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Effects of systemic glutamatergic manipulations on conditioned eyeblink responses and hyperarousal in a rabbit model of post-traumatic stress disorder

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Abstract

Glutamatergic dysfunction is implicated in many neuropsychiatric conditions including post-traumatic stress disorder (PTSD). Glutamate antagonists have shown some utility in treating PTSD symptoms while glutamate agonists may facilitate cognitive behavioral therapy outcomes. We have developed an animal model of PTSD, based on conditioning of the rabbit's eyeblink response, that addresses two key features: conditioned responses (CRs) to cues associated with an aversive event and a form of conditioned hyperarousal referred to as conditioning-specific reflex modification (CRM). The optimal treatment to reduce both CRs and CRM is unpaired extinction. The goals of the study were to examine whether treatment with the NMDA glutamate receptor antagonist ketamine could reduce CRs and CRM, and if the NMDA agonist d-cycloserine combined with unpaired extinction treatment could enhance extinction of both. Administration of a single-dose of subanesthetic ketamine had no significant immediate or delayed effect on CRs or CRM. Combining d-cycloserine with a single day of unpaired extinction facilitated extinction of CRs short-term while having no impact on CRM. These results caution that treatments may improve one aspect PTSD-symptomology while having no significant effects on other symptoms, stressing the importance of a multiple-treatment approach to PTSD and animal models that address multiple symptoms.

Keywords

classical conditioning; eyeblink conditioning; hyperarousal; post-traumatic stress disorder; fear conditioning; reflex modification; rabbit

Introduction

Glutamatergic dysfunction has been implicated as a factor in a wide range of neuropsychiatric conditions, including depression, schizophrenia, epilepsy, and Alzheimer's disease as well as anxiety/stress-based disorders like post-traumatic stress disorder (PTSD) (Nishi et al., 2015; Riaza Bermudo-Soriano et al., 2012; Zarate et al., 2010). The role of

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glutamatergic dysfunction in PTSD has been suggested by clinical research showing that drugs targeting glutamatergic neurotransmission can treat some aspects of PTSD symptomology or improve the efficacy of cognitive behavioral therapy. More specifically, glutamate antagonists have shown utility for treating active PTSD symptoms while glutamate agonists may serve as cognitive enhancers to improve cognitive behavioral therapy outcomes.

PTSD is a complex anxiety disorder in which previous exposure(s) to a traumatic event results in a multitude of symptoms including distress to cues that symbolize the trauma, alterations in arousal such as hypervigilance, and recurrent memories/flashbacks (American Psychiatric Association, 2013). A link between PTSD symptoms and glutamate dysfunction has been demonstrated by research showing that serum glutamate levels are positively associated with PTSD symptom severity (Nishi et al., 2015) and that glutamate levels are increased in the temporal cortex in PTSD patients, revealed by high-field brain proton magnetic resonance spectroscopy (Meyerhoff et al., 2014). Antagonism of glutamate receptors has been shown to reduce certain aspects of PTSD symptoms; for instance, a single sub-anesthetic dose of the NMDA antagonist ketamine reduced intrusive thoughts, avoidance symptoms, and hyperarousal (Feder et al., 2014). Because there is a significant learning and memory component to PTSD, owing to the development of conditioned emotional responses to cues or situations associated with past trauma (Lissek and van Meurs, 2014), many cognitive behavioral therapies for PTSD target the extinction of those responses. Such therapies can be facilitated by cognitive enhancers that target the glutamatergic system; for example, d-cycloserine, an NMDA agonist, has been well characterized as a drug that can enhance extinction learning both in animal models of anxiety as well as in a clinical setting (for review, see Davis et al., 2006).

We have developed an animal model of PTSD that addresses two of its key features: 1) the development of conditioned responses (CRs) to cues that are associated with an aversive event and 2) the development of hyperarousal (Burhans et al., 2008). This model is based on conditioning of the rabbit's nictitating membrane response (NMR), also known as eyeblink conditioning. As rabbits develop an eyeblink CR to a tone associated with a periorbital shock unconditioned stimulus (US), the reflexive eyeblink response to the shock, when tested by itself, changes and manifests as an exaggerated and more complex response, particularly to low intensity shocks that elicited little to no responding prior to conditioning. This change in the eyeblink reflex is termed conditioning-specific reflex modification or CRM. CRM is deemed "conditioning-specific" because rabbits that are exposed to tones and shock that are explicitly unpaired do not develop the same changes in reflexive responding. CRM, therefore, can be thought of as a form of conditioned hyperarousal. Some of the specific similarities between our model and PTSD are: 1) not all rabbits develop strong CRM just as not all humans exposed to trauma develop PTSD (Smith-Bell et al., 2012); 2) CRM can develop following an incubation period, just as onset of PTSD symptoms may be delayed after trauma exposure (Schreurs et al., 2011a); and 3) CRM is modulated by the amygdala, and amygdalar dysfunction is strongly implicated in PTSD (Burhans and Schreurs, 2008).

The CRM model of PTSD has shown potential for testing and furthering the understanding of both behavioral and pharmacological treatments of PTSD. This model highlights the challenge of PTSD treatment, which is finding a treatment that can address a multitude of symptoms simultaneously. We have found, for example, that presentations of the CS alone or US alone can reduce CRs to tone and CRM to shock, respectively, while sessions containing unpaired presentations of both the CS and US can reduce both at the same time (Schreurs et al., 2000). Importantly, results showed that the US intensity presented during this unpaired extinction treatment can be reduced eight-fold from that experienced during conditioning and still reduce CRM (Schreurs et al., 2011b). This result makes translation to behavioral therapy more feasible as it suggests that the presentation of a mild, innately stressful stimulus may help address hyperarousal symptoms during PTSD therapy. In terms of pharmacological treatments for PTSD, the CRM model has been shown to be sensitive to fluoxetine (Burhans et al., 2013) which is a member of the class of serotonin reuptake inhibitors (SSRIs) that are often recommended as the first-line pharmaceutical treatment of PTSD (Davidson, 2006; Ipser and Stein, 2012).

