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Appetitive memory reconsolidation depends upon NMDA receptor-mediated neurotransmission.

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Abstract

Memory persistence is a dynamic process involving the reconsolidation of memories after their reactivation. Reconsolidation impairments have been demonstrated for many types of memories in rats, and signalling at *N*-methyl-D-aspartate (NMDA) receptors appears often to be a critical pharmacological mechanism. Here we investigated the reconsolidation of appetitive pavlovian memories reinforced by natural rewards. In male Lister Hooded rats, systemic administration of the NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-*5H*-dibenzo{a,d}cyclohepten-5,10-imine maleate (MK-801, 0.1 mg/kg i.p.) either before or immediately following a brief memory reactivation session abolished the subsequent acquisition of a new instrumental response with sucrose conditioned reinforcement. However, only when injected prior to memory reactivation was MK-801 effective in disrupting the maintenance of a previously-acquired instrumental response with conditioned reinforcement. These results demonstrate that NMDA receptor-mediated signalling is required for appetitive pavlovian memory reconsolidation.

Introduction

Reconsolidation refers to the process that is disrupted when amnesia for an old memory is effected in a manner critically dependent upon the reactivation of that memory at the time of amnesic treatment (Lewis, Bregman, and Mahan, 1972; Nader, 2003). Such reactivation-dependent amnesia was initially demonstrated in rats using electroconvulsive shock treatment (Misanin, Miller, and Lewis, 1968; Schneider and Sherman, 1968), and has since been described in a wide variety of memory systems across a number of species (Child, Epstein, Kuzirian, and Alkon, 2003; Eisenberg and Dudai, 2004; Litvin and Anokhin, 2000; Pedreira, Perez-Cuesta, and Maldonado, 2002; Rose and Rankin, 2006; Sangha, Scheibenstock, and Lukowiak, 2003; Stollhoff, Menzel, and Eisenhardt, 2005; Suzuki, Josselyn, Frankland, Masushige, Silva, and Kida, 2004), including humans (Forcato, Burgos, Argibay, Molina, Pedreira, and Maldonado, 2007; Hupbach, Gomez, Hardt, and Nadel, 2007; Walker, Brakefield, Hobson, and Stickgold, 2003).

Beginning with the demonstration that the reconsolidation of conditioned fear memories in rats relies upon *de novo* protein synthesis in the basolateral amygdala (BLA)(Nader, Schafe, and Le Doux, 2000), the neural substrates of reconsolidation of many types of memories in rodents have been elucidated (Akirav and Maroun, 2006; Debiec, LeDoux, and Nader, 2002; Eisenberg, Kobil, Berman, and Dudai, 2003; Kelly, Laroche, and Davis, 2003; Lee, Everitt, and Thomas, 2004; Morris, Inglis, Ainge, Olverman, Tulloch, Dudai, and Kelly, 2006; Wang, Ostlund, Nader, and Balleine, 2005), though reactivation-dependent amnesia remains to be observed in certain experimental paradigms (Biedenkapp and Rudy, 2004; Hernandez and Kelley, 2004). While the study of fear memory reconsolidation may point to potential treatments for post-traumatic stress disorder (Debiec and LeDoux, 2006), much

attention has now turned to impairing drug memory reconsolidation as a treatment strategy for prolonging abstinence and preventing relapse in drug addiction (Bernardi, Lattal, and Berger, 2006; Lee, Di Ciano, Thomas, and Everitt, 2005; Lee, Milton, and Everitt, 2006a; Milekic, Brown, Castellini, and Alberini, 2006; Miller and Marshall, 2005; Milton, Lee, and Everitt, 2008; Valjent, Corbille, Bertran-Gonzalez, Herve, and Girault, 2006; Yim, Moraes, Ferreira, and Oliveira, 2006).

