The CB1 receptor antagonist AM251 impairs reconsolidation of pavlovian fear memory in the rat basolateral amygdala

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ABSTRACT

We have investigated the requirement for signaling at CB1 receptors in the reconsolidation of a previously consolidated auditory fear memory, by infusing the CB1 receptor antagonist AM251, or the FAAH inhibitor URB597, directly into the basolateral amygdala (BLA) in conjunction with memory reactivation. AM251 disrupted memory restabilisation, but only when administered post-reactivation. URB597 produced a small, transient enhancement of memory restabilisation when administered post-reactivation. The amnestic effect of AM251 was rescued by co-administration of the $GABA_A$ receptor antagonist bicuculline at reactivation, indicating that the disruption of reconsolidation was mediated by altered GABAergic transmission in the BLA. These data show that the endocannabinoid system in the BLA is an important modulator of fear memory reconsolidation and that its effects on memory are mediated by an interaction with the GABAergic system. Thus, targeting the endocannabinoid system may have therapeutic potential to reduce the impact of maladaptive memories in neuropsychiatric disorders such as post-traumatic stress disorder.

Keywords: behavioral science, cannabinoids, learning & memory, mood / anxiety / stress disorders, reconsolidation, conditioned fear, PTSD, rat

INTRODUCTION

Memory reconsolidation is the process by which a well-consolidated memory returns to a labile state and becomes susceptible to manipulation (Lewis, 1979; Nader, 2003). This process has been extensively investigated in the context of pavlovian conditioned fear memories, where pharmacological manipulation at memory reactivation can prevent (Dębiec *et al*, 2002; Milton *et al*, 2013; Nader *et al*, 2000) or enhance (Lee *et al*, 2006) the subsequent expression of the conditioned fear response. Thus, it has been argued that targeting the reconsolidation process may provide a novel means of disrupting maladaptive memories in neuropsychiatric disorders such as post-traumatic stress disorder (PTSD; Brunet *et al*, 2008; Schiller *et al*, 2010), persistently reducing symptoms of the disorder following only a single (or few) treatment sessions combining behavioral and pharmacological therapy. However, any amnestic agent used in the clinic ideally should not also have adverse side effects; therefore, identifying new drug targets for disrupting memory reconsolidation is of critical importance in translating these promising findings to the clinic.

Drugs that alter levels of endogenous endocannabinoids may provide a new drug target for treating psychiatric disorders using reconsolidation-based therapies. Growing evidence indicates a fundamental role for the endocannabinoid system in regulating memory consolidation (Campolongo *et al*, 2009; Hauer *et al*, 2011). However, less is known about endocannabinoid signaling in reconsolidation (De Oliveira Alvares *et al*, 2008; Kobilo *et al*, 2007), especially within the amygdala (Bucherelli *et al*, 2006; Lin *et al*, 2006). Furthermore, the involvement of endocannabinoid signaling in the reconsolidation process may not be straightforward, with apparently conflicting results having been reported in the literature: while agonism and antagonism at endocannabinoid receptors (CBRs) bidirectionally modulates the reconsolidation of aversive memories by respectively enhancing and impairing memory (De Oliveira Alvares *et al*, 2008; Suzuki *et al*, 2008), the CBR agonist, WIN55,212-2 *impairs* reconsolidation in fear-potentiated startle procedures after CS re-exposure (Lin *et al*, 2006). Treatment with the CBR subtype 1 (CB1R) antagonist rimonabant neither enhanced pavlovian fear memory nor resulted in amnesia (Suzuki *et al*, 2004); however, rather than indicating that reconsolidation is not dependent on CB1Rs, these data may instead reflect a requirement for

CB1Rs in the *destabilization* of memory (Suzuki *et al*, 2008). As the effects of blocking the restabilization of a reconsolidating memory can only be seen when memory destabilization has occurred (Ben Mamou *et al*, 2006; Milton *et al*, 2013), we hypothesized that the timing of the endocannabinoid manipulation may be critical in determining the behavioral outcome of the memory manipulation; we therefore predicted that if endocannabinoid signaling manipulations can disrupt memory destabilization or restabilization depending on treatment timing, amnesia would only be observed if the endocannabinoid system was targeted *after* memory reactivation. This is critically important for the future translation of reconsolidation-based therapies to the clinic.

Here, we have employed the widely-used pavlovian fear conditioning rodent model of PTSD that encompasses some of its key behavioural and physiological symptoms (Dębiec and LeDoux, 2004; Johansen *et al*, 2011; Mahan and Ressler, 2012) to test the hypotheses that: (i) the restabilization of an auditory fear memory would only be impaired if cannabinoid compounds were given following a memory reactivation session; (ii) that memory restabilization would be enhanced by increasing endocannabinoid signaling, (iii) that the requirement for endocannabinoids in reconsolidation would depend upon GABAergic transmission in the BLA. To investigate the necessity of CB1R activation for mnemonic processes, we used the CB1R antagonist AM251. However, as the use of drugs that directly bind and activate brain CBRs, such as WIN55,212-2 administered systemically, may ultimately be precluded from human clinical use by their abuse liability (Fattore *et al*, 2001), we chose to use the fatty-acid amide hydroxylase (FAAH) inhibitor URB597 (URB), which increases endogenous cannabinoid levels, which has no rewarding or reinforcing effects when given systemically (Piomelli *et al*, 2006). Therefore, animals with a well-consolidated auditory fear memory were infused with either URB or the CB1R antagonist AM251 (AM) into the BLA, either before or after fear memory reactivation. As endocannabinoids are hypothesized to regulate GABAergic signaling within the BLA (Azad *et al*, 2004), particularly at the $GABA_A$-subtype of receptor (Katona *et al*, 2001), we also investigated whether antagonism at GABAergic receptors could rescue any memory deficit induced by CB1R blockade.

