



## Adjunct treatment with ketamine enhances the therapeutic effects of extinction learning after chronic unpredictable stress

Denisse Paredes<sup>a</sup>, Anna R. Knippenberg<sup>a</sup>, Sarah E. Bulin<sup>a</sup>, Lydia J. Keppler<sup>a</sup>, David A. Morilak<sup>a,b,\*</sup>

<sup>a</sup> Department of Pharmacology and Center for Biomedical Neuroscience, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX, 78229, USA

<sup>b</sup> South Texas Veterans Health Care System, San Antonio, TX, 78229, USA

### ARTICLE INFO

#### Keywords:

Set shifting  
Fear extinction  
Ketamine  
Chronic stress  
Adjunct treatment

### ABSTRACT

Post-traumatic stress disorder (PTSD) is a debilitating illness characterized by dysfunction in the medial prefrontal cortex (mPFC). Although both pharmacological and cognitive behavioral interventions have shown some promise at alleviating symptoms, high attrition and persistence of treatment-resistant symptoms pose significant challenges that remain unresolved. Specifically, prolonged exposure therapy, a gold standard intervention to treat PTSD, has high dropout rates resulting in many patients receiving less than a fully effective course of treatment. Administering pharmacological treatments together with behavioral psychotherapies like prolonged exposure may offer an important avenue for enhancing therapeutic efficacy sooner, thus reducing the duration of treatment and mitigating the impact of attrition. In this study, using extinction learning as a rat model of exposure therapy, we hypothesized that administering ketamine as an adjunct treatment together with extinction will enhance the efficacy of extinction in reversing stress-induced deficits in set shifting, a measure of cognitive flexibility. Results showed that combining a sub-effective dose of ketamine with a shortened, sub-effective extinction protocol fully reversed stress-induced cognitive set-shifting deficits in both male and female rats. These effects may be due to shared molecular mechanisms between extinction and ketamine, such as increased neuronal plasticity in common circuitry (e.g., hippocampus-mPFC), or increased BDNF signaling. This work suggests that fast-acting drugs, such as ketamine, can be effectively used in combination with behavioral interventions to reduce treatment duration and potentially mitigate the impact of attrition. Future work is needed to delineate other pharmacotherapies that may complement the effects of extinction via shared or independent mechanisms.

### 1. Introduction

Stress-related psychiatric disorders, such as post-traumatic stress disorder (PTSD) and depression, exhibit dysfunction of the medial prefrontal cortex (mPFC) (Zubieta et al., 1999; Girotti et al., 2017). The mPFC regulates many processes disrupted in such disorders, such as avoidance behavior, fear modulation and cognitive bias (Padilla-Correa et al., 2019; Milad and Quirk, 2012; Yoshimura et al., 2010). Pharmacological treatments for PTSD and depression, such as selective serotonin reuptake inhibitors (SSRIs), are only moderately effective, and treatment resistance remains a problem (Bernardy and Friedman, 2015;

Janicak et al., 2010; Nutt, 2010). Antidepressants often fail to relieve cognitive deficits stemming from mPFC dysfunction (Shilyansky et al., 2016). Cognitive behavioral therapies (CBT) can reverse cognitive deficits (Bernhardt et al., 2021), yet only a small percentage of patients achieve full remission (Wiles et al., 2013). Prolonged exposure therapy (PE), a type of CBT used to treat PTSD, involves re-exposure to a traumatic event in a safe context to reduce the maladaptive stress response elicited by reminders of the event (Foa and Meadows, 1997). Although PE is one of the gold standard treatments for PTSD, dropout rates are high, with some studies of veterans reporting 24–68% attrition (Garcia et al., 2011; Hoge et al., 2014). Re-exposure provokes anxiety, and the

\* Corresponding author. Department of Pharmacology, Mail Code 7764 University of Texas Health Science Center, San Antonio 7703 Floyd Curl Drive, San Antonio, TX, 78229-3900, USA

E-mail addresses: [ParedesD@livemail.uthscsa.edu](mailto:ParedesD@livemail.uthscsa.edu) (D. Paredes), [rigodanzo@uthscsa.edu](mailto:rigodanzo@uthscsa.edu) (A.R. Knippenberg), [Bulin@uthscsa.edu](mailto:Bulin@uthscsa.edu) (S.E. Bulin), [Keppler@livemail.uthscsa.edu](mailto:Keppler@livemail.uthscsa.edu) (L.J. Keppler), [Morilak@uthscsa.edu](mailto:Morilak@uthscsa.edu) (D.A. Morilak).

<https://doi.org/10.1016/j.ynstr.2022.100468>

Received 15 February 2022; Received in revised form 9 June 2022; Accepted 5 July 2022

Available online 8 July 2022

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length of PE may deter patients with severe symptoms from completing treatment. PE typically entails 8–15 sessions (Karlin et al., 2010). Therefore, enhancing therapeutic efficacy of PE sooner, i.e., reducing the duration of treatment required for effective response, may improve patient outcomes.

Fear extinction (FE) in rodents bears similarity to PE in humans. FE involves repeated exposure to conditioned fear-provoking stimuli in a safe environment until fearful responding decreases (Milad and Quirk, 2002). FE therefore provides a model for preclinical researchers to study the neurobiological mechanisms of PE (Milad and Quirk, 2012). Use of FE as a therapeutic intervention in rats provides a model to investigate mechanisms underlying the beneficial effects of PE (Paredes and Morilak, 2019; Pollak et al., 2008). Behavioral therapies and FE both activate regions of the brain such as the hippocampus, amygdala, and mPFC, that are affected by chronic stress (Andero and Ressler, 2012). Stress decreases responses evoked in the mPFC by stimulating the afferent from the medial dorsal thalamus (MDT), a region associated with strategy set shifting (Block et al., 2007). FE reverses stress-induced decreases in responsiveness of the mPFC to input from the MDT, promotes active coping behavior, similar to effects of PE in humans (Fucich et al., 2016; King et al., 2016), and reverses stress-induced deficits in set shifting, a form of cognitive flexibility mediated by the mPFC that is impaired in patients with PTSD and depression (Olff et al., 2014).

Acute low-dose administration of ketamine, a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist with rapidly-acting antidepressant effects, also improves mPFC function after stress, and is effective for some treatment-resistant patients (Zanos and Gould, 2018). Preclinical work demonstrates that ketamine can accelerate FE learning, and preliminary data with treatment-resistant patients suggest that PE efficacy may be improved by combination with ketamine (Rodriguez et al., 2016). Ketamine may enhance FE efficacy through converging mechanisms, as ketamine and FE share molecular processes in the mPFC necessary for their effects. Therapeutic effects of both ketamine and FE involve PI3 kinase-Akt signaling in the mPFC, (Duman et al., 2012; Paredes et al., 2022; Li et al., 2010). FE requires infralimbic (IL) glutamatergic activity and activity-dependent protein translation in the mPFC to reverse stress-induced cognitive deficits (Fucich et al., 2016, 2018). FE and ketamine restore stress-induced dysregulation of mPFC activity (Fucich et al., 2018; Etkin and Wager, 2007; Helpman et al., 2016; Yang et al., 2018; Jett et al., 2015). Both FE and ketamine require Brain Derived Neurotrophic Factor (BDNF) signaling and activity-dependent protein translation for their effects, and require plasticity in the ventral hippocampus (vHipp)-to-mPFC pathway (Paredes et al., 2022; Autry et al., 2011; Peters et al., 2010). Like FE, a single dose of ketamine (10 mg/kg) can reverse deficits induced by chronic unpredictable stress (CUS) on set shifting and active coping, and promote dendritic spine growth after stress (Jett et al., 2015; Moench et al., 2016; Zhang et al., 2019).

