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Combined administration of MK-801 and cycloheximide produces a delayed potentiation of fear discrimination memory extinction

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Abstract

Mixed evidence exists regarding the role of N-methyl-D-aspartate (NMDA) receptors in memory reconsolidation. We provide no evidence that NMDA receptors are involved with memory reconsolidation, but instead demonstrate that pre-reactivation systemic MK-801 injection, combined with post-reactivation intra-basolateral amygdala (BLA) cycloheximide infusion, produces a delayed potentiation of extinction learning. These data suggest that an interaction between NMDA antagonism and protein synthesis inhibition may enhance extinction by exerting effects outside of the intended reconsolidation manipulation window. The present work demonstrates a novel pharmacological enhancement of extinction, and underscores the importance of employing proper control procedures in reconsolidation research.

Keywords

reconsolidation; extinction; learning; memory; rat

It is generally well-accepted that memories are stabilized over time through a consolidation process (e.g. McGaugh, 2000). However, recent reconsolidation research has revealed that memories do not necessarily remain stable. Upon reactivation, a memory trace sometimes becomes labile and must be “reconsolidated” to become stable again (e.g., Misanin, Miller, & Lewis, 1968; Nader, Schafe, & LeDoux, 2000; Przybylski, Roulet, & Sara, 1999; Sara, 2000).

Reconsolidation can be conceptualized as a two-phase process (e.g. Lee, 2008). Memories must first become labile via a destabilization process. Memories then are “restabilized” or reconsolidated. It is during this labile window that amnesic manipulations, such as the administration of protein synthesis inhibitors can disrupt reconsolidation (e.g. Nader et al., 2000). This has been demonstrated across multiple neural systems using a variety of behavioral tasks, suggesting that it is an evolutionarily conserved process serving to update

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existing memories and preserve their relevance (Alberini, 2011; Dudai, 2006; Hupbach, Gomez, Hardt, & Nadel, 2007; Lee, 2009; Nader, & Hardt, 2009). It has been theorized, therefore, that reconsolidation allows for the modification of existing memories through plastic mechanisms without necessitating continuous reacquisition of learned behaviors (Alberini, 2011; Dudai, 2009; Forcato, Argibay, Pedreira, & Maldonado, 2009; Forcato, Rodríguez, Pedreira, & Maldonado, 2010; Lee, 2009; McKenzie & Eichenbaum, 2011; Sevenster, Beckers, & Kindt, 2012).

The consolidation and reconsolidation of aversive memories depends critically upon the amygdala, and formation of this association depends upon protein synthesis within the amygdala (e.g. Bailey, Kim, Sun, Thompson, & Helmstetter, 1999; Kochli, Thompson, Fricke, Postle, & Quinn, 2015; Kwapis, Jarome, Schiff, & Helmstetter, 2011; Maren, 2005; Schafe & LeDoux 2000). Once established, this association permanently supports the expression of the fear memory (Amano, Duvarci, Popa, & Paré, 2011; Davis, 2006; Gale et al., 2004; Fanselow & LeDoux, 1999; LeDoux, 1993). Amygdala-dependent memories are subject to updating via reconsolidation mechanisms, and the lateral/basolateral amygdala (BLA) has been widely demonstrated as a critical site of aversive reconsolidation-related plasticity (e.g. Brown, Wilson, Cocking, & Sorg, 2009; Duvarci, Nader, & LeDoux 2008; Gale et al., 2004; Milekic, Pollonini, & Alberini, 2007; Nader & Hardt, 2009; Nader et al., 2000).

