FENM (20 mg/kg) decreased the number of marbles buried when compared with saline. **(L, M)** In the EPM, memantine, (*R,S*)-ketamine, and FENM did not alter distance traveled or the time spent in the open arms and center of the maze. **(N)** In the NSF, memantine, (*R,S*)-ketamine, and FENM did not alter the latency to feed in the open arena. **(O, P)** Latency to feed and food eaten in the home cage were comparable across all drug groups. $n = 6$ male mice per group; error bars represent ± SEM; $^{**}p < .01$, $^{***}p < .001$. CFC, contextual fear conditioning; EPM, elevated plus maze; FENM, fluoroethylnormemantine; FST, forced swim test; K, (*R,S*)-ketamine; M, memantine; MB, marble burying; NSF, novelty-suppressed feeding; OF, open field; Sal, saline.

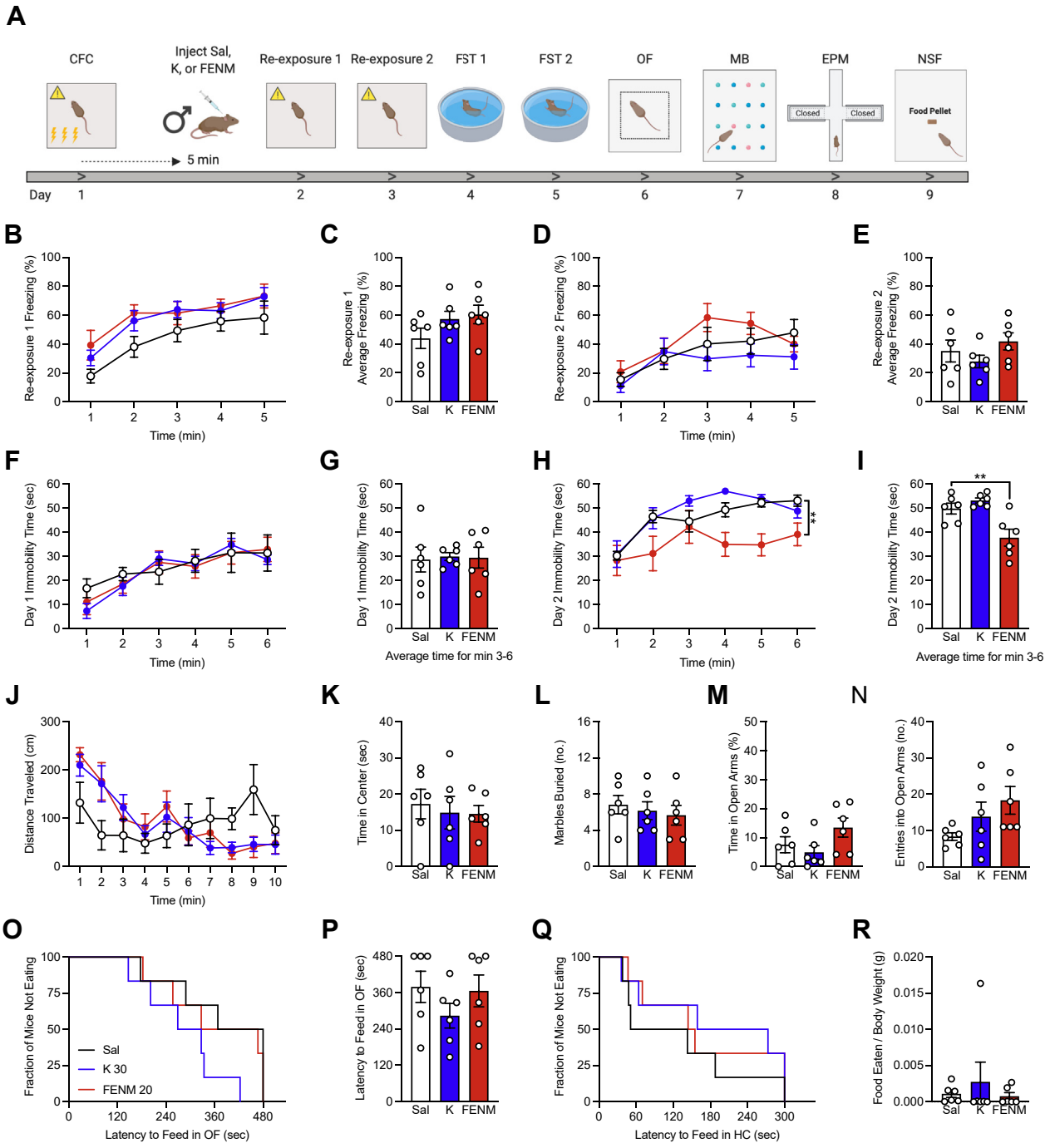
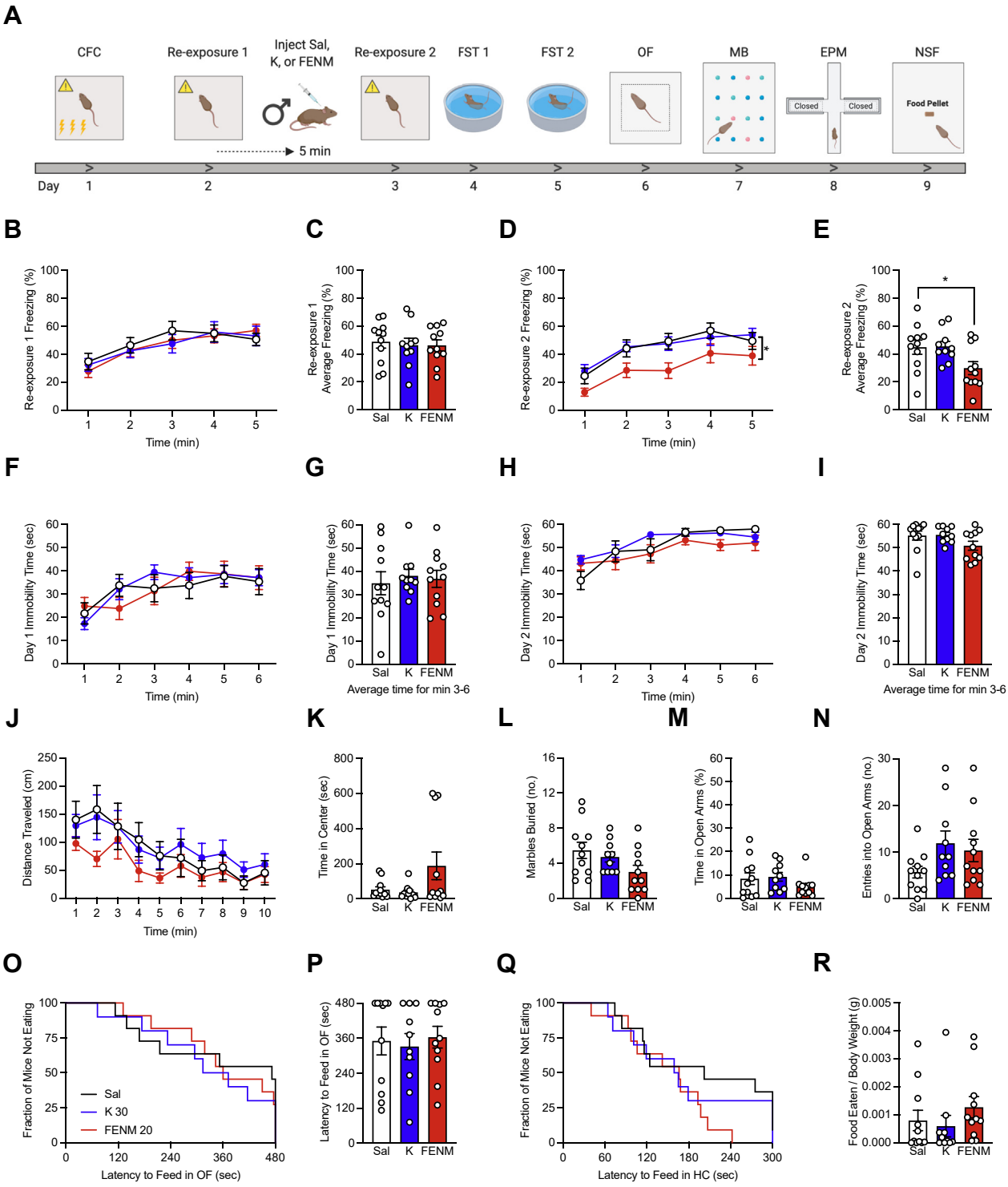