MATERIALS AND METHODS

Subjects

122 male Lister-Hooded rats (300-320g at the time of surgery, Charles River, Bicester, UK) were pair-housed on a reversed light-dark cycle (lights on at 1900 hrs). All subjects were fed 25 g per rat after behavioral procedures each day starting from the day of surgery; this amount of food maintains animals at a weight comparable to animals that receive food *ad libitum*. Water was available *ad libitum* except during the behavioral and infusion procedures. All procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

Surgery

Rats were anesthetized with a mixture (i.m.) of ketamine (80mg kg^{-1}; Ketaset, Pfizer, Walton-on-the-Hill, UK) and xylazine (10mg kg^{-1}; Rompun, Bayer, Newbury, UK) and implanted with bilateral guide cannulae (16mm, 24 gauge; Coopers Needle Works Ltd, Birmingham, UK) just dorsal to the BLA, as described previously (Milton *et al*, 2008) with co-ordinates of AP - 2.6 mm and ML ± 4.5 mm (from bregma), and DV – 5.6 mm (from dura). Stainless steel obdurators (Coopers Needle Works Ltd.) were inserted into both cannulae to maintain patency. A recovery period of at least 7 days was given prior to behavioral testing.

Drug infusions

Drugs were infused into the BLA using a syringe pump (Harvard Apparatus, Edenbridge, UK) and 5 µl Hamilton syringes, connected to injectors (28 gauge, projecting 2 mm beyond the guide cannulae; Bilaney, Sevenoaks, UK) by polyethylene tubing. All infusions were begun 30 seconds after the insertion of the injectors and performed over 2 minutes at a rate of 0.25 µl per min (total volume of 0.5 µl per side). The injectors were left in place for a further minute after the end of the infusion to allow the drugs to diffuse from the injection site. Although it is anticipated that the effects of infusions delivered at this rate and volume should be largely restricted to the BLA, we cannot exclude that the compounds may have also affected other amygdala nuclei. URB597 (Mor *et al*, 2004), a fatty-acid amide hydroxylase (FAAH) inhibitor

(URB; cyclohexylcarbamic acid 3´-carbamoyl-biphenyl-3-yl ester, 30 ng per 0.5 µl per side, Sigma-Aldrich, Dorset, UK), the CB1R antagonist AM251 (Lan *et al*, 1999; AM; N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3 carboxamide, 300 ng per 0.5 µl per side, Tocris, Bristol, UK) and $GABA_A$ receptor antagonist 1(S),9(R)-(−)-Bicuculline methiodide (BIC; 50 ng per 0.5 µl per side, Sigma-Aldrich) were dissolved in a vehicle (VEH) containing 5% polyethylene glycol, 5% Tween-80 and 90% saline. Dose-response curves for these drugs in fear memory tasks have been reported previously in the literature, so the doses used in the current work were chosen based on previous studies: the URB dose used is higher than the dose required to enhance the consolidation of inhibitory avoidance (IA) memory (Campolongo, 2010; Ratano *et al*, 2011); the AM dose is within the range that impairs olfactory fear conditioning (Tan *et al*, 2011) and higher than the dose that impairs the consolidation of IA memory (Campolongo *et al*, 2009); the dose of BIC impairs the consolidation of IA memory (Dickinson-Anson and McGaugh, 1997) and also the extinction of contextual fear memory (Berlau and McGaugh, 2006).

Histology

After behavioral testing was completed, rats received an overdose of sodium pentobarbital (Dolethal; Rhone-Merieux, Harlow, UK) and were transcardially perfused with 0.01 M PBS, followed by 4% paraformaldehyde (PFA). Brains were collected and stored in 4% PFA for at least 24 hours, before being transferred to 20% sucrose solution for cryoprotection prior to sectioning. The brains were sectioned coronally at 60 µm, stained with Cresyl Violet, and the cannulae placement assessment was conducted under light microscopy (Leica). Only subjects with the injectors located bilaterally within the BLA, and with no bilateral damage to the amygdala or any other area of the brain were included in the statistical analysis (**Figure 1**).

Behavioral procedures

Auditory fear conditioning was performed similarly to as described previously (Lee *et al*, 2005; Lee *et al*, 2006; Milton *et al*, 2013). Briefly, on Day 1, rats were habituated to the conditioning chamber (Med Associates, Sandown Scientific, Hampton, UK) for 2 hours and allowed to freely explore the context. On Day

2, the rats were placed in the same experimental context, and conditioned with two CS-US pairings. The CS was an auditory clicker (10 Hz, 80 dB, 60 s) and the US an electric footshock (0.5 mA, 1 s). The first CS-US pairing was presented after 35 ± 1 minute from the start of the session, followed by a 5 ± 1 minute interval when a second CS-US pairing was given. The conditioning session terminated 5 minutes after the last footshock delivery.

On Day 3, the fear memory was reactivated by re-exposing the rats to the conditioning chamber for a 2-min session, to a single presentation of the 60 s CS after 60 s of context exposure. All rats received an intra-BLA infusion of drug 30 minutes before or immediately (1-5 minutes) after the memory reactivation session. The timing of the pre-reactivation infusions was based on previous studies, where URB597 enhances stress-induced analgesia when administered intracranially 35 minutes prior to test (Hohmann *et al*, 2005) and AM251 blocks the effects of CB1R agonism on the tail flick test for at least an hour after intracranial infusion (Hasanein *et al*, 2007). Non-reactivated control groups underwent the same behavioral procedures, except that, on Day 3, the drug infusions were given in a novel room and they were not re-exposed to either the training context or the CS.

Testing took place 24 h (post-reactivation long-term memory, PR-LTM24h) and 8 days (PR-LTM8d) after reactivation to test long-term memory retention. Animals were returned to the conditioning chambers for a 2-min session where they received a single presentation of the 60-s CS after 60 s of context exposure. Freezing behavior was video-recorded, and conditioned freezing scored offline by an experimenter blind to treatment. Freezing is defined as the lack of movement except for breathing, and was assessed at 5 s intervals to give the percentage of time freezing. Freezing during the first minute of the testing session was assessed as measure of fear to the experimental context, and during the second as measure of fear to the CS.

Statistical Analysis

Data are presented as the mean ± s.e.m. and were analyzed using repeated measures ANOVA, with CS (Context *vs*. Cue) and Session (Reactivation *vs*. PR-LTM24h*vs*. PR-LTM8d) as within-subject factors, and Drug

(VEH *vs*. URB *vs*. AM) as between-subjects factors. Where the data violated the assumption of sphericity as assessed using Mauchly's test, a correction was applied; the Greenhouse-Geisser correction if $\varepsilon < 0.75$, and the Huynh-Feldt correction if $\varepsilon > 0.75$, as recommended by Cardinal & Aitken (2006). Where appropriate, further ANOVAs or pairwise comparisons were conducted; all pairwise comparisons were adjusted using the Šidák correction, which is a mathematically accurate form of the Bonferroni estimation (Cardinal *et al*, 2006).

