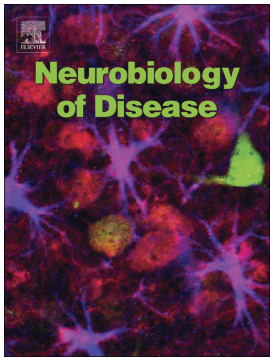


Ketamine accelerates fear extinction via mTORC1 signaling

Matthew J. Girgenti, Sriparna Ghosal, Dora LoPresto, Jane R. Taylor, Ronald S. Duman

PII: S0969-9961(16)30316-3
DOI: doi: 10.1016/j.nbd.2016.12.026
Reference: YNBDI 3893

To appear in: *Neurobiology of Disease*

Received date: 27 October 2016
Revised date: 15 December 2016
Accepted date: 29 December 2016

Please cite this article as: Matthew J. Girgenti, Sriparna Ghosal, Dora LoPresto, Jane R. Taylor, Ronald S. Duman , Ketamine accelerates fear extinction via mTORC1 signaling. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Ynbdi(2016), doi: 10.1016/j.nbd.2016.12.026

Ketamine accelerates fear extinction via mTORC1 signaling

Matthew J. Girgenti, PhD, Sriparna Ghosal, PhD, Dora LoPresto, BS, Jane R. Taylor, PhD, and Ronald S. Duman, PhD‡

Laboratory of Molecular Psychiatry, Center for Genes and Behavior, Department of Psychiatry, Yale University School of Medicine, New Haven, CT., USA, 06508

Number of Figures: 4

Key words: ketamine, fear extinction, mTOR, medial prefrontal cortex, PTSD
Running Title: Ketamine enhances fear extinction in rodents

‡ Address all correspondences to:
Dr. Ronald S. Duman
Elizabeth Mears and House Jameson Professor of Psychiatry and Pharmacology
Director, Abraham Ribicoff Research Facilities
Yale University School of Medicine
Phone: (203) 974-7726
E-mail: Ronald.duman@yale.edu

ABSTRACT

Impaired fear extinction contributes to the persistence of post-traumatic stress disorder (PTSD), and can be utilized for the study of novel therapeutic agents. Glutamate plays an important role in the formation of traumatic memories, and in the pathophysiology and treatment of PTSD, highlighting several possible drug targets. Recent clinical studies demonstrate that infusion of ketamine, a glutamate NMDA receptor antagonist, rapidly and significantly reduces symptom severity in PTSD patients. In the present study, we examine the mechanisms underlying the actions of ketamine in a rodent model of fear conditioning, extinction, and renewal. Rats received ketamine or saline twenty-four hours after fear conditioning and were then subjected to extinction-training on each of the following three days. Ketamine administration enhanced extinction on the second day of training (i.e., reduced freezing behavior to cue) and produced a long-lasting reduction in freezing on exposure to cue plus context 8 days later. Additionally, ketamine and extinction exposure increased levels of mTORC1 in the medial prefrontal cortex (mPFC), a region involved in the acquisition and retrieval of extinction, and infusion of the selective mTORC1 inhibitor rapamycin into the mPFC blocked the effects of ketamine on extinction. Ketamine plus extinction also increased cFos in the mPFC and administration of a glutamate-AMPA receptor antagonist blocked the effects of ketamine. These results support the hypothesis that ketamine produces long-lasting mTORC1/protein synthesis and activity dependent effects on neuronal circuits that enhance the expression of extinction and could represent a novel approach for the treatment of PTSD.

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a chronic and debilitating disorder with a life-time prevalence of 7.8% in the general population and higher in trauma-exposed groups (Kessler et al., 2008). PTSD is typically characterized as persistent re-experiencing of memories, avoidance of cues or situations that are reminiscent of the traumatic event, emotional numbing, and hyperarousal. PTSD symptoms, particularly those related to re-experiencing the traumatic event, may fall within the fear-conditioning paradigm of neurobiology. Alterations in fear conditioning and extinction learning are thought to play a role in the onset and maintenance of PTSD (Milad et al., 2007; Pitman et al., 2012).

Significant progress has been made in understanding the neurobiological basis of fear (Johansen et al., 2011; Kessler et al., 2008). Pavlovian fear conditioning is believed to take place at the convergence of neural circuits linking the amygdala, hippocampus and medial prefrontal cortex (mPFC) (Knapska et al., 2012; Milad et al., 2007; Pitman et al., 2012; Sierra-Mercado et al., 2011). Fear conditioning and extinction represent basic forms of associative learning that are highly conserved across species (Johnson et al., 2012). Trauma-exposed patients suffering from PTSD experience deficits in the extinction of learned fear associations when compared to those who do not develop PTSD (Holmes and Singewald, 2013; Lommen et al., 2013; Parsons and Ressler, 2013a). Therefore, animal models involving fear conditioning and extinction learning in rodents represent an ideal paradigm for preclinical assessments of PTSD and for identifying novel pharmacotherapies.

Antidepressants, particularly selective serotonin reuptake inhibitors, can reduce PTSD symptoms in humans (Zhang and Davidson, 2007) and fear in rodents (Karpova et al., 2011) when combined with extinction therapy. During extinction, repeated exposure to a cue previously associated with a fear-provoking event results in the gradual formation of a new memory that is thought to suppresses fear expression by establishing an inhibitory memory (Orsini and Maren, 2012). However, currently available antidepressants have several limitations, including slow onset of action (weeks to months) and low rates of efficacy, with only a subset of patients showing complete remission of PTSD symptoms (Ursano et al., 2004). These factors underscore the urgent need to develop new pharmacotherapies that enhance extinction and can provide a more persistent and rapid reduction in PTSD symptoms.

There is mounting evidence for a role of the excitatory neurotransmitter glutamate in stress responsiveness, the formation of traumatic memories, and the pathophysiology of PTSD, raising the possibility of identifying novel glutamatergic interventions for this disorder (Horn et al., 2016; Rasmussen, 2016; Riaza Bermudo-Soriano et al., 2012). Notably, recent clinical studies demonstrate that infusion of ketamine, a glutamate N-methyl-d-aspartate (NMDA) receptor antagonist, rapidly and significantly reduces symptom severity in PTSD patients (Feder et al., 2014). Moreover, administration of ketamine immediately after witnessing a traumatic event has been shown to prevent the enhancement of passive avoidance learning in mice (Ito et al., 2015).

The rapid actions of ketamine in behavioral models of depression and antidepressant response have been linked to increased synapse number and function in the mPFC (Duman and Aghajanian, 2012; Li et al., 2010). These studies also demonstrate that ketamine increases the mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway that regulates translation and synaptic protein synthesis (Duman and Aghajanian, 2012). Given the important role for neuroplasticity of the mPFC and its projections to amygdala in extinction learning we reasoned that the actions of ketamine in mPFC could also influence fear extinction (Orsini and Maren, 2012). The results of the current study show that ketamine improves extinction recall in adult rats and that this effect is mediated by activation of the mTORC1-dependent translation mechanisms in the mPFC.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing between 175-250 g and between 7-9 weeks of age were used for all experiments. Rats were pair housed in rectangular polypropylene cages with laboratory bedding and kept under standard conditions with a 12-h light/dark cycle. Ambient temperature in the vivarium was maintained at 21^{o}C. Food and water were available *ad libitum*, throughout the duration of the experiment. Animal use and procedures were in accordance with the National Institutes of Health guidelines and approved by the Yale University Animal Care and Use Committee.

