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Briana K. Chen, Gwenaëlle Le Pen, Adam Eckmier, Gilles Rubinstenn, Therese M. Jay, Christine A. Denny

Doctoral Program in Neurobiology and Behavior, Columbia University, New York, New York, USA (Ms Chen); Institute of Psychiatry and Neuroscience of Paris, INSERM U1266, Pathophysiology of Psychiatric Disorders, Université de Paris, Paris, France (Drs Le Pen, Mr Eckmier, and Dr Jay); ReST Therapeutics, Montpellier, France (Dr Rubinstenn); Division of Systems Neuroscience, Research Foundation for Mental Hygiene, Inc./New York State Psychiatric Institute, New York, New York, USA (Dr Denny); Department of Psychiatry, Columbia University Irving Medical Center, New York, New York, USA (Dr Denny).

Correspondence: Christine Ann Denny, PhD, Columbia University Irving Medical Center, NYSPI Kolb Research Annex, Room 777, 1051 Riverside Drive, Unit 87, New York, NY 10032 (cad2125@cumc.columbia.edu).

Abstract

Background: Memantine, a noncompetitive N-methyl-D-aspartate receptor antagonist, has been approved for use in Alzheimer's disease, but an increasing number of studies have investigated its utility for neuropsychiatric disorders. Here, we characterized a novel compound, fluoroethylnormemtantine (FENM), which was derived from memantine with an extra Fluor in an optimized position for in vivo biomarker labeling. We sought to determine if FENM produced similar behavioral effects as memantine and/or if FENM has beneficial effects against fear, avoidance, and behavioral despair.
Methods: We administered saline, FENM, or memantine prior to a number of behavioral assays, including paired-pulse inhibition, open field, light dark test, forced swim test, and cued fear conditioning in male Wistar rats.
Results: Unlike memantine, FENM did not produce nonspecific side effects and did not alter sensorimotor gating or locomotion. FENM decreased immobility in the forced swim test. Moreover, FENM robustly facilitated fear extinction learning when administered prior to either cued fear conditioning training or tone reexposure.
Conclusions: These results suggest that FENM is a promising, novel compound that robustly reduces fear behavior and may be useful for further preclinical testing.

Key Words: FENM, extinction learning, cued fear conditioning, PTSD, NMDAR antagonist

Introduction

N-methyl-D-aspartate (NMDA) receptor (NMDAR) function has been increasingly implicated in the pathophysiology of neuropsychiatric diseases, including mood disorders and Alzheimer's disease (AD) (Paul, 1997; Ravindran and Stein, 2009; Barkus et al., 2010; Steckler and Risbrough, 2012; Ates-Alagoz and Adejare, 2013; Lakhan et al., 2013; Liu et al., 2019). Expressed abundantly throughout the brain, NMDARs play a vital role in excitatory neurotransmission

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Significance Statement

N-methyl-D-aspartate (NMDA) receptors have been previously implicated as a potential target for the prevention and treatment of stress-related psychiatric disorders. Recently, a novel NMDA receptor antagonist, fluoroethylnormemantine (FENM), was derived from memantine. Here, using a variety of behavioral assays, we characterized the behavioral properties of FENM in rats. We found that FENM significantly reduced behavioral despair and facilitated extinction learning without altering sensorimotor function. Our findings indicate that FENM may be a suitable compound for further preclinical testing in a variety of stress-based behavioral assays.

and synaptic plasticity. Accordingly, disruption of NMDAR function, whether due to natural disease causes or pharmacological intervention, can significantly alter cognitive function and emotionality. A number of novel NMDAR antagonists have been demonstrated as efficacious treatments against a variety of neuropsychiatric disorders. For instance, (*R*,*S*)-ketamine is widely known for its rapid-acting, long-lasting antidepressant actions as well as its efficacy in treating treatment-resistant depression (Berman et al., 2000; Zarate et al., 2006; aan het Rot et al., 2010; Murrough et al., 2013). Moreover, intranasal (*S*)-ketamine (Spravato), a stereospecific derivative of (*R*,*S*)-ketamine that specifically targets NMDARs, was recently approved by the FDA as an adjunctive treatment for treatment-resistant depression and has shown strong, rapid efficacy for reducing symptoms of depression, including suicidality (Daly et al., 2018, 2019; Fedgchin et al., 2019; Popova et al., 2019; Fu et al., 2020; Ionescu et al., 2020; Nijs et al., 2020; Perez-Ruixo et al., 2020; Saad et al., 2020; Wajs et al., 2020; ClinicalTrials.gov, NCT0260929, NCT02094378, NCT02133001, NCT02343289, NCT02345148, NCT02606084, NCT02611505, NCT02674295, NCT02857777, NCT03298906). Previous studies have also shown that (*R*,*S*)-ketamine–induced NMDAR inhibition may also prevent stress-induced behavioral despair as well as attenuate learned fear when administered prior to stress (Amat et al., 2016; Brachman et al., 2016; McGowan et al., 2017; Dolzani et al., 2018; Mastrodonato et al., 2018; Pham et al., 2018; Chen et al., 2020b). Despite these promising studies, (*R*,*S*)-ketamine presents a challenge for clinical development because of its nonspecific side effects, including psychotropic actions and high abuse potential. Therefore, efforts are currently underway to develop NMDAR antagonists with similar efficacy but reduced side effects.

Interestingly, although many alternative NMDAR antagonists (e.g., rapastinel, MK-801) have shown promising antidepressant results in both rodents and humans, to date, these compounds have failed to show significant efficacy in reducing depressive symptoms compared with placebo controls in clinical trials (Ates-Alagoz and Adejare, 2013; Moskal et al., 2014; Newport et al., 2015; Gerhard et al., 2016; Yang et al., 2016; Kato et al., 2018; Kadriu et al., 2019; Kato and Duman, 2020; ClinicalTrials.gov, NCT02192099, NCT02267629, NCT02932943, NCT02943564, NCT02943577, NCT02951988, NCT03002077, NCT03352453, NCT03560518, NCT03575776, NCT03614156, NCT03668600, NCT03799900, NCT03814733, NCT03855865). One such drug is memantine, a noncompetitive open-channel NMDAR antagonist (Kishi et al., 2017a). Memantine is clinically used as a treatment for AD and has been shown to improve memory, awareness, and the ability to perform daily tasks (Kishi et al., 2017b). Although some early studies indicated that memantine may exhibit antidepressant efficacy, a longitudinal study showed that memantine is no better than placebo at managing symptoms of depression (Moryl et al., 1993; Kishi et al., 2017a). Therefore, despite its lack of antidepressant actions, memantine does exert pro-cognitive effects. Thus, examining analogues of memantine may reveal novel NMDAR antagonist compounds that can improve cognition as well as exhibit antidepressant and/or anxiolytic efficacy.