We have demonstrated that conditioned stimulus (CS)–sucrose memories also undergo reconsolidation, being dependent upon β -adrenergic signaling (Milton et al., 2008). While we have identified several mechanisms of addictive drug memory reconsolidation, such as β -adrenergic signaling and upregulation of the immediate-early gene *zif268*, in drug seeking procedures (Lee et al., 2005; Lee et al., 2006a; Milton et al., 2008), little is known about the reconsolidation of appetitive memories involving natural rewards. Therefore, we have employed the acquisition of a new response with sucrose conditioned reinforcement procedure (Lee et al., 2005) to investigate further the pharmacological mechanisms of appetitive CS–US memory reconsolidation, using the non-competitive N-methyl-*D*-aspartate (NMDA) receptor antagonist MK-801 to test the functional requirement of glutamatergic signaling at NMDA receptors. MK-801 has previously been demonstrated to impair memory reconsolidation in a variety of tasks (Kelley, Anderson, and Itzhak, 2007; Lee, Milton, and Everitt, 2006b; Przybylski and Sara, 1997). We also studied the effects of administration of MK-801 in conjunction with memory reactivation on the persistent responding for conditioned reinforcement that has been demonstrated for both natural- and drug-associated conditioned reinforcers (Di Ciano and Everitt, 2004a; Grimm, Hope, Wise, and Shaham, 2001).

Materials and methods

Subjects

The subjects were 84 experimentally naïve adult male Lister Hooded rats, weighing 250-300 g. 44 rats were used in Experiment 1 and 40 in Experiment 2. They were housed in pairs, in holding rooms maintained at 21°C on a reversed-light cycle (12 hours light: 12 hours dark; lights on at 19:00). Food was restricted to 15 g/day and water was freely available throughout the experiment. All procedures were conducted in accordance with the United Kingdom 1986 Animals (Scientific Procedures) Act (Project License PPL 80/1767).

Drug administration

(+)-MK-801 hydrogen maleate (Sigma, Poole, UK) was dissolved in sterile saline for intra-peritoneal injection (1 ml/kg). The dose of MK-801 (0.1 mg/kg) selected has previously been shown to impair the reconsolidation of conditioned fear memories (Lee et al., 2006b). On the final 2 days of training, rats were habituated to the intra-peritoneal injection procedure using the saline vehicle.

Behavioral procedures

Pavlovian acquisition

All procedures were carried out in 12 operant chambers (Med Associates, Laffayette, IN, USA) as described previously (Hellemans, Dickinson, and Everitt, 2006). During 9 days of training, the rats were placed individually in the operant chambers for 20 min. No levers were present during pavlovian acquisition. Each nosepoke response into the food magazine was reinforced by a 5-s elevation of the liquid dipper (1.0 ml of 10% sucrose). The CS light (right or left, counterbalanced) was illuminated during, and for 5 s after reward delivery (total 10 s presentation). Nosepoke responses made during the CS were recorded, but were not reinforced.

Acquisition of a new instrumental responses with conditioned reinforcement

To measure the conditioned reinforcing properties of the CS, established by the CS–sucrose association during conditioning, its ability to support the acquisition of a new response (ANR) was assessed. Two levers were extended into the chamber; a response on the lever located beneath the CS light (inactive lever) had no programmed consequence, whereas a response on the opposite (active) lever was followed by a 1-s illumination of the CS light, during which the house light was extinguished. Disrupting the reconsolidation of the CS–sucrose memory leads to a loss of the acquired conditioned reinforcing properties of the CS and hence failure to support the learning of the new instrumental response (discriminative responding on the active vs. inactive lever). Nosepoke responses had no programmed consequence, the liquid dipper was never activated, and the number of active and inactive lever presses was recorded during the 30 min sessions.

Memory reactivation

Experiment 1: to assess the effects of reactivation-related amnestic treatment upon the acquisition of the new instrumental response, the CS–sucrose memory was reactivated on the day after the final pavlovian training session. In a single 10-min session, the rats were returned to the operant chambers and received a presentation of the 10-s CS alone, following each nosepoke response. No sucrose was available. Rats were injected with MK-801 or the saline vehicle, either 30 min before the start of the reactivation session, or immediately after its termination. The effect of the treatment on the CS–sucrose memory was subsequently tested in 4 sessions of ANR, on days 1, 2, 5 & 8 after memory reactivation. Non-reactivated control groups were injected in

the holding room on the same day, and were immediately returned to their home cages without exposure to the memory reactivation session.

Experiment 2: to investigate the impact of the same treatments upon the maintenance of a previously-acquired new response with conditioned reinforcement, rats were first tested on the acquisition of responding for conditioned reinforcement for 6 sessions, or until they reached a criterion of at least 30 active lever responses in consecutive sessions. On the next day, they were subjected to a non-reactivation treatment, the injections of MK-801 and saline (rats randomly allocated to each treatment group) being administered without any behavioral session. The following day, a further test session for responding with conditioned reinforcement was conducted (non-reactivated test). The CS–sucrose memory was then reactivated in a manner identical to Experiment 1 (R1; 10-s CS presented contingent upon nosepoke response; rats receiving pre- or post-session injections), and tested in a subsequent session. Finally, as the previous treatments had no effect on the maintenance of responding with conditioned reinforcement, the rats were again subjected to the same treatments, but the CS–sucrose memory was reactivated in a normal 30-min ANR test session (R2), and the subsequent maintenance of responding with conditioned reinforcement was tested in 4 sessions over the following week.