Drugs

Ketamine hydrochloride (100mg/mL, Pfizer) was diluted to 10mg/ml in 0.9% (v/v) saline and administered at a volume of 1mL/kg. Rapamycin (0.02nmol, Cell signaling) was dissolved in dimethylsulfoxide (DMSO). NBQX (10mg/kg, Sigma) was dissolved in 0.9% (v/v) saline and administered at a volume of 1mL/kg by i.p. injection.

Apparatus

Fear conditioning was carried in four identical operant chambers (30 cm X 20 cm X 25 cm, Med Associates) constructed from aluminum and Plexiglas. These chambers were housed in a sound-attenuating box equipped with a ventilation fan and house light. The chambers contained a grid floor (19 parallel 0.48 cm diameter stainless steel rods, 1.6 cm apart) above a stainless steel waste pan. All rods were wired to a shock generator and scrambler (ENV-414S; Med Associates). A loudspeaker (ANL-926; Med Associates) was mounted in the chamber wall to provide the source of the auditory stimuli. A computer with a SmartCTL Interface System (DIG-700F; Med Associates) controlled the delivery of auditory stimuli and footshock. Each chamber was further equipped with a miniature high-speed Firewire monochromatic camera that was mounted on the inside of the cubicle door to permit recording of the testing sessions.

For extinction training and testing, the context was modified (context B) by using a smooth white acrylic insert instead of a grid floor and inserting a black plastic triangular insert into the chamber to alter both the color, texture, and shape of the chambers. Additionally, the house lights were kept off and a novel odor (e.g., 1% peppermint scented solution) was used to maximize discrimination from the original training context. There was ambient noise during fear conditioning, extinction, and extinction training.

Fear Conditioning Procedure

Rats were placed in the training context (context A) and after a 180 s acclimation period, they received seven pairings of the CS and US. The CS tone (78 dB, 2 kHz, 5 ms rise/fall time) was presented for 30 s and co-terminated with

a brief US footshock (1 s, 0.66 mA). The inter-tone interval (tone onset to next tone onset) ranged from 60 to 180 s with a mean onset of 94 s. The conditioning chambers were cleaned between subjects with 70% ethanol. The time-spent freezing during delivery of the CS tone was scored (CS freezing) and presented as the average across all CS-US pairings. Subjects were randomly assigned to either saline or ketamine groups, and were matched on CS tone freezing levels. The next day rats received a single injection of ketamine (10 mg/kg, i.p., 1 ml/kg) or 0.9 w/v saline.

Twenty-four hours after drug treatment, ketamine and vehicle-treated groups were placed in context B and extinction training began. Following a 3 min acclimation period, rats received 12 non-reinforced presentations of the CS (30 s, 78 dB, inter-tone interval: 60-90 s) in the novel context. Between animals the chambers were cleaned with a 1% peppermint scented solution. This procedure was repeated over the next 3 days to produce 4 separate days of extinction training. One week later spontaneous recovery and fear renewal was assessed by returning the subjects to context B (spontaneous recovery) and context A (training context, fear renewal).

Freezing, which is expressed as a percentage of time spent freezing during the 30-sec tone (unless otherwise noted), was measured using an automated computer analysis system (Video Freeze, SOF-843) and by blind hand scoring. Video signals were captured at a sampling rate of 30 frames per second and the software calculated the frame by frame change in grayscale values for each pixel. This value was compared to the grayscale values obtained when no animal was present in the chamber in order to obtain an “activity score” for each frame. Freezing was defined as sub-threshold activity (set at 18 activity units) for longer than 1 s. When compared with traditional hand scoring that defined freezing as the absence of all movement except that necessitated by respiration, a correlation of >.90 was found between the automated system and human observer values.

Surgery and Drug Infusions

After one week of habituation to the animal colony, rats were anesthetized with a ketamine-xylazine cocktail (100 mg/kg ketamine combined with 10mg/kg xylazine, i.p.) and placed into a stereotaxic apparatus. Previous studies have shown that anesthetic doses of ketamine do not elicit antidepressant effects (Autry et al., 2011; Li et al., 2010). Bilateral guide cannulas (26 Ga, Plastic One) were inserted into the medial prefrontal cortex as described previously(Li et al., 2010; 2011) (-0.9 mm anteriorposterior, ±-1.5 mm mediolateral, and -3.3 mm below dura, 30° angle). The cannula assembly was secured to the skull with four stainless steel screws and dental acrylic, and each animal was fitted with a dummy cannula to prevent the accumulation of debris.

Following a 7 to 9-day recovery period, rats were fear conditioned as described earlier. Twenty four hours after fear conditioning rapamycin (0.2 nmol, Cell Signaling) or DMSO were delivered in a 0.2 µl volume at a flow rate of 0.1 µl/min. A smaller volume of rapamycin was infused into the mPFC to ensure limited spread to surrounding brain structures (Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011). The infusion cannula was left in place for an additional 3 minutes after delivery before being slowly withdrawn to facilitate diffusion of the compound and to prevent back-filling of the guide. Treatments with rapamycin or DMSO were given 30 minutes before ketamine or saline injections (Li et al., 2011; 2010). After behavioral testing animals were sacrificed and perfused with 4% paraformaldehyde and post fixed with sucrose. Sections of perfused brains (20um) were Nissl stained and examined for cannula placements and animals with incorrect placement were eliminated from the behavioral analysis.

Western Blotting

Brain tissue was collected 90 minutes after presentation of the last tone during the extinction recall test on day 2. The amygdala and medial prefrontal was carefully dissected and frozen on dry ice and stored at -80ºC until processing. Dissected samples were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA,

1 mM sodium vanadate, 10 mM NaF, and 1X protease inhibitor cocktail. Protein concentration was determined by BCA assay (Pierce Biotechnology). For Western blotting, equal amounts of protein (20 µg) were loaded and separated on a 7.5% SDS-PAGE gel. After electrophoresis, the proteins were electrically transferred to nitrocellulose membranes. Following electro-transfer, membranes were blocked for 1 hr in 5% bovine serum albumin in PBS-T (PBS + 0.1% Tween-20) and incubated overnight at 4°C with primary antibody. The following primary antibodies were used: p-mTOR (1:1000, Millipore), mTOR (1:1000, Millipore), p-ERK/ERK1 (1:1000, Millipore), ERK1/ERK2 (1:1000, Millipore), p-AKT (1:1000, Millipore), AKT (1:1000, Millipore), p-p70s6K (1:2000, Millipore) and p70s6K, (1:10000, Millipore), cFos (1:1000, Santa Cruz) and Gapdh (1:10000, Abcam). Following incubation, membranes were washed in TBS-T and incubated for 1 h with an appropriate peroxidase-labeled secondary antibody (1:10000; Vector Laboratories). Bands were visualized with enhanced chemluminescence and exposed to Hyblot CL autoradiography film (Denville Scientific Inc.). Membranes were stripped (2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 6.8) for 30 min at 50-55 ºC and then received several washes with PBS-T. The stripped membranes were placed in blocking solution for 1 hr and incubated with a primary antibody direct against the total levels of the respective protein (non-phosphorylated) as a protein loading control.