In this study, we hypothesized that adjunct treatment with ketamine will enhance the efficacy of extinction in reversing stress-induced deficits in set shifting. To test this, we developed a sub-effective extinction protocol, reducing the number of tone exposures used in our fully effective extinction model. This was then used in combination with a sub-effective dose of ketamine to test for enhanced efficacy in reversing stress-induced deficits in cognitive set shifting. As ketamine is known to share mechanisms with extinction, we suggest that showing the sub-effective extinction model to be sensitive to the beneficial effects of adjunct treatment with ketamine would serve as proof-of-principle to validate the future use of this model to test novel candidates for adjunct therapy. Portions of this work have been presented in abstract form (Paredes et al., 2017).

## 2. Materials & methods

**Animals:** 354 adult male and naturally cycling female Sprague Dawley rats (Envigo, Houston, TX, 225–249 g for both sexes) were used.

Rats were initially group-housed in same-sex cages (3 per cage) on a 12/12 h light cycle (lights on at 07:00) with food and water *ad lib*. After 1-week acclimation, they were single-housed prior to beginning experimental treatment. Experiments took place during the light portion of the cycle. All procedures were in accordance with NIH guidelines and approved by the UTHSA Institutional Animal Care and Use Committee.

### 2.1. Fear conditioning and extinction

**I. Habituation:** Two days before chronic unpredictable stress (CUS) procedures, rats were habituated to two contexts in sound attenuating chambers for 15 min per context. Context A consisted of square metal walls and a metal grid floor connected to a shock generator (Harvard Apparatus, model H13-15). Context B consisted of a chamber with green and white striped vinyl floor and circular white vinyl walls.

**II. Fear Conditioning:** One day before CUS, rats were placed in Context A and received four pairings of a tone (10 kHz, 75 dB, 20 s) co-terminus with a footshock (0.8 mA, 0.5 s), with an inter-trial interval of 120 s. Fear responses were quantified as percent of time freezing during each tone using FreezeView software (ActiMetrics #ACT-100, Coulbourn Instruments). Tone control rats were exposed to the same tones without footshock.

**III. Fear Extinction:** Three days post-CUS, all rats received fear extinction treatment. Rats were placed in Context B and exposed to either 16 or 8 tones without footshock, with an average inter-trial interval of 120 s. Tone controls were treated identically; because they had not been fear-conditioned, no extinction learning occurred for them in this session.

**IV. Retention:** In experiments for which extinction retention was assessed, 24 h after fear extinction, animals were placed in Context B and the same procedure used for extinction was used to test extinction retention.

### 2.2. Chronic unpredictable stress (CUS)

The CUS protocol was adapted from (Fucich et al., 2016; Bondi et al., 2010). A different acute stressor was applied each day (14 days for males, 21 days for females), including the following: 30 min restraint, 1 h shake, 24 h wet bedding, 15 min footshock, 10 min tail pinch, 15 min warm swim, 10 min cold swim, overnight lights-on. We have reported previously that 14 days of CUS does not induce set shifting deficits in females, but 21-day CUS induces a deficit in females comparable to that observed in males after 14 days CUS (Paredes et al., 2022; Bulin et al., 2020).

### 2.3. Attentional set-shifting test (AST)

AST was utilized to measure cognitive flexibility specifically on the extradimensional set shifting task (Fucich et al., 2016). A week before testing, rats were food restricted to ~66% of average daily intake (7 g for females, 12 g for males). Rats were trained and tested in a white plastic arena containing two terracotta pots separated by a Plexiglas wall at the end opposite the start box. The AST entails three days, with one day inserted between training and testing to allow for extinction and/or acute ketamine administration 24 h before testing:

**I. Habituation:** Rats were trained to dig in pots filled with sawdust to retrieve a 1/3 piece of Honey Nut Cheerio (General Mills Cereals, Minneapolis, MN, USA).

**II. Training:** Rats were trained to locate the Cheerio in one of two pots by discriminating cues in two stimulus dimensions (an odor applied to the rim of each pot and the digging medium filling each pot).

**III. Treatment:** Rats received either a single extinction session, a single acute injection of ketamine (0.3, 1.0, 3.0 or 10.0 mg/kg, i.p.)

or saline vehicle, or a combined treatment of acute drug injection given immediately after extinction.

**IV. Testing:** Rats were tested on a series of discrimination tasks in which a criterion of six consecutive correct responses was required to proceed to the next task (Table 1). Rats started the simple discrimination task with either medium or odor as the relevant stimulus dimension and proceeded through the subsequent tasks (i.e. complex discrimination, reversal learning, intradimensional shift, and second reversal) leading to formation of a cognitive set. In the extradimensional (ED) set-shifting task, the previously relevant dimension (e.g. odor), was irrelevant and the previously irrelevant dimension (e.g., medium) became relevant, indicating the location of the Cheerio, thus requiring a cognitive set shift.

## 2.4. In vivo electrophysiology

Three days after the end of CUS, rats underwent fear extinction or tone control treatment, or ketamine injections. 24 hr after treatment, local field potentials (LFPs) evoked by stimulation of the mediodorsal thalamus (MDT) were recorded in the mPFC. Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic apparatus. A bipolar twisted electrode was placed in the left MDT (AP: -2.6, ML: +0.9, DV: -5.0 mm), and a recording electrode in the left mPFC (AP: + 3.0, ML: +0.6, DV: -4.0–4.2 mm). The MDT was stimulated in 100  $\mu$ A increments from 100 to 600  $\mu$ A for 5 min (30 pulses, 260  $\mu$ s pulse width, 0.1 Hz). LFPs were amplified (gain 5000x; low cutoff 0.3 Hz, high cutoff 1000 Hz) and digitized (Power Lab; ADInstruments). mPFC response was measured as the amplitude between the peak of the first negative deflection (N1), occurring approximately 5–8 msec after stimulation, and the peak of the subsequent positive deflection (P2), at approximately 15–18 msec (Herry et al., 1999).

## 2.5. Experiment 1

We have shown that 16-tone extinction fully reverses stress-induced deficits in both set-shifting and afferent-evoked responses in mPFC (Paredes and Morilak, 2019; Fucich et al., 2016, 2018). Thus, to establish a sub-effective extinction protocol (e.g., to model a partial course of PE treatment), we reduced the number of tone exposures during extinction from the standard 16 tones to 8 tones. We chose 8 tones because in our standard extinction procedure, freezing decreases over

**Table 1**  
**Stages of the Attentional Set Shifting Test (AST).** Order of discrimination stages used for AST. All data shown are from the extra dimensional (ED) set shifting task.