Recently, a novel radiolabeled compound, [^{18}F]-fluoroethylnormemantine (FENM), was derived from memantine as a novel positron emission tomography (PET) tracer (Salabert et al., 2015, 2018). With a K_i of $3.5^{10\text{-}6}$ M and high lipophilicity (logD = 1.93), [^{18}F]-FENM stabilized 40 minutes after injection with approximately 0.4% of the original dose present in the brain. Combined ex vivo autoradiography and immunohistochemistry indicated that [^{18}F]-FENM strongly colocalizes with NMDARs in the cortex and cerebellum. Intriguingly, this colocalization is blocked by injection of (*R*,*S*)-ketamine, suggesting that FENM exhibits a lower affinity to the NMDAR receptor than (*R*,*S*)-ketamine. Moreover, because both compounds bind to phencyclidine sites in the NMDAR channel pore, these data suggest that the compounds may also exert similar behavioral effects. However, while the antidepressant-like effects of FENM remain unknown, recent data indicate that FENM enhances cognitive function and exerts neuroprotective properties in a mouse model of AD (Couly et al., 2020). In this study, Couly and colleagues found that FENM reversed deficits in long-term memory, navigation, and place learning, and object recognition in a pharmacological model of AD. Interestingly, compared with the effects of memantine, the authors showed that FENM improved spatiotemporal orientation in the Hamlet test while memantine did not affect behavior. FENM's behavioral actions were found to correspond with a reduction in inflammatory cytokines and neuronal cell loss in the hippocampus. Thus, although FENM shows potential for enhancing cognition and protecting age-related brain impairments, it is still unknown whether the drug can reverse stress-related maladaptive behaviors.

Here, we aimed to characterize the effects of FENM on avoidance behavior, behavioral despair, and extinction learning in rats. A single injection of saline, FENM, or memantine was administered acutely prior to a range of behavioral assays, including paired-pulse inhibition (PPI), open field (OF), forced swim test (FST), and cued fear conditioning (FC) and extinction. FENM did not affect sensorimotor behavior or avoidance behavior. FENM decreased behavioral despair in the FST and reduced fear behavior when administered at a variety of timepoints prior to cued FC and extinction training. These data indicate that FENM is a novel compound with robust fear-reducing properties and is a promising candidate for preclinical development as a potential treatment for fear-related disorders.

METHODS

For a full description of Methods, please refer to the Supplemental Methods.

Rats

Three hundred male Wistar rats were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Rats were ordered at 250–274 g and were tested after 1 week of acclimatization. Rats were kept 2 per cage in a 12-hour-light/-dark (6:00 AM–6:00 PM) colony room at 22°C. Food and water were provided ad libitum. Rats were used once per behavioral experiment. All procedures were approved by a local ethics committee (French Ministry of Health and Research Authorization N°APAFIS#3571-2015091614517975v7) in accordance with the European Communities Council Directive (2010/63/EU) and the European Union guidelines.

Drugs

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

FENM

FENM was administered in a single dose at 1, 3, 5, 10, or 20 mg/kg of body weight. FENM was generated by M2i (Saint-Cloud, France).

Memantine

Memantine was administered in a single dose at 1, 3, or 10 mg/kg of body weight. Memantine was provided by M2i (Saint-Cloud, France) and purchased from TCI Chemicals (Tokyo, Japan).

Behavioral Methods

All behavioral tests were performed in the active phase.

PPI

The apparatus consisted of 4 startle chambers (SRLAB, San Diego Instruments, CA) containing a transparent Plexiglas tube (diameter 8.2 cm, length 20 cm) mounted on a Plexiglas frame within a ventilated enclosure. Acoustic noise bursts were presented via a speaker mounted 24 cm above the tube. Throughout the session, a background noise level of 68 dB was maintained. A piezoelectric accelerometer mounted below the frame detected and transduced motion within the tube. Startle amplitudes were defined as the average of 100 1-ms stabilimeter readings collected from stimulus onset. Rats were run in groups of 4. Each rat was put into the PPI chamber for a 5-minute acclimatization period with a 68-dB background noise. Following this period, 10 startle pulses (120 dB, 40-ms duration) were presented with an average inter-trial interval of 15 seconds. Then, no stimulus (background noise, 68 dB), prepulses alone (72, 76, 80, or 84 dB, 20-millisecond duration), startle pulses alone, and prepulses followed (80 millisecond later) by startle pulses, were presented 10 times, randomly distributed over the next 32 minutes. The percentage of PPI induced by each prepulse intensity was calculated as: 100[(SP – SPP)/SP], with SP being the average startle amplitude following the startle pulses and SPP being the average startle response following the combination of a certain prepulse and the startle pulse.

OF

The OF test was conducted in an arena (43.2 cm × 43.2 cm) with transparent acrylic walls and white floor (Med Associates Inc., St. Albans, VT). Rats were individually placed in the OF arena for 120 minutes. Locomotor activity was monitored using a two 16-beam infrared system. Data from the first 30 minutes were included in analysis. Velocity and total distance travelled were analyzed using the Activity Monitor software (Med Associates).

Light Dark Test (LDT)

The apparatus for the light/dark test consisted of a box (40 cm × 40 cm × 32 cm) divided into dark (black walls, floor and roof, 5 lux) and light (white walls and floor, no roof, 600 lux) compartments of equal volume (40 × 40 × 32 cm). The compartments were connected via a small opening (10 × 10 cm) enabling transition between the 2 boxes. Rats were placed in the light compartment, and the time spent in each compartment during the 10 minutes test was assessed online via a video camera located above the box. Behavior was automatically analyzed using video tracking software (View Point, Lyon, France).

FST

For the FST, rats were individually placed into glass cylinders (40 cm high, 18 cm in diameter) containing 28 cm of water at 23°C ±2°C for 15 minutes (pre-swim) and then gently dried and returned to their home cages. They were placed again in the cylinders 24 hours later, and the 6-minute FST was conducted. All sessions were recorded with an automated video tracking system (View Point). The total duration of immobility as well as bursting were measured throughout the second trial.

Cued FC

Cued FC occurred in a standard conditioning chamber (Context A) (VCF-007, Med Associates) kept inside a sound-attenuating cubicle. This chamber had the internal dimensions of 30 × 24 × 33 cm, with aluminum sidewalls, an opaque polycarbonate rear wall, and a transparent Plexiglas door. The grid floor was connected to a shock scrambler and shock generator, and approximately 0.2 mL of 1% ammonium hydroxide was put in the collection pan. The chamber was dark except for 2 infrared light sources located above the training box (NIR200, Med Associates). Rats received 3 tone-shock pairings at 180 seconds, 381 seconds, and 582 seconds, following placement in the chamber and the launching of the Med Associates program. Each tone was 20 seconds long and each shock, which followed the tone, lasted 1 second. Each foot shock was 1.0 mA based on prior pilot studies.

Extinction

Thirty minutes before either extinction session, rats were injected with saline or FENM and placed into context B. Context B was the same darkened chamber as Context A but with a white plastic floor and curved wall inserts scented with 1% acetic acid in the collection pan. Each extinction session had twenty-four 20-second tone presentations, separated by 35 seconds. Videos were scored by hand by an experimenter blind to treatment group to avoid software-induced false freezing. Freezing was quantified over the entire session.

Statistical Analysis

All data were analyzed using Prism 7.0 and 8.0 (GraphPad Software, La Jolla, CA). Alpha was set to .05 for all analyses. Data were confirmed as normally distributed with equal variance using a Shapiro-Wilk normality test and by calculating the variance. A 1-way, 2-way, or repeated-measures ANOVA (RMANOVA) was conducted where appropriate. Post-hoc analysis was conducted when the overall ANOVA (e.g., drug) was significant or when the interaction between the 2 factors (e.g., drug × tone or drug × time) was significant. Post-hoc Dunnett's tests were used

to determine the statistical significance of multiple experimental groups against a single control group. All statistical tests and *P* values are listed in supplementary Table 1.