RESULTS

Pre-reactivation infusion of URB597 and AM251 affected neither retrieval nor reconsolidation of pavlovian fear memory

In the first experiment, the FAAH inhibitor URB597 (URB) or the CB1R antagonist AM251 (AM) or vehicle (VEH) were bilaterally infused into the BLA 30 minutes prior to memory reactivation. All experimental groups had previously conditioned to the CS, as all rats showed a greater freezing response to the CS than to the context during the reactivation session [$F_{1,25}$=41.6, p<0.001, η^2=0.63]. As shown in **Figure 2**, rats in all experimental groups froze similarly to the CS during the test sessions [Drug: F<1], and though conditioned freezing reduced across the test sessions [Session: $F_{2,50}$ =5.83, p=0.005, η^2=0.19], this extinction was the same for all experimental groups [F<1]. Furthermore, analyzing the memory reactivation session alone showed that there were no acute effects of the drugs on the expression of conditioned freezing [F<1]. Thus, neither URB nor AM affected memory retrieval or reconsolidation when given prior to memory reactivation.

AM251 infused immediately post-reactivation disrupted, whereas URB597 slightly and transiently enhanced, fear memory reconsolidation

AM infused into the BLA immediately after memory reactivation disrupted fear memory reconsolidation as assessed at test (**Figure 3**). All rats had previously conditioned to the CS, and all groups showed a greater

fear response to the CS than to the context during the reactivation session [$F_{1,29}$=50.5, p<0.001, η^2=0.64]. However, animals that received AM immediately post-reactivation showed less freezing at test, 24h later, than animals receiving URB or VEH [Drug: $F_{2,29}$= 6.00, p<0.01, η^2=0.29] across both test sessions [Session: $F_{2,29}$=4.78, p<0.05, η^2=0.14] and all experimental groups [Drug x Session: $F_{4,58}$=5.53, p<0.001, η^2=0.28]. Rats infused with AM immediately after the reactivation session froze less at test 24h after reactivation than they had during the reactivation session [PR-LTM24h *vs.* reactivation, p=0.011; PR-LTM8d *vs.* reactivation, p=0.001].

Rats infused with URB post-reactivation showed *higher* levels of freezing at the 24h test than they had shown during the memory reactivation session [PR-LTM24h *vs.* reactivation, p=0.041], but this increased fear response to the CS did not persist to the 8d test [PR-LTM8d *vs.* reactivation, p=0.96]. Thus, intra-BLA infusion of URB may have produced a small, transient enhancement of memory reconsolidation, while the antagonist AM persistently disrupted reconsolidation of pavlovian fear memory.

The effects of intra-BLA AM251 and URB597 on memory reconsolidation were dependent on memory reactivation

To determine whether the effects of URB and AM on fear memory persistence were reactivation-dependent, separate groups of rats were infused with URB, AM or VEH, without undergoing memory reactivation (**Figure 4**). All rats previously conditioned to the CS, as they froze more during the CS presentation than to the context 48h after conditioning [$F_{1,21}$= 23.5, p<0.001, η^2=0.53]. Though the experimental groups did not differ from each other [Drug: $F_{2,21}$=0.02, p=0.98], the URB-infused group did not extinguish responding across the two test sessions, unlike the AM and VEH groups [Drug x Session: $F_{2,21}$=4.57, p<0.05, η^2=0.30]. While there was a reduction in freezing at the 8d test for animals infused with VEH [PR-LTM24h *vs.* PR-LTM8d, p<0.05] and AM [PR-LTM24h *vs.* PR-LTM8d, p<0.01], the URB-treated animals did not reduce their freezing behavior [PR-LTM24h *vs.* PR-LTM8d, p > 0.05]. Furthermore, the non-reactivated AM-treated group showed greater freezing at the 24hr test than animals that had received AM

following memory reactivation [**Figure 3**; $p<0.05$] indicating that the amnesia produced by AM was reactivation-dependent. Thus, 48h and 9d after conditioning, the fear memory was still intact, and the amnesia produced by AM was only seen when the drug was given in conjunction with memory reactivation.

The disruption of reconsolidation produced by AM251 was blocked by the administration of the $GABA_A$ receptor antagonist bicuculline

In order to better understand the persistent amnesia produced by post-reactivation AM administration, we investigated the interaction of endocannabinoid and GABAergic signaling within the amygdala. To test the hypothesis that the amnesia produced by the CBR antagonist AM was mediated by increased GABA transmission, animals were infused with the $GABA_A$ receptor antagonist bicuculline (BIC), alone or in conjunction with AM at reactivation (**Figure 5**). As before, all rats previously conditioned to the CS, and froze more during the CS presentation than to the context during the reactivation session [$F_{1,34}=184.0$, $p<0.001$, $\eta^2=0.84$]. All rats showed equivalent levels of freezing during the reactivation session [$F_{3,34}=1.27$, $p=0.30$] and AM infusions post-reactivation resulted in amnesia at tests 24h later [Drug: $F_{3,34}=3.12$, $p<0.05$, $\eta^2=0.22$; Session: $F_{2,68}=55.5$, $p<0.001$, $\eta^2=0.62$; Session x Drug: $F_{6,68}=4.31$, $p<0.001$, $\eta^2=0.28$] though administration of BIC alone had no effect relative to VEH [$p > 0.99$]. Freezing was reduced at test relative to reactivation for rats that had received either AM [PR-LTM24h *vs.* reactivation, $p<0.001$; reactivation *vs.* PR-LTM8d, $p<0.001$], BIC alone [PR-LTM24h *vs.* reactivation, $p=0.011$; PR-LTM8d *vs.* reactivation, $p=0.001$] or AM+BIC [PR-LTM8d *vs.* reactivation, $p=0.002$]. Post hoc tests showed that at test, AM-treated rats froze less than VEH-treated [$p=0.029$] and BIC-treated rats [$p=0.017$] and, importantly, less than animals receiving AM+BIC [$p=0.045$]. Thus, the disruptive effect on memory reconsolidation induced by the blocking CB1Rs was replicated, and this disruption of reconsolidation could be prevented by antagonism at $GABA_A$ receptors. These results indicate that the endocannabinoid signaling-mediated disruption of CS-fear memory reconsolidation depends upon the consequent increase in GABAergic transmission in the BLA.