Because the symptomology of PTSD is complex and the exact role of glutamatergic dysfunction in PTSD in humans is not well-understood, glutamatergic manipulations in animal models of PTSD may provide important additional insight into the role of glutamate in PTSD. The goal of the following two experiments was to examine whether the CRM model is sensitive to effects of glutamatergic manipulation. Experiment 1 tested a scenario relevant to the Feder et al. (2014) PTSD clinical trial with the NMDA antagonist ketamine, which demonstrated a significant reduction in PTSD symptom severity for up to two weeks following a single, subanesthetic dose of ketamine. The hypothesis tested was whether a single, subanesthetic dose of ketamine could reduce already established PTSD-like symptoms in our CRM model of PTSD in rabbits. In Experiment 2, we examined whether unpaired extinction treatment could enhance extinction of PTSD-like symptoms if combined with the partial NMDA agonist, d-cycloserine. Previous work in our CRM model has shown that simultaneous extinction of CRs to tone and CRM to shock can be achieved with three to six days of unpaired extinction treatment but may be worsened if only one day of extinction occurs (Schreurs et al., 2011b). In Experiment 2, we chose to examine whether d-cycloserine could improve the efficacy of a single day of unpaired extinction with weak shock, in order to avoid possible floor effects that may occur if tested with three or six days of extinction.

Methods

Subjects

A total of 69 subjects were used in two experiments (n=28 and n=41 for Experiments 1 and 2, respectively). The subjects were male, New Zealand White rabbits (*Oryctolagus cuniculus*), 2-3 months of age weighing approximately 1.9-2.3 kg upon delivery from the supplier (Charles River, Saint-Constant, Canada). The rabbits were housed in individual cages on a 12 hour light-dark cycle and given free access to food and water. They were maintained in accordance with the guide for the care and use of laboratory animals issued by the National Institutes of Health, and the research was approved by the West Virginia

University Animal Care and Use Committee. One rabbit was removed at the start of Experiment 1 due to a failure to adapt to restraint.

Apparatus

The apparatus and recording procedures for conditioning of the NMR have been detailed by Schreurs and Alkon (1990) who modeled their apparatus based on those described by Gormezano (Coleman and Gormezano, 1971; Gormezano, 1966). Rabbits were restrained in a Plexiglas box placed inside a sound-attenuating, ventilated chamber (Coulbourn Instruments, Allentown, PA; Model E10-20). Inside the chamber, a stimulus panel containing a speaker and houselight (10-W, 120 V) was mounted at a 45° angle 15 cm anterior and dorsal to the rabbit's head. An exhaust fan created a constant ambient noise level of 75 dB inside the chamber. Periorbital electrical stimulation was delivered by a programmable two-pole stimulator (Colbourn Instruments, Model E13-35) via stainless steel Autoclip wound clips (Stoelting, Wood Dale, IL) that were positioned 10 mm ventral and 10 mm posterior to the dorsal canthus of the right eye. Stimulus delivery, data collection, and analysis were all accomplished using the LabVIEW software system (National Instruments, Austin, TX).

The NMRs were transduced by a potentiometer (Novotechnik US Inc., Southborough, MA; Model P2201) connected at one end, via a freely moving ball and socket joint, to an L-shaped lever containing a hook that attached to a 6-0 nylon loop sutured into but not through the nictitating membrane (NM). At the other end, the potentiometer was connected to a 12-bit analog-to-digital converter (5-ms sampling rate, 0.05-mm resolution), and individual A/D outputs were stored on a trial-by-trial basis for subsequent analysis.

Procedure

One week following arrival, rabbits were acclimated to restraint by placing them in restrainers for 30 minutes while under close supervision. On subsequent days, rabbits in both Experiments received the same initial training sequence of one session per day in the following order: adaptation, US pretest, six days of classical delay NMR conditioning, and US post test (Post1). For adaptation, subjects were prepared for delivery of the periorbital shock US and NMR recording and then adapted to the training chambers, without CS or US presentations, for an amount of time equivalent to subsequent training sessions (80 min). For pretest and post tests, subjects received 80 trials of US presentations with an average inter-trial interval (ITI) of 60 s (range 50-70 s). Each US presentation was one of 20 combinations of periorbital shock intensity (0.1, 0.3, 0.5, 1.0, or 2.0 mA) and duration (10, 25, 50, or 100 ms), and these 20 unique USs were presented in four separately randomized blocks, with the restriction that the same intensity or duration could not occur more than three times in succession. For delay conditioning, each session consisted of 80 trials of paired presentations of a 400 ms, 1 kHz, 82 dB CS that co-terminated with a 100 ms, 2 mA US (300 ms interstimulus interval). The paired CS-US trials were presented with an average ITI of 60 s.

For Experiment 1, investigating effects of ketamine on established CRs and CRM, one day following Post1, rabbits were given a ketamine or saline injection without a training session.

One day after the drug injection, rabbits received a second US post test (Post2) followed the next day by a CS-alone test (CS Test1). A third US post test (Post3) and second CS-alone test (CS Test2) occurred on consecutive days one week later. The CS test, used to measure retention of the CR to the tone CS, consisted of 80 presentations of the tone CS alone with an average ITI of 60 s. The first and second CS Tests measured immediate and longer term effects, respectively, of ketamine on the CR.