Figure 2. FENM is also an effective antidepressant after a shorter intertrial interval between stress and behavioral despair assays. **(A)** Experimental design. **(B–E)** During CFC re-exposures 1 and 2, all groups of mice exhibited comparable freezing. **(F, G)** On day 1 of the FST, all groups exhibited comparable immobility time. **(H, I)** On day 2 of the FST, FENM, but not (*R,S*)-ketamine, significantly reduced immobility time when compared with saline mice. **(J, K)** In the OF, all groups of mice traveled a comparable distance traveled and spent a comparable amount of time in the center of the arena. **(L)** In the MB task, all groups of mice buried a comparable number of marbles. **(M, N)** Behavior was comparable across all groups in the EPM. **(O, P)** In the NSF, (*R,S*)-ketamine and FENM did not alter the latency to feed in the open arena. **(Q, R)** Latency to feed and food eaten in the HC were comparable across all groups. $n = 6$ male mice per group; error bars represent ± SEM; $^{**}p < .01$. CFC, contextual fear conditioning; EPM, elevated plus maze; FENM, fluoroethylnormemantine; FST, forced swim test; HC, home cage; K, (*R,S*)-ketamine; MB, marble burying; NSF, novelty-suppressed feeding; OF, open field; Sal, saline.


A
CFC
Re-exposure 1
Inject Sal, K, or FENM
Re-exposure 2
FST 1
FST 2
OF
MB
EPM
NSF
5 min
Closed
Closed
Food Pellet
Day 1 2 3 4 5 6 7 8 9
B
Re-exposure 1 Freezing (%)
Time (min)
C
Re-exposure 1 Average Freezing (%)
Sal K FENM
D
Re-exposure 2 Freezing (%)
Time (min)
E
Re-exposure 2 Average Freezing (%)
Sal K FENM
F
Day 1 Immobility Time (sec)
Time (min)
G
Day 1 Immobility Time (sec)
Sal K FENM
Average time for min 3-6
H
Day 2 Immobility Time (sec)
Time (min)
I
Day 2 Immobility Time (sec)
Sal K FENM
Average time for min 3-6
J
Distance Traveled (cm)
Time (min)
K
Time in Center (sec)
Sal K FENM
L
Marbles Buried (no.)
Sal K FENM
M
Time in Open Arms (%)
Sal K FENM
N
Entries into Open Arms (no.)
Sal K FENM
O
Fraction of Mice Not Eating
Sal
K 30
FENM 20
Latency to Feed in OF (sec)
P
Latency to Feed in OF (sec)
Sal K FENM
Q
Fraction of Mice Not Eating
Latency to Feed in HC (sec)
R
Food Eaten / Body Weight (g)
Sal K FENM

RESULTS

FENM Is Antidepressant When Administered After Exposure to Stress

First, we aimed to test whether FENM, a novel NMDAR antagonist, could be antidepressant when administered after exposure to stress. Saline, memantine (10 mg/kg), (*R,S*)-ketamine (30 mg/kg), or FENM (10, 20, or 30 mg/kg) was administered 5 minutes after CFC (Figure 1A). Drug dosing was chosen based on results from previous studies (16,21,32–34). Freezing was comparable between all groups during CFC training and re-exposure (Figure 1B–D). FENM (20 mg/kg) reduced immobility time on day 1 of the forced swim test (FST) (Figure 1E). On FST day 2, all drugs and doses tested, with the exception of memantine, reduced immobility time compared with saline (Figure 1F, G), indicating that (*R,S*)-ketamine and FENM, but not memantine, decrease stress-induced behavioral despair.

We next assayed locomotion and stress-induced avoidance, perseverative, and hyponeophagia behavior. Behavior was comparable between all groups in the open field (OF), elevated plus maze (EPM), and novelty-suppressed feeding (NSF) assays (Figure 1H–J, L–P). In the marble burying (MB) task, FENM (20 mg/kg) significantly reduced the number of marbles buried (Figure 1K). These data indicate that (*R,S*)-ketamine and FENM, but not memantine, may be administered after exposure to stress to decrease stress-induced behavioral despair and that FENM reduces stress-induced perseverative behavior in male mice.

Emerging evidence demonstrates the importance of testing novel therapeutics in both sexes (21,35,36). To test whether FENM is effective in female mice, we administered FENM (10 or 20 mg/kg) 5 minutes after CFC training (Figure S1A in Supplement 1). FENM did not alter learned fear behavior (Figure S1B–E in Supplement 1) but significantly reduced behavioral despair on day 2, but not on day 1, of the FST (Figure S1F–I in Supplement 1). All other behaviors assayed were not significantly altered by FENM administration (Figure S1J–Q in Supplement 1). These data demonstrate that, as in male mice, FENM is also effective against behavioral despair in female mice when administered after exposure to stress.

Next, we sought to determine the acute actions of FENM administered after stress. Saline, (*R,S*)-ketamine (30 mg/kg), or FENM (20 mg/kg) was administered 5 minutes after CFC (Figure 2A). Here, CFC re-exposure occurred 1 day after CFC training. The dose of FENM was chosen based on results from Figure 1. Freezing was comparable between all groups during CFC training and re-exposures 1 and 2 (Figure 2B–E). On FST day 2, but not day 1, FENM, but not (*R,S*)-ketamine, significantly reduced immobility time compared with saline (Figure 2F–I). Behavior in all other assays were not altered by FENM or (*R,S*)-ketamine when compared with saline (Figure 2J–Q). These data indicate that FENM, but not (*R,S*)-ketamine, is effective against stress-induced behavioral despair when the interval between stress and behavioral testing is reduced in male mice.