Statistical Analysis

The variance of lever pressing tends to increase in proportion to the mean (Dickinson and Dawson, 1987; Winer, 1991), and so the data were checked for homogeneity of variance. All raw lever press data failed to conform to homogeneity of variance requirements of ANOVA and so were square root transformed prior to statistical analysis. Data are thus presented as mean + SEM square root lever presses. Data were also checked for sphericity, and the Greenhouse-Geisser correction was used as

appropriate. As the behavior of rats injected with saline either prior to or following memory reactivation did not differ, these were collapsed into a single saline control group. Planned comparisons included an analysis of active vs inactive lever responses for each group, and a significance level of $p < 0.05$ was selected for all analyses.

Results

Acquisition of the new instrumental response

Administration of MK-801 resulted in a reactivation-dependent impairment in the acquisition of a new instrumental sucrose seeking response measured subsequently. The reactivated control saline-treated group learned to respond on the active lever for the CS over the four sessions of acquisition (1, 2, 5, and 8 days after reactivation), and responding was significantly higher than on the inactive lever (Fig. 1). In contrast, the reactivated MK-801 treated groups made many fewer responses on the active lever than control rats. Moreover, rats injected with MK-801 at memory reactivation showed no preference subsequently for the active lever over the inactive lever for up to 8 days after reactivation. The impairments in the acquisition of a new response were critically dependent upon reactivation of the CS-drug memory, since rats that were injected with MK-801, but with the memory reactivation session omitted, readily learned the new instrumental response with conditioned reinforcement. Saline and MK-801 treated rats in the nonreactivated condition thus showed a strong preference for the active lever over the inactive lever (Fig 1C). An overall comparison of reactivated and nonreactivated groups revealed a reactivation-dependent effect of treatment upon discriminated responding across all four test sessions, which indicates a persistent impairment in learning the new response.

Importantly, there was no difference between the groups in overall (inactive and active) lever-pressing activity or nosepoke responses during the test sessions (data not shown; Reactivation x Treatment: p 's>0.22; Reactivation x Treatment x Session: p 's>0.68), which reveals that there was no deficit in general motivation or activity. Furthermore, the reactivation dependence of the impairment demonstrates that MK-801 had no nonspecific effects on lever pressing performance. Prior to the conditioned reinforcement test, all groups acquired the nosepoke response for sucrose reinforcement, and the total number of CS–sucrose pairings was similar across all groups (data not shown; all group means between 262 and 269.4 pairings; $F_{(4,43)}=1.47$, $p>0.23$). Importantly, therefore, conditioning of the CS–sucrose association was equivalent in all groups. Finally, during the reactivation session all groups received similar numbers of nonreinforced CS exposures (Fig. 2). Therefore, the impairments in the acquisition of a new response cannot be attributed to prior differences in conditioning, CS exposure or extinction.

Performance of the acquired instrumental response with conditioned reinforcement

All groups acquired the new instrumental response with sucrose conditioned reinforcement prior to treatment (Fig. 3: baseline responding). Following stabilization of responding, the injection of MK-801 on a behavioural rest day had no effect on subsequent lever pressing (Fig. 3: test). Furthermore, MK-801, administered either pre-trial or post-trial, had no effect when injected in conjunction with a memory reactivation session in which the CS was presented contingent upon the original nosepoke sucrose-taking response (Fig. 4: R1; levers were not present; on average 13.9 CS presentations during the reactivation session with no effect of treatment, $F<1$). Only when the CS–sucrose memory was reactivated through contingent

presentations of the CS upon the new lever press response was an effect observed (Fig. 4: R2; on average 27.3 CS presentations during the reactivation session with no effect of treatment, $F < 1$). Under these conditions, MK-801 administered pre-trial but not post-trial resulted in a reactivation-dependent impairment of subsequent discriminated responding. The impairment in the MK-801 pre-trial group was persistent and continued to be observed during 3 further sessions up to 8 days following memory reactivation (Fig 5).