For Experiment 2, investigating the effects of d-cycloserine combined with one day of unpaired extinction, on the day following Post1, rabbits were given a d-cycloserine or saline injection followed by a single session of unpaired extinction with a weak shock. One day after the combined treatment, rabbits received a second US post test (Post2), followed by a third US post test (Post3) one week later. One day after Post3, rabbits received a CS Test. For both experiments, Post2 measured the more immediate effects of the drug on CRM while Post3 measured the longer term effects. Immediate effects of d-cycloserine on the CR were measured on CS-alone trials during extinction, and longer term effects were measured during the CS Test the following week. The unpaired extinction session consisted of 80 presentations of the tone CS and 80 presentations of a reduced intensity 0.3-mA periorbital US (100 ms) that were explicitly unpaired and presented in a pseudorandom order. The average ITI for the unpaired session was reduced to 30 s in order to match the session length of the other training sessions (approximately 80 minutes).

Conditioned responses (CRs) of the NMR were defined as any extension of the NM exceeding 0.5 mm that was initiated following CS onset but prior to US onset. For US testing, a UR was defined as any extension of the NM exceeding 0.5 mm that was initiated within 300 ms following US onset. The definition of the UR was based on prior observations that responses to the US following CS-US pairings had onset latencies within the same range as CRs (Schreurs et al., 2000). The amplitude of the response was calculated as the maximum extension of the NM in millimeters. Onset latency of the response was the latency in ms from stimulus onset to when the NM rose 0.1 mm above baseline while peak latency was the latency in ms from stimulus onset until maximum NM extension occurred. Area of the response was calculated as the total area of the response curve (arbitrary units, au) from stimulus onset until the end of the trial (trial length = 2000 ms). For URs during US testing, two additional measures were calculated in order to overcome the statistical limitations of empty data cells produced by subthreshold responses to periorbital shock, particularly at the lower intensities and durations. These measures, magnitude of the response amplitude and magnitude of the response area, included the amplitudes and areas of all NMRs above baseline regardless of whether the 0.5 mm criterion was met (Garcia et al., 2003). A significant pre- to post test increase in any of the UR response measures as a function of classical conditioning is a defining feature of CRM. To increase the sensitivity for detection of CRM and to follow the convention of previous CRM studies, data were collapsed across duration at the five US intensities, and CRM analyses were focused on the first 20-trial US sequence, where the strongest CRM is observed (Schreurs et al., 2000). To examine the shape and timing of NMRs during US tests, response topographies were generated at each US intensity by averaging across rabbits and across US durations within each experimental group.

Drug Injections

For Experiment 1, Ketamine HCl was obtained from Henry Schein as an injectable solution (100 mg/ml). The purchased 100 mg/ml solution and additional 50 mg/ml and 25 mg/ml solutions, prepared by diluting with 0.9 % sterile saline, were utilized for injection doses of 20, 10, and 5 mg/kg, respectively, in order to equate injection volume ($\bar{X} = 0.5$ ml). The day following Post1, rabbits were transported to the room in which behavioral training took place, and ketamine (5, 10, or 20 mg/kg) or vehicle (0.9% saline) was injected intramuscularly. Rabbits were returned to transport containers and monitored for 30 minutes prior to returning to home cages. Sedative effects on posture were observed for all ketamine doses and were typically characterized as drooping heads and/or leaning to one side; however, these gross effects dissipated within 30 minutes or less. Ketamine was administered 24 h prior to CRM testing (Post2) because initial pilot testing at the 10 mg/kg and 20 mg/kg doses revealed sensorimotor side effects on the NMR that lasted beyond 30 minutes. An additional concern was that ketamine has been previously shown to dose-dependently increase intraocular pressure in rabbits at both anesthetic and subanesthetic doses, with effects lasting several hours (Bar-Ilan and Pessah, 1986).

For Experiment 2, solutions of 12.5, 25, and 50 mg/ml of d-cycloserine (pure USP; AppliChem, St. Louis, MO) dissolved in 0.9% sterile saline were prepared for injection doses of 3, 6, and 12 mg/kg, respectively, in order to equate injection volume ($\bar{X} = 0.56$ ml). This range of doses was selected as it has been previously demonstrated that 6 mg/kg of d-cycloserine is an effective dose for facilitating eyeblink conditioning in rabbits (Thompson and Disterhoft, 1997). The day following Post1, rabbits were transported to the room in which behavioral training took place, and d-cycloserine (3, 6, or 12 mg/kg) or vehicle (0.9% saline) was injected intramuscularly. Rabbits were returned to transport containers and monitored for approximately 20 minutes prior to being prepared for the unpaired extinction training session, which was started after a total of 30 minutes had elapsed following drug injections.

Statistical analysis

Unless described otherwise, experimental group data were analyzed by repeated measures analysis of variance (ANOVA, SPSS 21), with p values corrected using the procedures of Huynh-Feldt for violations of the sphericity assumption. Planned and follow-up comparisons were Bonferroni corrected for the number of comparisons.

Results

Experiment 1

Classical Delay Conditioning—The left side of Figure 1 shows the average percentage of CRs to the tone CS across the six days of classical delay conditioning in rabbits in Experiment 1. All rabbits acquired the delay conditioning task with the exception of one rabbit whose data were eliminated for failure to reach a learning criterion of 80% CRs by the last day of conditioning. Final n's per group were 6, 7, 6, and 7 for saline and 5, 10, and 20 mg/kg ketamine drug groups, respectively. There were no differences in acquisition rate or level between groups, as evidenced by a significant effect of Training Day [$F(5,110) = 90.8$,

$p < 0.001$] with no effects or interactions involving Drug Group. Bonferroni-corrected planned comparisons showed that CRs significantly increased from the first to second day of conditioning ($p < 0.01$) and then remained at a similar, high level throughout the subsequent sessions. By the sixth day of delay conditioning, rabbits had mean CRs in excess of 95% ($\bar{X} = 97.52 \pm 1.25$ SEM).