Discrimination stage	Dimensions		Example combinations	
	Relevant	Irrelevant	(+)	(-)
<b>Simple</b>	Odor		Clove/ sawdust	Nutmeg/ sawdust
<b>Compound</b>	Odor	Medium	Clove/raffia Clove/yarn	Nutmeg/yarn Nutmeg/ raffia
<b>Reversal 1</b>	Odor	Medium	Nutmeg/ raffia Nutmeg/yarn	Clove/raffia Clove/yarn
<b>Intradimensional shift</b>	Odor	Medium	Rosemary/ wood balls Rosemary/ plastic beads	Cinnamon/ plastic beads Cinnamon/ wood balls
<b>Reversal 2</b>	Odor	Medium	Cinnamon/ plastic beads Cinnamon/ wood balls	Rosemary/ wood balls Rosemary/ plastic beads
<b>Extradimensional shift (ED)</b>	Medium	Odor	Velvet/ citronella Velvet/thyme	Crepe/thyme Citronella

the first 8 tones, after which it plateaus for the remaining 8 tones (see Figs. 1B, 2B and 4A). Thus, rats will have achieved the same within-session extinction learning after 8 tones as those receiving 16 tones, and the final 8 tones in the standard procedure may represent “overtraining”. In experiment 1, we tested if extinction retention was worse after 8-tone extinction, implying less plasticity. 30 rats (16 males, 6–10/group; 14 females, 6–8/group) were used in two groups (16-tone and 8-tone extinction).

## 2.6. Experiment 2

We next investigated the effect of 8-tone extinction compared to 16-tones on evoked responses in the mPFC of stressed rats, tested 24 h after extinction. 68 rats (33 males, 7–10/group; 35 females, 6–10/group) were used in 4 groups: nonstress 16-tone extinction (NS Ext-16), CUS-tone control, CUS Ext-16, and CUS 8-tone extinction (Ext-8).

In a separate cohort of rats, we tested effects of 8-tone extinction compared to 16-tones in reversing stress-induced deficits in set-shifting on the AST. 49 rats (27 males, 5–11/group; 22 females, 5–6/group) were used in 4 groups (NS Ext-16, CUS-tone control, CUS Ext-16, and CUS Ext-8).

## 2.7. Experiment 3

To inform our dose selection for the combined treatment of sub-effective extinction and sub-effective ketamine, we investigated the dose-dependent effects of ketamine on MDT-evoked LFPs in the mPFC of stressed rats. 74 rats (39 males, 5–10/group; 35 females, 5–11/group) were used in 5 groups. All groups were CUS-treated and injected acutely with one of 4 doses of ketamine (0.3, 1.0, 3.0, or 10.0 mg/kg) or saline vehicle 24 h prior to testing. We and others have previously shown 10 mg/kg to be a fully effective dose (Duman et al., 2012; Jett et al., 2015).

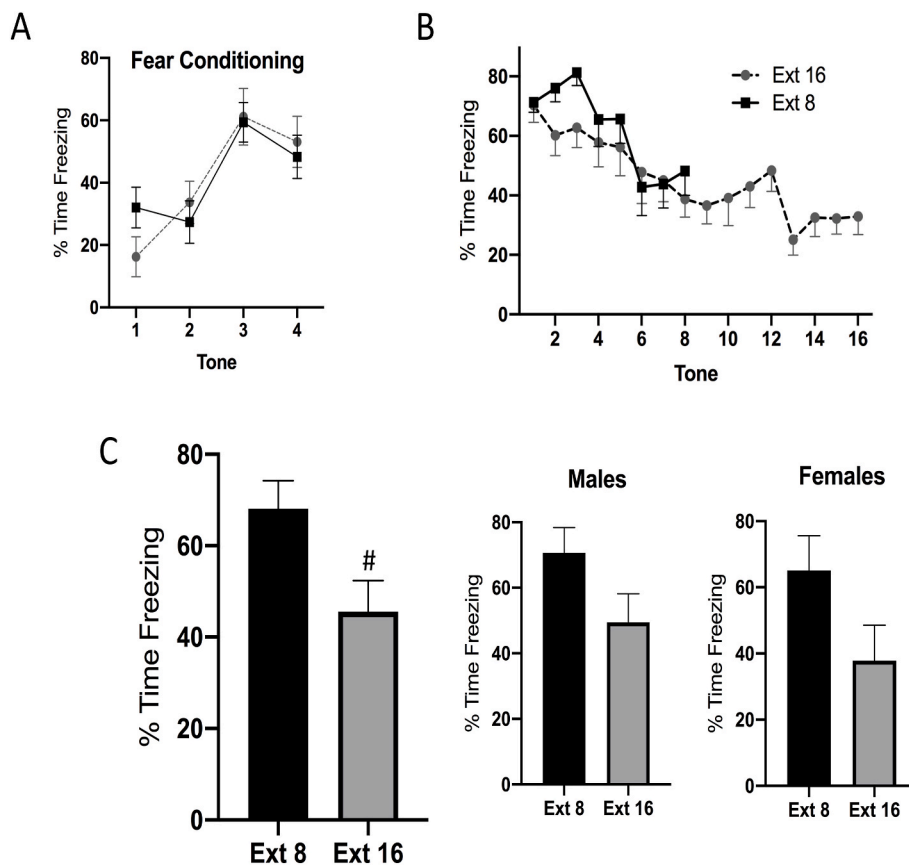
We reported previously that 10 mg/kg ketamine effectively reversed stress-induced deficits in set-shifting (Jett et al., 2015). Thus, in other rats, we tested lower doses of ketamine on set-shifting to identify a sub-effective dose to test in combination with 8-tone extinction. 69 rats (39 males, 4–9/group; 30 females, 4–6/group) were used in 6 groups (non-stressed saline controls, and CUS-treated groups given either saline or 0.3, 1.0, 3.0, or 10.0 mg/kg ketamine).

## 2.8. Experiment 4

Based on the results of the preceding experiments, we tested the efficacy of adjunct treatment combining sub-effective 8-tone extinction and sub-effective 1.0 mg/kg ketamine in reversing stress-induced set-shifting deficits. 41 rats (17 males, 4–5/group; 24 females, 5–7/group) were used in 4 groups (CUS-tone control + saline, CUS-Ext 8 + saline, CUS-tone control + 1.0 mg/kg ketamine, CUS-Ext 8 + 1.0 mg/kg ketamine). Ketamine was administered immediately upon completion of the extinction session to avoid confounding behavioral effects of having drug on board during extinction (some ataxia was noted, even at 1 mg/kg), and to coincide with the activation of the BDNF receptor TrkB after extinction (Paredes et al., 2022).

## 2.9. Experiment 5

Our electrophysiological data showed that both 8-tone extinction and 1 mg/kg ketamine alone partially rescued MDT-evoked responses in the mPFC attenuated by stress, albeit not to the same extent as the behaviorally effective treatments of 16-tone extinction and 10 mg/kg ketamine. Thus, it is possible that in combination, 1 mg/kg plus 8-tone extinction would fully restore MDT-evoked responses in the mPFC. An additive enhancement by 8-tone extinction plus 1 mg/kg ketamine may be one mechanism by which adjunct treatment reverses stress-induced deficits on set shifting. To test this hypothesis, MDT-evoked responses were recorded in mPFC of 23 rats (12 males, 11 females) in 3 groups



**Fig. 1. 8-Tone extinction produces less retention compared to 16-tone extinction.** (A) Fear conditioning curves showing % time freezing during tone presentation for 16-tone and 8-tone extinction groups. (B) Fear extinction curves during 16-tone and 8-tone extinction. Data include both males (6–10/group) and females (6–8/group). (C) Extinction retention is worse after 8-tone extinction compared to 16-tone extinction (# $p < 0.05$ ). Middle and right graphs show male and female data, respectively. Bars represent SEM.

(CUS-tone control + saline, CUS-Ext 8 + saline, CUS- Ext 8 + 1.0 mg/kg ketamine).