Reconsolidation of associative fear memories depends upon N-methyl-D-aspartate (NMDA) glutamate receptors and their downstream intracellular signaling cascades (Ben Mamou, Gamache, & Nader, 2006; Lee, Milton, & Everitt, 2006; Tronson, Wiseman, Olausson, & Taylor, 2006; Wang, de Oliveira Alvares, & Nader, 2009). A body of evidence suggests that while NMDA receptors (NMDARs) are critical for the reconsolidation-related destabilization of memory, they are not necessary for the reconsolidation of memories per se. For example, when Ben Mamou and colleagues administered intra-BLA infusions of NMDAR antagonists AP-5 or ifenprodil prior to memory reactivation, it prevented a deficit induced by intra-BLA infusions of the protein synthesis inhibitor, anisomycin (2006). Similarly, pre-reactivation administration of the NMDA receptor antagonist, MK-801, prevented the destabilization of a methamphetamine-associated contextual memory in mice (Yu, Huang, Chang, & Gean, 2016). In addition, it was recently shown that calmodulin-dependent protein kinase II (CaMKII), a well-studied enzyme downstream of NMDARs, gates induction of reconsolidation processes via regulation of proteasome activity (Jarome, Ferrara, Kwapis, & Helmstetter, 2016). However, another body of evidence suggests that NMDAR antagonism is sufficient to disrupt reconsolidation per se. This has been demonstrated across a variety of types of memory, including appetitive (Flavell & Lee 2013; Milton et al., 2012; Reichelt, Exton-McGuinness, & Lee, 2013), drug (Alaghband & Marshall, 2013; Exton-McGuinness, Lee, & Reichelt, 2015; Tedesco, Mutti, Auber, & Chiamulera, 2014), contextual fear (Heath et al., 2015), cued fear (Lee et al., 2006), conditioned food aversion (in a snail model; Nikitin, Solntseva, & Kozyrev, 2016) and spatial (Przybylski & Sara, 1997) paradigms. These results are at odds with work demonstrating NMDAR antagonism prevents destabilization, but has no effect on memory retrieval or reconsolidation (e.g. Ben Mamou et al., 2006).

The present work initially aimed to test whether systemic administration of MK-801 would disrupt memory destabilization or memory reconsolidation per se in a context fear discrimination paradigm. Prior to memory reactivation, rats were injected systemically with MK-801 or vehicle. Immediately following reactivation, rats received intra-BLA infusions of the protein synthesis inhibitor, cycloheximide, or vehicle. If MK-801 blocks memory destabilization, rats injected with MK-801 would be protected from a cycloheximide-induced memory deficit. However, if MK-801 was sufficient to disrupt memory reconsolidation per se, all groups injected with MK-801 would display a memory deficit, regardless of post-reactivation infusion and, perhaps, rats given both MK-801 and cycloheximide would show the greatest deficit. A control experiment in which animals received identical drug concentrations at identical time points in the absence of memory reactivation was included to determine whether the observed effects were specific to memory reconsolidation processes.

Method

Subjects

Experimentally naïve adult male Long-Evans rats (Envigo, Indianapolis, IN) were used for all experiments. All rats were pair housed in standard colony caging on a 14:10-h light:dark cycle and given *ad libitum* access to food and water. The rats were handled for one minute per day for five consecutive days prior to surgery. All procedures were performed during the light cycle and were approved by the Miami University Institutional Animal Care and Use Committee in accordance with the NIH Guidelines for the Care and Use of Experimental Animals.

Apparatus

Animals underwent conditioning in two distinct contexts. Context A consisted of four chambers (32.4 x 25.4 x 21.6 cm; MED-Associates, Inc., Georgia, VT). The ceiling and front door of each chamber are made of clear Plexiglas, the back wall is white Plexiglas and the two side walls are aluminum. The floor consists of 19 equally spaced stainless steel rods. The grid floor in each chamber is wired to a shock generator and scrambler (MED-Associates, Inc.). The conditioning chambers were wiped down with an odorless 5% sodium hydroxide solution and scented with 50% vanilla flavor (Kroger) solution. The chamber was brightly lit (125 lux) by a light box located above the conditioning chamber. Context B chambers (32.4 x 25.4 x 21.6 cm; MED-Associates, Inc.) are located in a different experimental room and are distinct from Context A. They consist of a Plexiglas floor and an opaque Plexiglas equilateral triangular insert. The context was cleaned and scented with distilled white vinegar (Kroger). The light box above the chamber provided near-infrared lighting (0 lux). The rats were continuously monitored by a progressive scan video camera with a visible light filter (MED-Associates, Inc.) connected to a computer in the experimental room running Video-Freeze software (MED-Associates, Inc.) designed for automated assessment of defensive freezing (see Anagnostaras et al., 2010).