Effects of Ketamine on Conditioned Responses to the Tone Conditioned

Stimulus—The effects of a single ketamine injection on CRs to the tone CS can be seen on the right side of Figure 1. Analysis comparing the last day of delay conditioning (D6) with the first and second CS Tests (CS Test1, CS Test2) indicated a significant effect of Session [$F(2,44) = 56.0$, $p < 0.001$] with no significant effects or interactions involving Drug Group. Corrected post-hoc comparisons indicated a significant decrease in CRs at CS Test1 from the terminal level exhibited on D6 ($p < 0.001$), and a further decrease from CS Test1 to CS Test2 ($p < 0.01$). To examine CR retention more specifically without the confounding effects of repeated CS-alone presentations producing extinction, an additional analysis examined just the first ten trials of the CS Test (boxed panels of Figure 1) and compared with D6 performance. Again, there was a significant effect of Session [$F(2,44) = 15.98$, $p < 0.001$] with no significant effects or interactions involving Drug Group. Follow-up comparisons indicated that there was no reduction of CRs in the first ten trials of CS Test1 compared to D6, demonstrating that all groups showed a high level of CR retention. During the first ten trials of CS Test2, CRs were reduced relative to both D6 ($p < 0.001$) and CS Test1 ($p < 0.01$). There were also no significant effects or interactions involving Drug Group when each CS Test session was analyzed by 10 trial blocks.

Conditioned Hyperarousal—To determine the initial amount of conditioned hyperarousal prior to ketamine treatment, Pretest to Post1 comparisons were conducted to measure the US intensities and UR parameters for which CRM occurred, characterized as a significant Pretest to Post1 increase. Figure 2 shows the frequency of the NMR in response to the five US intensities while the topographies depicted in Figure 3 show changes in the amplitude, area, and timing of the NMR. For UR frequency (%URs), CRM was indicated by a significant interaction of US Test and Intensity [$F(4,88) = 11.15$, $p < 0.001$]. Planned comparisons indicated that Pretest to Post1 increases occurred at the 0.3 and 0.5 mA intensities (p 's < 0.001). CRM was also indicated by a significant US Test by Intensity interaction for both magnitude of the amplitude (MAmp) [$F(4,88) = 9.07$, $p < 0.001$] and magnitude of the area (MArea) [$F(4,88) = 4.92$, $p < 0.01$]. Similar to results for %URs, significant Pretest to Post1 increases were found at the 0.3 mA and 0.5 mA intensities for both MAmp ($p < 0.01$; $p < 0.001$, respectively) and MArea (p 's < 0.01). Onset and peak latency of the UR were analysed for each US intensity separately, owing to the limitation of empty data cells (see Methods). While there was no CRM for onset latency, an increase from Pretest to Post1 was found for peak latency at the 0.5 mA intensity, indicated by a significant main effect of US Test [$F(1,18) = 7.81$, $p < 0.05$]. For all analyses of all UR parameters, there were no significant effects or interactions involving Drug Group, demonstrating that there were no significant differences in baseline responding during Pretest or CRM between groups prior to drug treatment.

Effects of Ketamine on Conditioned Hyperarousal—The UR topographies in Figure 3 show the change in the size of the amplitude, area, and timing of the NMR one day (Post2, red line) and one week (Post3, green line) following ketamine or saline injection. For all groups, regardless of whether receiving saline or ketamine, it appeared that CRM was not diminished following drug treatment and may have even been enhanced, particularly in the 5 mg/kg ketamine group when tested one week following the injection. Analyses comparing Pretest with Post Tests 1-3 did not reveal any significant main effects or interactions involving Drug Group. Focusing analyses on the intensities for which CRM was found prior to drug treatment (0.3, 0.5 mA), again there were no significant effects or interactions involving Drug Group. Analyses did, however, confirm the observation that CRM was maintained one day and one week following drug treatment. For %URs (see Figure 2), there was a significant main effect of US Test [$F(3,66) = 14.41$, $p < 0.001$] but no significant Test $\times$ Intensity interaction, with Bonferroni corrected post-hoc comparisons indicating that %URs for Post Tests 1-3 were all higher than Pretest (p 's < 0.001) but not different from each other. Similarly, for MArea there was a significant main effect of US Test [$F(3,66) = 6.71$, $p < 0.01$] but not a significant interaction with Intensity, with post-hoc tests showing that area of the NMR was greater at Posts 1-3 compared with Pretest (p 's < 0.01) but not different across post tests. For MAmp there was a significant interaction of US Test and Intensity [$F(3,66) = 7.87$, $p < 0.01$] with Bonferroni-corrected planned comparisons showing that the amplitude of the NMR for Posts 1-3 was higher than Pretest at both the 0.3 and 0.5 mA intensities and also further increased from Post1 to Post2 (p 's < 0.05). At Post3, CRM at 0.5 mA returned to the level observed at Post1 ($p < 0.05$).

Experiment 2

Classical Delay Conditioning—The left side of Figure 4 shows the average percentage of CRs to the tone CS across the six days of classical delay conditioning. All rabbits acquired the delay conditioning task with the exception of two rabbits whose data were eliminated for failure to reach a learning criterion of 80% CRs by the last day of conditioning. Final n's per group were 9, 10, 10, and 10 for saline and 3, 6, and 12 mg/kg d-cycloserine drug groups, respectively. There were no differences in acquisition rate or level between groups, as evidenced by a significant effect of Training Day [$F(5,175) = 189.5$, $p < 0.001$] with no significant effects or interactions involving Drug Group. Bonferroni-corrected planned comparisons showed that CRs significantly increased from the first to second day of conditioning ($p < 0.001$) and plateaued for the remaining sessions. By the sixth day of delay conditioning, rabbits had mean CRs of 96% ($\bar{X} = 95.98 \pm 0.86$ SEM).