### 2.10. Statistical analyses

To compare extinction curves, repeated measures ANOVA was used on the first 8 tones. For retention, student's t-test was used to compare freezing during the first tone in the retention test. One way ANOVA was used to compare trials to criterion on the extradimensional set shifting task between groups, with pairwise comparisons using the Holm-Sidak test. Least-Sums-of-Squares F-test was used to compare stimulus-response curves for MDT-evoked local field potentials in the mPFC, with pairwise comparisons, determined *a priori*, with the Bonferroni correction. For all primary analyses, males and females were analyzed together. Data were then analyzed and shown separately by sex, although these experiments were not explicitly powered to test sex differences. Symbols in the figures denote significant comparisons as follows: \*significant vs non-stressed controls (stress effect); +significant vs CUS-saline or CUS-tone controls (treatment effect); #significant differences between extinction conditions or drug doses.

## 3. Results

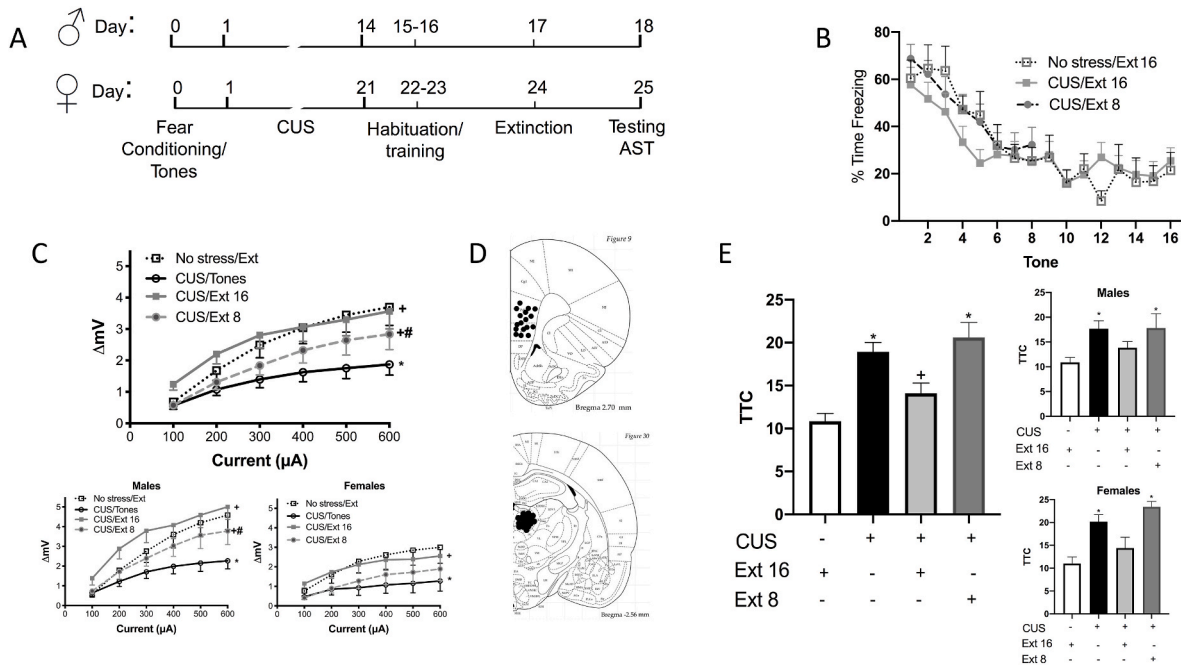
### 3.1. Extinction retention is attenuated after 8-tone extinction compared to 16-tone extinction

We have previously established that 16-tone extinction is effective in reversing stress-induced set shifting deficits. To develop a sub-effective model of extinction to reverse stress-induced cognitive impairments, we tested whether extinction retention was worse after 8-tone extinction compared to the fully effective 16-tone extinction. Extinction retention is considered a behavioral measure of learning-induced plasticity.

Therefore, an impairment in extinction retention in the 8-tone extinction group compared to the 16-tone extinction group may indicate dampened learning. Fear conditioning (Fig. 1A) and within-session freezing during the first 8 tones of extinction (Fig. 1B) did not differ between groups (extinction:  $F_{1,27} = 1.095$ ,  $p > 0.05$ ). Freezing on tone 1 of retention, measured 24 h after extinction, was higher in rats that underwent 8-tone extinction ( $t_{28} = 2.581$ ,  $p < 0.05$ , Fig. 1C). Analyzed separately, males and females showed similar effects, although these comparisons were not powered to test sex differences and were not significant ( $t_{14} = 1.539$ ,  $p > 0.05$ ,  $t_{12} = 2.087$ ,  $p > 0.05$ ). Therefore, extinction memory retention, but not within-session extinction, was attenuated in the 8-tone extinction group.

### 3.2. 8-Tone extinction has a partial effect on stress-attenuated MDT-evoked LFPs in mPFC

We have demonstrated that 16-tone extinction reverses stress-induced attenuation of evoked LFPs in the mPFC. We reasoned that because 8-tone extinction rats exhibit impaired extinction retention compared to 16-tone extinction rats, 8-tone extinction may produce some learning-induced plasticity processes that partially reverse stress-induced attenuation in mPFC responsivity. Within-session freezing during the first 8 tones of extinction did not differ between fear-conditioned groups ( $F_{2,43} = 1.066$ ,  $p > 0.05$ ; Fig. 2B). Stimulus-response curves for MDT-evoked local field potentials in the mPFC differed between groups (sexes combined:  $F_{4,306} = 10.30$ ,  $p < 0.0001$ , males:  $F_{4,150} = 13.79$ ,  $p < 0.0001$ , females:  $F_{4,150} = 4.615$ ,  $p < 0.05$ , Fig. 2C). In the aggregate analysis, CUS-tones attenuated mPFC responsivity (CUS-tones vs NS Ext-16 ( $p < 0.0001$ ), which was reversed by 16-tone extinction ( $p < 0.0001$ ), and 8-tone extinction ( $p < 0.05$ ). However, CUS Ext-16 also differed from CUS Ext-8 ( $p < 0.05$ ). Pairwise group comparisons for males revealed differences between CUS-tones



**Fig. 2.** 8-Tone extinction does not reverse stress-induced deficits in MDT-evoked response in the mPFC.

(A) Experimental timeline for male and female rats. (B) Fear extinction curves for 16- and 8-tone extinction were not different over the first 8 tones. (C) Effects of 16- and 8-tone extinction on stress-attenuated MDT-evoked responses in the mPFC of males and females. The effect of stress (CUS-tones vs NS Ext-16,  $*p < 0.0001$ ) was reversed by 16-tone extinction ( $^+p < 0.0001$ ). There was a partial effect of 8-tone extinction ( $+p < 0.05$ ), in that it was also significantly different from that of Ext-16 ( $\#p < 0.05$ ). In males (bottom left), there was an effect of stress ( $*p < 0.05$ ) that was reversed by 16-tone extinction ( $^+p < 0.0001$ ), and partially reversed by 8-tone extinction ( $^+p < 0.001$ ), which also differed from 16-tone extinction ( $\#p < 0.01$ ). In females, there was an effect of stress ( $*p < 0.05$ ) that was reversed by 16-tone extinction ( $^+p < 0.001$ ). The effect of 8-tone extinction was not significant in females. However, in females, 8-tone extinction also did not differ from 16-tone extinction. (D) Recording electrode placements in the mPFC (top) and stimulating electrode placements in the MDT (bottom). Graphics are from the atlas of Paxinos and Watson (Paxinos and Watson, 2006). (E) CUS induced a deficit in set shifting compared to nonstress controls ( $*p < 0.0001$ ). The CUS-induced deficit in set-shifting was reversed by 16-tone extinction ( $^+p < 0.05$ ), but not 8-tone extinction; the full stress effect was still evident in the CUS 8-tone extinction group ( $*p < 0.0001$  vs nonstress controls). In both males (5–11/group) and females (5–6/group), there was an effect of stress ( $*p < 0.05$ ). The stress effect was attenuated by 16-tone extinction, similar to the therapeutic effect seen in the combined groups, but pairwise comparisons in the sexes analyzed separately were not significant. By contrast, the stress effect was unchanged by 8-tone extinction in either sex ( $*p < 0.05$ ). Bars represent SEM.