Surgery

Rats were anesthetized and skulls leveled by equating lambda and bregma in the dorsal ventral plane. Guide cannulae (22-gauge; Plastics One, Roanoke, VA) were lowered into the brain bilaterally targeting the BLA using the following coordinates: BLA (AP -3.0 mm, ML ± 5.3 mm, DV -7.9 mm) relative to bregma (Paxinos & Watson, 1998). Four skull screws and dental acrylic were used to secure the guide cannulae within the skull. Obturators were placed into the guide cannulae to prevent debris from entering. Following surgery, the rats were given two subcutaneous injections: 3 ml of 0.9% saline for rehydration and 5 mg/kg/ml of Rimadyl to reduce pain and inflammation. Following surgery, the rats were placed into a recovery cage on a heating pad until they fully awoke from anesthesia. Post-operative care was performed for five consecutive days after surgery. Rimadyl (5 mg/kg/ml; s.c.) and saline (0.9%; 3 ml; s.c.) were administered at 24 and 48 hours post-surgery.

Procedure

Experiment 1—Over nine days, rats underwent daily four-minute behavioral training sessions in a 2:1 CTX– to CTX+ ratio to minimize overtraining (see Figure 1A). Reinforced exposures consisted of a three-minute pre-shock period, a two-second footshock (1mA), and a 58-second post-shock period. Non-reinforced exposures had a duration of four minutes. In one context (CTX+), the rat always received a footshock. In the other context (CTX–), the rat never received a footshock. On day 10, rats underwent a non-reinforced exposure to the CTX+. On days 11 through 15, rats underwent daily non-reinforced extinction sessions in the CTX+. On day 16, rats underwent a non-reinforced session in the CTX–. All reactivation, extinction, and testing consisted of a four-minute non-reinforced exposure to the appropriate context except for the first extinction session in experiment 1, which was three minutes. Freezing was defined as a complete cessation of movement besides that required for respiration (e.g., Fanselow, 1980). Freezing was quantified continuously during the first 3 minutes of each behavioral session and expressed as a percentage of time spent freezing.

Experiment 2—Experiment two followed identical procedures to experiment 1, except that rats received drug injections and infusions in the colony room in the absence of a memory reactivation session. Additionally, rats underwent six days of extinction training (in order to equate with the one reactivation and five extinction sessions of experiment 1). Following extinction training, rats underwent three days of reinforced exposures to the former CTX– followed by one non-reinforced exposure to the former CTX– (see Figure 2A). Rather than the complete factorial design used in experiment 1, experiment 2 consisted of three treatment groups: vehicle/vehicle, MK-801/cycloheximide, and a no surgery/injection/infusion control group. The no surgery/injection/infusion group was included to demonstrate “normal” acquisition and extinction using these parameters. This group allowed for the assessment of the combined possible contributions of decremental effects of cannulation, intracranial infusions, i.p. injections, and associated stress.

Drugs

The NMDA receptor antagonist, MK-801 (Toronto Research Chemicals, Inc.), was injected at dose of 0.1mg/kg dissolved in sterile saline administered at a volume of 1ml/kg i.p. 30 minutes prior to reactivation (Heath et al., 2015). The protein synthesis inhibitor, cycloheximide (50mg/ml; Sigma-Aldrich, Inc.), was dissolved in 50%DMSO/50%aCSF and infused bilaterally into the BLA immediately following reactivation (Kochli et al., 2015). Injectors (28-gauge; Plastics One, Roanoke, VA) were connected to 10 µl Hamilton syringes using clear polyethylene tubing. The injectors were inserted into the cannulae so that they extended 1 mm below the guide. Infusions were delivered via an infusion pump (KD Scientific, Inc., Holliston, MA) at a rate of 0.1µl/min for 5 min, with 2 min following infusion to allow for diffusion. In control rats, the vehicle was infused into the same location at the same rate and duration.

Histology

Following behavioral testing, rats were anesthetized and infused with Cresyl Violet acetate (10% in distilled water; Sigma-Aldrich, Inc.) into each site using the same volume as drug infusions. Rats were then perfused with 0.9% saline followed by 10% formalin. Brains were sliced in 50 µm coronal sections through the entire extent of the amygdala. Sections were stained with 0.5% thionin (Sigma-Aldrich, Inc.) as described previously (Kochli et al., 2015). Infusion locations were verified using a light microscope by an observer who was blind to the condition and behavior of each animal.

Results

Experiment 1

Discrimination performance was determined by comparing time spent freezing in CTX+ and CTX- at the end of training (training days eight and nine, respectively). A paired-samples t-test revealed that animals froze significantly more to CTX+ (day 8) than to CTX- (day 9) [$t(37) = 12.66, p < 0.001$]. Difference score did not vary as a function of group assignment [$p < 0.05$]. Animals that did not spend at least 25% more time freezing in CTX+ than CTX-, demonstrating a failure to form the context discrimination, were excluded from subsequent analyses.