FENM Does Not Alter Behavioral Despair in Nonstressed Mice

We then sought to test whether FENM could alter behavioral despair in nonstressed mice. Saline or FENM (20 mg/kg) was administered 1 hour before the start of the FST (Figure S2A in Supplement 1). Both groups of mice had comparable immobility during day 1 (Figure S2B, C in Supplement 1) and day 2 (Figure S2D, E in Supplement 1) of the FST. These data indicate that FENM does not alter behavioral despair in nonstressed male mice.

FENM Decreases Hyponeophagia in Nonstressed Mice

To test whether FENM altered avoidance and perseverative behavior in nonstressed mice, saline or FENM (20 mg/kg) was administered 1 hour before the start of the OF (Figure S3A in Supplement 1). FENM did not significantly alter behavior in the OF, MB, and EPM tests. Figure S3B–I in Supplement 1). FENM decreased the latency to feed in the NSF arena but not in the home cage when compared with saline administration (Figure S3J–L in Supplement 1). Both groups of mice ate a comparable amount of food in the home cage (Figure S3M in Supplement 1). These data indicate that FENM does not alter avoidance or perseverative behavior but decreases hyponeophagia in nonstressed male mice.

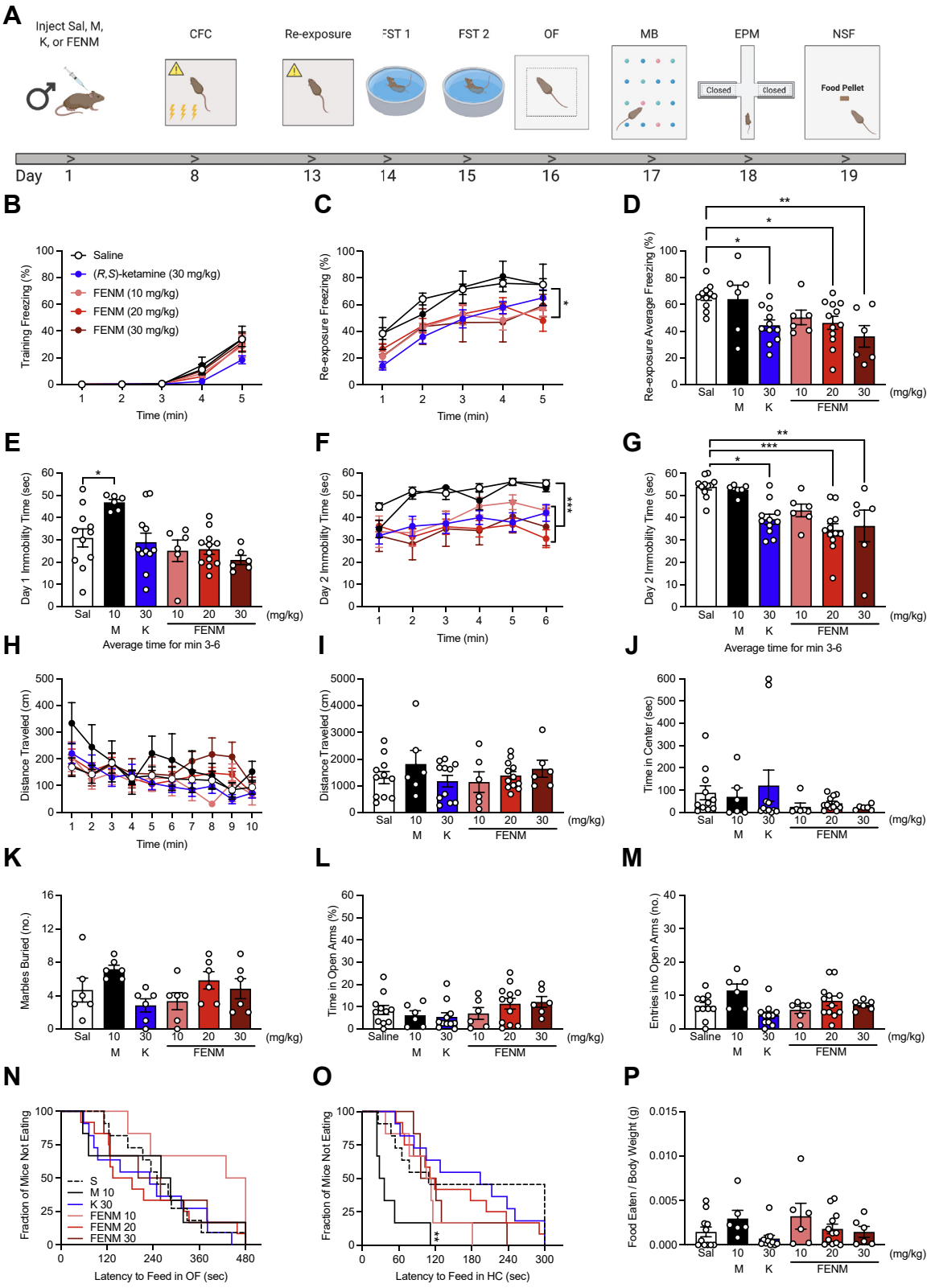
FENM Is Not Effective When Administered Before an Extinction Trial

We then aimed to determine whether FENM could also facilitate fear extinction. Saline, (*R,S*)-ketamine (30 mg/kg), or FENM (20 mg/kg) was administered 5 minutes before re-exposure 1 (Figure S4A in Supplement 1). Behavior was comparable across groups during all behavioral tests (Figure S4B–Q in Supplement 1). These data indicate that (*R,S*)-ketamine and FENM do not facilitate context extinction when administered before the first context re-exposure in male mice.

FENM Facilitates Extinction Learning

Subsequently, we sought to determine whether FENM could facilitate extinction if administered after an extinction trial.

Figure 3. FENM attenuates learned fear when administered after an extinction trial. **(A)** Saline, (*R,S*)-ketamine (30 mg/kg), or FENM (20 mg/kg) was administered 5 minutes after re-exposure 1 (24 hours before re-exposure 2). **(B, C)** During re-exposure 1, freezing was comparable across all groups. **(D, E)** During re-exposure 2, FENM, but not (*R,S*)-ketamine, significantly decreased fear expression when compared with saline. During day 1 **(F, G)** and day 2 **(H, I)** of the FST, all groups exhibited a comparable immobility time. **(J–L)** In the OF and MB assays, behavior was comparable across all groups. **(M, N)** In the EPM, the time spent in and entries into the open arms was comparable in all groups. In the NSF, FENM and (*R,S*)-ketamine did not alter the latency to eat in **(O, P)** the NSF arena or **(Q)** the HC. **(R)** All groups ate a comparable amount of food. n = 7–11 male mice per group; error bars represent ± SEM; $^{*}p < .05$. CFC, contextual fear conditioning; EPM, elevated plus maze; FENM, fluoroethylnormemantine; FST, forced swim test; HC, home cage; K, (*R,S*)-ketamine; MB, marble burying; NSF, novelty-suppressed feeding; OF, open field; Sal, saline.