and NS Ext-16 ( $p < 0.0001$ ), CUS Ext-16 ( $p < 0.0001$ ), and CUS Ext-8 ( $p < 0.05$ ). CUS Ext-8 was also significantly different from CUS Ext-16 ( $p < 0.05$ ). Hence, 8-tone extinction partially reversed the stress-induced attenuation of evoked LFPs, but not to the same extent as 16-tone extinction. Pairwise group comparisons for females revealed differences between CUS-tones and NS Ext-16 ( $p < 0.0001$ ), and CUS Ext-16 ( $p < 0.05$ ), but not between CUS-Tones and CUS Ext-8. However, CUS Ext-8 also did not differ from CUS Ext-16. Therefore, together these results indicate a partial effect of 8-tone extinction on stress-compromised mPFC responsivity. Electrode placement was confirmed histologically (Fig 2D); 4 rats were excluded from analysis due to poor recordings.

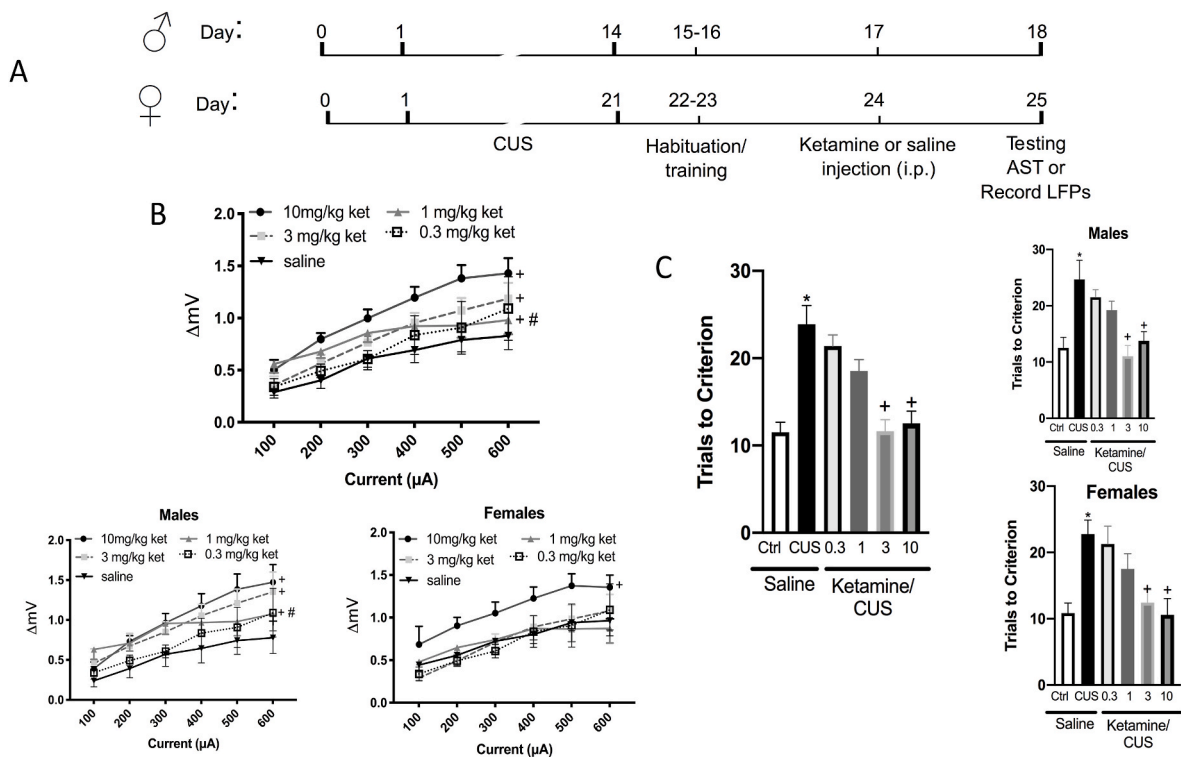
### 3.3. 8-Tone extinction does not reverse set shifting deficits after chronic stress

We next tested if 8-tone extinction, like 16-tone extinction, was effective in reversing stress-induced deficits on set shifting performance. One-way ANOVA showed group differences in set-shifting ( $F_{3,45} = 15.13$ ,  $p < 0.0001$ , Fig. 2E). Pairwise comparisons revealed a stress effect (NS Ext-16 vs CUS-tones,  $p < 0.0001$ ). Ext-16 reversed the effects of stress on set shifting (CUS-tones vs CUS Ext-16,  $p < 0.05$ ). The Ext-8 group was not significantly different from CUS-tones, and was significantly different from NS controls ( $p < 0.0001$ ). Male data analyzed separately showed a significant group difference ( $F_{3,23} = 4.720$ ,  $p < 0.05$ ). Pairwise comparisons revealed a stress effect, NS Ext-16 vs CUS-tones ( $p < 0.05$ ), which was not reversed by CUS Ext-8. Female data analyzed separately revealed a group difference ( $F_{3,18} = 11.30$ ,  $p <$

$0.05$ ). In females, pairwise comparisons revealed a stress effect (NS Ext-16 vs CUS-tones,  $p < 0.05$ ), which was not reversed by 8 tone extinction (CUS Ext-8 vs CUS tones,  $p > 0.05$ ). The CUS Ext 16 group did not differ from the non-stressed group ( $p > 0.05$ ). In sum, 8-tone extinction was not effective in restoring CUS-induced set shifting deficits. Thus, 8-tone extinction can be used as a tool to model sub-effective extinction that does not reverse stress-induced set shifting deficits.

### 3.4. Dose-dependent effects of ketamine on MDT-evoked LFPs in the mPFC of stressed rats

Ketamine at 10 mg/kg fully restored mPFC function compromised by chronic stress (Zhang et al., 2019). Therefore to identify a sub-effective ketamine dose that did not fully restore mPFC function, we measured MDT-evoked LFPs in the mPFC following chronic stress. Stimulus-response curves for MDT-evoked local field potentials in the mPFC differed between groups ( $F_{8,434} = 7.618$ ,  $p < 0.0001$ , Fig. 3A). Pairwise comparisons revealed differences between saline and 10.0 mg/kg ( $p < 0.0001$ ), 3.0 mg/kg ( $p < 0.05$ ), and 1.0 mg/kg ketamine ( $p < 0.05$ ). However, 1.0 mg/kg was also different from 10.0 mg/kg ( $p < 0.05$ ). Saline was not different from 0.3 mg/kg. For male data analyzed separately, there was a significant group effect ( $F_{8,260} = 4.928$ ,  $p < 0.0001$ ), with differences between saline and 10.0 mg/kg, 3.0 mg/kg and 1.0 mg/kg ketamine ( $p < 0.0001$ ,  $p < 0.05$ ,  $p < 0.05$ , respectively) but not 0.3 mg/kg 1.0 mg/kg was also different from 10.0 mg/kg ( $p < 0.05$ ). In females, there was a significant group effect ( $F_{8,200} = 4.344$ ,  $p < 0.05$ ), with differences between saline and 10.0 mg/kg ketamine ( $p < 0.0001$ ), but not between saline and 0.3 mg/kg, 1.0 mg/kg, or 3.0 mg/kg