Five animals were excluded as per discrimination criterion. An additional six animals were excluded from analysis due to misplaced cannula. Thus, a total of 33 animals were included in the data analyses (see Figure 1B). The final number of animals in each group was as follows: vehicle/vehicle = 6, vehicle/cycloheximide = 10, MK-801/vehicle = 9, MK-801/cycloheximide = 8.

Across the nine days of discrimination training, all rats show an increase in freezing to CTX+ and an initial increase followed by a decrease in freezing to CTX- (Figure 1C). By the end of discrimination training, a paired-samples t-test revealed that animals froze significantly more to CTX+ (day 8) than to CTX- (day 9) [$t(32) = 16.56, p < 0.001$]. During the reactivation session, animals injected with MK-801 thirty minutes prior froze significantly less than those injected with vehicle, [$t(31) = 2.92, p < 0.01$] (see Figure 1D). A mixed-factor

ANOVA revealed an extinction day x injection interaction [$F(4,116) = 2.45, p = 0.05$]. *A priori* planned comparisons revealed that on days four and five of extinction, animals in the MK-801/cycloheximide condition froze significantly less than those in the vehicle/vehicle condition [$ps < 0.05$]. However, neither MK-801 alone nor cycloheximide alone were significantly different from vehicle controls at any point throughout extinction testing [$ps > 0.05$] (see Figure 1E). A two-way ANOVA revealed no differences among groups during the CTX- test [$p > 0.05$] (see Figure 1F). Repeated measures ANOVAs were performed to analyze freezing during each 30 sec time bin across each day of extinction. These data are presented as additional content in Supplementary Information online (see Figure S1).

Experiment 2

Discrimination performance was determined by comparing time spent freezing in CTX+ and CTX- at the end of training (training days eight and nine, respectively). A paired-samples t-test revealed that animals froze significantly more to CTX+ (day 8) than to CTX- (day 9) [$t(32) = 10.01, p < 0.001$]. Difference score did not vary as a function of group assignment [$ps < 0.05$]. Animals that did not spend at least 25% more time freezing in CTX+ than CTX-, demonstrating a failure to form the context discrimination, were excluded from subsequent analyses.

Eight animals were excluded as per discrimination criterion. An additional two animals were excluded from analysis due to misplaced cannula. Thus, a total of 25 animals were included in the data analyses (see Figure 2B). The number of animals in each group was as follows: vehicle/vehicle = 7, MK-801/cycloheximide = 8, no surgery = 10.

Across the nine days of discrimination training, all rats show an increase in freezing to CTX+ and an initial increase followed by a decrease in freezing to CTX- (Figure 2C). By the end of discrimination training, a paired-samples t-test revealed that animals froze significantly more to CTX+ (day 8) than to CTX- (day 9) [$t(24) = 14.65, p < 0.001$]. A mixed-factor ANOVA revealed an extinction day x condition interaction [$F(5,150) = 1.90, p < 0.05$]. *A priori* planned comparisons revealed that on days three, four, and five of extinction, animals in the MK-801/cycloheximide condition froze significantly less than those in the vehicle/vehicle and no surgery conditions [$ps < 0.05$]. Importantly, vehicle/vehicle and no surgery conditions did not differ over the course of extinction [$ps > 0.05$] (see Figure 2D). A mixed-factor ANOVA revealed no effect of condition over the course of acquisition of context fear re-training [$p > 0.05$]. A two-way ANOVA revealed no differences among groups during the re-training test [$p > 0.05$] (see Figure 2E). Repeated measures ANOVAs were performed to analyze freezing during each 30 sec time bin across each day of extinction. These data are presented as additional content in Supplementary Information online (see Figure S2).

Discussion

The present results show that pre-reactivation injections of MK-801 attenuate freezing during the reactivation session. This is not a surprising finding, as other studies have demonstrated that NMDA receptor antagonism results in impairment in the retrieval of aversive memories (e.g., Lee et al., 2006, but see Ben Mamou et al., 2006). It is worth noting that there is evidence to suggest that a deficit in freezing during the reactivation session may be

attributable to motoric effects of MK-801, as this drug is known to increase activity in an open field test (e.g. Danysz, Essmann, Bresink, & Wilk, 1994; Hasegawa, Inoue, Kawaminami, & Fujita, 2016). While it is impossible to directly assess whether this deficit is attributable to motoric or mnemonic effects (see Godsil, Stefanacci, & Fanselow, 2005), the distinction is irrelevant to the present work as the drug is no longer present during the subsequent behavioral tests of memory extinction.