The intensity of the protein bands was quantified using image analysis software (ImageJ 1.35, National Institute of Mental Health). For each blot the background signal was determined by tracing an unlabeled area adjacent to each band and subtracting this value from the target band. Resultant values were normalized to the average signal for the total (non-phosphorylated) protein levels (also background adjusted) to reduce inter- and intra-gel variability.

Statistics

Statistics were performed using GraphPad Prism 6.05 for OSX. All data are expressed as the mean ± SEM. Sample sizes were based on previous behavioral and biochemical studies conducted in our laboratory. Data for

signaling proteins with two groups were analyzed by *Student's t*-test and for four groups by two way ANOVA. For all extinction experiments repeated-measures ANOVA were used. Significant group differences were only reported if protected by significant effects or interactions with ANOVA. In all cases, if a statistically significant interaction was found, additional comparisons were calculated. Bonferroni *post hoc* analysis was performed where appropriate.

RESULTS

Ketamine Enhances Fear Extinction Learning

Sprague-Dawley rats (200 grams) were fear conditioned using 7 pairs of a neutral tone (conditioned stimulus, CS) that co-terminated with an aversive stimulus- footshock (0.6mA) (unconditioned stimulus, US)(n=16-19). Twenty-four hours after fear conditioning, the animals were administered ketamine (10 mg/kg, i.p.) or saline. This is the same ketamine dose that produces rapid antidepressant actions in rodent models(Li et al., 2010). It should be noted that the active form of ketamine is fully metabolized after 24 hours (Zarate et al., 2012). The next day the animals were subjected to extinction training in a different context (context B) from the fear conditioning for 3 consecutive days (Figure 1a). On the first day of extinction training, fear-related freezing was similar across both groups with no significant differences observed. However, during the second day of extinction training, freezing levels were significantly reduced in rats receiving ketamine compared to the saline group (repeated measures [RM] ANOVA, treatment effect, $F_{(18,70)}$=6.255, p<0.05), suggesting that ketamine had strengthened the formation of the extinction memory acquired the day before (Figure 1b). This effect persisted in freezing levels measured in the

third day of extinction with a significant treatment effect in Block 1 (RM ANOVA, treatment effect $F_{(1,70)}$=6.255, *post hoc* p<0.01). This persistent ketamine effect is consistent with the reported rapid and sustained induction of synaptic number and function in the mPFC (Li et al., 2010). To better illustrate the enhancement of extinction, we show average freezing for each day in both ketamine and saline treated conditions (Figure 1B, last panel).

Conditioned responses that have been previously extinguished can spontaneously recover over time. We next determined if ketamine's enhancement of extinction learning was stable and could reduce spontaneous recovery and renewal of conditioned fear memory. One week after the end of extinction training, rats were returned to the extinction context and exposed to a single 30-second tone. There was no significant difference in freezing between the ketamine- and saline-treated rats during spontaneous recovery (Figure 1c, left panel). Twenty-four hours later, animals were returned to the training context and exposed to a single 30-second tone. While saline-treated rats showed a clear return of fear with exposure to the original context plus cue, ketamine-treated rats showed a significant reduction in fear renewal, even at this time point of 8 days after the last day of extinction (Figure 1c, middle panel) (t=2.897, df=18, *p < 0.05, *Student's* t-test). To discriminate whether freezing was a result of contextual learning we measured the percentage of freezing during the first 3 minutes the animals were placed in the original context. We found no significant difference in the percent freezing between groups as a result of exposure only to the original context (Figure 1c, right panel), suggesting a combination of cue and

context is necessary to elicit fear. Taken together, these results show that a single dose of ketamine can enhance the recall of fear extinction as well as reduce the recovery of fear responses following extinction training.

Ketamine plus fear extinction increases mTORC1 signaling in the mPFC

Ketamine is reported to increase mTORC1 signaling in the mPFC (Li et al., 2010). Here, we examined the influence of ketamine and extinction training on mTORC1 signaling by measuring levels of the phosphorylated and activated forms of mTOR and p70S6K. Levels of mTORC1 signaling were analyzed in synaptoneurosome-enriched extracts of mPFC that were isolated immediately after day 2 of extinction training (Figure 2a). We found that rats (n=6) receiving ketamine before extinction, compared to saline plus extinction training, displayed a robust and significant two-fold increase in levels of p-p70S6K in mPFC (t=2.32, df=10, p<0.05); there was no significant difference in levels of p-mTOR (t=1.092, df=10, p=0.30) (Figure 2b).

We also examined the regulation of two upstream pathways, extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) that converge to regulate mTORC1 signaling. Ketamine plus extinction compared to saline plus extinction training significantly increased the phosphorylated and activated forms of ERK (including ERK1 and ERK2) and Akt in the mPFC. Levels of p-ERK were increased almost 2 fold (t=3.11, df=10, p<0.05), and there was a small but significant effect on p-Akt (t=4.171, df=10, p<0.01) (Figure 2b). These data

provide evidence for activation of upstream kinases in the actions of ketamine plus extinction.

Inhibition of mTORC1 Signaling in the mPFC Blocks ketamine Enhancement of Fear Extinction

The rapid synaptic and antidepressant actions of ketamine require mTORC1 signaling (Li et al., 2010). To determine if mTORC1 signaling is required for ketamine enhancement of fear extinction, we infused the selective inhibitor, rapamycin (0.2nmol) into the mPFC 30 minutes prior to ketamine. Similar to our previous studies (Li et al., 2010, 2011) we targeted the interface between the prelimbic and infralimbic subregions of the mPFC. Rats (n=6) were subjected to fear extinction training 24 and 48 hrs later as in the earlier studies (Figure 3a). First, we assessed the biochemical efficacy of rapamycin infusion by analysis of p-p70S66 kinase in dissections of mPFC to determine if ketamine induction of this phospho-protein was blocked by rapamycin. We confirmed that ketamine + DMSO infusion increased the phosphorylation of p70S6 Kinase compared to saline + DMSO groups (t=10.16, df= 4, $^{**}p<0.001$), and this effect was completely blocked by rapamycin infusion (t=3.84, df=4, $^{*}p<0.01$) (Figure 3b).

We also found that rapamycin infusion into the mPFC completely blocked ketamine enhancement of extinction recall on the second day (RM ANOVA, treatment effect, $F_{(3,100)}=37.01$, df=3, $p<0.0001$, *post hoc* ketamine vs. ketamine + rapamycin, Blocks 2, 4, and 5, $p<0.01$) (Figure 3c). We also observed a

significant decrease in the percent of freezing one day after ketamine treatment on the first day of extinction in this cohort of animals. The reason for the difference with the results in Figure 1 is not clear but could be related to surgical cannula implantation and the stress associated with this procedure, which could enhance fear conditioning and increase the sensitivity to ketamine. We performed histological analysis to confirm cannula placement. Four animals from each cohort were set a side to assess the biochemical effect of rapamycin infusion. We performed western blots for p-p70S66 kinase to confirm that activity was blocked. We confirmed that ketamine + DMSO infusion induced phosphorylation of p70S6 Kinase compared to saline + DMSO groups ($t=10.16$, $df= 4$, $^{**}p<0.001$). Rapamycin blocked the phosphorylation of p70S6 kinase in the ketamine + rapamycin groups compared to the ketamine + DMSO groups ($t=3.84$, $df=4$, $^{*}p<0.01$) .Taken together, these data demonstrate a requirement for mTORC1 signaling in the enhancement of fear extinction learning after ketamine.