**Fig. 3.** Dose-dependent effects of ketamine on stress-induced deficits in MDT-evoked mPFC response and cognitive set shifting. (A) Experimental timeline for male and female rats. (B) In stressed rats, 10.0 mg/kg, 3.0 mg/kg and 1.0 mg/kg restored mPFC responsivity to MDT stimulation ( $^+p < 0.05$ ). 0.3 mg/kg was not different from saline. However, 1.0 mg/kg was also different from 10.0 mg/kg ( $^{\#}p < 0.05$ ), indicating a partial effect. In males (left inset; 5–10/group), 1.0 mg/kg, 3.0 mg/kg and 10.0 mg/kg all restored stress-compromised mPFC response compared to saline ( $^+p < 0.0001$ ). 1.0 mg/kg was also different from 10.0 mg/kg ( $^{\#}p < 0.05$ ). In females (right inset, 5–11/group), only 10.0 mg/kg differed from saline ( $^+p < 0.0001$ ). (C) CUS induced a deficit in set shifting ( $^*p < 0.001$ ), that was reversed by ketamine at 3.0 mg/kg and 10.0 mg/kg ( $^+p < 0.002$ ), but not 0.3 mg/kg or 1.0 mg/kg. Similarly, in males (top inset, 4–9/group), there was a stress effect ( $^*p < 0.05$ ), that was reversed only by 3.0 mg/kg and 10.0 mg/kg ketamine ( $^+p < 0.01$ ). CUS induced a set-shifting deficit in females (4–6/group, bottom inset,  $^*p < 0.05$ ), that was also reversed by 3.0 mg/kg and 10 mg/kg ( $^+p < 0.05$ ), but not 0.3 mg/kg or 1.0 mg/kg. Bars represent SEM.

(all  $p > 0.05$ ). Thus, 1.0 mg/kg ketamine had a partial and inconsistent effect on stress-compromised afferent-evoked responses in mPFC. 8 rats were excluded due to poor recordings.

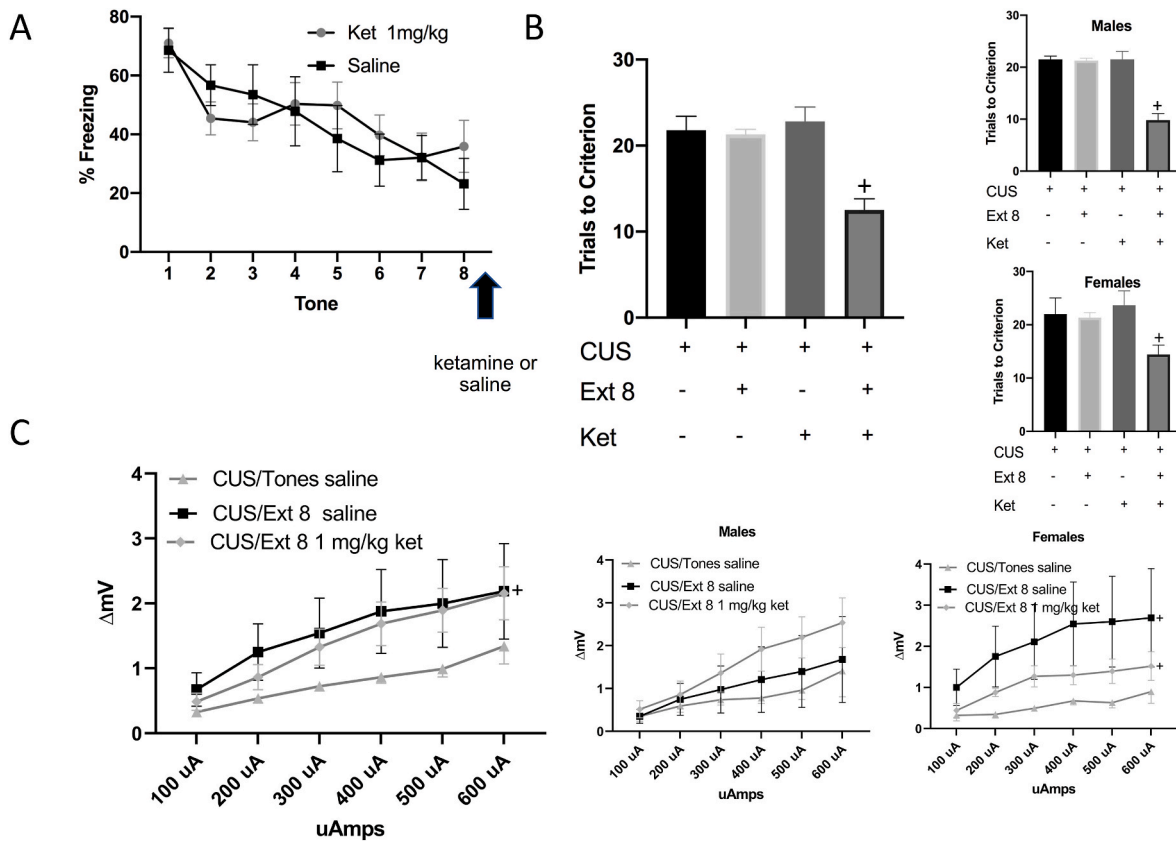
### 3.5. Dose-dependent effects of ketamine in reversing stress-induced set-shifting impairments

Our group has demonstrated that an acute injection of ketamine at 10 mg/kg effectively reverses stress-induced deficits in set shifting (Jett et al., 2015). In order to identify doses of ketamine that were not fully effective, we tested the effects of various doses of ketamine (0.3, 1, 3, 10 mg/kg) on set shifting after stress. The goal was to select a sub-effective dose of ketamine to test in adjunct with 8-tone extinction. In the aggregate analysis, ANOVA revealed a group difference in set-shifting ( $F_{5,63} = 12.90$ ,  $p < 0.0001$ , Fig. 3B). Pairwise comparisons revealed a stress effect (NS saline vs CUS-saline,  $p < 0.0001$ ). Ketamine reversed the effect of stress at doses of 3.0 mg/kg and 10.0 mg/kg ( $p < 0.0001$  for both) but not at 0.3 mg/kg or 1.0 mg/kg. Analyzed separately, male data showed a significant group difference ( $F_{5,33} = 6.592$ ,  $p < 0.0001$ ). Pairwise comparisons revealed a stress effect (NS-saline vs CUS-saline,  $p < 0.05$ ). Ketamine reversed the effect of stress at doses of 3.0 mg/kg and 10.0 mg/kg (CUS-saline vs CUS 10 mg/kg; CUS saline vs 3 mg/kg,  $p < 0.05$  for both) but not at 0.3 mg/kg or 1.0 mg/kg. Female data showed similar effects, with a significant group difference ( $F_{5,24} = 5.615$ ,  $p < 0.05$ ). In females, ketamine reversed the effect of stress at doses of 3.0 mg/kg and 10.0 mg/kg ( $p < 0.05$ ) but not at 0.3 mg/kg or 1.0 mg/kg. In sum, 3.0 and 10.0 mg/kg ketamine effectively reversed stress-induced set-shifting deficits, and 1.0 mg/kg was ineffective.