DISCUSSION

We have demonstrated that antagonism at CB1Rs prevents fear memory reconsolidation and may offer a promising therapeutic strategy in the treatment of post-traumatic stress disorder (PTSD), while also elucidating the mechanism by which CB1R antagonism exerts its effects on anxiety. Although there are other animal models, such as the single-prolonged stressor, predator-based psychosocial stress and predator scent stress models (see Daskalakis *et al*, 2013, for review) that capture different aspects of PTSD, we have shown that blocking CB1Rs within the BLA disrupted the reconsolidation of a pavlovian CS-fear memory, resulting in persistent loss of fear evoked by the subsequent presentation of the CS, a change in behavior widely suggested to model a desirable outcome for PTSD treatment (Dębiec and LeDoux, 2006; Parsons and Ressler, 2013; Schiller *et al*, 2010). This disruption of memory reconsolidation, which persisted for at least 8d after CS re-exposure, occurred if and only if the CB1R antagonist AM251 was infused locally in BLA immediately *after* retrieval; administration 30 minutes *prior* to memory reactivation did not result in an amnestic effect during the test sessions, and AM251 administration in the absence of memory reactivation did not produce amnesia. Non-reactivated groups tended to show lower levels of conditioned fear at test than animals that had been reactivated, supporting the hypothesized function for reconsolidation of memory strengthening. However, although freezing was reduced in the non-reactivated groups, it was still higher than in amnesic animals; therefore, the requirement for AM251 treatment *and* reactivation is more consistent with a blockade of memory restabilization than insensitivity of measurement. By contrast to the amnesia produced by AM251 given after reactivation, infusion into the BLA of the FAAH inhibitor URB597 in conjunction with memory reactivation resulted in a minor, transient enhancement of conditioned freezing. Whether a greater – or more sustained – effect would be observed with a higher dose of URB597 remains to be established, though it should also be considered that drugs targeting the endocannabinoid system often have biphasic effects (Metna-Laurent *et al*, 2012). Furthermore, we found that the memory impairment induced by post-reactivation CB1R antagonism could be prevented by co-infusion of the $GABA_A$ receptor antagonist bicuculline, suggesting that

endocannabinoid-mediated signaling affects reconsolidation via modulation of GABAergic transmission in the amygdala.

The data presented here are consistent with previous work indicating a role for CB1Rs in the plasticity underlying emotional memory, and help to account for some of the apparent inconsistencies in the previous literature. CB1Rs are required for the extinction, though interestingly not the consolidation (Arenos *et al*, 2006; Marsicano *et al*, 2002), of conditioned fear memory for context-shock associations (Suzuki *et al*, 2004) and tone-shock associations (Marsicano *et al*, 2002) and enhancing CB1R transmission enhances the extinction of fear memory (Chhatwal *et al*, 2005). However, while it has previously been shown that *antagonists* at CB1Rs disrupt the consolidation of inhibitory avoidance memory (Campolongo *et al*, 2009), consolidation of contextual fear conditioning is impaired by the *activation* of CB1Rs within the hippocampus (Maćkowiak *et al*, 2009). We hypothesize that these apparently discrepant findings are due to the timing of the amnestic treatment. We suggest that CB1Rs are required for the process of memory destabilization, as has been shown for contextual fear memories (Suzuki *et al*, 2008). Although extinction is usually conceptualized as the learning of a new, inhibitory 'CS-no US' memory (Bouton, 1991), there is evidence that there are some changes in synaptic strength in the amygdala, which are required for long-term storage of the original CS-fear memory (Gale *et al*, 2004), following extinction training (see Barad *et al*, 2006, for review). Our finding that blocking CB1Rs prior to memory reactivation did *not* result in amnesia is consistent with the blockade of memory destabilization (Suzuki *et al*, 2008) which, it should be noted, is a process that is doubly dissociable from memory *retrieval* (Milton et al, 2013). Blockade of memory destabilization would prevent memory reconsolidation from being engaged, and consequently prevent the effects of any treatment that might enhance (e.g. FAAH) or disrupt (e.g. AM251) reconsolidation (Lee, 2008). Mechanistically, this effect may be mediated through indirect actions on BLA pyramidal neurons. CB1Rs are located on GABAergic interneurons within the BLA (Katona *et al*, 2001) and act presynaptically to reduce GABAergic transmission onto BLA pyramidal neurons (Azad *et al*, 2003; Katona *et al*, 2001). Thus, we hypothesize that CB1R antagonism should act to disinhibit GABAergic interneurons, increasing the inhibition

of the pyramidal neurons, and therefore preventing the neuronal activity, mediated through GluN2B-containing NMDARs (Milton *et al*, 2013), that is required for memory destabilization. This is a speculative hypothesis and the interactions between endocannabinoid and glutamatergic signaling in fear memory reconsolidation remain to be investigated.

When administered after memory reactivation, CB1R antagonism led to a blockade of memory restabilization that was dependent on GABAergic signaling. We hypothesize that after memory reactivation, when the destabilization process has already occurred, the disinhibition of GABAergic interneurons through CB1R antagonism acts again to inhibit pyramidal neurons, but this time largely affecting GluN2A-containing NMDARs, which are required for memory restabilization (Milton *et al*, 2013). Thus, CB1R antagonism should result in amnesia similar to that observed following the administration of drugs that enhance GABAergic signaling (Zhang and Cranney, 2008). This hypothesis is supported by our finding that antagonism at $GABA_A$ receptors rescued the AM251-induced deficit. Unlike previous work, we did not observe amnesia when BIC was administered alone; however, previous work targeting GABAergic signaling in reconsolidation (Bustos *et al*, 2006; Zhang *et al*, 2008) has used systemic administration of GABAergic receptor antagonists rather than intracerebral infusions, as were used here. Furthermore, whether the transient memory enhancement produced by URB597 administration is also dependent upon GABAergic mechanisms remains a subject for future research.

We would argue that an alternative view – that the time dependence of the manipulation was simply due to the drugs being ineffective during the memory reactivation session when administered 30 minutes beforehand – is unlikely, as both URB597 and AM251 have been shown to produce behavioral effects when administered intracranially at earlier time points relative to the behavioral session (Hasanein *et al*, 2007; Hohmann *et al*, 2005). Therefore, we suggest that the difference in the effects of CB1R antagonism – impairing destabilization or restabilization of the fear memory – depends more critically upon treatment timing.