Ketamine Enhancement of Extinction: Induction of cFos and influence of AMPA Receptor blockade

There is a growing consensus that the mPFC and the amygdala are critically involved in the acquisition and retrieval of fear extinction (Liberzon and Sripada, 2008; Quirk and Mueller, 2007). We measured levels of cFos, a marker of neuronal activity, to determine if ketamine plus extinction influences neural activation of mPFC and amygdala. cFos protein levels were examined in crude nuclear extracts of tissue taken 90 minutes after the final tone presentation on

the second day of extinction training (Figure 4a). Compared to saline-treated rats undergoing extinction, ketamine plus extinction increased cFos protein levels in mPFC (*Student's* t-test, t=3.66, df=10, *$p < 0.01$) (n=6) (Figure 4b). cFos levels in ketamine-treated amygdala trended toward a decrease but were not significantly different (n.s., t=2.36, df=10, $p= 0.06$) (Figure 4b). These results support earlier findings that increased mPFC activity through exposure-related training leads to reduction of fear-related amygdala processing that is associated with improved extinction learning and recall (Cho et al., 2013; Holmes et al., 2012; Milad and Quirk, 2002) (Giustino and Maren, 2015; Marek et al., 2013).

The synaptic and antidepressant actions of ketamine require glutamate a-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid receptors (AMPA) receptors (Li et al., 2010; Maeng et al., 2008). To determine whether AMPA receptors are also required for the enhancement of extinction learning by ketamine, we pretreated rats (n=10) with a selective AMPA receptor inhibitor, 2,3-dihydroxy-6-nitro-7sulfamoyl-benzol(f)quinoxaline-2,3-dione (NBQX) 10 minutes before ketamine (Figure 4c)(Li et al., 2010). This dose and method of treatment has been shown to block glutamate AMPA receptors and has been shown to attenuate the effects of ketamine in rodent models of depression and antidepressant response (Li et al., 2010; Maeng et al., 2008). Twenty-four hours later, extinction training was conducted as described above, over the next 2 days. As observed in Figure 1, during the first day of extinction training, fear-related freezing was similar across all groups. During the second day of extinction, we observed significant reduction in cue induced freezing in rats

receiving ketamine compared to the saline group. Pretreatment with NBQX produced a partial but significant blockade of the effects of ketamine (Figure 4d) (*RM ANOVA, treatment effect, $F_{(2,132)}$=5.574, df=2, p<0.05; *post hoc* ketamine treated vs. NBQX + ketamine treated, p<0.05 for blocks 2 and 3; *post hoc* ketamine + NBQX vs. saline, p>0.05 for all blocks). Taken together, these data point to the importance of neuronal activity and AMPA receptor dependent actions of the effects of ketamine on fear extinction.

CONCLUSION

The rapid-acting antidepressant effects of ketamine in rodent models and clinical trials have been well established (Berman et al., 2000; Li et al., 2010; Zarate et al., 2006), and recent studies demonstrate the efficacy of ketamine for the treatment of PTSD (Feder et al., 2014). Blockade of NMDA receptors by ketamine increases the number and function of synapses in the mPFC (Li et al., 2010), a region that also plays a critical role in the acquisition and retrieval of extinction (Knapska et al., 2012; Orsini and Maren, 2012; Quirk and Mueller, 2007). Our findings demonstrate that a single dose of ketamine enhances the recall of extinction learning. It is also possible that ketamine inhibits reconsolidation of fear memory and thereby reduces freezing during extinction training, a possibility that cannot be ruled out at the present time. On day 2 of extinction training we observe a large change in freezing levels between the ketamine and saline treated animals. This observation suggests the possibility that ketamine enhances extinction memory. Moreover, fear renewal was

decreased in ketamine treated animals 7 days later. These findings demonstrate a rapid and long-lasting enhancement of fear extinction that could be mediated by ketamine stimulation of mTORC1 signaling pathways and sustained synaptic actions in the mPFC (Hoeffer and Klann, 2010).

Mechanistic studies demonstrate that ketamine activates the mTORC1 cascade in the mPFC and that the synaptic and antidepressant behavioral responses are dependent on mTORC1 signaling (i.e blocked by rapamycin)(Li et al., 2010). We found that extinction in animals receiving ketamine increased the phosphorylated and activated form of p70S6K in the mPFC, as well as levels of the upstream kinases p-ERK and p-Akt. A role for mTORC1 was directly tested by infusions of rapamycin into the mPFC prior to ketamine administration, which completely blocked ketamine enhancement of extinction. Previous studies demonstrate that ketamine increases the number and function of spine synapses in layer V pyramidal neurons in the mPFC and that these effects are also dependent on mTORC1 signaling(Li et al., 2010). Together these findings support the hypothesis that ketamine's enhancement of extinction results from increased synaptic function in the mPFC. Increased synaptic number and function could enhance synaptic connectivity of mPFC with target regions that control fear conditioning and extinction such as the amygdala (Etkin and Wager, 2007; Mahan and Ressler, 2012).

We also found that ketamine administration increases levels of cFos in the mPFC after extinction training, indicating an increase in neuronal activation during the enhancement of extinction training. Previous studies demonstrate that

ketamine increases glutamate release in the mPFC (Moghaddam et al., 1997) and increases levels of cFos (Fuchikami et al., 2015), presumably via disinhibition of GABA interneuron activity (Duman and Aghajanian, 2012). Previous reports also indicate that the rapid antidepressant actions of ketamine require activation of glutamate AMPA receptors (Li et al., 2010; Maeng et al., 2008). In the current study we observed that pretreatment with the AMPA receptor antagonist NBQX partially blocked ketamine-induced enhancement of extinction. Together these studies indicate a role for glutamate-AMPA receptor activity in the actions of ketamine on fear extinction.

Several lines of evidence suggest that glutamatergic neurotransmission plays a critical role in the pathogenesis of anxiety and fear disorders (Bergink, 2004; Millan, 2003; Riaza Bermudo-Soriano et al., 2012). Fear extinction involves neurocircuitry of at least three main brain areas: the amygdala, mPFC, and the hippocampus (Myers et al., 2010; Quirk and Mueller, 2007). Within the mPFC, afferents from glutamatergic pyramidal neurons in the infralimbic subregion activate glutamatergic neurons in the basolateral nucleus of the amygdala (BLA) that synapse onto inhibitory GABAergic interneurons. This connection is thought to gate signaling from the BLA to the central nucleus of the amygdala. In this circuit, fear extinction overrides fear conditioning via strengthening of those synapses. This inhibitory drive is powerful enough to overcome the excitatory responses associated with a pre-existing fear conditioning circuit (Herry et al., 2008; Parsons and Ressler, 2013b). It is possible that the glutatmate burst caused by ketamine and the subsequent

increased synaptic connectivity strengthens these infralimbic mPFC synapses resulting in enhancement of fear extinction. Future studies will be required to test the role of infralimbic mPFC in the enhancement of fear extinction by ketamine, and to differentiate effects on prelimbic mPFC, which has been implicated in the formation of fear conditioning (Quirk and Mueller, 2007).