RESULTS

Memantine, But Not FENM, Decreases Prepulse Inhibition in Rats

Reduced PPI is indicative of deficient sensorimotor gating and is present in various neuropsychiatric disorders, including schizophrenia and Tourette's syndrome. It has previously been shown that memantine can impair normal PPI startle responses (Swerdlow et al., 2009). We therefore sought to determine whether FENM would affect sensorimotor gating similarly to memantine. Rats were administered saline, memantine (10 mg/kg), or FENM (5, 10, or 20 mg/kg). Thirty minutes later, rats were tested in a PPI assay (Figure 1a). Memantine, but not FENM, reduced prepulse inhibition of the startle response compared with saline controls (Figure 1b; RMANOVA drug: $F_{(4,55)}=2.94$, $P=.0283$, pulse: $F_{(3,165)}=68.35$, $P<.0001$, drug × pulse: $F_{(12,165)}=0.5241$, $P=.8970$; Dunnett's multiple comparisons: Sal vs memantine (10 mg/kg): $P=.0103$, Sal vs FENM (5 mg/kg): $P=.4591$, Sal vs FENM (10 mg/kg): $P=.7847$; Sal vs FENM (20 mg/kg): $P=.9824$). When mean freezing was averaged across the entire trial, memantine, but not FENM, significantly lowered the mean startle response (Figure 1c; ANOVA drug: $F_{(4,55)}=2.94$, $P=.0283$; Dunnett's multiple comparisons: Sal vs memantine (10 mg/kg) $P=.0103$, Sal vs FENM (5 mg/kg) $P=.4591$, Sal vs FENM (10 mg/kg) $P=.7847$, Sal vs FENM (20 mg/kg) $P=.9824$). These results indicate that FENM does not alter sensorimotor gating like its parent compound memantine.

FENM Does Not Alter Locomotion or Avoidance Behavior in Rats

Memantine has previously been shown to reduce ambulatory activity and rearing when administered acutely before the OF (Kos and Popik, 2005). Here, we sought to determine if FENM, similarly to its parent compound, also altered locomotor behavior. Rats were administered saline, memantine (10 mg/kg), or FENM (5, 10, or 20 mg/kg) and 30 minutes later placed into an OF (Figure 2a). Memantine, but not FENM, decreased ambulatory [Figure 2b; ANOVA: drug $F_{(4,55)}=14.19$, $P<.0001$; Dunnett's multiple comparisons: Sal vs memantine (10 mg/kg) $P<.0001$, Sal vs FENM (5 mg/kg) $P=.8133$, Sal vs FENM (10 mg/kg) $P=.9259$, Sal vs FENM (20 mg/kg) $P=.0939$], vertical [Figure 2c; ANOVA: drug $F_{(4,55)}=14.430$, $P<.0001$; Dunnett's multiple comparisons: Sal vs memantine (10 mg/kg) $P<.0001$, Sal vs FENM (5 mg/kg) $P=.9720$, Sal vs FENM (10 mg/kg) $P=.1678$, Sal vs FENM (20 mg/kg) $P=.9993$], and stereotypic [Figure 2d; ANOVA: $F_{(4,55)}=10.86$, $P<.0001$; Dunnett's multiple comparisons: Sal vs memantine (10 mg/kg) $P<.0001$, Sal vs FENM (5 mg/kg) $P=.9933$, Sal vs FENM (10 mg/kg) $P=.95$, Sal vs FENM (20 mg/kg) $P=.9998$] counts in the OF. All groups spent a comparable amount of time in the center (Figure 2e; ANOVA: drug $F_{(4,55)}=0.449$, $P=.4487$). These data show that, unlike memantine, FENM does not alter locomotion.

Next, to further expand on on the locomotor effects, we utilized an LDT. Here, rats were again administered saline, memantine (1, 3, or 10 mg/kg), or FENM (1, 3, or 10 mg/kg) 30 minutes before the LDT (supplementary Figure 1a). The time spent in the light compartment did not differ between the groups (supplementary Figure 1b; ANOVA: drug $F_{(6,76)}=2.13$, $P=.0595$). As observed in the OF assay, memantine (10 mg/kg), but not FENM, significantly reduced the number of transitions between the light and dark compartments [supplementary Figure 1c; ANOVA: drug $F_{(6,76)}=2.314$, $P=.0416$; Dunnett's multiple comparisons: Sal

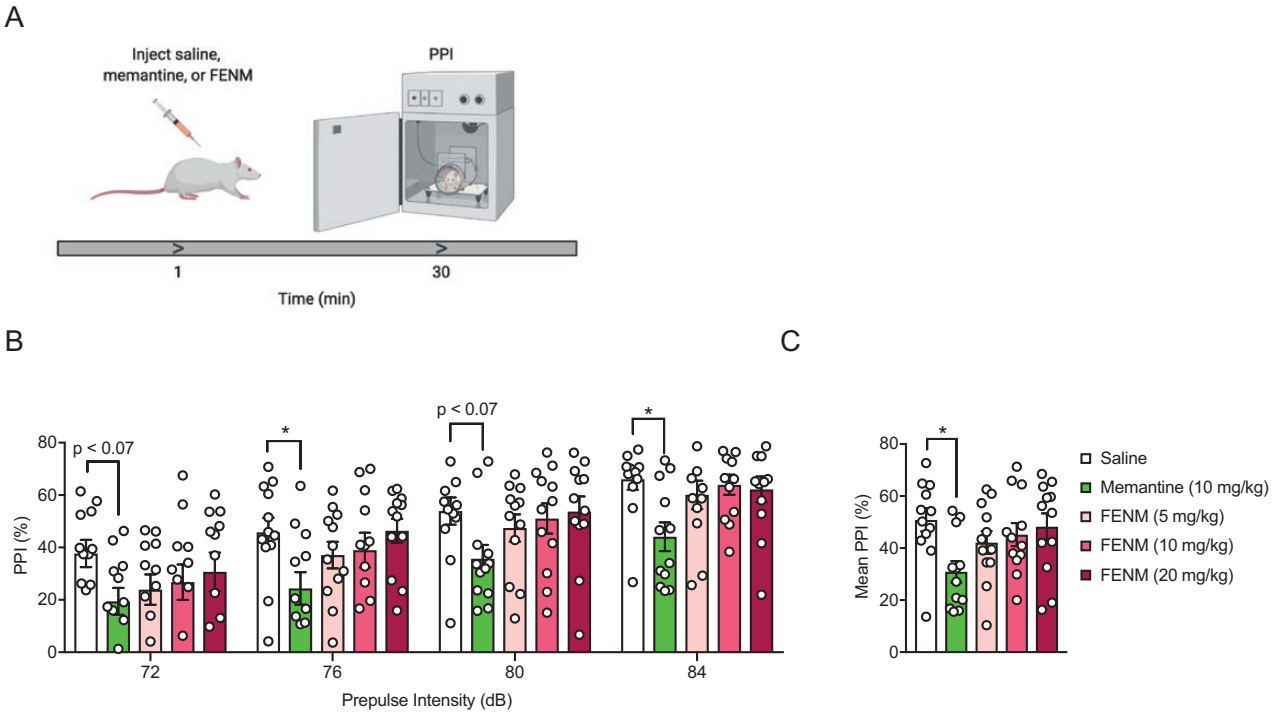

Figure 1. Fluoroethylnormemantine (FENM) does not alter the startle response during pre-pulse inhibition. (a) Experimental protocol. (b) Memantine consistently reduced the startle response at multiple stimulus intensities compared with saline-administered rats. FENM (5, 10, or 20 mg/kg) did not alter the startle response. (c) Memantine, but not FENM, significantly reduced mean freezing compared with the saline control group (n = 12 male rats/group). Error bars represent ± SEM. **P < .01. Abbreviation: PPI, paired-pulse inhibition.