Importantly, MK-801 given pre-R2 had no effect on overall (inactive and active) lever-pressing activity or nosepoke responses during the test sessions (data not shown; Reactivation x Treatment: $F < 1$), which reveals that there was no deficit in general motivation or activity. Furthermore, the reactivation dependence of the impairment demonstrates that MK-801 had no nonspecific effects on lever pressing performance. Finally, during the R2 reactivation session all groups received similar numbers of nonreinforced CS exposures (data not shown; $F < 1$), and so the MK-801-induced impairment in the acquisition of a new response cannot be attributed to prior differences in CS exposure or extinction.

Discussion

The present results demonstrate that appetitive pavlovian associations reinforced by natural rewards undergo memory reconsolidation after their reactivation. We used an acquisition of a new response (ANR) procedure that measures the conditioned reinforcing properties of a sucrose-associated CS to investigate the pharmacological mechanisms of appetitive CS–US memory reconsolidation. We found that systemic administration of the NMDA receptor antagonist MK-801 resulted in a reactivation-dependent impairment in the subsequent ANR. The amnesic effect of MK-801 was

equally profound whether administered 30 min prior to the reactivation session or immediately following its termination. However, only when given prior to memory reactivation was MK-801 effective in reducing the maintenance of responding with conditioned reinforcement once the instrumental response had already been established.

The ANR procedure tests specifically the conditioned reinforcing properties of appetitive conditioned stimuli (Mackintosh, 1974). The acquisition of a new instrumental response with conditioned reinforcement depends upon the prior explicit pairing of stimuli with a rewarding US (Parkinson, Roberts, Everitt, and Di Ciano, 2005; Taylor and Robbins, 1984), and so a failure of rats to acquire the new instrumental response may reflect disruption of one or more of several processes. An inability to acquire new instrumental associations may disrupt ANR, as would a failure to retrieve the previously learned CS–US association. However, given that in the present study the amnesic treatment was administered 24 hours before the first session of ANR, and that its deleterious effects were critically dependent upon the memory reactivation session, these do not provide explanations of possibly acute effects of MK-801 on learning and retrieval during the ANR sessions. Instead, the most parsimonious account of the present data is that MK-801 impaired the reconsolidation of the CS–US memory. Indeed this interpretation explains our previous results (Lee et al., 2005; Milton et al., 2008), contrary to the misinterpretation adopted by Milekic et al. (2006) that the impairment in learning the new instrumental response reflects a deficit in consolidation rather than reconsolidation. Nevertheless, there remains the unresolved question of whether reconsolidation impairments, including those observed here, reflect a long-term deficit in memory storage or retrieval.

The conditioned reinforcing properties of appetitive stimuli, as measured here by their ability to support the learning of a new instrumental response, can be dissociated neurally from other acquired properties of appetitive conditioned stimuli. For example, whereas conditioned reinforcement is dependent upon the basolateral, but not central nuclei of the amygdala (Burns, Robbins, and Everitt, 1993; Robledo, Robbins, and Everitt, 1996), the acquired incentive and motivational properties of CSs, as measured in autoshaping and pavlovian-instrumental transfer studies depend specifically on the central nucleus of the amygdala (Hall, Parkinson, Connor, Dickinson, and Everitt, 2001; Parkinson, Robbins, and Everitt, 2000). Therefore a likely primary locus of action of MK-801 is the BLA, consistent with both the finding that CS–cocaine memory reconsolidation was impaired by infusions of the β -adrenergic receptor antagonist propranolol and an antisense oligodeoxynucleotide for *Zif268* directly into the BLA (Lee et al., 2005; Lee et al., 2006a; Milton et al., 2008). Nevertheless, the amnesic effects of MK-801 may be mediated by actions at other neural sites in addition to the BLA.

Reactivation of both a conditioned fear memory and a CS–cocaine association results in an upregulation of *Zif268* expression in the core region of the nucleus accumbens as well as in the BLA (Hall, Thomas, and Everitt, 2001; Thomas, Arroyo, and Everitt, 2003; Thomas, Hall, and Everitt, 2002). As *Zif268* expression in the BLA has been shown to be necessary for the reconsolidation of both types of memories (Lee et al., 2005), it nevertheless remains possible that functional plasticity in the nucleus accumbens core is involved in memory reconsolidation. Therefore, glutamatergic signalling may also be required in the nucleus accumbens, and hence the amnesic effect of systemically administered NMDA receptor antagonists might be mediated not only by the BLA but also by the nucleus accumbens core. This is

consistent both with a report of impaired drug memory reconsolidation in a morphine conditioned place preference procedure after infusions of the protein synthesis inhibitor anisomycin into the nucleus accumbens (Milekic et al., 2006), and with the anatomical and functional connectivity between the BLA and the nucleus accumbens core that is necessary for the conditioned reinforcing effects of appetitive CSs (Di Ciano and Everitt, 2004b).