There has been much discussion about the timing of administration of amnestic agents relative to memory reactivation in studies of reconsolidation (Finnie and Nader, 2012; Schiller and Phelps, 2011). We suggest that there is no theoretical requirement for treatment timing that determines whether reconsolidation is targeted or not; instead, the focus should be on whether the process of memory destabilization or restabilization is being targeted. Some amnestic agents, such as non-subtype selective NMDA receptor antagonists, prevent memory restabilization but not destabilization (Lee *et al*, 2006; Milton *et al*, 2008; Milton *et al*, 2013) and pre-reactivation administration is more effective than post-reactivation administration because NMDA receptor antagonism results in amnesia by blocking fast excitatory neurotransmission events at the point of reactivation. For other amnestic agents, such as protein synthesis inhibitors (Milekic and Alberini, 2002; Nader *et al*, 2000), where the onset of the process to be inhibited is slower, post-reactivation administration is effective at disrupting restabilization. In this context, the effects of manipulating endocannabinoid signaling are unusual and of particular interest, because they can result in *opposite* effects on memory processes depending on the timing of antagonist administration. For example, for the consolidation of inhibitory avoidance memory, blocking CB1Rs in rat hippocampus and BLA with AM251 post-training induces impairments in avoidance behavior (De Oliveira Alvares *et al*, 2008), but pre-training administration of AM251 *facilitates* consolidation (De Oliveira Alvares *et al*, 2008). A better understanding of the molecular mechanisms that underlie memory destabilization and restabilization, and how they interact with different neurotransmitter systems, such as the endocannabinoid system, will be more beneficial to future reconsolidation studies and translation to the clinic than assertions that all treatments must be given post-reactivation in order to avoid effects on the separate and dissociable process of memory retrieval.

The data presented here clarify the requirement for endocannabinoid signaling in memory reconsolidation, and also indicate a novel target for the disruption of maladaptive memories that contribute to the persistence of psychiatric disorders such as PTSD (Brewin *et al*, 1996). Although additional investigations in

other animal models of PTSD, and with systemic administration of the compounds used here, would be informative and would facilitate translation to the clinic, our data indicate that antagonizing CB1Rs after memory reactivation may allow the disruption of old, well-established fear memories, reducing the persistence of the physiological and behavioral anxiety symptoms that characterize PTSD.

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FIGURE LEGENDS

Figure 1. Representation of cannulae placements within the BLA. The placements for individual experiments are shown separately, and coordinates are from bregma. For each experiment (***A,*** pre-reactivation administration; ***B,*** post-reactivation administration; ***C***, administration without reactivation; ***D,*** combined endocannabinoid and GABAergic manipulations) the white circles represent the vehicle group and the dark circles represent the AM group. The gray circles represent URB group (***A, B, C***), the gray squares represents the BIC group (***D***), and the dark squares represent AM+BIC group (***D***). This figure was modified, with permission, from Paxinos and Watson (2004).

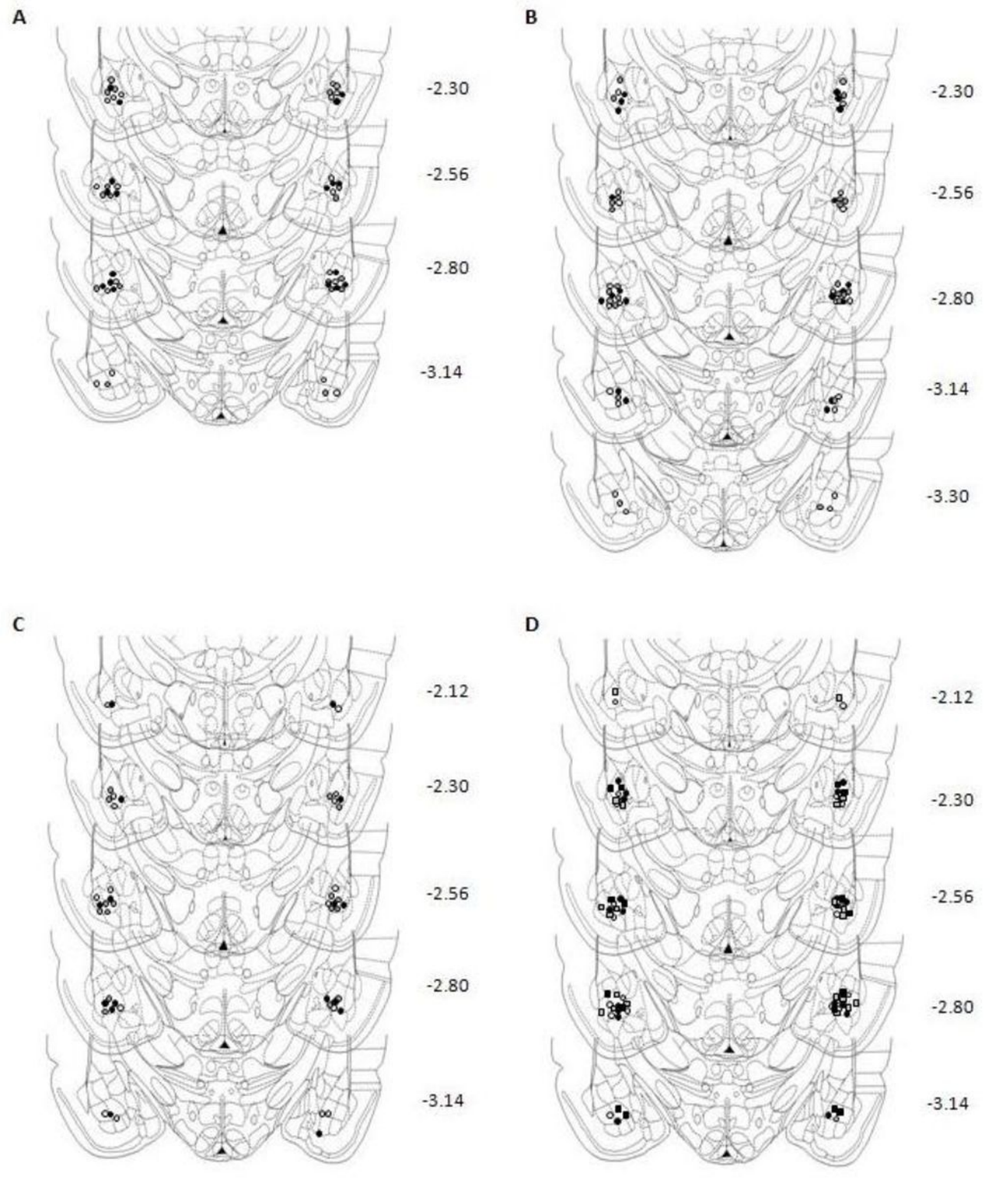
Figure 2. Effects of the FAAH inhibitor URB and the CB1 receptor antagonist AM on CS–fear memory reconsolidation. Administration of URB or AM prior to memory reactivation had no effect on the retrieval of the CS–fear memory at reactivation and did not alter expression of freezing response at tests conducted 24h or 8d later. Group sizes were VEH, *n*=9; URB, *n*=10; AM, *n*=9.