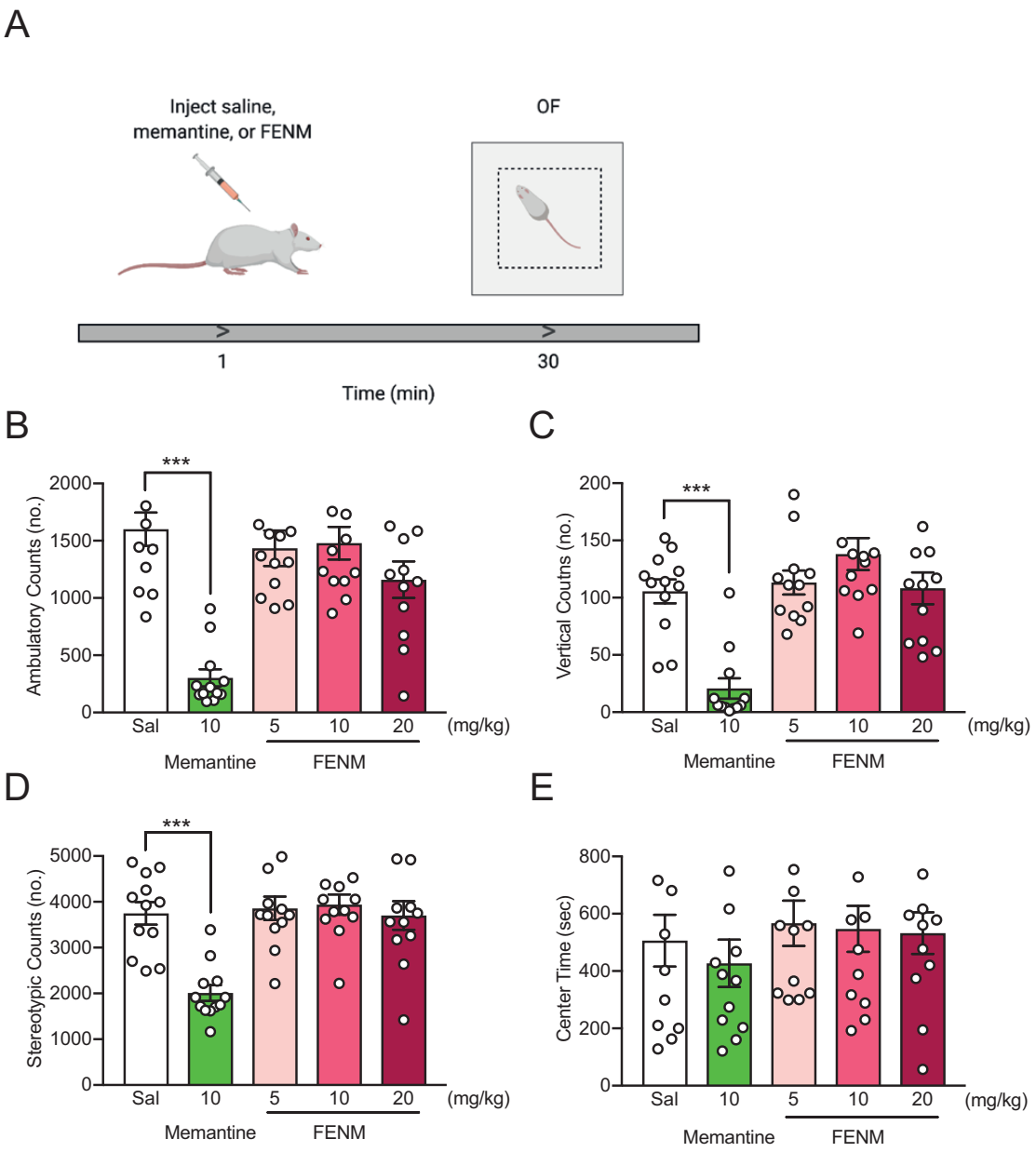

Figure 2. Fluoroethylnormemantine (FENM) does not alter locomotion or exploratory behavior in the open field. (a) Experimental protocol. (b–d) Memantine, but not FENM, significantly reduced locomotion, rearing, and stereotypic behavior, respectively, in the open field (OF) compared with saline-administered rats. (e) Time in the center of the OF was comparable across all drug groups. (n = 12 male rats/group). Error bars represent ± SEM. ***$P < .0001$. Abbreviation: OF, open field.

vs memantine (1 mg/kg) $P = .5480$, Sal vs memantine (3 mg/kg) $P = .0533$, Sal vs memantine (10 mg/kg) $P = .0174$, Sal vs FENM (1 mg/kg) $P = .8739$, Sal vs FENM (3 mg/kg) $P = .9781$, Sal vs FENM (10 mg/kg) $P = .3863$]. These data suggest that FENM does not significantly alter avoidance behavior.

FENM Decreases Behavioral Despair in Rats

Previous data suggest that memantine may reduce behavioral despair in rats (Moryl et al., 1993). Here, we sought to test if FENM altered behavioral despair (Figure 3a). Male Wistar rats were given a 15-minute preswim trial, which functioned as an initial stressor (Porsolt et al., 1977). Twenty-four hours later, saline, memantine (1, 3, or 10 mg/kg), or FENM (1, 3, or 10 mg/kg) was injected, and 30 minutes later, a second FST session was administered for 6 minutes. Here, drug dosages for both memantine and FENM were chosen in accordance with a previous study indicating that lower doses of memantine (e.g., 1, 3, or 10 mg/kg) can reduce behavioral despair in rodents (Almeida et al., 2006). Consistent with this publication, memantine (1, 3, and 10 mg/kg) decreased immobility time [Figure 3b and d; RMANOVA: drug $F_{(6,77)} = 3.649$, $P = .0031$, time $F_{(5,385)} = 68.02$, $P < .0001$, drug × time $F_{(30,385)} = 1.503$, $P = .0461$; Dunnett's multiple comparisons: Sal vs memantine (1 mg/kg) $P = .0435$, Sal vs memantine (3 mg/kg) $P = .1678$, Sal vs memantine (10 mg/kg) $P = .0007$; ANOVA: drug $F_{(6,77)} = 4.558$, $P = .0005$; Dunnett's multiple comparisons: Sal vs memantine (1 mg/kg) $P = .0133$, Sal vs memantine (3 mg/kg) $P = .0283$, Sal vs memantine (10 mg/kg) $P = .0001$] and