It is of note that the reactivation-dependent amnesia for the CS–sucrose memory was observed following reexposure to the CS alone, consistent with our previous studies with cocaine-associated memories (Lee et al., 2005; Milton et al., 2008). In contrast, use of the conditioned place preference procedure to investigate appetitive drug-related memory reconsolidation has led to conflicting results regarding the stimulus reexposure requirements of reactivation-dependent amnesia (Bernardi et al., 2006; Milekic et al., 2006; Miller and Marshall, 2005; Valjent et al., 2006; Yim et al., 2006). While two studies found that reexposure to the place preference apparatus alone was sufficient (Bernardi et al., 2006; Miller and Marshall, 2005), the others suggested that the reactivation requirements were more stringent, including re-exposure to the US. Therefore, in general it has been more difficult to demonstrate memory reconsolidation deficits using a place preference procedure rather than one that explicitly measures conditioned reinforcement. This may, therefore, contribute to the suitability of the ANR procedure for observing reactivation-dependent amnesia, as the new instrumental response cannot be acquired through alternative mechanisms, such as pavlovian approach or contextual influences. In contrast, conditioned place preference might be mediated by conditioned approach to, or conditioned reinforcement by, both discrete and contextual stimuli.

When rats were allowed to acquire the new instrumental response prior to amnesic treatment, MK-801 was effective in reducing subsequent lever pressing when injected prior to the memory reactivation session. Furthermore, the amnesia was dependent upon specific parameters of memory reactivation. Stimulus reexposure achieved through returning to the original training situation, where it was contingent upon the nosepoke sucrose taking response, albeit in extinction, was not effective. However, a markedly lower number of CS presentations was delivered than for the ANR acquisition experiment (on average 13.7 vs. 23.5), likely to be a result of progressive extinction of the nosepoke response as the new instrumental response is learned over several sessions. When the CS–sucrose memory was reactivated by CS presentations contingent upon the acquired lever-press response, a clear effect of pre-activation MK-801 was observed. The mean number of CS presentations during that ANR reactivation session was 25.3, suggesting that a threshold of CS presentation is required sufficiently to reactivate the memory and render it subject to disruption. This account is consistent with a previous study of contextual fear conditioning (Suzuki et al., 2004). However, the present data also indicate that it is not the length of CS presentation that is critical in reactivating the CS–sucrose memory, but rather the number of punctate CS presentations, as the absolute duration of CS presentation during the successful reactivation was only 25 s compared to 137 s of the long CS presentations delivered upon nosepoke responses.

Whereas MK-801 administered either 30 min prior to or immediately after memory reactivation impairs the conditioned reinforcing properties of the CS measured in the later acquisition of a new response phase, only pre-activation treatment with MK-801 impaired the subsequent persistent responding with conditioned reinforcement. The failure of post-trial MK-801 to induce amnesia in the

latter setting may simply be a consequence of the longer instrumental session required to reactivate the memory. The sessions of responding with conditioned reinforcement were 30 min long, compared to the 10-min nosepoke reactivation session. Therefore, the timing of the post-trial MK-801 injection relative to the start of the reactivation session is delayed by 20 min in the performance experiment as compared to the acquisition study. This account would suggest that not only is some continued NMDA receptor-mediated neural transmission required following reactivation in order to reconsolidate the memory, as evidenced by the amnesic effect of post-reactivation NMDA receptor antagonism here in the acquisition experiment and elsewhere (Akirav and Maroun, 2006; Przybylski and Sara, 1997; Torras-Garcia, Lelong, Tronel, and Sara, 2005), but there is a limited time window during which disruption of this activity can impair the reconsolidation process.

In summary, the conditioned reinforcing properties of an appetitive CS previously associated with sucrose reinforcement undergo reconsolidation in a manner dependent upon NMDA receptor signalling. Thus antagonism of the NMDA receptor at memory reactivation results in the persistent inability both to acquire a new instrumental response with conditioned reinforcement, and to maintain previously learned responding with conditioned reinforcement.