Figure 3. Effects of the FAAH inhibitor URB597 (30ng/0.5µl) and the CB1 receptor antagonist AM251 (300ng/0.5µl) on CS–fear memory reconsolidation. Administration of URB597 (30ng/0.5µl) immediately after the reactivation session produced a small, transient enhancement of CS–fear memory reconsolidation

at 24h, but not 8d, after reactivation. AM251 (300ng/0.5µl) persistently impaired memory reconsolidation when compared with vehicle and URB597 (30ng/0.5µl)-treated rats after both 24h and 8d after the reactivation session. Data are presented as means ± SEM. Group sizes were VEH, *n*=10; URB597 (30ng/0.5µl), *n*=12; AM251 (300ng/0.5µl), *n*=10.

Figure 4. Effects of the FAAH inhibitor URB597 (30ng/0.5µl) and the CB1 receptor antagonist AM251 (300ng/0.5µl) on CS–fear memory reconsolidation in rats not exposed to the memory reactivation session. Administration of URB597 (30ng/0.5µl) or AM251 (300ng/0.5µl) in absence of memory reactivation had no effect on the retrieval of the CS–fear memory both 24h and 8d after administration. Data are presented as means ± SEM. Group sizes were VEH, *n*=8; URB597 (30ng/0.5µl), *n*=8; AM251 (300ng/0.5µl), *n*=8.