There are currently only two FDA approved drug treatments for PTSD, the SSRI's paroxetine and sertraline, highlighting the need for additional pharmacotherapies for this disorder. As with most classical antidepressants, the SSRIs require several weeks of treatment to produce a therapeutic response, and even then have limited efficacy for the treatment of PTSD symptoms. Several lines of clinical evidence support the therapeutic potential of glutamatergic agents. Soldiers with severe burns that had received perioperative ketamine during hospitalization were found to have lower incidence of developing PTSD (McGhee et al., 2008). Moreover, a recent clinical trial demonstrated that ketamine infusion was associated with significant and rapid reduction in PTSD symptoms when compared to midazolam (Feder et al., 2014). Although d-cycloserine (DCS), a partial NMDA agonist, was ineffective for reducing fear relapse (Woods and Bouton, 2006), it was found to augment fear extinction learning in rodents and humans (Riaza Bermudo-Soriano et al., 2012; Scheeringa and Weems, 2014). DCS had mixed efficacy for reducing PTSD symptoms in a randomized placebo-controlled study (de Kleine et al., 2012; Rothbaum et al., 2014). These findings indicate that glutamate NMDA receptor blocking agents have potential for treating PTSD and related disorders, and the

current study provides evidence that this may occur in part via activity-dependent mTORC1 signaling in the mPFC. Development of novel ketamine-like agents with fewer side effects is a major focus of antidepressant drug development efforts and could also provide new, safer and more effective treatments for PTSD and other fear-related disorders.

Funding and Disclosure

This work is supported by NIMH R01MH93897 (RSD), the State of Connecticut, and Yale University. The authors list the following interests: R.S. Duman has consulted and/or received research support from Naurex, Lilly, Forest, Johnson & Johnson, Taisho, and Sunovion. The remaining authors, M.J. Girgenti, S. Ghosal, D. LoPresto, and J.R. Taylor have no competing financial interests.

REFERENCES

Autry, A.E., Adachi, M., Nosyreva, E., Na, E.S., Los, M.F., Cheng, P.-F., Kavalali, E.T., Monteggia, L.M., 2011. NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. Nature 475, 91–95. doi:10.1038/nature10130

Bergink, V., 2004. Glutamate and anxiety. European Neuropsychopharmacology 14, 175–183. doi:10.1016/S0924-977X(03)00100-7

Berman, R.M., Cappiello, A., Anand, A., Oren, D.A., Heninger, G.R., Charney, D.S., Krystal, J.H., 2000. Antidepressant effects of ketamine in depressed patients. Biol. Psychiatry 47, 351–354.

Cho, J.-H., Deisseroth, K., Bolshakov, V.Y., 2013. Synaptic encoding of fear extinction in mPFC-amygdala circuits. Neuron 80, 1491–1507. doi:10.1016/j.neuron.2013.09.025

de Kleine, R.A., Hendriks, G.-J., Kusters, W.J.C., Broekman, T.G., van Minnen, A., 2012. A randomized placebo-controlled trial of D-cycloserine to enhance exposure therapy for posttraumatic stress disorder. Biol. Psychiatry 71, 962–968. doi:10.1016/j.biopsych.2012.02.033

Duman, R.S., Aghajanian, G.K., 2012. Synaptic dysfunction in depression:

potential therapeutic targets. Science 338, 68–72. doi:10.1126/science.1222939

Etkin, A., Wager, T.D., 2007. Functional neuroimaging of anxiety: a meta-analysis of emotional processing in PTSD, social anxiety disorder, and specific phobia. Am J Psychiatry 164, 1476–1488. doi:10.1176/appi.ajp.2007.07030504

Feder, A., Parides, M.K., Murrough, J.W., Perez, A.M., Morgan, J.E., Saxena, S., Kirkwood, K., aan het Rot, M., Lapidus, K.A.B., Wan, L.-B., Iosifescu, D., Charney, D.S., 2014. Efficacy of Intravenous Ketamine for Treatment of Chronic Posttraumatic Stress Disorder 71, 681. doi:10.1001/jamapsychiatry.2014.62

Giustino, T.F., Maren, S., 2015. The Role of the Medial Prefrontal Cortex in the Conditioning and Extinction of Fear. Front Behav Neurosci 9, 298. doi:10.3389/fnbeh.2015.00298

Herry, C., Ciocchi, S., Senn, V., Demmou, L., Müller, C., Lüthi, A., 2008. Switching on and off fear by distinct neuronal circuits. Nature 454, 600–606. doi:10.1038/nature07166

Hoeffer, C.A., Klann, E., 2010. mTOR signaling: at the crossroads of plasticity, memory and disease. Trends in Neurosciences 33, 67–75. doi:10.1016/j.tins.2009.11.003

Holmes, A., Fitzgerald, P.J., MacPherson, K.P., DeBrouse, L., Colacicco, G., Flynn, S.M., Masneuf, S., Pleil, K.E., Li, C., Marcinkiewcz, C.A., Kash, T.L., Gunduz-Cinar, O., Camp, M., 2012. Chronic alcohol remodels prefrontal neurons and disrupts NMDAR-mediated fear extinction encoding. Nature Publishing Group 15, 1359–1361. doi:10.1038/nn.3204

Holmes, A., Singewald, N., 2013. Individual differences in recovery from traumatic fear. Trends in Neurosciences 36, 23–31. doi:10.1016/j.tins.2012.11.003

Horn, S.R., Charney, D.S., Feder, A., 2016. Understanding resilience: New approaches for preventing and treating PTSD. Exp. Neurol. doi:10.1016/j.expneurol.2016.07.002

Ito, W., Erisir, A., Morozov, A., 2015. Observation of Distressed Conspecific as a Model of Emotional Trauma Generates Silent Synapses in the Prefrontal-Amygdala Pathway and Enhances Fear Learning, but Ketamine Abolishes those Effects 1–10. doi:10.1038/npp.2015.100

Johansen, J.P., Cain, C.K., Ostroff, L.E., LeDoux, J.E., 2011. Molecular Mechanismsof Fear Learning and Memory. Cell 147, 509–524. doi:10.1016/j.cell.2011.10.009

Johnson, L.R., McGuire, J., Lazarus, R., Palmer, A.A., 2012. Pavlovian fear memory circuits and phenotype models of PTSD. Neuropharmacology 62, 638–646. doi:10.1016/j.neuropharm.2011.07.004

Karpova, N.N., Pickenhagen, A., Lindholm, J., Tiraboschi, E., Kulesskaya, N., Agustsdottir, A., Antila, H., Popova, D., Akamine, Y., Sullivan, R., Hen, R., Drew, L.J., Castren, E., 2011. Fear Erasure in Mice Requires Synergy Between Antidepressant Drugs and Extinction Training. Science 334, 1731–1734. doi:10.1126/science.1214592

Kessler, R.C., Sonnega, A., Hughes, M., Nelson, C.B., 2008. Posttraumatic Stress Disorder in the National Comorbidity Survey 1–13.