### 3.6. Adjunct treatment combining sub-effective extinction and sub-effective ketamine results in fully effective reversal of set-shifting deficits after stress

The results from our previous experiments demonstrate that a) 8 tone extinction did not reverse stress-induced set shifting deficits but did induce some plasticity in the mPFC, b) ketamine at 1 mg/kg did not reverse stress-induced set shifting deficits in both males and females, while 1 mg/kg induced electrophysiological changes in males but not females. Thus, we tested whether combining sub-effective 8-tone extinction and sub-effective ketamine (1 mg/kg) would effectively reverse stress-induced deficits in set shifting. Within-session freezing during the first 8 tones of extinction did not differ between fear-conditioned groups ( $F_{1,20} = 0.09339$ ,  $p > 0.05$ ; Fig. 4A). In the aggregate analysis, ANOVA revealed group differences in set-shifting ( $F_{3,37} = 13.52$ ,  $p < 0.0001$ ,  $p < 0.05$ , Fig. 4B). Pairwise comparisons revealed that the adjunct treatment group, CUS-Ext 8 + 1.0 mg/kg ketamine, was significantly different from all other groups, effectively reversing the detrimental effect of stress on set-shifting ( $p < 0.05$  for all comparisons). Male data analyzed separately showed a significant group effect ( $F_{3,13} = 29.69$ ,  $p < 0.0001$ ). The CUS-Ext 8 + 1.0 mg/kg group was significantly different from all other groups ( $p < 0.0001$  for all comparisons). In females, a significant group effect was also detected ( $F_{3,20} = 3.909$ ,  $p < 0.05$ ), and pairwise comparisons showed a difference between the adjunct treatment group and CUS-tones 1 mg/kg ( $p < 0.05$ ). These results show that adjunct treatment combining a sub-effective dose of ketamine with sub-effective extinction enhanced efficacy and reversed the stress-induced set-shifting impairment.

We initially selected 8-tone extinction for adjunct treatment because,



**Fig. 4. Adjunct treatment with 8-tone extinction plus 1.0 mg/kg ketamine reverses stress-induced set shifting deficits.** (A) Extinction curves did not differ between groups. Ketamine injection was given immediately after extinction. (B) Combined treatment with 8-tone extinction plus 1.0 mg/kg ketamine significantly improved set-shifting in stressed rats ( $^{\dagger}p < 0.001$ ). Neither 8-tone extinction alone, nor 1.0 mg/kg ketamine alone had any effect on set-shifting in stressed rats. Similarly, in stressed males (upper inset, 4–5/group), only combined treatment with Ext-8 + 1.0 mg/kg ketamine significantly improved set-shifting in stressed rats ( $p < 0.0001$ ). Likewise, in females (lower inset,  $n = 5$ –7/group), only combined treatment with Ext-8 + 1.0 mg/kg ketamine differed from CUS 1.0 mg/kg ketamine ( $p < 0.0001$ ). (C) Ketamine does not enhance the effects of 8-tone extinction on evoked LFPs in the mPFC. CUS-tones + saline is different from both CUS 8-tones + saline ( $^{\dagger}p < 0.005$ ), and CUS 8-tones + 1.0 mg/kg ketamine ( $^{\dagger}p < 0.005$ ). Ketamine did not enhance the effects of 8-tone extinction (CUS 8-tones saline vs. CUS 8-tones 1.0 mg/kg ketamine,  $p = 0.6674$ ). In males (middle, 3–5/group), no differences were detected between groups. In females (right, 3–5/group), CUS-tones saline is different from both CUS 8-tones saline and CUS 8-tones ketamine ( $^{\dagger}p < 0.001$ ). Bars represent SEM.

as a group, rats achieved the same final level of extinction after 8-tones as they did after 16-tones, but without the continued training that the 16-tone groups received. However, it is possible that 8-tone extinction might be more or less effective depending on the degree of final extinction achieved in individual rats. To address this, we used results from the rats exposed to 8-tone extinction alone in experiments 2 and 4 to test for a correlation of the average final freezing on tones 7 and 8 with the trials to criterion on the subsequent set-shifting task. There was no correlation ( $r_{(25)} = -0.2021$ ,  $p = 0.3325$ ).

### 3.7. Effects of adjunct treatment combining sub-effective extinction and sub-effective ketamine on MDT-evoked responses in mPFC

Our behavioral data demonstrated that sub-effective 8-tone extinction in combination with sub-effective 1.0 mg/kg ketamine was fully effective in reversing stress-induced deficits in set shifting. Therefore, we tested whether the combination of these sub-effective treatments also had additive electrophysiological effects, fully restoring stress-attenuated responsivity of the mPFC to MDT afferent input. Stimulus-response curves for MDT-evoked local field potentials in the mPFC differed between groups ( $F_{4,132} = 6.207$ ,  $p = 0.001$ ; Fig. 4C). Pairwise comparisons showed CUS-tones + saline to be different from both CUS 8-tones + saline ( $p = 0.0002$ ), and CUS 8-tones + 1.0 mg/kg ketamine ( $p = 0.0001$ ). However, ketamine did not further enhance the effects of 8-tone extinction (CUS 8-tones + saline vs. CUS 8-tones 1.0 mg/kg +

ketamine,  $p = 0.6674$ ). Therefore, additive enhancement of mPFC responsivity to afferent input from the MDT is not a mechanism by which adjunct treatment with sub-effective extinction and sub-effective ketamine fully rescues stress-induced set-shifting deficits.

## 4. Discussion

The present study demonstrates that adjunct treatment with ketamine enhanced the efficacy of a sub-effective extinction treatment, fully reversing stress-induced deficits in cognitive set-shifting. This study validates a model of sub-effective extinction that does not reverse set shifting deficits after stress, but induces some plasticity in the mPFC. This sub-effective model can therefore be used to identify pharmacotherapies that may enhance the therapeutic effects of behavioral therapy in patients that receive incomplete duration of treatment.

Premature dropout is a major problem for PE, with some studies reporting a quarter of patients leave treatment before session 3 out of 10–12 (Kehle-Forbes et al., 2016). Patients may avoid experiencing the distress of re-exposure, and thereby receive an incomplete course of PE therapy. The present study demonstrates that adjunct treatment with ketamine enhanced the efficacy of a sub-effective extinction treatment, fully reversing stress-induced cognitive deficits. This suggests that rapidly-acting drugs such as ketamine may be effective as adjunct treatment when the duration of behavioral therapy is sub-optimal. Thus, an attenuated course of PE treatment that may otherwise be ineffective

could be made effective by adjunct treatment with a rapid-acting pharmacological agent such as ketamine. These findings are consistent with preliminary clinical results suggesting improved efficacy of PE combined with ketamine, and more broadly, that pharmacotherapy combined with CBT improves response in treatment-resistant patients (Wiles et al., 2013; Shiroma et al., 2020).