Effects of Combined D-cycloserine and Extinction Treatment on Extinction of Conditioned Responses—The immediate effects of an injection of d-cycloserine combined with a single session of unpaired extinction with a weak shock can be seen in the middle of Figure 4, which shows CR data for both the entire extinction session as well as blocks of twenty trials of tone CS presentations. The data suggest that CRs were lowest in the highest dose drug group (12 mg/kg), with the block data suggesting that this was a result of enhanced extinction of CRs to the tone CS rather than an overall decrease in CRs as a result of a retention or expression deficit. Initial analyses that included all drug doses did not show any significant drug effects. Analysis comparing the final day of delay conditioning

with the unpaired extinction session confirmed that significant extinction of CRs took place during the unpaired extinction session, as indicated by a significant main effect of Session [$F(1,35) = 45.78$, $p < 0.001$]. Analysis of the 20-trial blocks of the extinction session indicated a significant effect of Block [$F(3,105) = 21.72$, $p < 0.001$], with planned comparisons showing that CRs decreased from the first to the second block of CS presentations ($p < 0.001$) but did not significantly decrease further during the remainder of the session.

Contrast analysis (Psy, University of New South Wales) was conducted to focus comparisons on the 12 mg/kg d-cycloserine group with saline controls, and with the 3 and 6 mg/kg drug dose groups combined, for the unpaired extinction session. CRs were significantly lower in the 12 mg/kg group compared to the other drug doses across the entire extinction session [$F(1,35) = 5.56$, $p < 0.05$]. Importantly, analysis of blocks indicated the difference was only significant during the second [$F(1,35) = 6.83$, $p < 0.05$] and third blocks [$F(1,35) = 6.32$, $p < 0.01$] of CS presentations, suggesting enhanced within-session extinction in the highest dose group rather than a retention or expression deficit. Comparison with the saline controls mirrored the comparison with the lower drug doses, indicating reduced CRs in the 12 mg/kg group for the second [$F(1,35) = 5.51$, $p < 0.05$] and third blocks [$F(1,35) = 4.79$, $p < 0.05$] of the extinction session.

The remaining level of CRs one week after treatment with d-cycloserine combined with a single unpaired extinction session is shown on the right side of Figure 4. Data shown are for the entire CS Test session as well as four 20-trial blocks of CS presentations. The within-session enhancement of extinction in the 12 mg/kg d-cycloserine drug group was no longer present one week later, as all groups showed a similar level of CRs at the start of the session and a similar rate of further extinction as the session of CS-alone presentations progressed. There was a significant effect of Block [$F(3,105) = 9.40$, $p < 0.001$] but no significant effects involving Drug Group. Corrected planned comparisons indicated that there was a significant decrease in CRs from the first to second block of CS-alone presentations ($p < 0.05$).

The last 20 CS presentations during the unpaired extinction session were compared with the first 20 trials of the CS Test were done to measure retention of extinction. There were no significant effects involving Drug Group, but there was a trend for a significant effect of Session [$F(1, 35) = 3.60$, $p = 0.066$], indicating that CRs were higher during the beginning of the CS Test compared to the terminal level at the end of extinction. These findings therefore reflect some recovery of the CR after the first extinction session.

The US-alone trials of the unpaired extinction session were analyzed to determine if there were any sensory effects of d-cycloserine on the UR. The URs for the entire session as well as 20-trial blocks of US presentations are shown in the blocked panel of Figure 4. Although there was a suggestion of a dose-dependent decrease in the frequency of the UR, there were no significant drug effects. There was a significant effect of Block [$F(3,105) = 15.86$, $p < 0.01$], with planned comparisons indicating that URs decreased from the first to second ($p < 0.01$) and the second to third blocks ($p = 0.05$). All groups therefore displayed significant decreases in UR frequency over the extinction session, suggesting some habituation to the

US. There were also no significant differences between the saline controls and drug groups on the amplitude, area, and timing of the UR.

Conditioned Hyperarousal—To determine the initial amount of conditioned hyperarousal prior to d-cycloserine combined with unpaired extinction treatment, Pretest to Post1 comparisons were conducted to measure the US intensities and UR parameters for which CRM occurred. Figure 5 shows the frequency of the NMR in response to the five US intensities, while the amplitude, area, and timing of the NMR are depicted in the topographies in Figure 6. For %URs, CRM was indicated by a significant interaction of US Test and Intensity [$F(4, 140) = 13.13$, $p < 0.001$] with corrected planned comparisons showing Pretest to Post1 increases at the 0.3 ($p < 0.001$), 0.5 ($p < 0.001$), and 1.0 mA ($p < 0.05$) intensities. CRM was also indicated by significant Pretest to Post1 increases for MAmp [$F(4, 140) = 6.90$, $p < 0.001$] at the 0.3 ($p = 0.01$) and 0.5 mA ($p < 0.05$) intensities and for onset latency at the 0.5 mA intensity [$F(1, 25) = 6.44$, $p < 0.05$]. There was also a significant US Test and Intensity interaction for MArea [$F(1, 140) = 4.81$, $p < 0.001$], and although there were Pretest to Post1 increases in amplitude at the 0.3 and 0.5 mA intensities, the comparisons were not significant. For all analyses involving Pretest to Post1 comparisons, there were no significant effects or interactions involving Drug Group, demonstrating that there were no significant differences in CRM between groups prior to drug treatment. Analysis of baseline responding at Pretest revealed no significant group differences, with the exception of a difference in onset latency at the 2.0 mA intensity [$F(3, 35) = 4.93$, $p < 0.01$] due to subjects later assigned to the 12 mg/kg group having a longer latency to respond compared to the 6 mg/kg d-cycloserine group ($p < 0.01$).