A
Inject Sal, M, K, or FENM
CFC
Re-exposure
FST 1
FST 2
OF
MB
EPM
NSF
Closed
Closed
Food Pellet
Day 1 8 13 14 15 16 17 18 19
B
Training Freezing (%)
Saline
(R,S)-ketamine (30 mg/kg)
FENM (10 mg/kg)
FENM (20 mg/kg)
FENM (30 mg/kg)
Time (min)
C
Re-exposure Freezing (%)
Time (min)
D
Re-exposure Average Freezing (%)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
E
Day 1 Immobility Time (sec)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
Average time for min 3-6
F
Day 2 Immobility Time (sec)
Time (min)
G
Day 2 Immobility Time (sec)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
Average time for min 3-6
H
Distance Traveled (cm)
Time (min)
I
Distance Traveled (cm)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
J
Time in Center (sec)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
K
Marbles Buried (no.)
Sal 10 30 10 20 30 (mg/kg)
M K FENM
L
Time in Open Arms (%)
Saline 10 30 10 20 30 (mg/kg)
M K FENM
M
Entries into Open Arms (no.)
Saline 10 30 10 20 30 (mg/kg)
M K FENM
N
Fraction of Mice Not Eating
S
M 10
K 30
FENM 10
FENM 20
FENM 30
Latency to Feed in OF (sec)
O
Fraction of Mice Not Eating
Latency to Feed in HC (sec)
P
Food Eaten / Body Weight (g)
Sal 10 30 10 20 30 (mg/kg)
M K FENM

Here, we administered saline, (*R,S*)-ketamine, or FENM 5 minutes after CFC re-exposure 1 (Figure 3A). During CFC training and re-exposure 1 (Figure 3B, C), freezing was comparable across all groups. Interestingly, during re-exposure 2, FENM, but not (*R,S*)-ketamine, significantly decreased fear expression when compared with saline (Figure 3D, E). FENM did not alter all other behaviors tested (Figure 3F–Q). Overall, these data suggest that FENM is not effective when administered before extinction training but may be effective for attenuating fear expression when administered after an extinction trial in male mice.

FENM Is Prophylactic Against Learned Fear and Stress-Induced Behavioral Despair

We have previously reported that (*R,S*)-ketamine administration 1 week before exposure to stress results in prophylactic efficacy (16,18–22). To determine whether FENM could also be prophylactic against stress, saline, memantine (10 mg/kg), (*R,S*)-ketamine (30 mg/kg), or FENM (10, 20, or 30 mg/kg) was administered 1 week before CFC (Figure 4A). Freezing was comparable across all groups during CFC training (Figure 4B). During CFC re-exposure, (*R,S*)-ketamine (30 mg/kg) and FENM (20 and 30 mg/kg) administration decreased fear expression (Figure 4C, D). On FST day 1, memantine (10 mg/kg) increased immobility time when compared with saline controls (Figure 4E). However, on FST day 2, (*R,S*)-ketamine (30 mg/kg) and FENM (20 and 30 mg/kg), but not memantine (10 mg/kg), significantly reduced immobility time when compared with saline-administered mice (Figure 4F, G). These data show that both (*R,S*)-ketamine and FENM, but not memantine, attenuate learned fear and decrease stress-induced behavioral despair when administered as a prophylactic.

In the OF, MB, and EPM, no drugs tested significantly altered behavior (Figure 4H–M). Memantine, but not (*R,S*)-ketamine or FENM, reduced latency to feed in the home cage during the NSF (Figure 4N–P). Together, these data indicate that, in the same manner as (*R,S*)-ketamine, FENM is a robust prophylactic against learned fear and stress-induced behavioral despair but not stress-induced avoidance behavior.

To examine whether FENM exerts prophylactic efficacy in females, we administered a single dose of saline or FENM (10 or 20 mg/kg) in female 129S6/SvEv mice. One week later, mice were administered the same behavioral protocol shown in Figure 4A (Figure S5A in Supplement 1). Unlike in male mice, FENM did not alter freezing during CFC training or re-exposure (Figure S5B–E in Supplement 1). FENM at 20 mg/kg, but not 10 mg/kg, significantly reduced behavioral despair on FST day 2 but not on FST day 1 (Figure S5F–I in Supplement 1). FENM did not alter any other behaviors in female mice (Figure S5J–Q in Supplement 1). Our results indicate that FENM may be an efficacious prophylactic against behavioral despair but does not affect learned fear in female 129S6/SvEv mice.

FENM Is Not Prophylactic Against Stress-Induced Behavioral Despair When Administered 5 Minutes Before Exposure to Stress