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Figure 1. MK-801 impaired the subsequent acquisition of a new sucrose seeking response. Active and inactive lever presses were compared over four testing sessions for both reactivated (**A, B**) and non-reactivated (**C**) conditions. **A**, MK-801 administered 30 min prior to memory reactivation impaired the acquisition of discriminated responding in a reactivation-dependent manner (Treatment x Reactivation x Lever: $F_{(1,32)}=8.56, p<0.01$; Treatment x Reactivation x Lever x Session: $F<1$; Treatment x Reactivation: $F_{(1,32)}=1.54, p>0.22$). MK-801 treated rats did not respond more on the active than the inactive lever (Lever: $F<1$; Lever x Session: $F_{(2,21)}=2.09, p>0.14$). **B**, MK-801 administered immediately after memory reactivation impaired the acquisition of discriminated responding in a reactivation-dependent manner (Treatment x Reactivation x Lever: $F_{(1,30)}=6.76, p<0.02$; Treatment x Reactivation x Lever x Session: $F<1$; Treatment x Reactivation: $F<1$). MK-801 treated rats did not respond more on the active than the inactive lever (F 's <1). Data presented as mean \pm SEM.

Figure 2. Number of CS presentations during the memory reactivation session. All groups received similar numbers of CS presentations ($F_{(2,27)}=1.74, p>0.19$; Saline vs. MK-801 pretrial: $F_{(1,16)}=4.39, p>0.05$). Data presented as mean + SEM.

Figure 3. MK-801 administered in the absence of memory reactivation had no effect on subsequent performance of responding with conditioned reinforcement. Rats were previously trained to acquire the new instrumental response with sucrose conditioned reinforcement. When they had reached a stable level of responding (baseline) the rats were injected with MK-801 or saline and then tested on the next day (test).

Figure 4. Only MK-801 administered pre-reactivation impaired subsequent maintenance of a sucrose seeking response with conditioned reinforcement. Following the non-reactivation test, rats were rebaselined. The experimental timeline (**A**) shows that the CS–sucrose memory was reactivated contingently upon the original sucrose taking nosepoke response (**R1**) prior to a test 24 hr later. Following a further period of rebaselining, the CS–sucrose memory was reactivated again, this time contingently upon the acquired active lever press response (**R2**), and was tested 24 hr later. Rats were administered with saline (**B**), MK-801 pretrial (**C**) or MK-801 posttrial (**D**). Active and inactive lever presses were compared for pre-reactivation baseline and test. When the CS was presented contingently upon the original nosepoke sucrose-taking response during memory reactivation (**R1**), none of the treatments affected subsequent discriminated responding (Treatment x Reactivation x Session x Lever: F 's<1). However, MK-801 administered 30 min prior to a memory reactivation session in which the CS was presented contingently upon the acquired lever press response (**R2**) impaired the maintenance of discriminated responding in a reactivation-dependent manner (Treatment x Reactivation x Session x Lever: $F_{(1,28)}=6.21, p<0.02$; Treatment x Reactivation x Session: $F<1$). In contrast, MK-801 administered post-R2 had no effect on subsequent responding (Treatment x Reactivation x Session x Lever: p 's>0.28). Data presented as mean + SEM. * $p<0.05$.

Figure 5. MK-801 treated rats are persistently impaired in responding with sucrose conditioned reinforcement. Active and inactive lever presses were compared for rats treated with saline (**A**) or MK-801 (**B**) prior to memory reactivation. Discriminated responding was significantly impaired across all four test sessions (Treatment x

Lever: $F_{(1,12)}=18.74, p<0.01$; Treatment x Session x Lever: $F<1$). Data presented as mean + SEM.