Effects of Combined D-cycloserine and Extinction Treatment on Conditioned Hyperarousal—The UR topographies in Figure 6 show the change in amplitude, area, and timing of the NMR one day (Post2, red line) and one week (Post3, green line) following treatment with d-cycloserine combined with a single day of unpaired extinction with a weak shock. At Post2, CRM appeared to be diminished by extinction treatment, regardless of whether rabbits received saline or drug, although the effect may have been weakest in the lowest drug group (3 mg/kg). At Post3, the reduction in CRM was diminished, and there was a suggestion that CRM was even enhanced compared to the initial CRM assessment (Post1) in the drug groups. Analyses comparing Pretest with Post Tests1-3 did not reveal any significant main effects or interactions involving Drug Group. Analyses were then focused on the intensities for which CRM was strongest for % URs (0.3, 0.5, 1.0 mA), MAmp, and MArea (0.3, 0.5 mA). Again, there were no significant main effects or interactions involving Drug Group. Overall reduction of CRM following one day of extinction treatment was confirmed by a significant effect of Test for % URs [$F(3,105) = 22.31$, $p < 0.001$], MAmp [$F(3,105) = 12.29$, $p < 0.001$], and MArea [$F(3,105) = 7.40$, $p < 0.001$], with corrected post-hoc comparisons indicating that CRM was reduced from Post1 to Post2 for MAmp, MArea (p 's < 0.001), and a trend for reduction in % URs ($p = 0.056$). Corrected post-hoc tests also indicated that the reduction in CRM was diminished when assessed one week later as there were significant increases from Post2 to Post3 for all three parameters (p 's < 0.001). The observed Post1 to Post3 enhancement was not statistically supported.

Discussion

Effects of Ketamine effects on PTSD-like symptoms

Treatment with a single, subanesthetic dose of ketamine did not produce any significant immediate or prolonged reduction in PTSD-like symptoms. Instead, CRs to tone and conditioned hyperarousal remained at a high level in all groups, regardless of drug treatment, and there was a subtle enhancement of conditioned hyperarousal in the lowest dose group one week following treatment. These findings therefore suggest that expression of PTSD-symptoms in the CRM paradigm is not ameliorated by acute treatment with ketamine, and conditioned hyperarousal possibly may even be worsened by treatment after a delay.

Although ketamine has shown some promise in treatment of PTSD in the clinic (Feder et al., 2014), there is also evidence that ketamine may not be an ideal drug treatment, especially for certain aspects of PTSD symptomology. There have been mixed reports, for example, on the benefits of ketamine treatment received by traumatic accident and burn victims, with some reporting that it can decrease prevalence of PTSD (McGhee et al., 2008) while others report that it can increase the severity of PTSD diagnostic scores (Schonenberg et al., 2005, 2008; Winter and Irle, 2004). Tests of subanesthetic ketamine in healthy volunteers have reported dissociative and psychosis-like effects that mimic schizophrenia (Krystal et al., 1994; Morgan et al., 2004) as well as biomarkers of stress such as increased cortisol, impairment of stress-sensitive memory function, and transient changes in the activity of the hypothalamic pituitary adrenal axis revealed by brain imaging (Khalili-Mahani et al., 2015). Another study in healthy subjects showed that memory for a conditioned aversive cue could be enhanced if reactivated under the influence of ketamine (Corlett et al., 2013). Considering these findings, ketamine may produce unpredictable results depending upon the state of the patient at the time of treatment. For example, if the psychosis-like and stress-like reactions to ketamine trigger a re-experiencing of a traumatic event or if a patient has other active PTSD symptoms, there is a risk that ketamine may strengthen the traumatic memory through reconsolidation. Results from this study additionally caution that hypervigilance symptoms in particular may not improve and possibly can be worsened by ketamine treatment after time passes after treatment. It should be noted that Feder and colleagues did report a decrease in hyperarousal symptoms 24 h after ketamine treatment (Feder et al., 2014), but the effect appeared to lessen across the days following treatment.

We have previously demonstrated that the development of conditioned hyperarousal is accompanied by an increase in heart rate response to the periorbital shock, an effect that is strongest for the lower intensity shocks for which CRM is also strongest (Schreurs and Smith-Bell, 2005). These findings support the existence of an anxiety/fear component to conditioned hyperarousal. There are conflicting reports on whether ketamine is anxiolytic or anxiogenic in preclinical animal models. When subanesthetic ketamine is administered shortly before testing, contrasting anxiolytic (Engin et al., 2009; Zhang et al., 2015) and anxiogenic effects (da Silva et al., 2010; Silvestre et al., 1997) have been reported. In fear-conditioning studies, ketamine administered during fear memory reactivation has shown to both reduce (Duclot et al., 2016) and enhance conditioned fear (Honsberger et al., 2015). If we consider the magnitude of conditioned hyperarousal a reflection of an anxiogenic or fear

state, our results suggest ketamine has no immediate effect on anxiety-like behavior, adding to the inconsistency within the literature. One possibility is that ketamine may have had an effect in our model if stronger reactivation of the trauma memory was induced, such as by placing subjects back in the conditioning chambers and briefly re-exposing them to training stimuli shortly after ketamine administration. However, the mixed results in the literature make it difficult to predict whether stronger memory reactivation under ketamine would yield an increase or decrease in PTSD-like symptoms.