Figure 5. Effects of the CB1 receptor antagonist AM251 (300ng/0.5µl) or the $GABA_A$ receptor antagonist bicuculline (BIC 50ng/0.5µl) on CS–fear memory reconsolidation. Administration of AM251 (300ng/0.5µl) immediately after the reactivation session persistently impaired the CS–fear memory both 24h and 8d after the reactivation session. Data are presented as means ± SEM. Group sizes were VEH, *n*=10; AM251 (300ng/0.5µl), *n*=10; BIC (50ng/0.5µl), *n*=10; AM251 (300ng/0.5µl) + BIC (50ng/0.5µl), *n*=8.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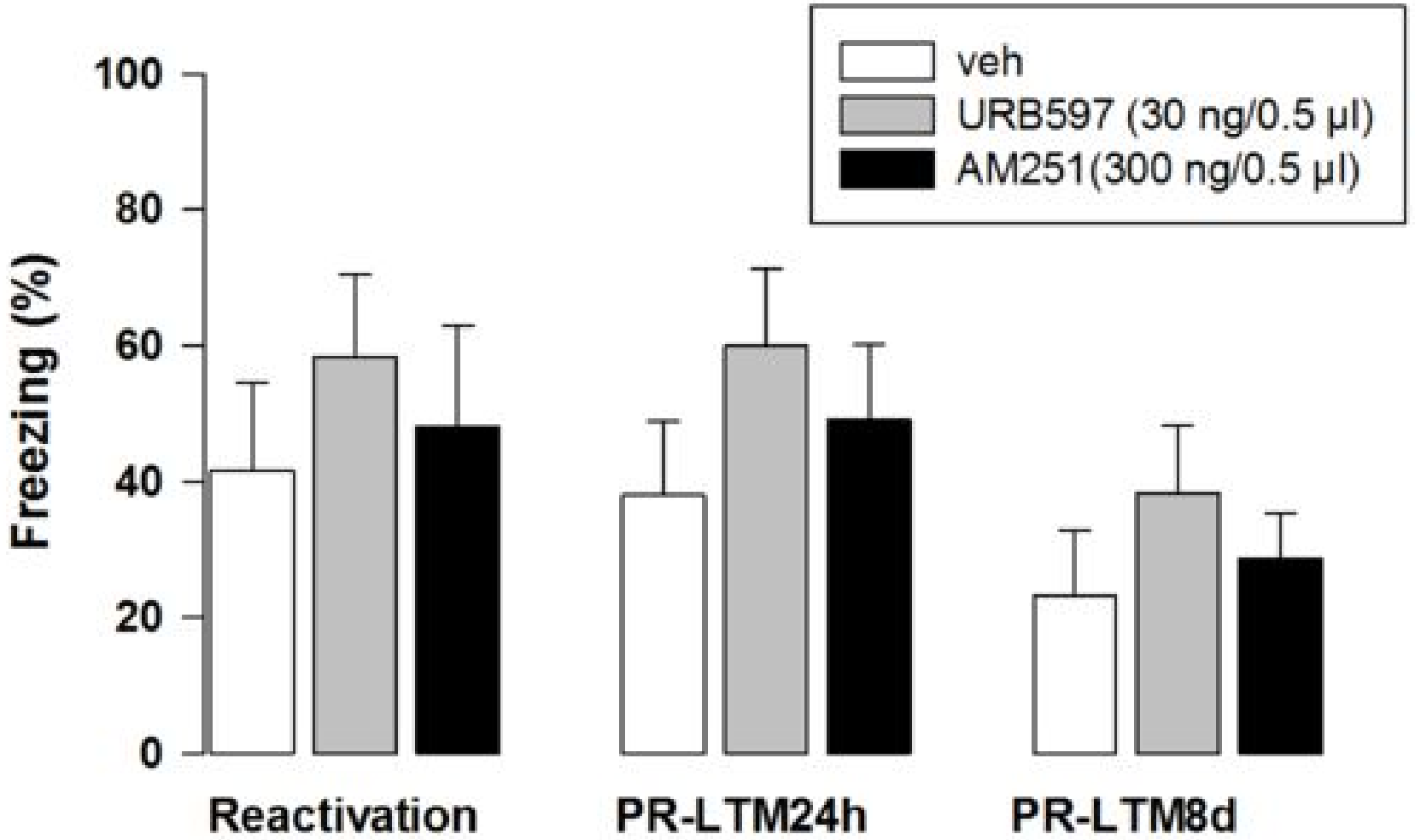