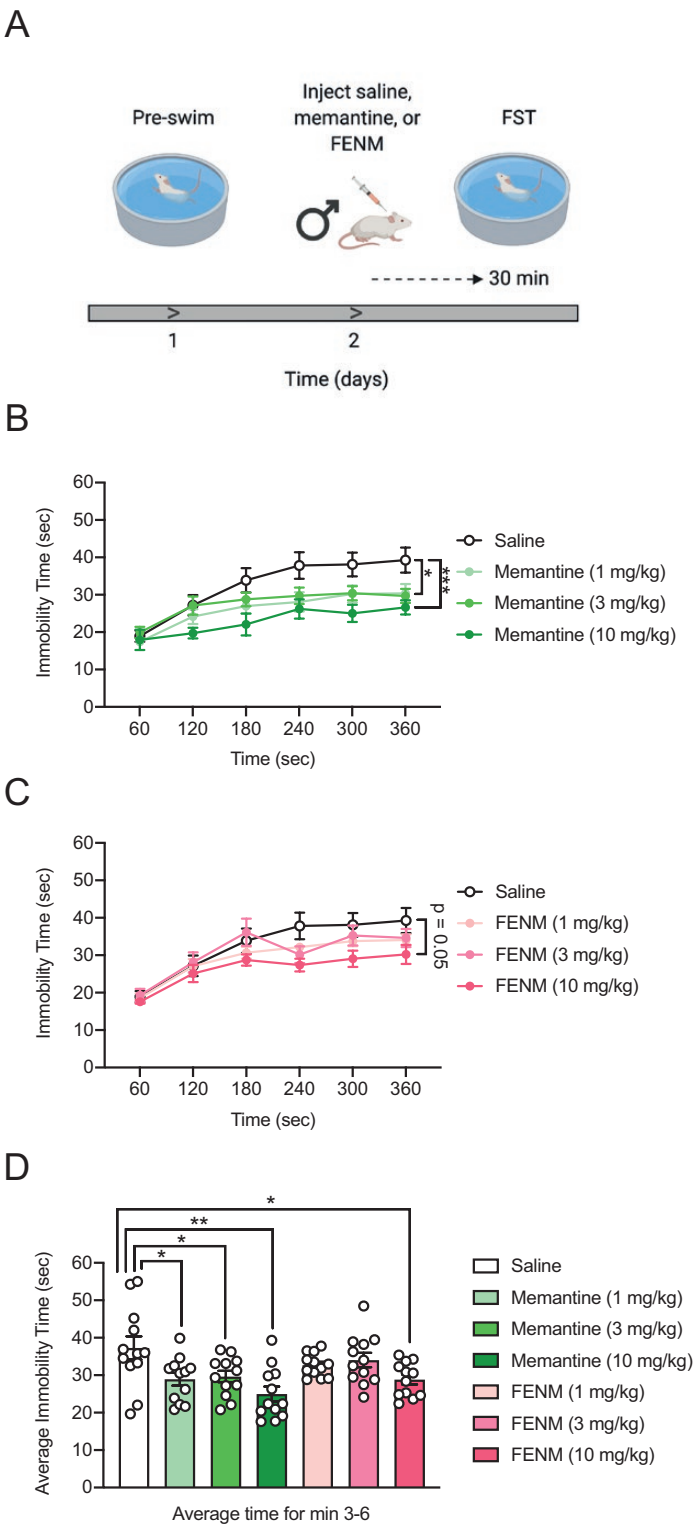

Figure 3. Fluoroethylnormemantine (FENM) reduces behavioral despair in the forced swim test. (a) Experimental protocol (b) Memantine at all doses significantly reduced immobility time in the forced swim test (FST) compared with saline-administered rats. (c) FENM (1 and 3 mg/kg) did not alter immobility time compared with saline. FENM (10 mg/kg) reduced overall immobility time, but this decrease was not significant. (d) Memantine (1, 3, and 10 mg/kg) and FENM (10 mg/kg) significantly reduced mean immobility time, averaged over minutes 3–6, in the FST. (n = 12 male rats/group). Error bars represent ± SEM. *$P < .05$, **$P < .01$, ***$P < .0001$. Abbreviation: FST, forced swim test.

increased bursting duration in the FST (supplementary Table 1) (Almeida et al., 2006). FENM (10 mg/kg) exerted a trending reduction in overall immobility time and was effective in reducing mean immobility time (Figure 3c–d; RMANOVA: drug $F_{(6,77)} = 3.649$, $P = .0031$, time $F_{(5,385)} = 68.02$, $P < .0001$, drug × time $F_{(30,385)} = 1.503$, $P = .0461$. Dunnett's multiple comparisons: Sal vs FENM (1 mg/

kg) $P = .5903$, Sal vs FENM (3 mg/kg) $P = .9161$, Sal vs FENM (10 mg/kg) $P = .0504$; ANOVA: drug $F_{(6,77)} = 4.558$, $P = .0005$; Dunnett's multiple comparisons: Sal vs FENM (1 mg/kg) $P = .3384$, Sal vs FENM (3 mg/kg) $P = .6842$, Sal vs FENM (10 mg/kg) $P = .0125$] as well as increasing bursting duration (supplementary Table 1). These data indicate that FENM is effective at reducing behavioral despair.

FENM Attenuates Learned Fear and Facilitates Extinction Learning in Rats

Next, we aimed to determine whether FENM could have effects in attenuating fear behavior when administered at various timepoints before or after stress or extinction learning. In these experiments, rats were not administered memantine due to previous data indicating that memantine does not alter fear learning or memory in wild-type rodents during cued FC (Dong et al., 2008; Ishikawa et al., 2016). Rats were administered a single injection of saline or FENM (5, 10, or 20 mg/kg) 30 minutes prior to 3-shock cued FC. The next 2 days, rats were placed into a novel context and reexposed to the conditioned stimulus. During both tests 1 and 2, freezing was assayed in rats as a measure of learned fear behavior (Figure 4a).

During the training session, there was no significant difference in freezing across all groups (Figure 4b–c; RMANOVA: drug $F_{(3,28)} = 1.219$, $P = .3212$, time $F_{(3,84)} = 178.2$, $P < .0001$, drug × time $F_{(9,84)} = 1.574$, $P = .1364$. 2-way ANOVA: drug $F_{(3.112)} = 1.562$, $P = .2027$, tone $F_{(3,112)} = 161.5$, $P < .0001$, drug × tone $F_{(9,112)} = 1.426$, $P = .1852$). During tone test 1, one day after training, rats administered FENM at 5 and 10 mg/kg exhibited significantly reduced freezing compared with controls during tones 9–16 [Figure 4d–e; RMANOVA: drug $F_{(3,28)} = 11.62$, $P < .0001$, time $F_{(24,672)} = 11.6$, $P < .0001$, drug × time $F_{(72,672)} = 6.451$, $P < .0001$; Dunnett's multiple comparisons: Sal vs FENM (5 mg/kg) $P = .0001$, Sal vs FENM (10 mg/kg) $P < .0001$, Sal vs FENM (20 mg/kg) $P = .6682$; 2-way ANOVA: drug $F_{(3,112)} = 11.62$, $P < .0001$, tone $F_{(3,112)} = 21.87$, $P < .0001$; drug × tone $F_{(9,112)} = 9{,}659$, $P < .0001$; Dunnett's multiple comparisons: Sal vs FENM (5 mg/kg) $P = .0178$, Sal vs FENM (10 mg/kg) $P < .0001$, Sal vs FENM (20 mg/kg) $P = .9040$]. Rats given FENM (20 mg/kg) showed reduced freezing during tones 1–8 but increased freezing during tones 17–24 compared with saline controls (Figure 4e). The following day, during tone test 2, FENM at 10 and 20, but not 5 mg/kg, significantly reduced overall freezing compared with saline [Figure 4f; RMANOVA: drug $F_{(3,28)} = 46.46$, $P < .0001$, time $F_{(24,672)} = 15.35$, $P < .0001$, drug × time $F_{(72,672)} = 4.664$, $P < .0001$; Dunnett's multiple comparisons: Sal vs FENM (5 mg/kg) $P = .9857$, Sal vs FENM (10 mg/kg) $P < .0001$, Sal vs FENM (20 mg/kg) $P < .0001$]. Rats administered FENM at 10 and 20 mg/kg froze significantly less than saline-administered rats during all epochs of the testing session [Figure 4g; 2-way ANOVA: drug $F_{(3,112)} = 66.81$, $P < .0001$, tone $F_{(3,112)} = 41.62$, $P < .0001$; drug × tone $F_{(9,112)} = 4.666$, $P < .0001$; Dunnett's multiple comparisons: Sal vs FENM (5 mg/kg) $P = .7523$, Sal vs FENM (10 mg/kg) $P < .0001$, Sal vs FENM (20 mg/kg) $P < .0001$]. Rats in the FENM (5 mg/kg) group showed enhanced freezing during tones 1–8 but reduced freezing during tones 9–16 compared with saline controls (Figure 4g). These data show that FENM administered prior to stress can attenuate learned fear in male rats in a dose- and time-specific manner.