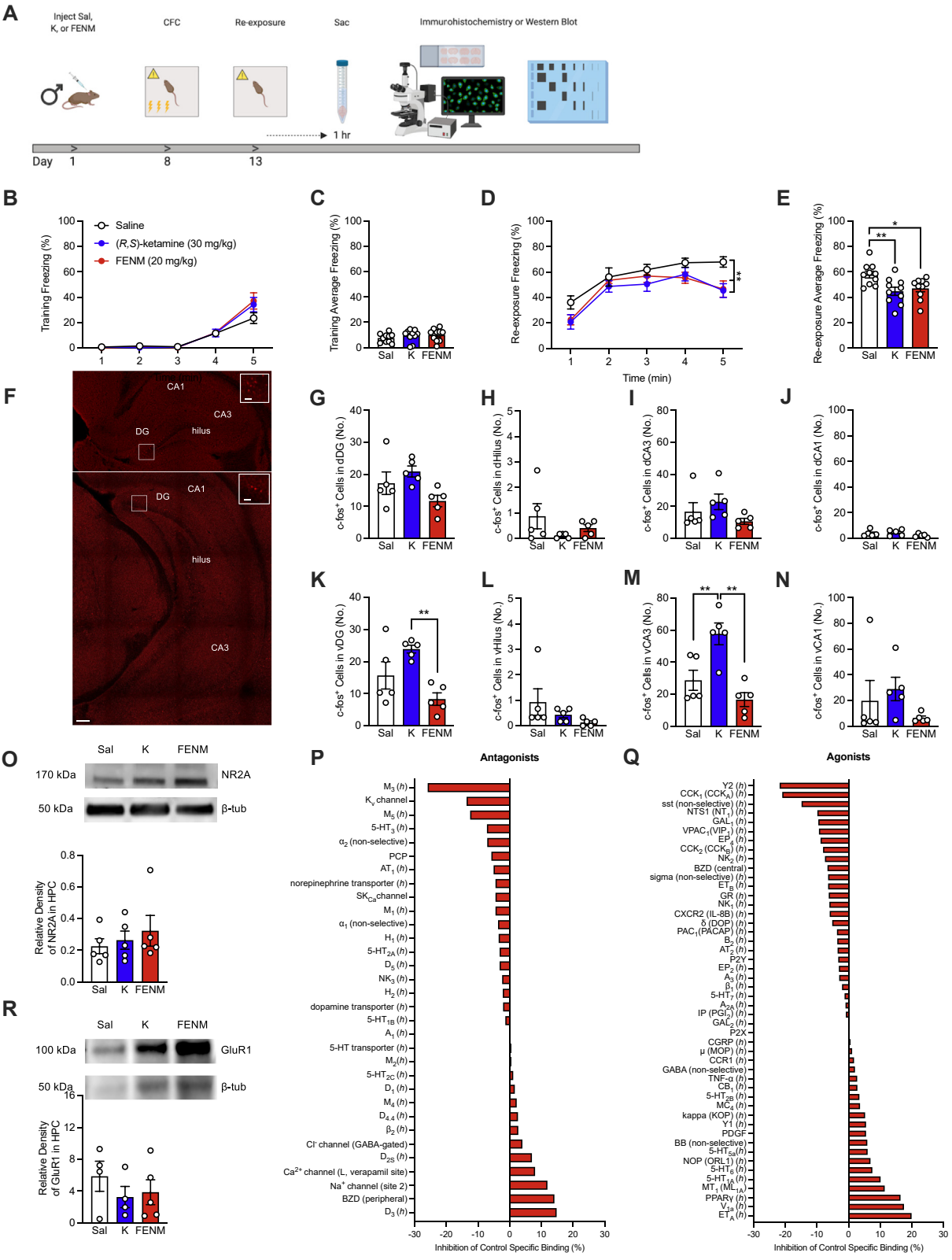
We next aimed to determine whether FENM could be effective as a prophylactic when administered shortly before CFC stress. Saline, (*R,S*)-ketamine (30 mg/kg), or FENM (20 mg/kg) was administered 5 minutes before CFC (Figure S6A in Supplement 1). During CFC training, (*R,S*)-ketamine significantly increased freezing when compared with saline and FENM (Figure S6B in Supplement 1). This increase in freezing behavior during training may be due to the anesthetic and/or psychotropic properties of (*R,S*)-ketamine. FENM did not result in alterations in freezing behavior during CFC training. During CFC re-exposures 1 and 2, (*R,S*)-ketamine, but not FENM, significantly decreased fear expression (Figure S6C–D in Supplement 1). These results suggest that in (*R,S*)-ketamine-treated mice, the decrease in freezing behavior during testing is most likely due to (*R,S*)-ketamine's anesthetic properties during training, resulting in ineffective encoding of the CFC context.

On FST day 2, but not day 1, (*R,S*)-ketamine, but not FENM, significantly decreased immobility time when compared with saline (Figure S6E–H in Supplement 1). In the OF, MB task, and EPM, (*R,S*)-ketamine and FENM did not significantly alter behavior (Figure S6I–L in Supplement 1). In the NSF, (*R,S*)-ketamine increased the latency to feed in the open arena compared with FENM-administered mice (Figure S6M, N in Supplement 1). However, latency to feed and food eaten in the home cage were comparable between all drug groups (Figure S6O, P in Supplement 1). These data indicate that while (*R,S*)-ketamine and FENM exhibit prophylactic efficacy, they do not reduce learned fear and prevent stress-induced behavioral despair and avoidance behavior when administered 5 minutes before stress.

(*R,S*)-Ketamine, but Not FENM, Selectively Increases Neural Activity in vCA3

Previously, we showed that prophylactic (*R,S*)-ketamine may attenuate learned fear by increasing neural activity in vCA3 (18). To determine whether FENM has similar effects, male mice were administered a single dose of saline, (*R,S*)-ketamine (30 mg/kg), or FENM (20 mg/kg) 1 week before 3-shock CFC. Five days later, mice were re-exposed to the training context

Figure 4. FENM attenuates learned fear and protects against stress-induced behavioral despair when administered 1 week before CFC. **(A)** Experimental protocol. **(B)** Freezing was comparable across all groups during CFC training. **(C, D)** (*R,S*)-ketamine (30 mg/kg) and FENM (20 and 30 mg/kg) significantly decreased fear expression. **(E)** On FST day 1, memantine (10 mg/kg) significantly increased immobility time when compared with saline-administered mice. **(F, G)** On day 2 of the FST, (*R,S*)-ketamine and FENM (20 and 30 mg/kg) significantly reduced immobility time when compared with saline controls. **(H–J)** In the OF, (*R,S*)-ketamine and FENM did not alter distance traveled or time spent in the center of the arena. **(K)** In the MB task, all groups buried a comparable number of marbles. **(L, M)** (*R,S*)-ketamine and FENM did not alter distance traveled or time spent in the open arms and center of the EPM. **(N)** Memantine, (*R,S*)-ketamine and FENM did not alter latency to feed in the NSF. **(O)** Memantine significantly reduced latency to feed in the HC during the NSF. **(P)** Food eaten in the NSF was comparable across all drug groups. n = 5–12 male mice per group; error bars represent ± SEM; $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. CFC, contextual fear conditioning; EPM, elevated plus maze; FENM, fluoroethylnormemantine; FST, forced swim test; HC, home cage; K, (*R,S*)-ketamine; M, memantine; MB, marble burying; NSF, novelty-suppressed feeding; OF, open field; Sal, saline.


A
Inject Sal, K, or FENM
CFC
Re-exposure
Sac
Immurohistochemistry or Western Blot
1 hr
Day 1 8 13
B
Saline
(R,S)-ketamine (30 mg/kg)
FENM (20 mg/kg)
Training Freezing (%)
C
Training Average Freezing (%)
Sal K FENM
D
Re-exposure Freezing (%)
Time (min)
E
Re-exposure Average Freezing (%)
F
CA1
CA3
DG
hilus
G
c-fos+ Cells in dDG (No.)
H
c-fos+ Cells in dHilus (No.)
I
c-fos+ Cells in dCA3 (No.)
J
c-fos+ Cells in dCA1 (No.)
K
c-fos+ Cells in vDG (No.)
L
c-fos+ Cells in vHilus (No.)
M
c-fos+ Cells in vCA3 (No.)
N
c-fos+ Cells in vCA1 (No.)
O
170 kDa
NR2A
50 kDa
β-tub
Relative Density of NR2A in HPC
R
100 kDa
GluR1
Relative Density of GluR1 in HPC
P
Antagonists
Inhibition of Control Specific Binding (%)
Q
Agonists
Inhibition of Control Specific Binding (%)

and sacrificed 1 hour later. Immunohistochemistry was used to quantify expression of c-fos across the HPC (Figure 5A). As previously shown, (*R,S*)-ketamine and FENM significantly reduced freezing upon re-exposure when compared with saline controls (Figure 5B–E). The number of c-fos$^+$ neurons was comparable across all groups throughout the dorsal HPC, as well as in the ventral dentate gyrus, ventral hilus, and vCA1 (Figure 5F–L, N). However, prophylactic (*R,S*)-ketamine, but not FENM, significantly increased c-fos expression in vCA3 of the HPC (Figure 5M). These data indicate that although both drugs attenuate learned fear, FENM, unlike (*R,S*)-ketamine, does not alter activity in vCA3 during fear memory retrieval.