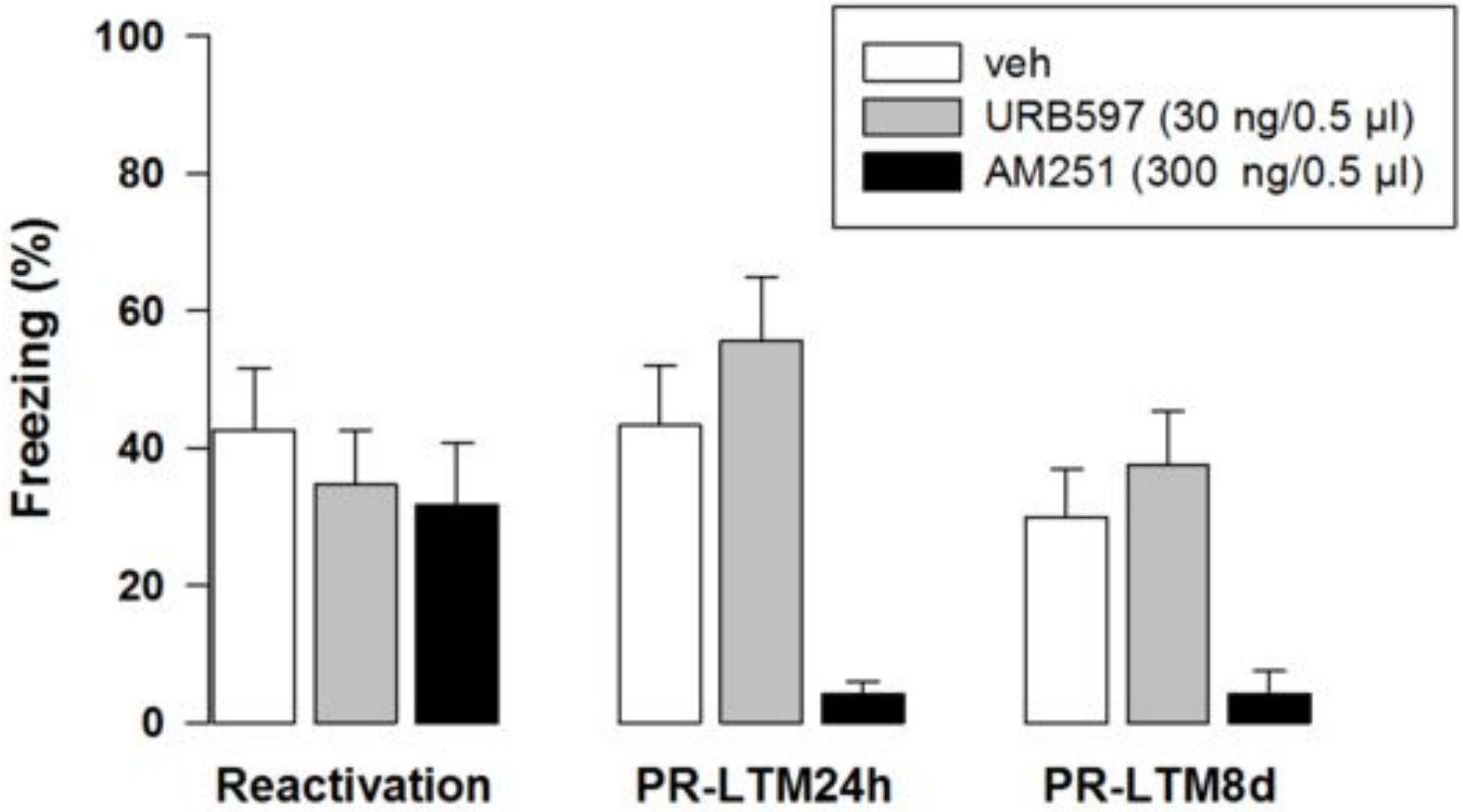
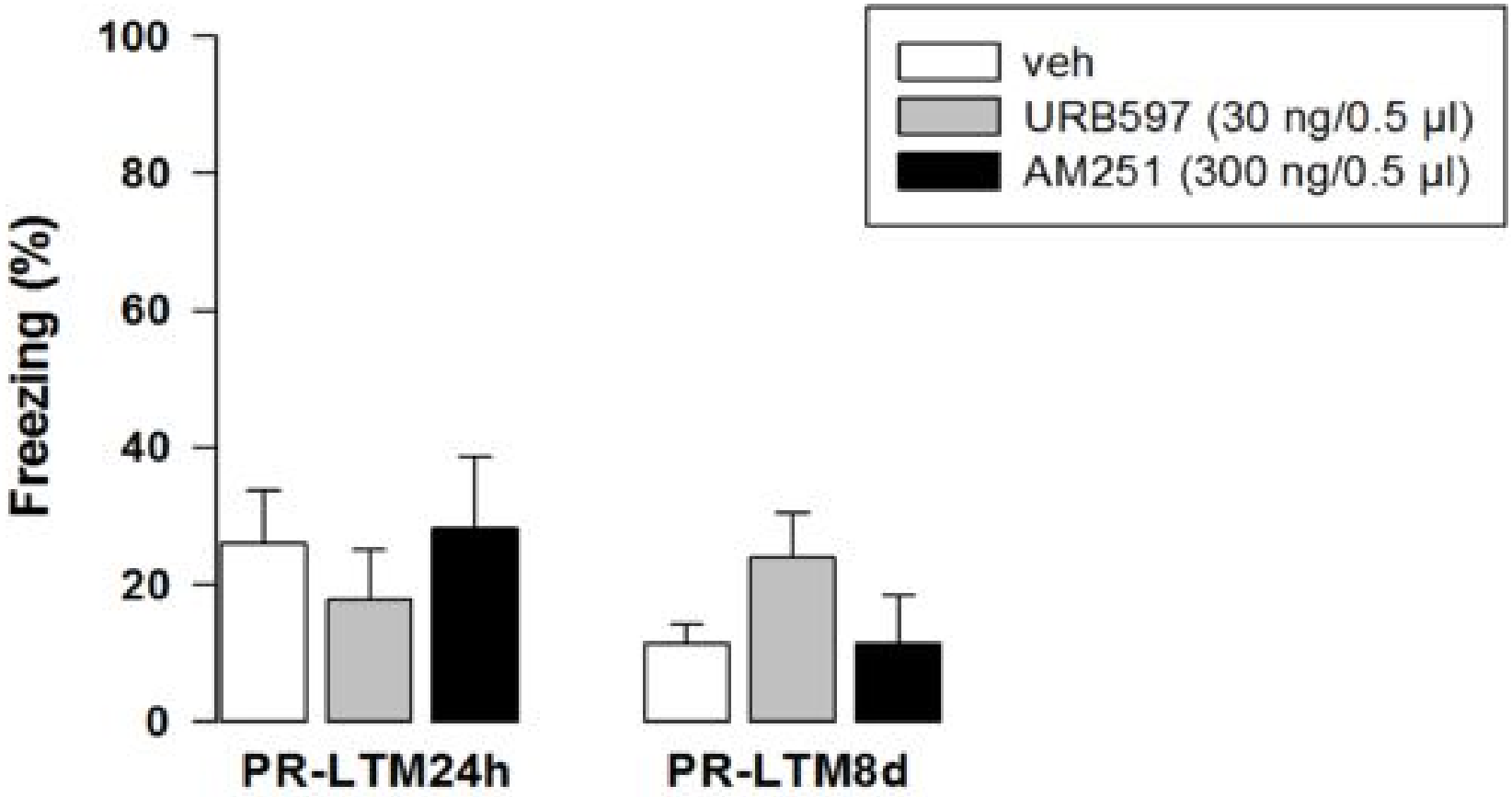
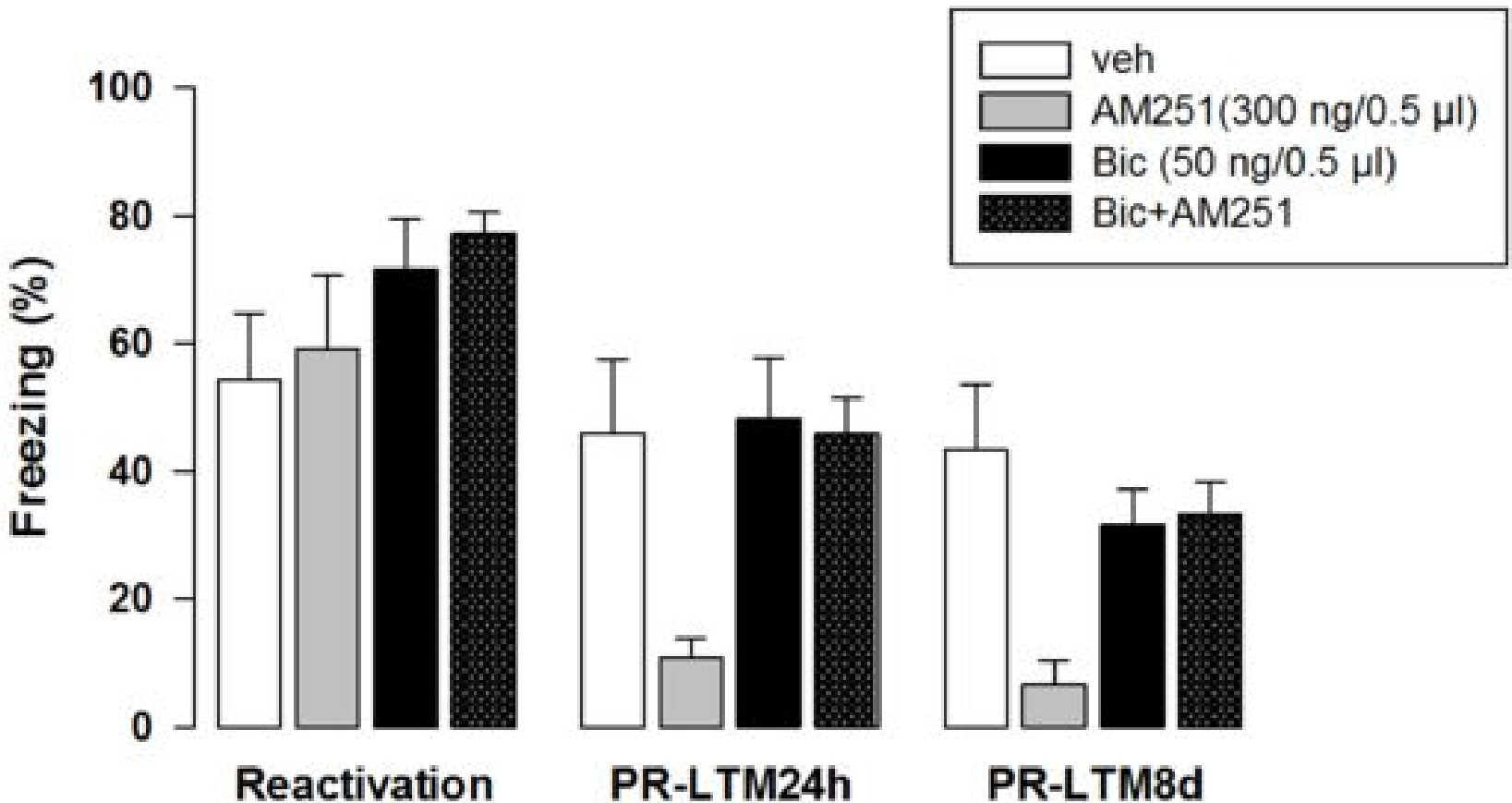

veh
URB597 (30 ng/0.5 µl)
AM251(300 ng/0.5 µl)
Freezing (%)
100
80
60
40
20
0
Reactivation
PR-LTM24h
PR-LTM8d


100
80
60
40
20
0
Freezing (%)
veh
URB597 (30 ng/0.5 µl)
AM251 (300 ng/0.5 µl)
Reactivation
PR-LTM24h
PR-LTM8d


100
80
60
40
20
0
Freezing (%)
PR-LTM24h
PR-LTM8d
veh
URB597 (30 ng/0.5 µl)
AM251 (300 ng/0.5 µl)


veh
AM251(300 ng/0.5 µl)
Bic (50 ng/0.5 µl)
Bic+AM251
Freezing (%)
100
80
60
40
20
0
Reactivation
PR-LTM24h
PR-LTM8d