Knapska, E., Macias, M., Mikosz, M., Nowak, A., Owczarek, D., Wawrzyniak, M., Pieprzyk, M., Cymerman, I.A., Werka, T., Sheng, M., Maren, S., Jaworski, J., Kaczmarek, L., 2012. Functional anatomy of neural circuits regulating fear and extinction. Proc. Natl. Acad. Sci. U.S.A. 109, 17093–17098. doi:10.1073/pnas.1202087109

Laurent, V., Westbrook, R.F., 2009. Inactivation of the infralimbic but not the prelimbic cortex impairs consolidation and retrieval of fear extinction. Learn. Mem. 16, 520–529. doi:10.1101/lm.1474609

Li, N., Lee, B., Liu, R.J., Banasr, M., Dwyer, J.M., Iwata, M., Li, X.Y., Aghajanian, G., Duman, R.S., 2010. mTOR-Dependent Synapse Formation Underlies the Rapid Antidepressant Effects of NMDA Antagonists. Science 329, 959–964. doi:10.1126/science.1190287

Li, N., Liu, R.-J., Dwyer, J.M., Banasr, M., Lee, B., Son, H., Li, X.-Y., Aghajanian, G., Duman, R.S., 2011. Glutamate N-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. Biol. Psychiatry 69, 754–761. doi:10.1016/j.biopsych.2010.12.015

Liberzon, I., Sripada, C.S., 2008. The functional neuroanatomy of PTSD: a critical review. Prog. Brain Res. 167, 151–169. doi:10.1016/S0079-6123(07)67011-3

Lommen, M.J.J., Engelhard, I.M., Sijbrandij, M., van den Hout, M.A., Hermans, D., 2013. Pre-trauma individual differences in extinction learning predict posttraumatic stress. Behav Res Ther 51, 63–67. doi:10.1016/j.brat.2012.11.004

Maeng, S., Zarate, C.A., Du, J., Schloesser, R.J., McCammon, J., Chen, G., Manji, H.K., 2008. Cellular mechanisms underlying the antidepressant effects of ketamine: role of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors. Biol. Psychiatry 63, 349–352. doi:10.1016/j.biopsych.2007.05.028

Mahan, A.L., Ressler, K.J., 2012. Fear conditioning, synaptic plasticity and the amygdala: implications for posttraumatic stress disorder. Trends in Neurosciences 35, 24–35. doi:10.1016/j.tins.2011.06.007

Marek, R., Strobel, C., Bredy, T.W., Sah, P., 2013. The amygdala and medial prefrontal cortex: partners in the fear circuit. J. Physiol. (Lond.) 591, 2381–2391. doi:10.1113/jphysiol.2012.248575

McGhee, L.L., Maani, C.V., Garza, T.H., Gaylord, K.M., Black, I.H., 2008. The correlation between ketamine and posttraumatic stress disorder in burned service members. J Trauma 64, S195–8– Discussion S197–8. doi:10.1097/TA.0b013e318160ba1d

Milad, M.R., Quirk, G.J., 2002. Neurons in medial prefrontal cortex signal memory for fear extinction. Nature 420, 70–74. doi:10.1038/nature01138

Milad, M.R., Wright, C.I., Orr, S.P., Pitman, R.K., Quirk, G.J., Rauch, S.L., 2007. Recall of fear extinction in humans activates the ventromedial prefrontal cortex and hippocampus in concert. Biol. Psychiatry 62, 446–454.

doi:10.1016/j.biopsych.2006.10.011
Millan, M.J., 2003. The neurobiology and control of anxious states. Prog. Neurobiol. 70, 83–244.
Myers, K.M., Carlezon, W.A., Davis, M., 2010. Glutamate Receptors in Extinction and Extinction-Based Therapies for Psychiatric Illness. Neuropsychopharmacology 36, 274–293. doi:10.1038/npp.2010.88
Orsini, C.A., Maren, S., 2012. Neural and cellular mechanisms of fear and extinction memory formation. Neuroscience & Biobehavioral Reviews 36, 1773–1802. doi:10.1016/j.neubiorev.2011.12.014
Parsons, R.G., Ressler, K.J., 2013a. Implications of memory modulation for post-traumatic stress and fear disorders. Nature Publishing Group 16, 146–153. doi:10.1038/nn.3296
Parsons, R.G., Ressler, K.J., 2013b. Implications of memory modulation for post-traumatic stress and fear disorders. Nature Publishing Group 16, 146–153. doi:10.1038/nn.3296
Pitman, R.K., Rasmusson, A.M., Koenen, K.C., Shin, L.M., Orr, S.P., Gilbertson, M.W., Milad, M.R., Liberzon, I., 2012. Biological studies of post-traumatic stress disorder 1–19. doi:10.1038/nrn3339
Quirk, G.J., Mueller, D., 2007. Neural Mechanisms of Extinction Learning and Retrieval. Neuropsychopharmacology 33, 56–72. doi:10.1038/sj.npp.1301555
Rasmussen, K.G., 2016. Has psychiatry tamed the “ketamine tiger?” Considerations on its use for depression and anxiety. Prog. Neuropsychopharmacol. Biol. Psychiatry 64, 218–224. doi:10.1016/j.pnpbp.2015.01.002
Riaza Bermudo-Soriano, C., Perez-Rodriguez, M.M., Vaquero-Lorenzo, C., Baca-Garcia, E., 2012. New perspectives in glutamate and anxiety. Pharmacol. Biochem. Behav. 100, 752–774. doi:10.1016/j.pbb.2011.04.010
Rothbaum, B.O., Price, M., Jovanovic, T., Norrholm, S.D., Gerardi, M., Dunlop, B., Davis, M., Bradley, B., Duncan, E.J., Rizzo, A., Ressler, K.J., 2014. A randomized, double-blind evaluation of D-cycloserine or alprazolam combined with virtual reality exposure therapy for posttraumatic stress disorder in Iraq and Afghanistan War veterans. Am J Psychiatry 171, 640–648. doi:10.1176/appi.ajp.2014.13121625
Scheeringa, M.S., Weems, C.F., 2014. Randomized placebo-controlled D-cycloserine with cognitive behavior therapy for pediatric posttraumatic stress. J Child Adolesc Psychopharmacol 24, 69–77. doi:10.1089/cap.2013.0106
Sierra-Mercado, D., Padilla-Coreano, N., Quirk, G.J., 2011. Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. Neuropsychopharmacology 36, 529–538. doi:10.1038/npp.2010.184
Ursano, R.J., Bell, C., Eth, S., Friedman, M., Norwood, A., Pfefferbaum, B., Pynoos, J.D.R.S., Zatzick, D.F., Benedek, D.M., McIntyre, J.S., Charles, S.C., Altshuler, K., Cook, I., Cross, C.D., Mellman, L., Moench, L.A., Norquist, G., Twemlow, S.W., Woods, S., Yager, J., Work Group on ASD and PTSD, Steering Committee on Practice Guidelines, 2004. Practice guideline for the treatment of patients with acute stress disorder and posttraumatic

stress disorder. Am J Psychiatry.
Woods, A.M., Bouton, M.E., 2006. D-cycloserine facilitates extinction but does not eliminate renewal of the conditioned emotional response. Behav. Neurosci. 120, 1159–1162. doi:10.1037/0735-7044.120.5.1159
Zarate, C.A., Brutsche, N., Laje, G., Luckenbaugh, D.A., Venkata, S.L.V., Ramamoorthy, A., Moaddel, R., Wainer, I.W., 2012. Relationship of ketamine's plasma metabolites with response, diagnosis, and side effects in major depression. Biol. Psychiatry 72, 331–338. doi:10.1016/j.biopsych.2012.03.004
Zarate, C.A., Singh, J.B., Carlson, P.J., Brutsche, N.E., Ameli, R., Luckenbaugh, D.A., Charney, D.S., Manji, H.K., 2006. A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. Arch. Gen. Psychiatry 63, 856–864. doi:10.1001/archpsyc.63.8.856
Zhang, W., Davidson, J.R., 2007. Post-traumatic stress disorder: an evaluation of existing pharmacotherapies and new strategies. Expert Opin Pharmacother 8, 1861–1870. doi:10.1517/14656566.8.12.1861