We then sought to test whether FENM could alter fear behavior when administered after cued FC, prior to extinction learning. Rats were administered a 3-shock cued FC training session. The next day, rats were administered a single injection of saline or FENM (5, 10, or 20 mg/kg). Thirty minutes later, rats were placed into a novel context and reexposed to the conditioned stimulus. The third day, rats were reexposed to the conditioned stimulus. Freezing behavior was quantified during all training and testing sessions (Figure 5a). During training, there was no difference in freezing between all groups (supplementary Table 1). The following day, during tone test 1, FENM at 5 mg/kg, but no other doses, significantly reduced overall freezing compared with saline [Figure 5b; RMANOVA: drug $F_{(3,27)} = 6.298$, $P = .0022$, time $F_{(24,648)} = 6.808$, $P < .0001$, drug × time $F_{(72,648)} = 4.643$, $P < .0001$; Dunnett's multiple comparisons: Sal vs FENM (5 mg/kg) $P = .0424$, Sal vs FENM (10 mg/kg) $P = .3284$, Sal vs FENM (20 mg/kg) $P = .4934$]. During tones 9–16, but no other epochs, rats administered FENM (5 and 20 mg/kg) froze significantly less than saline-administered control rats [Figure 5c; 2-way ANOVA: drug $F_{(3,108)} = 4.723$, $P = .0039$, tone $F_{(3,108)} = 9.777$, $P < .0001$; drug × tone $F_{(9,108)} = 6.892$, $P < .0001$; Dunnett's multiple comparisons: Sal vs FENM (5 mg/kg) $P = .0376$, Sal vs FENM (10 mg/kg) $P = .7276$, Sal vs FENM (20 mg/kg) $P = .3011$]. Subsequently, during tone test 2, FENM (5 or 10 mg/kg) attenuated freezing during tones 9–16 while FENM (20 mg/kg) reduced freezing during the last half of the testing session [Figure 5d–e; RMANOVA: drug $F_{(3,27)} = 6.802$, $P = .0015$, time $F_{(24,648)} = 18.81$, $P < .0001$, drug × time $F_{(72,648)} = 2.906$, $P < .0001$; Dunnett's multiple comparisons: Sal vs FENM (5 mg/kg) $P = .1890$, Sal vs FENM (10 mg/kg) $P = .654$, Sal vs FENM (20 mg/kg) $P = .0007$; 2-way ANOVA: drug $F_{(3,108)} = 8.452$, $P < .0001$, tone $F_{(3,108)} = 78.04$, $P < .0001$; drug × tone $F_{(9,108)} = 3.822$, $P = .0003$; Dunnett's multiple comparisons: Sal vs FENM (5 mg/kg) $P = .1318$, Sal vs FENM (10 mg/kg) $P = .5836$ Sal vs FENM (20 mg/kg) $P < .0001$].

Finally, we sought to test whether FENM could affect fear behavior when administered during extinction learning. Rats were administered a 3-shock cued FC training session. The next day, rats were placed into a novel context and reexposed to the conditioned cues. On the third day, rats were given a single injection of saline or FENM (5, 10, or 20 mg/kg) 30 minutes prior to a second testing session (Figure 5f). There was no significant overall difference freezing during cued FC or during tone test 1 (supplementary Table 1). However, in tone test 2, FENM (10 and 20 mg/kg) significantly reduced freezing throughout the test compared with control saline-administered rats [Figure 5g–h; RMANOVA: drug $F_{(3,29)} = 50.01$, $P < .0001$, time $F_{(24,696)} = 14.98$, $P < .0001$, drug × time $F_{(72,696)} = 2.126$, $P < .0001$; Dunnett's multiple comparisons: Sal vs FENM (5 mg/kg) $P = .5038$, Sal vs FENM (10 mg/kg) $P < .0001$, Sal vs FENM (20 mg/kg) $P < .0001$; 2-way ANOVA: drug $F_{(3,116)} = 76.14$, $P < .0001$, tone $F_{(3,116)} = 39.6$, $P < .0001$; drug × tone $F_{(9,116)} = 4.368$, $P < .0001$; Dunnett's multiple comparisons: Sal vs FENM (5 mg/kg) $P = .8588$, Sal vs FENM (10 mg/kg) $P < .0001$, Sal vs FENM (20 mg/kg) $P < .0001$]. Overall, these data suggest that FENM can facilitate extinction learning and reduce fear behavior when administered either before or after stress or extinction learning but may be more effective in attenuating learned fear when administered directly prior to reexposure.

Discussion

In this study, we aimed to test the behavioral effects of FENM administration on sensorimotor gating, avoidance behavior, behavioral despair, and fear behavior in rats. Our experiments show that FENM can effectively reduce behavioral despair as well as facilitate extinction learning without altering locomotion or sensorimotor behavior. These results show that FENM exerts antidepressant-like and fear-attenuating properties. FENM was also effective in attenuating learned fear when administered acutely prior to FC, indicating that it may also be leveraged as a resilience-enhancing prophylactic. Together, our findings indicate that FENM, a novel NMDAR antagonist, may be