Another possible reason for the lack of a strong effect of ketamine treatment in the current study is that effects of ketamine would be more apparent if testing for PTSD-like symptoms occurred after only a short delay, rather than 24 and 48 h later. However, our concerns about ketamine having sensory-motor effects on the eyeblink response precluded us from examining more immediate effects of ketamine (see Methods). Of importance is that others have reported effects of ketamine on anxiety-like or fear conditioned behaviors when tested a day after treatment (Babar et al., 2001; Honsberger et al., 2015), suggesting that any effects ketamine may have should last at least 24 hs. In addition, clinical reports of ketamine given to PTSD patients also mention effects lasting beyond one day (Feder et al., 2014).

Effects of d-cycloserine combined with extinction treatment on PTSD-like symptoms

The highest dose of d-cycloserine combined with a single day of unpaired extinction treatment enhanced within-session extinction of CRs to the tone CS, but the effect was shortlived as it was no longer present one week later. None of the doses of d-cycloserine tested enhanced extinction of CRM. All groups including saline-injected controls showed some reductions in CRM when tested a day after combined treatment, although the lowest dose of d-cycloserine made extinction slightly *less* effective. All rabbits, regardless of drug administered, showed the return of robust CRM one week following combined treatment, demonstrating no delayed effect of d-cycloserine on retention of conditioned hyperarousal. There were also no effects of d-cycloserine on CRs measured a week after treatment, suggesting that any within-session enhancement of extinction learning did not translate to a longer-term enhancement of extinction retention. These results suggest that acute treatment with d-cycloserine has the potential to enhance extinction in the short term of only one aspect of our model -CRs to stimuli associated with trauma- while having either no or a worsening effect on conditioned hyperarousal.

In previous work, we observed that one day of unpaired extinction with a weak periorbital shock worsened conditioned hyperarousal symptoms, cautioning to the danger of not completing a full course of treatment (Schreurs et al., 2011b). However, this finding is inconsistent with the results presented here, as we found that one day of extinction did produce some short-term reduction in hyperarousal, in drug groups as well as the saline control group. However, the robust CRM demonstrated one week following treatment does add some support to the idea that a single day of extinction is an ineffective treatment. There were some procedural differences between the current study and the previous report that may account for the inconsistencies. In the former study, heart rate data were collected during CRM testing, which involves additional handling in order to expose the chest and attach wound clips that are used to record electrocardiogram data. Heart rate recordings were

not made during conditioning or extinction training, constituting a possible context or state change between acquisition and extinction versus CRM testing. Another difference is that owing to the closure of a former vending source, rabbits from the current study are from a different breeding stock of New Zealand rabbits that receive different protocols prior to arrival, including increased acclimation to handling. In fact, we have found significant differences in learning and the strength of CRM between rabbits from the two vender sources (unpublished data), suggesting that differences in response to extinction treatment are also possible.

In both clinical research and animal models, d-cycloserine treatment combined with exposure therapy or extinction treatment has yielded mixed results (for review, see Fitzgerald et al., 2014). One consistent finding across human and animal work is that the success of d-cycloserine treatment appears to be conditional on the current fear state or level of extinction learning that has already taken place. For example, in a study on patients with generalized social anxiety disorder, clinical improvement from one exposure therapy session to the next was enhanced if subjects reported low fear at the end of the session but not if they reported high fear (Smits et al., 2013). Similarly, in studies in rats, d-cycloserine was only beneficial when subjects exhibited some extinction learning (Bouton et al., 2008), and enhancement could be improved if more trials were presented under the drug (Bouton et al., 2008; Lee et al., 2006). In another example, d-cycloserine treatment in mice did not facilitate extinction in an extinction-resistant strain but did in strains able to demonstrate some extinction learning (Sartori et al., 2016). Our findings mirror these results as we demonstrated a within-session enhancement of CR extinction with the highest d-cycloserine dose that did not develop until an excess of 20 extinction trials were presented. Importantly, these studies in rodents also demonstrated that effects of d-cycloserine did not last long-term or prevent fear renewal (Bouton et al., 2008; Sartori et al., 2016). In agreement, our results from the extinction retention test one week later in the highest dose group showed no carryover of the extinction enhancement but rather showed an increase in responding at the beginning of the retention test compared to the terminal level at the end of extinction. Since this observation was specific to the highest dose of d-cycloserine, one possibility is that it may reflect a state-dependent form of fear renewal as a result of a change in internal context from extinction (drug) to retesting (no drug); however, spontaneous recovery of responding due to the passage of time is also a consideration (Bouton, 2002).

There are several reasons why the current study may have failed to demonstrate facilitation of extinction of conditioned hyperarousal. Because even saline controls demonstrated a decrease in CRM following one day of unpaired extinction, the possibility of a floor effect cannot be ruled out. Another conclusion is that systemic d-cycloserine simply does not successfully target neurochemical systems or neural substrates that may be involved in CRM extinction. The preponderance of evidence for d-cycloserine facilitating extinction is based on extinction of fear to trauma-associated cues, which in our model is most relatable to the development of the CR to the tone CS associated with the shock US, but does not necessarily apply to CRM. Although development of CRs and CRM have shown some interdependence, we also have found a dichotomy can exist, particularly in terms of extinction of those responses (Burhans et al., 2008). For example, we can extinguish CRs but not CRM with tone alone presentations, but conversely, can extinguish CRM but not CRs

with shock alone presentations. Unpaired extinction is successful in simultaneously extinguishing both because it combines the two separate treatments, suggesting that two separate extinction processes may be going on, possibly with distinct neural and/or neurochemical substrates. In support of this hypothesis, we have demonstrated that the central nucleus of the amygdala is crucial for the expression of CRM but not CRs (Burhans and Schreurs, 2008). Results presented here add to this dichotomy by showing that a systemic NMDA agonist may facilitate extinction of CRs while having no effect on CRM. In further support, work in a rodent model of hyperarousal has also demonstrated that NMDA receptor manipulation can affect conditioned fear responses without affecting fear sensitization (Siegmund and Wotjak, 2007).