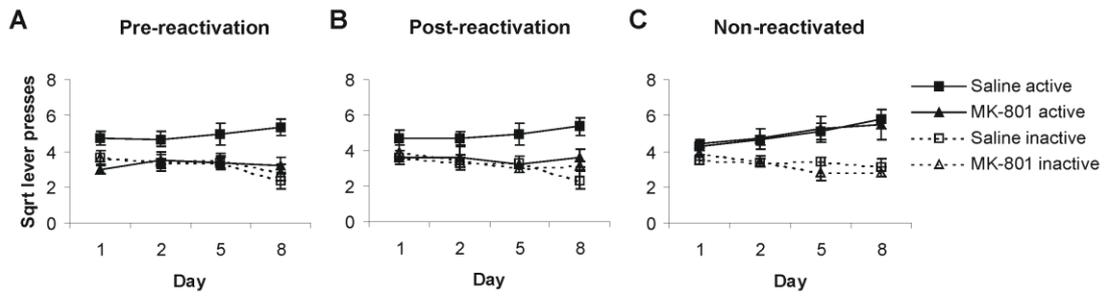


Fig. 1

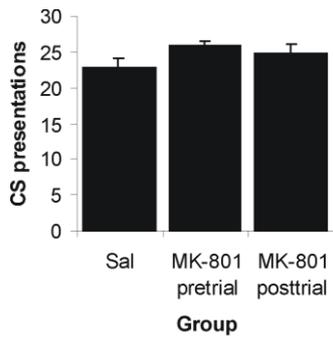


Fig. 2

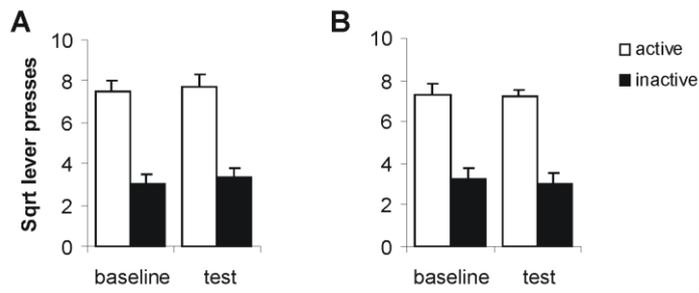


Fig. 3

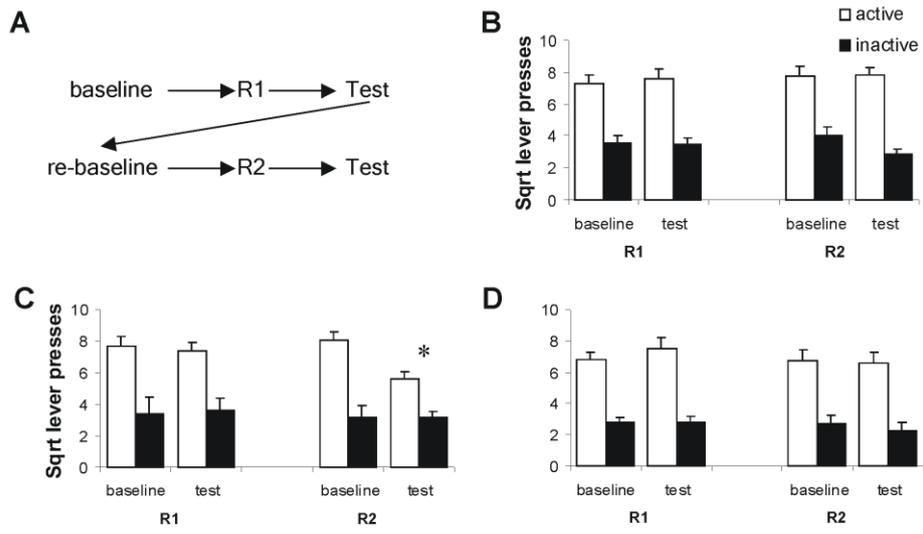


Fig. 4

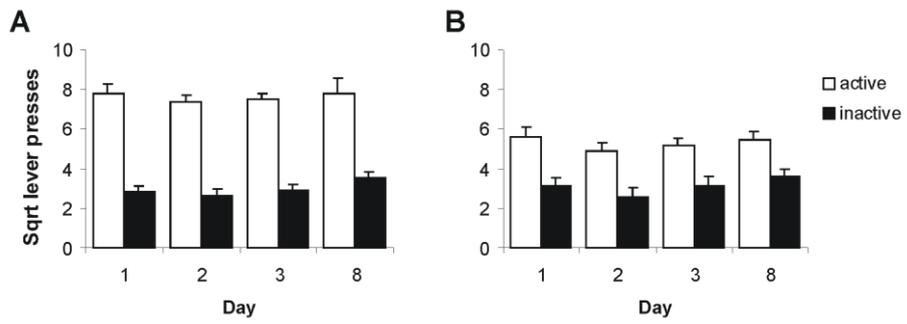


Fig. 5