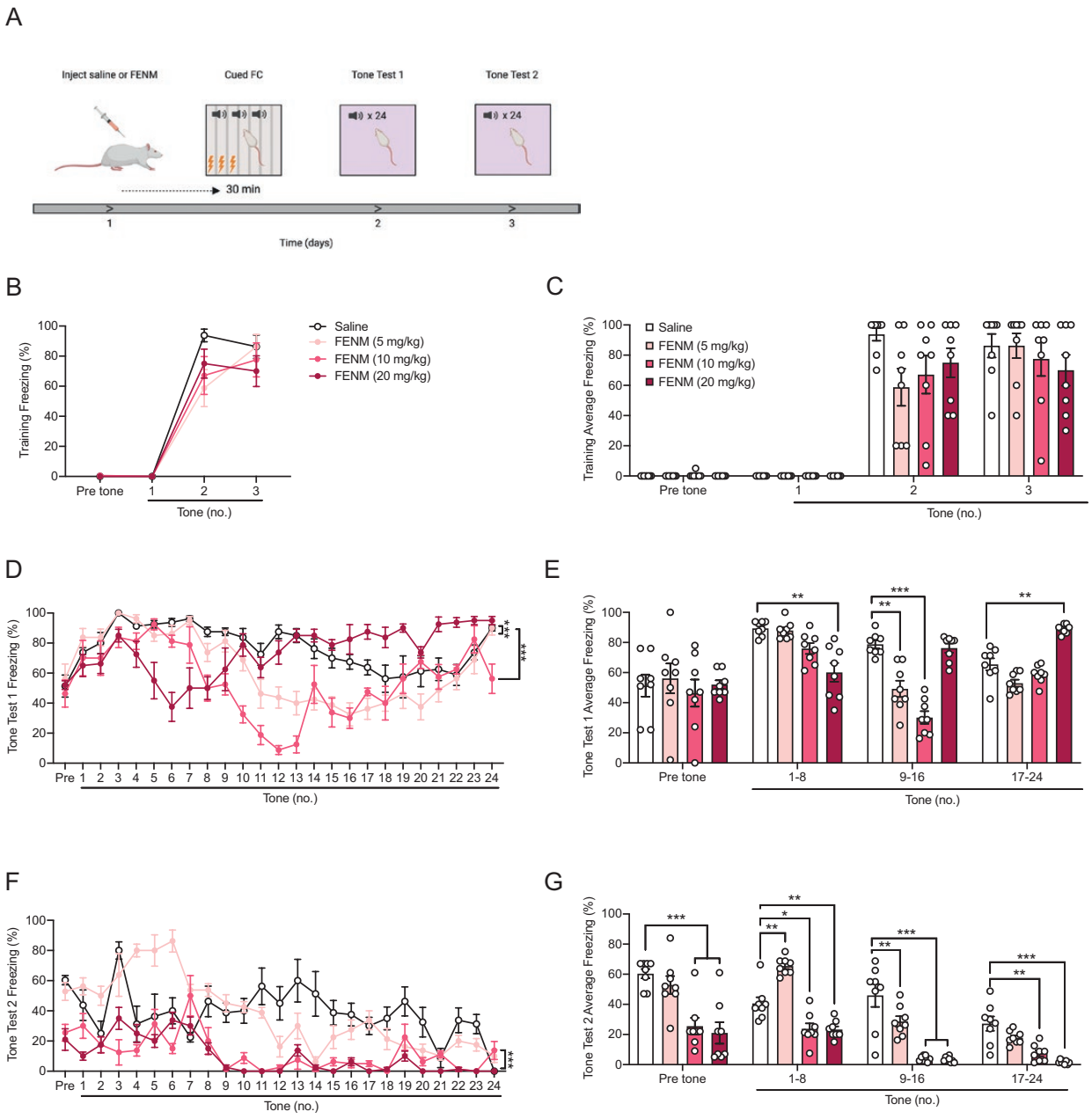

Figure 4. Fluoroethylnormemantine (FENM) attenuates learned fear when administered directly before cued FC. (a) Rats were administered a single injection of saline or FENM (5, 10, or 20 mg/kg) 30 minutes prior to 3-shock cued FC. The next 2 days, rats were placed into a novel context and reexposed to the conditioned stimulus. During both tests 1 and 2, freezing was assayed in rats as a measure of learned fear behavior. (b–c) In the training session, freezing was comparable across all groups. (d–e) During test 1, FENM (5, 10, and 20 mg/kg) reduced freezing to the conditioned tones at various time points compared with the saline control group. FENM (20 mg/kg) increased freezing compared with saline during the last epoch of the test. (f–g) During test 2, at every time point, FENM (10 and 20 mg/kg) attenuated learned fear compared with the saline control group. FENM (5 mg/kg) increased freezing during tones 1–8, but reduced fear behavior during tones 9–16 (n = 8 male rats/group). Error bars represent ± SEM. *P < .05, **P < .01, ***P < .0001. Abbreviations: FC, fear conditioning; Sal, saline.

a suitable candidate for further preclinical testing in a variety of stress-related behavioral assays.

Numerous studies have documented the efficacy of NMDAR antagonists in both treating and preventing stress-related psychiatric disorders. These compounds include, but are not limited to, (*R*,*S*)-ketamine, MK-801, and rapastinel (Kadriu et al., 2019). Notably, many of these compounds elicit rapid antidepressant responses in both rodent and human models but are not reported to exhibit anxiolytic properties (Moskal et al., 2014; Newport et al., 2015; Kadriu et al., 2019). A number of these novel compounds are also being tested in clinical trials as either adjunctive or standalone treatments for various depressive disorders, but, to date, many have failed to show significant efficacy in reducing depressive symptoms relative to placebo groups (Kadriu et al., 2019; ClinicalTrials.gov, NCT02192099, NCT02267629, NCT02932943, NCT02943564, NCT02943577, NCT02951988, NCT03002077, NCT03352453, NCT03560518, NCT03575776, NCT03614156, NCT03668600, NCT03799900, NCT03814733, NCT03855865). Additionally, (*R*,*S*)-ketamine and group II metabotropic glutamate receptor antagonists have been