Conclusions

The CRM model of PTSD highlights two key features of PTSD symptomology: CRs to cues associated with trauma and a form of conditioned hyperarousal referred to as CRM. The advantage of a PTSD model that addresses more than one feature is the ability to compare and contrast how different treatments, particularly pharmacological treatments, impact different symptoms. Here we demonstrate that our model shows some sensitivity to systemic glutamatergic manipulation, but has differential effects on CRs versus CRM. Our first important finding was that administration of a single-dose of subanesthetic ketamine had no immediate effect on CRs or CRM, but suggested enhancement of CRM one week later with the highest dose of ketamine. These results add caution to recent findings of a potential benefit of ketamine treatment in PTSD, results that are difficult to interpret due to the common comorbidity of depression and PTSD. Our findings suggest that even though ketamine may be beneficial to PTSD symptoms, particularly those that overlap or interact with depression-like symptoms, ketamine may not improve or may even increase hyperarousal/hypervigilance symptoms. Our second major result was that combining unpaired extinction treatment with d-cycloserine facilitated extinction of CRs in the short-term while having little impact on CRM. Translating these findings to the clinic, they illustrate that although d-cycloserine may facilitate exposure therapy, it may provide little to no benefit to hyperarousal symptoms, stressing the importance of incorporating additional treatments that can reduce hyperarousal. Overall, our findings demonstrate that there is no one size fits all treatment approach to PTSD, owing to the complexity of symptoms, and that pharmacological as well as cognitive behavioral treatments must be tailored towards addressing multiple symptoms, which may require combining multiple behavioral and pharmacological treatments.

The findings presented here do not support a strong role for the glutamatergic system in mediating the expression or extinction of the conditioned hyperarousal aspect of our model. Few current models of PTSD explicitly test for behaviors related to hyperarousal; therefore, there is a great need for research that elucidates the mechanisms mediating its acquisition, expression, and extinction. Continuation of the work in our lab, as well as others that are examining fear sensitization (Perusini et al., 2016; Poulos et al., 2015; Siegmund and Wotjak, 2007), are important steps toward creating animal models of PTSD that reflect the multiple symptomology and complexity of the disorder and can be used to optimize treatments.

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Figure 1.

The mean percentage ($\pm$ SEM) of conditioned responses (CRs) to the tone conditioned stimulus (CS) during six daily sessions of delay conditioning and two CR retention tests consisting of CS-alone presentations (CS Test1, 2). The right inset panels show the first ten trials for each CS Test. On a separate day where no training occurred (Drug Inject), rabbits received saline (open circle) or 5 mg/kg (grey circle), 10 mg/kg (dark grey triangle), or 20 mg/kg ketamine (black square).

Figure 2.

Mean ($\pm$ SEM) percentage of unconditioned responses for the first 20 trials of presentations of the periorbital shock unconditioned stimulus (US) during pretest (Pretest, diagonal striped bar), the post test following delay conditioning (Post1, black bar), the post test following ketamine treatment (Post2, white bar), and a final post test one week later (Post3, gray bar). Bar graphs are shown for the five US intensities (0.1, 0.3, 0.5, 1.0, 2.0 mA) presented during US testing, collapsed across duration.

Figure 3.

Averaged response topographies for the unconditioned response to the periorbital shock unconditioned stimulus (US) during the first 20 trials of the US pretest (Pretest, black dotted line), the post test following delay conditioning (Post1, black line), the post test following drug injection (Post2, red line), and a final post test one week later (Post3, green line).

Topographies are shown at the five unconditioned stimulus intensities (2.0, 1.0, 0.5, 0.3, 0.1 mA) presented during US testing, collapsed across duration.

Figure 4.

The mean percentage ($\pm$ SEM) of conditioned responses (CRs) to the tone conditioned stimulus (CS) during six daily sessions of delay conditioning, one session of unpaired extinction with weak shock combined with saline or d-cycloserine treatment, and a CR retention test consisting of CS-alone presentations eight days later (CS Test). CRs to the tone CS during extinction and the CS Test session are shown averaged across the entire session (AVG) and in blocks of 20 CS-alone trials for both the extinction and CS Test sessions. The top inset panel shows the mean percentage ($\pm$ SEM) of unconditioned responses (URs) to the 0.3 mA shock presented during extinction, averaged across the entire session and in blocks of 20 US-alone trials. Prior to the unpaired extinction session, rabbits received saline (open circle) or 3 mg/kg (grey circle), 6 mg/kg (dark grey triangle), or 12 mg/kg d-cycloserine (black square).

Figure 5.

Mean ($\pm$ SEM) percentage of unconditioned responses for the first 20 trials of presentations of the periorbital shock unconditioned stimulus (US) during pretest (Pretest, diagonal striped bar), the post test following delay conditioning (Post1, black bar), the post test following saline or d-cycloserine treatment combined with a single session of unpaired extinction with weak shock (Post2, white bar), and a final post test one week later (Post3, gray bar). Bar graphs are shown for the five US intensities (0.1, 0.3, 0.5, 1.0, 2.0 mA) presented during US testing, collapsed across duration.

Figure 6.

Averaged response topographies for the unconditioned response to the periorbital shock unconditioned stimulus (US) during the first 20 trials of the US pretest (Pretest, black dotted line), the post test following delay conditioning (Post1, black line), the post test following saline or d-cycloserine treatment combined with a single session of unpaired extinction with weak shock (Post2, red line), and a final post test one week later (Post3, green line). Topographies are shown at the five unconditioned stimulus intensities (2.0, 1.0, 0.5, 0.3, 0.1 mA) presented during US testing, collapsed across duration.