Next, we hypothesized that both drugs may alter expression of NMDAR or AMPAR subunits in the HPC. Western blotting was used to quantify levels of NR2A, a subunit of NMDARs, and GluR1, a subunit of AMPARs (Figure 5A). These subunits were chosen because of their high expression levels in adult rodent HPC (37,38). We found that hippocampal NR2A and GluR1 subunit expression was comparable across all groups, indicating that neither FENM nor (*R,S*)-ketamine alters total expression of NMDAR or AMPAR subunits in the HPC during re-exposure (Figure 5O, R).

In order to determine whether FENM targeted receptors apart from the NMDAR, drug binding was assayed using in vitro radioligand binding assays. Because previous data indicated that FENM specifically targets NMDARs and can be displaced by (*R,S*)-ketamine administration, we investigated whether a submicromolar affinity may exist for other targets that had not been previously identified (29). At 1×10^{-7} M, FENM did not induce inhibition of control specific binding ±25%, indicating that the compound does not significantly bind to other receptors apart from the NMDAR (Figure 5P, Q).

FENM Attenuates Large-Amplitude AMPAR-Mediated Bursts in CA3

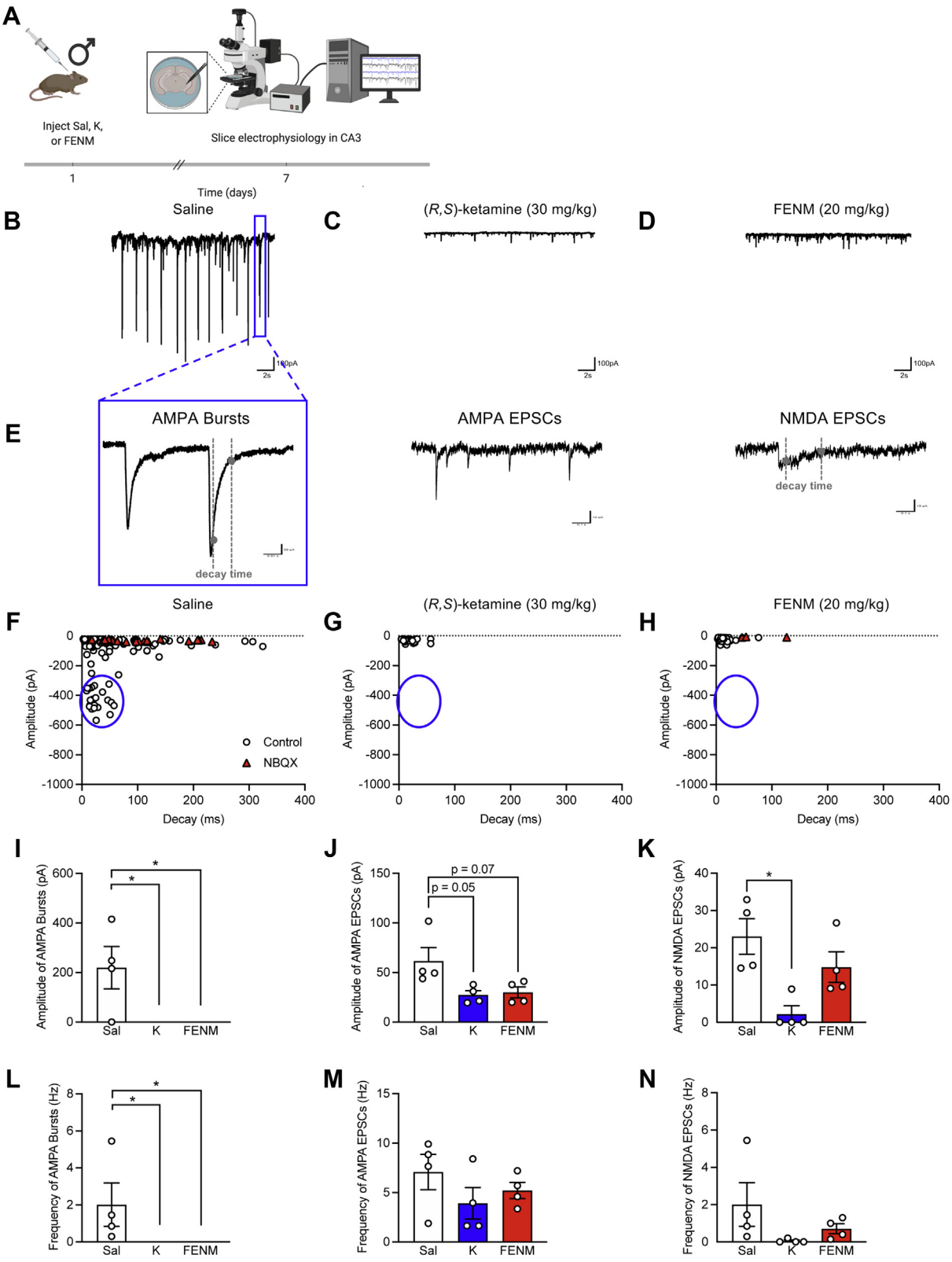
Finally, we aimed to investigate electrophysiological mechanisms that may contribute to FENM's behavioral effects. We previously demonstrated that (*R,S*)-ketamine, its stereospecific metabolite (2*R*,6*R*)-hydroxynorketamine ([2*R*,6*R*]-HNK), and the serotonin receptor type IV (5-HT$_4$R) agonist prucalopride, all of which are prophylactic against stress, robustly attenuate large-amplitude bursts of spontaneous AMPAR-mediated activity in vCA3 of the HPC (21,22). Here, male mice were injected with saline, (*R,S*)-ketamine (30 mg/kg), or FENM (20 mg/kg). One week later, whole-cell voltage clamp recordings of spontaneous excitatory postsynaptic currents (EPSCs) were performed in hippocampal vCA3 pyramidal cells (Figure 6A, E–G). Saline-administered mice displayed spontaneous large-amplitude bursts of AMPAR-mediated EPSCs as well as small-amplitude NMDAR-mediated EPSCs, which were revealed by bath application of NBQX, an AMPAR blocker (Figure 6B, H). Both large-amplitude AMPAR-mediated bursts and small-amplitude NMDAR-mediated EPSCs were attenuated in mice administered (*R,S*)-ketamine (Figure 6C, I). In mice administered FENM, the large AMPAR-mediated bursts were also robustly diminished, but small-amplitude NMDAR-mediated EPSCs were present, similar to saline controls (Figure 6D, G). The amplitude and frequency of AMPAR-mediated bursts was significantly attenuated in both experimental groups (Figure 6K, N). There was a trending, but not significant, decrease in the amplitude, but not frequency, of smaller AMPAR-mediated currents in both experimental groups (Figure 6L, O). (*R,S*)-ketamine-, but not FENM-treated, mice exhibited a significant reduction in NMDAR-mediated EPSC amplitude (Figure 6M). The frequency of NMDAR-mediated currents remained comparable across all groups (Figure 6P). These data show that, similar to previously studied prophylactic compounds, FENM significantly reduces large-amplitude AMPAR-mediated bursts in hippocampal vCA3 1 week after administration (21,22). Our results reinforce the emerging data indicating that targeting AMPAR-mediated neural activity in hippocampal vCA3 may be critical for altering resilience to stress.