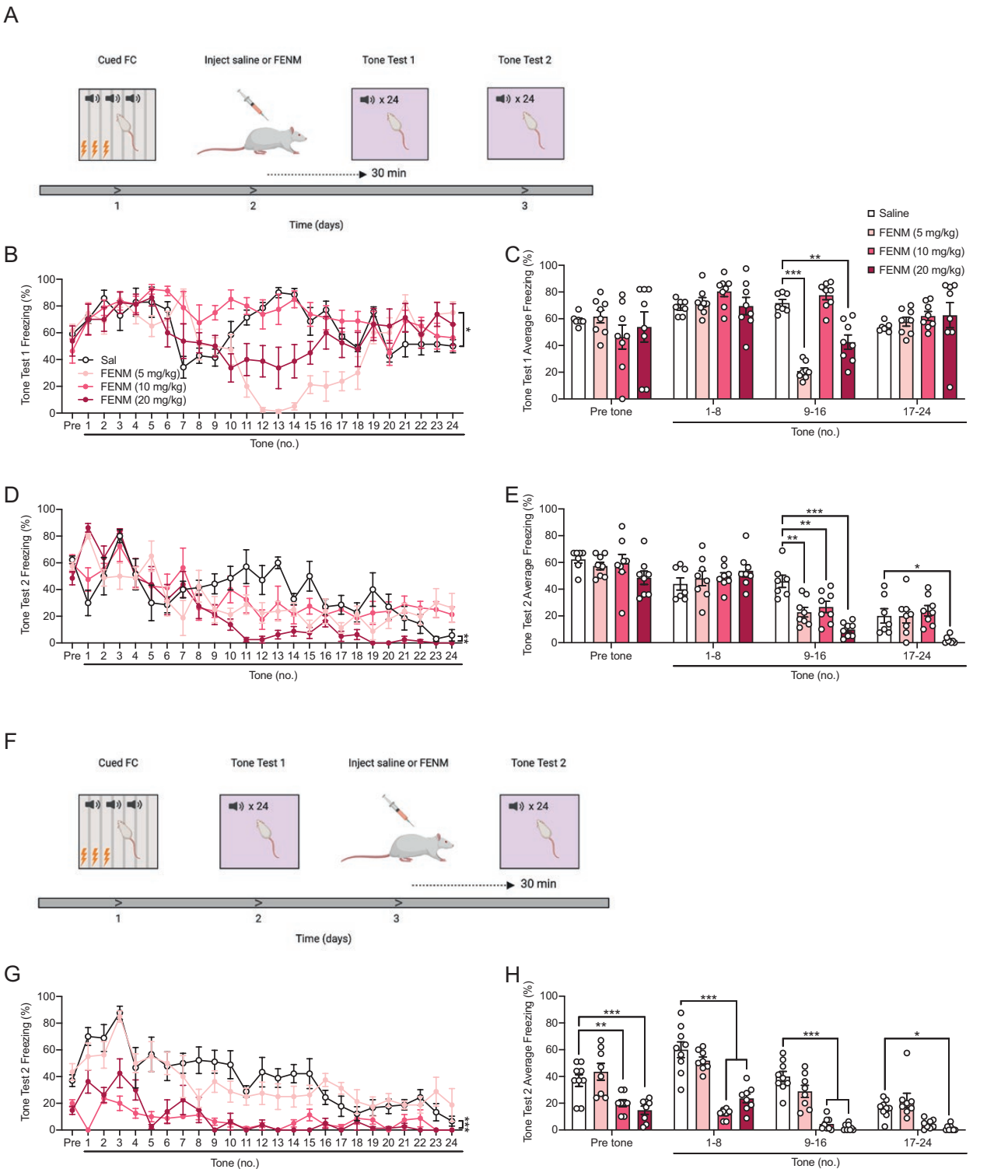

Figure 5. Fluoroethylnormemantine (FENM) facilitates extinction learning. (a) Experimental protocol. (b–c) Freezing prior to the tones and during tones 1–8 was comparable between all the drug groups. FENM at 5 and 20, but not 10 mg/kg, significantly reduced freezing during tones 9–16 compared with saline-administered rats. (d) In tone test 2, FENM (20 mg/kg) significantly reduced overall freezing compared with the control saline group. (e) All doses of FENM significantly reduced freezing during tones 9–16. FENM (20 mg/kg) also reduced freezing during tones 17–24. (f) Experimental protocol. (g–h) During test 2, FENM (5 mg/kg) did not alter fear behavior compared with saline controls. FENM (10 mg/kg) reduced freezing during the pre-tone epoch and during tones 1–16. FENM (20 mg/kg) significantly reduced freezing throughout the entire testing session. (n = 7–9 male rats/group). Error bars represent ± SEM. *P < .05, **P < .01, ***P < .0001. Abbreviations: FC, fear conditioning; sal, saline.

shown to exhibit prophylactic properties against stress-induced behavioral despair (Highland et al., 2019). Our results, which demonstrate that FENM is antidepressant and may reduce fear, further indicate that targeting NMDARs may be an important strategy to both treating and preventing stress-related psychiatric illness.

Previous research has demonstrated a number of mechanisms by which NDMAR modulation can relieve depressive-like symptoms (Zanos et al., 2018; Kadriu et al., 2019). It is important, however, to note that these mechanisms are not mutually exclusive and may work in a complementary fashion to achieve overall effects on behavior. Notably, NDMAR antagonism can rapidly upregulate the translation of various proteins and neurotrophic factors in the brain, such as brain-derived neurotrophic factor, which supports synaptic plasticity in regions of the brain, including the prefrontal cortex (Björkholm and Monteggia, 2016; Zanos and Gould, 2018; Zanos et al., 2018). This mechanism has been shown to be necessary for the rapid-acting antidepressant effects of (*R*,*S*)-ketamine and could potentially contribute to the rapid-acting effects of other NMDAR antagonists, including FENM (Björkholm and Monteggia, 2016). (*R*,*S*)-ketamine-mediated NMDAR antagonism has also been shown to reconfigure brain-wide neural networks to restore homeostatic metabolic processes as well as synchronize gamma oscillatory activity by reducing excessive NMDAR-dependent neurotransmission (Wang and Arnsten, 2015; Arnsten et al., 2016; Lv et al., 2016; Kadriu et al., 2019; Nugent et al., 2019). Future studies are therefore necessary to determine whether these and other candidate processes contribute to the neurobiological actions of FENM.

Intriguingly, we also found that FENM, in addition to its antidepressant properties, facilitated extinction learning. In our experiments, when administered at a variety of timepoints prior to both cued FC and extinction training, FENM exerted long-lasting reductions in fear behavior in a dose-specific manner. We speculate that FENM may alter the recall of fear memory traces, ultimately leading to a rapid and long-lasting attenuation of behavioral fear responses. Moreover, FENM was recently shown to improve spatial learning and object recognition as well as reduce spatiotemporal disorientation in a rodent model of AD, suggesting that the results we observed during cued FC were specific to fear expression (Couly et al., 2020). Future studies will be necessary to test whether FENM can alter fear memory traces to attenuate learned fear. Overall, our experiments indicate that, following further preclinical testing, FENM may be effective as a treatment for fear-related disorders such as posttraumatic stress disorder or specific phobias.

Importantly, FENM did not alter sensorimotor gating during PPI or locomotion in the OF acutely after injection. These results are in direct contrast with FENM's parent compound memantine, which significantly reduced startle response and locomotion 30 minutes after injection. Our data suggest that FENM does not have significant, nonspecific side effects that could confound the data we collected in the FST and cued FC assays. These results show that FENM may be a suitable candidate for further preclinical development. Future studies are, however, necessary to determine potential nonspecific behavioral effects of the compound.

Despite these promising data, it is still unknown if FENM can exhibit antidepressant and fear-attenuating properties in females. We and others have previously shown that both antidepressant and prophylactic drugs may be efficacious at different doses in male and female preclinical models (Franceschelli et al., 2015; Chen et al., 2020b). Additionally, we have also shown that prophylactic compounds that attenuate learned fear in male mice, such as (*R*,*S*)-ketamine, (2*S*,6*S*)-hydroxynorketamine, an (*R*,*S*)-ketamine metabolite, and RS-67,333, a type IV serotonin receptor agonist, fail to alter fear learning or expression in female mice (Chen et al., 2020a, 2020b). Thus, we aim to investigate the prophylactic and fear-reducing efficacy of FENM in females in future studies.

Overall, this series of experiments characterizes a novel NMDAR antagonist, FENM, that exhibits antidepressant properties and facilitates extinction learning in preclinical rat models. The data support the NMDAR as a critical target to better treat and prevent a variety of stress-induced behaviors. Ultimately, further study of this and similar compounds may lead to more efficacious interventions to reduce the burden of stress-related psychiatric disease.

Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

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Statement of Interest

B.K.C., G.R., and C.A.D. are named on provisional and nonprovisional patent applications for the prophylactic use of (*R*,*S*)-ketamine, FENM, and related compounds against stress-related psychiatric disorders. Since the start of 2020, C.A.D. has received honoraria for speaking for Janssen, Janssen Southeast Asia, North American Neuromodulation Society, and Albany Medical College. G.L.P., A.E., and T.M.J. have nothing to disclose. FENM was provided for this study as a gift from ReST Therapeutics.

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