Interestingly, there were no significant differences in freezing between any groups on day one of extinction. Methodologically, this session is identical to a non-reinforced CTX+ test typically used to assess the result of reconsolidation-related amnesic manipulations. During this session, animals exhibited uniformly high levels of freezing (~80%). It is not until days four and five of extinction that effects became detectable. Animals injected with MK-801 prior to reactivation and infused with cycloheximide immediately following reactivation froze significantly less than vehicle/vehicle controls on days four and five. Similarly, in experiment 2, non-reactivated animals that were administered MK-801 and cycloheximide froze significantly less than vehicle/vehicle and non-surgery controls on days three, four, and five of extinction.

In experiment 2, extinction training was followed with three daily acquisition sessions in the former CTX-. Every day the rats received a single footshock in order to observe an acquisition curve across days. Non-reactivated control animals were able to acquire and express contextual fear to the former CTX- equally, regardless of the pharmacological manipulations they had received previously. This demonstrates that effects observed are not attributable to manipulations interfering with rats' ability to acquire and/or express conditioned fear responses. Additionally, no-surgery control animals did not differ from vehicle/vehicle animals in their acquisition, extinction, or retraining performance, suggesting that stress and/or tissue damage caused by cannulation procedures did not influence behavioral performance.

Protein synthesis inhibition and NMDAr antagonism are typically sufficient to disrupt memory reconsolidation when administered alone (e.g., Nader et al., 2000; Heath et al., 2015, respectively). Indeed, an identical dose of MK-801 administered systemically prior to memory reactivation was sufficient to disrupt memory reconsolidation in a contextual fear paradigm (Heath et al., 2015). Our previous work has demonstrated that intra-amygdalar administration of cycloheximide using identical surgical coordinates and infusion parameters was sufficient to disrupt auditory and contextual fear consolidation (Kochli et al., 2015). In the present work, neither MK-801 nor cycloheximide alone was sufficient to disrupt reconsolidation, and effects produced were only detectable following extended extinction training. Indeed, observed effects are independent of reconsolidation processes, as a similar pattern of results is observed in no-reactivation control animals. This suggests that some amnesic manipulations may have lingering effects well beyond the intended manipulation window. It is unclear by what mechanism MK-801 and intra-BLA cycloheximide interact to enhance the formation of an extinction memory several days following administration.

Conclusion

In conclusion, the present data suggest that combined administration of systemic MK-801 and local intra-BLA infusion of cycloheximide produces an additive effect in the enhancement of context fear discrimination memory extinction. This effect does not appear to be a reconsolidation-related phenomenon, as the enhancement of extinction occurs in non-reactivated control animals. Further, the effect is not attributable to an impaired ability to acquire or express contextual fear. While the mechanisms governing this effect remain unclear, the present work underscores the need for proper control procedures in reconsolidation research, especially when working with novel behavioral and/or pharmacological approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Experiment timeline. (B) Cannula placement for all animals included in analysis (atlas images modified from The Rat Brain in Stereotaxic Coordinates (3rd ed.), Bregma -2.56mm and Bregma -3.14mm, by G. Paxinos & C. Watson, 1997, San Diego, CA: Academic Press, Inc with permission from Elsevier). The number of animals in each group was as follows veh/veh = 6, veh/cyclo = 10, MK-801/veh = 9, MK-801/cyclo = 8; N = 33. (C–F) The percentage of time spent freezing during each training session (C), the reactivation session (D), each extinction session (E), and the CTX– test (F). Bars represent ± 1 SEM.

Figure 2.

(A) Experiment timeline. (B) Cannula placement for all animals included in analysis (atlas images modified from The Rat Brain in Stereotaxic Coordinates (3rd ed.), Bregma -2.56mm and Bregma -3.14mm, by G. Paxinos & C. Watson, 1997, San Diego, CA: Academic Press, Inc with permission from Elsevier). The number of animals in each group was as follows: veh/veh = 7, MK-801/cyclo = 8, no surgery = 10; N = 25. (C-E) The percentage of time spent freezing during each training session (C), each extinction session (D), and retraining (E). Bars represent ± 1 SEM.