DISCUSSION

Here, we characterized FENM, an NMDAR antagonist with antidepressant and prophylactic efficacy. We found that 1) FENM exhibits antidepressant-like properties in male and female mice exposed to stress, 2) FENM suppresses hyponeophagia in nonstressed male mice, 3) FENM attenuates fear when administered after extinction in male mice, 4) FENM is prophylactic when administered 1 week before exposure to stress in both sexes, and 5) FENM reduces large-amplitude AMPAR-mediated bursts in hippocampal vCA3 1 week after administration.

We previously reported that (*R,S*)-ketamine is prophylactic when administered 1 week before social defeat, learned helplessness, CFC, chronic corticosterone, and inflammatory stressors (16–18,20). Recently, we found that (2*R*,6*R*)-HNK, a metabolite of (*R,S*)-ketamine, and 5-HT$_4$R agonists are also effective prophylactics (21,22). Moreover, Gould *et al.* reported that group II metabotropic glutamate receptor (mGlu$_{2/3}$) antagonists are also protective against stress (21,22,39). As we

Figure 5. FENM does not bind to additional receptors nor alter neural activity or protein expression during fear retrieval. **(A)** Behavioral protocol. Male mice were given a single administration of saline, (*R,S*)-ketamine (30 mg/kg), or FENM (20 mg/kg) 1 week before three-shock CFC training. Five days later, mice were re-exposed to the training context. One hour after re-exposure, mice were sacrificed, and brain tissue was collected for use in either immunohistochemistry or Western blotting analysis. **(B, C)** Mice in all groups froze at comparable levels during CFC training. **(D, E)** Upon re-exposure, (*R,S*)-ketamine– and FENM-administered mice exhibited significantly less freezing when compared with saline controls. **(F)** Representative images of c-fos immunohistochemical staining in the (top) dorsal hippocampus and (bottom) ventral hippocampus. **(G–J)** Levels of c-fos$^+$ neurons were comparable across all groups in the dDG, dorsal hilus, dCA3, and dCA1. **(K, L)** All groups exhibited comparable levels of c-fos$^+$ neurons in the vDG and ventral hilus. **(M)** Mice administered (*R,S*)-ketamine, but not FENM, had significantly higher numbers of c-fos$^+$ neurons in vCA3. **(N)** c-fos expression was comparable across all groups in the vCA1. **(O, R)** Western blot analysis revealed comparable expression of NR2A and GluR1 subunits of the NMDA receptor and AMPA receptor, respectively, in the hippocampus. **(P, Q)** FENM did not significantly bind to any targets tested, indicating that the compound exerts selective affinity for the NMDA receptor. Error bars represent ± SEM; scale bars = 250 or 25 μm (insets); *$p < .05$, **$p < .01$. β-tub, beta-tubulin; CA, cornu ammonis; CFC, contextual fear conditioning; dCA, dorsal CA; dDG, dorsal DG; DG, dentate gyrus; FENM, fluoroethylnormemantine; K, (*R, S*)-ketamine; Sac, sacrifice; Sal, saline; vCA, ventral CA; vDG, ventral DG.


A
Inject Sal, K, or FENM
Slice electrophysiology in CA3
1
7
Time (days)
B
Saline
C
(R,S)-ketamine (30 mg/kg)
D
FENM (20 mg/kg)
100pA
2s
E
AMPA Bursts
AMPA EPSCs
NMDA EPSCs
decay time
F
Saline
G
(R,S)-ketamine (30 mg/kg)
H
FENM (20 mg/kg)
Amplitude (pA)
Decay (ms)
Control
NBQX
I
Amplitude of AMPA Bursts (pA)
J
Amplitude of AMPA EPSCs (pA)
p = 0.07
p = 0.05
K
Amplitude of NMDA EPSCs (pA)
L
Frequency of AMPA Bursts (Hz)
M
Frequency of AMPA EPSCs (Hz)
N
Frequency of NMDA EPSCs (Hz)
Sal
K
FENM

verified here that FENM exerts selective affinity for the NMDAR, our data support emerging evidence that NMDARs are a key target, not only for the treatment, but also for the prevention of stress-related disorders (40). NMDAR activity is intricately linked to synaptic plasticity and, accordingly, plays a significant role in fear memory (41,42). Because NMDARs act as detectors of synchronous activity, altering baseline NMDAR function could lead to modulations in neural network function, particularly in brain regions involved in fear learning (43). Ultimately, this modification could prove beneficial in buffering neurobiological responses to stress (42). Indeed, (*R,S*)-ketamine has been previously shown to restore homeostatic metabolic processes, reconfigure brain-wide neural network activity, and synchronize gamma oscillatory activity by reducing excessive NMDAR-dependent neurotransmission (44–46). Further research is necessary to determine whether FENM can modulate neural network dynamics similarly to (*R,S*)-ketamine.

On a molecular level, previous studies indicate that NMDAR antagonism may reduce behavioral despair through a number of candidate mechanisms (47,48). One such proposal, the disinhibition hypothesis, posits that NMDAR antagonism on inhibitory interneurons in the prefrontal cortex may lead to an overall increase in excitatory neurotransmission and cause a corresponding activation of postsynaptic AMPARs (49–52). Previous data show that NMDAR antagonism may directly inhibit the GluN2B subunit in extrasynaptic NMDARs, leading to a desuppression of mTOR (mechanistic target of rapamycin) function to induce protein synthesis (53–56). Additionally, NMDAR inhibition may suppress spontaneous NMDAR-mediated neurotransmission, leading to the inhibition of elongating factor 2 kinase (eEF2K) activity and enhancing translation of BDNF (brain-derived neurotrophic factor) (57,58). Finally, NMDAR antagonism may also inhibit neuronal bursting in the lateral habenula, leading to an acute downregulation of behavioral despair (59). While these studies have specifically used (*R,S*)-ketamine as an NMDAR antagonist, future studies will determine whether FENM exerts similar neurobiological actions to transduce its behavioral effects.

Notably, we found that although FENM reduced behavioral despair in both sexes, it decreased fear and perseverative behavior in male but not female mice. These data are consistent with previous studies demonstrating that (*R,S*)-ketamine reduces behavioral despair in both sexes but does not alter fear or perseverative behavior in female rodents (21,24). Across species, males and females exhibit distinct behaviors during fear learning and recall (60). For instance, female rats fail to exhibit learned helplessness, and female mice display higher levels of fear generalization than male mice (61,62). Similarly, neuroimaging data indicate that men and women utilize distinct brain circuitry to process stressful cues (63–65). Based on our data, NMDAR antagonism may be a more effective target in attenuating fear-related pathologies in males than in females. Further study is necessary to determine critical targets for reducing fear-related maladaptive behavior in females.