Individuals with psychiatric disorders display perseverative, rigid thinking that maintains and exacerbates their symptoms. CBT and PE engage cognitive flexibility to implement cognitive restructuring and re-evaluation of cognitive biases. PTSD is associated with reduced ventromedial mPFC activation, a region that mediates cognitive flexibility (Hayes et al., 2012). FE potentiates mPFC neural activity during extinction recall (Milad and Quirk, 2002), ergo FE may prime mPFC responsiveness to enable more flexible cognitive processing. Surprisingly, our electrophysiological data showed that 1.0 mg/kg ketamine did not enhance the effects of 8-tone extinction on MDT-evoked LFPs in the mPFC. Therefore, the mechanism of adjunct treatment combining sub-effective extinction and sub-effective ketamine does not depend on additive restoration of stress-compromised MDT-mPFC responsiveness. However, this does not rule out the possibility of enhanced mPFC responsiveness to another afferent input, such as that from the ventral hippocampus (vHipp). Indeed, FE memory consolidation requires activation of the vHipp and the infralimbic subregion of mPFC. Likewise, antidepressant-like effects of ketamine require activity in the vHipp-mPFC pathway (Carreno et al., 2016). Thus, vHipp plasticity is another possible site of convergence for the mechanisms of ketamine and extinction, and is a possible substrate for additive enhanced responsiveness. We have shown phosphorylation of the BDNF receptor TrkB at the Y515 site in the IL 30 min post-FE, and the necessity of Akt and Erk signaling in IL immediately after FE for its effects on set shifting after CUS (Paredes et al., 2022). Therefore, we chose to administer ketamine immediately after the sub-effective extinction treatment, to coincide with the timing of TrkB receptor activation and initiation of consolidation processes, which include Akt and Erk signaling. Our electrophysiological data suggest the 8-tone FE treatment partially restored evoked responses in the mPFC, which may serve to prime the stressed mPFC to respond more robustly to a second treatment that may otherwise be ineffective on its own.

FE and ketamine share overlapping mechanisms, making ketamine an ideal candidate with which to test the sensitivity of the sub-effective extinction model to detect the ability of adjunct treatment to enhance the therapeutic efficacy of extinction. BDNF-associated plasticity has been well-documented to play a role in the mechanisms of both FE and ketamine (Paredes et al., 2022; Autry et al., 2011; Peters et al., 2010; Pattwell et al., 2012). Glutamate signaling triggers the production of BDNF, and BDNF subsequently alters neuronal sensitivity to glutamate by phosphorylating NMDAR and upregulating AMPAR expression and trafficking (Mattson, 2008). Both ketamine and FE treatment activate the BDNF receptor, TrkB (Paredes et al., 2022; Carreño et al., 2011), and induce phosphorylation of proteins associated with BDNF signaling, such as extracellular regulated protein kinase (Erk) and ribosomal protein S6 (Fucich et al., 2016; Li et al., 2010). Such pathways are necessary for long term potentiation (LTP), memory consolidation, and structural synaptic changes (Li et al., 2010; Schafe et al., 2000; Mullen et al., 2012). Ketamine at high doses interferes with fear memory and extinction consolidation (Clifton et al., 2018; Duclot et al., 2016), while lower doses (10 mg/kg) facilitate memory consolidation (Radford et al., 2018). The effects of ketamine on fear memory depend on route of administration as well as dose (Choi et al., 2020). Ketamine at low doses, given intraperitoneally, accelerates FE learning via mTORC1 signaling, a downstream target of BDNF receptor activation (Girgenti et al., 2017). In addition to converging signaling mechanisms, FE and ketamine elicit neuronal plasticity in overlapping circuits. Moreover, unlike typical antidepressants, ketamine reaches effective micromolar concentrations in the brain rapidly (Casarotto et al., 2021), and improves symptoms in a rapid and sustained manner (Hashimoto, 2020; Price et al., 2009),

making it a viable candidate for adjunct treatment with behavioral therapy.

However, the fact that FE and ketamine share certain mechanisms does not exclude the possibility that independent mechanisms may also be responsible for the enhanced effectiveness of adjunct treatment with extinction plus ketamine. Although ketamine acts primarily via the NMDAR, it has also been shown that antidepressant effects of ketamine require activation of opioid receptors and dopamine D2/D3 receptors (Li et al., 2015; Williams et al., 2018). Systemic administration of ketamine also impacts many brain regions not directly involved in extinction learning. Therefore, it is possible that a sub-effective course of extinction learning initiates a degree of plasticity in the mPFC that does not reach a threshold necessary for behavioral impact, but that plasticity may be enhanced by the effects of ketamine acting through either shared mechanisms or unshared mechanisms that nonetheless can independently influence the same mPFC circuitry.

Ketamine did not enhance responsiveness of the mPFC to afferent input from the MDT in females at 3.0 or 1.0 mg/kg. We also show that ketamine at 3.0 and 10.0 mg/kg reversed stress-induced deficits in set shifting in both males and females. This may seem at odds with reports showing greater sensitivity of female rodents to the antidepressant effects of ketamine on the Forced Swim Test (FST) (Wright and Kabbaj, 2018). However, other studies have found discrepancies between the effects of therapeutic doses of ketamine in behavioral measures of depression and functional changes after stress in the mPFC. Indeed, the same dose of ketamine rescued depression-like behavior in male and female rats, while spine density measures remained unchanged in stressed females after ketamine (Sarkar and Kabbaj, 2016). Our data also do not exclude the possibility that lower doses of ketamine may enhance the responsiveness of the mPFC from another input, such as the vHipp. In females, but not males, chronic stress affects dendritic morphology of vHipp neurons, (Morales Rico et al., 2015). Thus, the vHipp is sexually dimorphic in the context of stress and response to treatment (Galea et al., 1997; Shors et al., 2001).

Although ketamine decreases symptoms in a rapid and sustained manner, it unfortunately has many other characteristics, including abuse potential, that limit its use in treating psychiatric disorders. This is especially relevant for patients with PTSD and depression, which are often comorbid with substance use disorders (Kilpatrick et al., 2003). Other therapies that can mimic the cellular and circuit-level effects of ketamine (e.g., activate the vHipp-mPFC pathway, promote BDNF signaling), but lack abuse-related or psychotomimetic effects (Carreno et al., 2017) may be preferable for adjunct treatment in patients with substance abuse history. Future studies using the sub-effective extinction platform may identify other pharmacotherapies that complement the effects of FE via either shared or independent mechanisms to enhance neural plasticity and therapeutic efficacy.

## Funding and disclosure

Dr. Paredes, Ms. Knippenberg, Dr. Bulin and Ms. Keppeler have no competing interests. Dr. Morilak receives in-kind research support from Lundbeck that has no relation to the work presented in this paper. This work was supported by research grant MH053851 from the National Institute of Mental Health, National Institutes of Health; by Merit Award 1101BX003512 from the U.S. Department of Veterans Affairs Biomedical Laboratory Research and Development Program; and by a grant from the William and Ella Owens Medical Research Foundation, none of which had any role in study design, data collection, analysis or interpretation, nor in the preparation or decision to submit this paper for publication. The contents of this paper do not represent the views of the Department of Veterans Affairs or the U.S. Government.

## CRediT authorship contribution statement

**Denisse Paredes:** performed data collection, analysis and



interpretation, wrote and edited the manuscript. **Anna R. Knippenberg**: contributed to data collection, interpretation, and edits to the manuscripts. **Sarah E. Bulin**: contributed to data collection, interpretation, and edits to the manuscripts. **Lydia J. Keppler**: contributed to data collection, interpretation, and edits to the manuscripts. **David A. Morilak**: contributed to experimental design and conception, data interpretation, editing and final approval of the manuscript.

### Declaration of competing interest

Dr. Paredes, Mrs. Knippenberg, Dr. Bulin and Mrs. Keppler have no competing interests. Dr. Morilak receives in-kind research support from Lundbeck that has no relation to the work presented in this paper.

### Acknowledgements

We thank Christina George and Sarah Way for technical assistance.

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