Figure Legends

Figure 1: Ketamine accelerates extinction learning and reduces recovery of fear memory with time. **(a)** Schematic of the fear conditioning protocol used to examine the effects of ketamine administration on fear extinction learning. Rats (n=15 per group) underwent fear conditioning (7 CS/7US pairings) in context A and the next day (24 hr later) received either saline or ketamine (10 mg/kg, i.p.) 24 hr before extinction. Extinction training involved 12 presentations of the tone alone in a different context (context B) over the course of 3 days. **(b)** Ketamine-treated rats showed significantly reduced freezing on the second and third blocks of the second session of training. By the third extinction session, there were no significant differences in freezing levels between the groups. *P<.05 (saline vs. ketamine). Average freezing percentages for blocks 1-4 are plotted for each day and condition. **(c)** One week later spontaneous recovery (first panel) and fear renewal (second panel) were assessed by returning rats to the extinction context (context B) or the original context (context A), respectively. Context B (first panel) elicited no difference in the response to the cue (tone). However, when the animals were returned to the original context (A) (second panel) and presented the cue, the ketamine-treated animals exhibited reduced fear induced freezing compared to the saline-treated animals. The animals exhibited no difference in freezing behavior during the first 3 minutes of fear renewal (third panel), suggesting that it is not simply a response to being put back into the original context. Freezing data is shown in blocks of 2 trials. Results are the mean ± S.E.M., n=16-19, RM ANOVA, treatment effect, $F_{(18,198)}$=5.087, P<0.05.

Figure 2: Influence of ketamine and fear extinction on mTORC1 signaling. **(a)** Rats underwent fear conditioning (7CS/ 7US pairings) in context A and the next day received ketamine or saline 24 hrs before extinction. Fear extinction training and recall was carried out over the next two days. Rats (n=6 per group) were sacrificed 90 mins after testing for extinction. **(b)** Levels of the phosphorylated and activated forms of mTOR, p70S6K, ERK, and Akt and total levels of each were analyzed by western blot in synaptoneurosome fractions of the mPFC. Representative Western immunoblot bands for each phosphoprotein and total protein are shown on the right. Results are the mean ± S.E.M., n = 6 per group. *P < 0.05 (Student's t-test).

Figure 3: Inhibition of mTORC1 signaling in the mPFC blocks ketamine enhancement of fear extinction. **(a)** Schematic of fear conditioning protocol to examine the effect of mTORC1 inhibition on ketamine's enhancement of extinction learning. Cannulas were placed into mPFC and one week after surgery, and rats were subjected to fear conditioning, rapamycin infusion ± ketamine, and extinction training exactly as conducted in previous studies. Rapamycin was infused (0.2 nmol) into the mPFC (n=8-10 per group) 30 minutes before ketamine treatment. **(b**) Levels of p-pS6Kinase were reduced in mPFC after rapamycin infusion. Results are ±S.E.M., n=4. *Students* t-test (t=3.8, df=4, *p<0.01**)** Levels of p-pS6Kinase were increased in mPFC after treatment with ketamine and sham infusion of DMSO (n=4. *Students* t-test (t=10.16, df=4, p<0.001)) **(c)** In this experiment ketamine administration produced a significant

enhancement of extinction on both training days, possibly due to stress associated with cannula surgeries, and rapamycin infusion completely blocked the effect of ketamine on extinction. Freezing data is shown in blocks of 2 trials. Results are the mean ± S.E.M., n = 6. RM ANOVA, treatment effect $F_{(5,100)}$=2.659, ketamine + DMSO vs. rapamycin + ketamine, rapamycin + saline, and DMSO + saline.

Figure 4: Influence of ketamine and fear extinction on and cFos expression; inhibition of enhanced extinction by AMPA receptor blockade. **(a)** Rats underwent fear conditioning (7CS/ 7US pairings) in context A and the next day received ketamine or saline 24 hrs before extinction. Fear extinction training and recall was carried out over the next two days. Rats (n=6 per group) were sacrificed 90 mins after testing for extinction. **(b)** Levels of cFos protein in the crude nuclear fractions of mPFC and amygdala were also determined; levels of GAPDH were determined to control for loading. Results are the mean ± S.E.M., n = 6 per group. *P < 0.05 (Student's t-test). **(c)** Schematic of fear conditioning protocol to examine the effect of glutamate AMPA receptor blockade on ketamine-induced enhancement of extinction. Rats (n=10) underwent fear conditioning, NBQX ± ketamine administration, and extinction training as described for the first experiment. **(d)** Pretreatment (30 min) with NBQX (10 mg/kg, i.p.) partially blocked ketamine (10 mg/kg, i.p.) enhancement of extinction on day 2 during the second block. Freezing data is shown in blocks of 2 trials. Results are the mean ± S.E.M., n = 10. RM ANOVA, treatment effect, $F_{(9,132)}$=5.574, ketamine treated vs. NBQX + ketamine treated, n.s. RM ANOVA, treatment effect, $F_{(8,40)}$=0.34, ketamine + NBQX vs. saline.