When administered after stress, FENM exerted a more immediate reduction in behavioral despair compared with (*R,S*)-ketamine. These results suggest that both compounds may enhance resilience if administered directly after stress exposure and may therefore exhibit clinically relevant significance in preventing affective disorders. Our data indicate that although both compounds act as rapid-acting antidepressants, the effects of (*R,S*)-ketamine may require more time to manifest in mice compared with FENM. While FENM's acute actions are still unknown, (*R,S*)-ketamine is known to exert its rapid antidepressant effects by upregulating BDNF expression (66). We hypothesize that FENM may upregulate BDNF signaling faster than (*R,S*)-ketamine, leading to a more rapid manifestation of its antidepressant actions. Further studies are therefore necessary to examine the acute neurobiological actions of FENM.

In our study, we found that FENM and (*R,S*)-ketamine exert distinct effects on neural activity in the hippocampus despite resulting in similar reductions in fear behavior. Our findings are consistent with previous results from our lab showing that prophylactic (*R,S*)-ketamine alters memory traces underling fear memory retrieval but not encoding (18). Indeed, many other studies have demonstrated that (*R,S*)-ketamine administration significantly alters c-fos expression throughout the brain, although increased numbers of c-fos$^+$ cells are typically associated with increased freezing responses (67–69). It has previously been demonstrated at (*R,S*)-ketamine administration enhances cognitive flexibility and restores active coping behaviors (70). These data suggest that increased vCA3 c-fos expression may activate additional downstream signaling and plasticity-related mechanisms that contribute to the protective effects of (*R,S*)-ketamine, thereby contributing to a reduction in fear behavior upon re-exposure (70–73). Future studies will

Figure 6. Similar to (*R,S*)-ketamine, FENM reduces AMPAR-mediated bursts in hippocampal CA3. **(A)** Mice were administered saline, (*R,S*)-ketamine (30 mg/kg), or FENM (20 mg/kg) 1 week before whole-cell voltage clamp electrophysiology. Representative EPSCs in a **(B)** saline-administered, **(C)** (*R,S*)-ketamine–administered, and **(D)** FENM-administered mouse. **(E)** Representative expanded view of a large-amplitude AMPAR-mediated burst, smaller-amplitude AMPAR-mediated EPSC, and NMDAR-mediated EPSC. Decay time was measured from 10%–90% of the peak amplitude. **(F)** Scatter plot indicating that a saline-administered mouse displayed large bursts of AMPAR-mediated EPSCs in addition to small-amplitude NMDAR-mediated currents, which were revealed by washing in NBQX. **(G)** Large-amplitude AMPAR-mediated bursts and NMDAR-mediated EPSCs were attenuated in (*R,S*)-ketamine mice. **(H)** In FENM-administered mice, large-amplitude AMPAR-mediated bursts were also robustly attenuated, but NMDAR-mediated EPSCs were not significantly altered. **(I)** Large AMPAR-mediated burst amplitude was attenuated in experimental groups compared with saline controls. **(J)** There was a trending but not significant reduction in the mean amplitude of spontaneous AMPAR-mediated currents in (*R,S*)-ketamine– and FENM-administered mice. **(K)** The mean amplitude of NMDAR-mediated currents was significantly reduced in (*R,S*)-ketamine–, but not in FENM-administered mice. **(L)** AMPAR-mediated bursts were blocked by both (*R,S*)-ketamine and FENM drug administration. **(M)** The mean frequency of all AMPAR-mediated EPSCs within a 20-second recording period was not significantly altered by drug administration. **(N)** The frequency of NMDAR-mediated EPSCs was comparable across all groups. $n = 4$ cells per group; error bars represent ± SEM; $^*p < .05$. AMPAR, AMPA receptor; CA3, cornu ammonis area 3; EPSC, excitatory postsynaptic current; FENM, fluoroethylnormemantine; K, (*R, S*)-ketamine; NMDAR, NMDA receptor; Sal, saline.

examine the neurocircuitry underlying prophylactic (*R,S*)-ketamine and FENM's effects to determine whether these actions in the hippocampus impact brain-wide neural activity.

Finally, our study provides further evidence that prophylactic compounds may alter AMPAR-mediated signaling in vCA3 to enhance resilience. We have previously identified various prophylactic compounds, including (*R,S*)-ketamine, its metabolite (2*R*,6*R*)-HNK, and the 5-HT_4R agonist prucalopride, that block large, spontaneous AMPAR-mediated bursts in vCA3 1 week after administration (21,22). Similarly, FENM attenuated AMPAR-mediated bursts of excitatory activity in vCA3. It is possible that these compounds decrease the amount of glutamate packaged within vesicles at dentate gyrus granule cell terminals or reduce the number of vesicles released from the spontaneous vesicle pool, thus attenuating AMPAR-mediated bursts (74). Alternatively, these compounds could also result in postsynaptic modifications of AMPARs on vCA3 neurons (75). The overall reduction in large-amplitude AMPAR activity in vCA3 could alter synaptic homeostasis in downstream areas, such as vCA1, to improve the encoding of contextual stimuli, reduce potential for fear generalization, and enhance resilience to stress (76). However, a more thorough electrophysiological characterization is required to determine the exact mechanisms contributing to our findings.

Overall, the present study has identified a novel NMDAR antagonist that is efficacious at preventing and treating stress-induced fear, behavioral despair, and avoidance behavior in both sexes. These data reinforce the NMDAR as a key target for regulating different stress-related behaviors. Future studies may lead to a deeper understanding of how NMDAR antagonists can modulate a variety of stress-related maladaptive behaviors.

ACKNOWLEDGMENTS AND DISCLOSURES

The research reported in this article was supported by grants from the NIMH (Grant No. F31 MH121023 [to BKC]), the NIA (Grant Nos. K01 AG054765 [to VML] and R56 AG058661 and R21 AG064774 [to CAD]), the NICHD (Grant No. R01 HD101402 [to CAD]), and the NINDS (Grant No. R21 NS114870 [to CAD]). This work was funded by a grant from Columbia Technology Ventures (to BKC, CAD). We thank Philippe Guerret for the provision of FENM.

We thank Dr. Jonathan Javitch and Cory Langreck for comments on the project and experimental assistance. We also thank Dr. Ron Katz, Dr. Denis David, and members of the Denny Laboratory for insightful comments on this project and manuscript.

BKC, JCM, GR, and CAD are named on provisional and nonprovisional patent applications for the prophylactic use of (*R,S*)-ketamine, fluoroethylnormemantine, and/or related compounds against stress-related psychiatric disorders. Since the start of 2020, CAD has received honoraria for speaking for Janssen, Janssen Southeast Asia, North American Neuromodulation Society, and Albany Medical College. All other authors report no biomedical financial interests or potential conflicts of interest.

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Received Aug 4, 2020; revised Apr 13, 2021; accepted Apr 30, 2021.

Supplementary material cited in this article is available online at https://doi.org/10.1016/j.biopsych.2021.04.024.

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