Figure 1

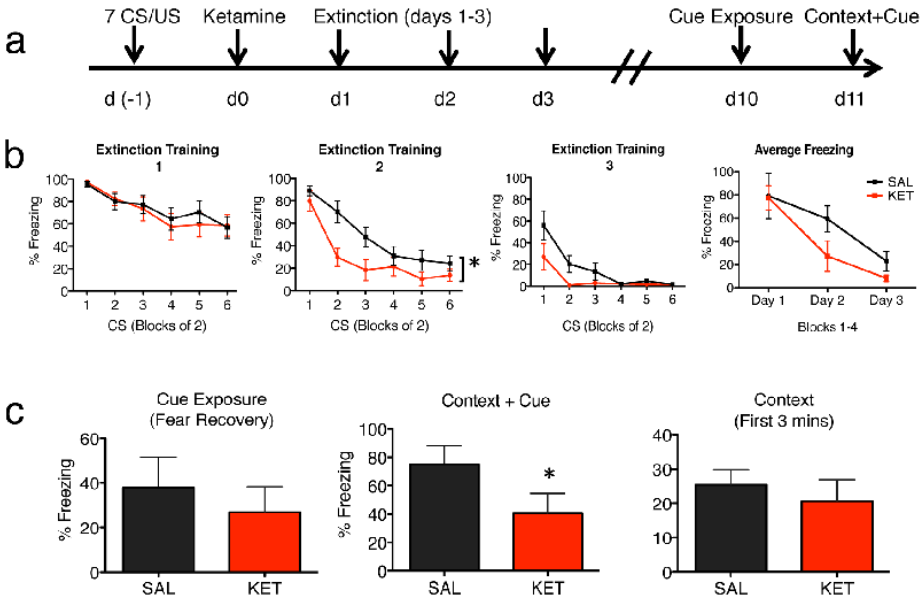


Figure 2

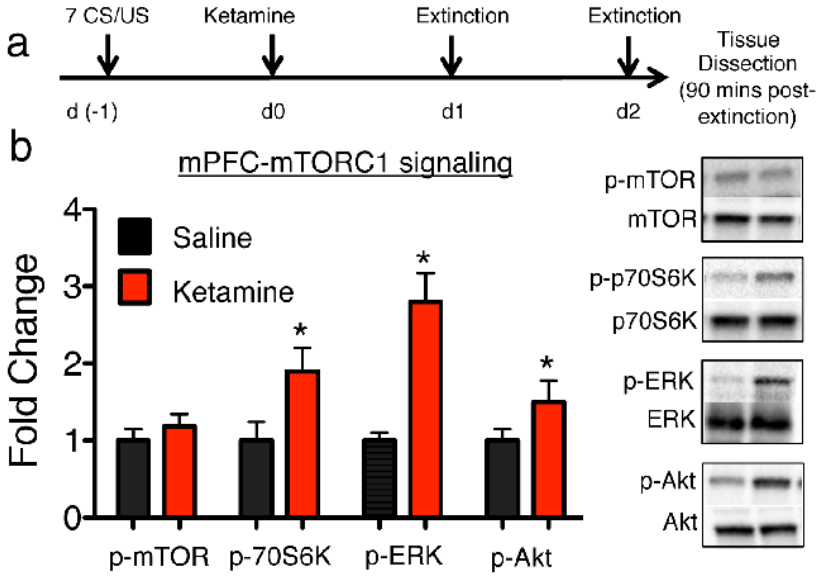


Figure 3

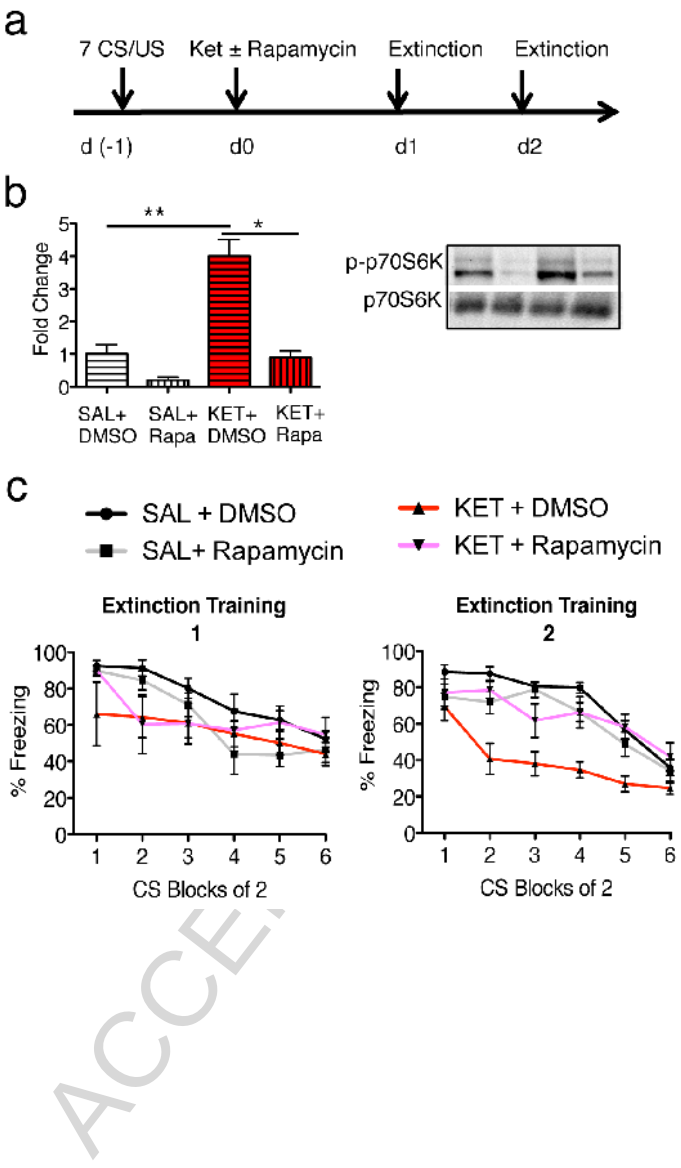
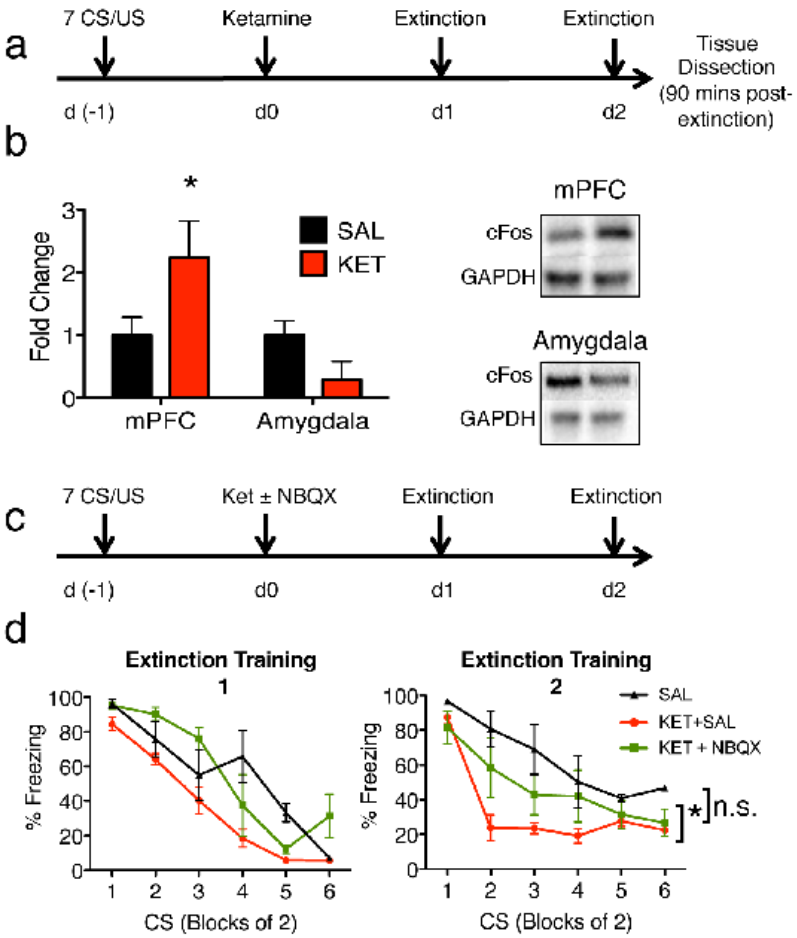


Figure 4



Highlights

1. New treatments for PTSD are desperately needed. Glutamatergic signaling is thought to play an important role in the formation of traumatic memories and in the onset of PTSD.
2. Ketamine accelerates extinction learning in a model of fear conditioning.
3. mTORC signaling and AMPA receptor signaling are required for ketamine's effect on extinction learning.
4. Inhibition of mTORC signaling blocks ketamine's effect on extinction learning.