Inhibition of alpha7 nicotinic receptors in ventral hippocampus selectively attenuates reinstatement of morphine-conditioned place preference and changes in AMPA binding

Abstract:
Recurrent relapse is a major problem in treating opiate addiction. Pavlovian conditioning plays a role in recurrent relapse whereby exposure to cues learned during drug intake can precipitate relapse to drug taking. α7 nicotinic acetylcholine receptors (nAChRs) have been implicated in attentional aspects of cognition and mechanisms of learning and memory. In this study we have investigated the role of α7 nAChRs in morphine-conditioned place preference (morphine-CPP). CPP provides a model of associative learning that is pertinent to associative aspects of drug dependence. The α7 nAChR antagonist methyllycaconitine (MLA; 4 mg/kg s.c.) had no effect on the acquisition, maintenance, reconsolidation or extinction of morphine-CPP, but selectively attenuated morphine-primed reinstatement of CPP, in both mice and rats. Reinstatement of morphine-CPP in mice was accompanied by a selective increase in [3H]-AMPA binding (but not in [3H]-MK801 binding) in the ventral hippocampus that was prevented by prior treatment with MLA. Administration of MLA (6.7 µg) directly into the ventral hippocampus of rats prior to a systemic priming dose of morphine abolished reinstatement of morphine-CPP, whereas MLA delivered into the dorsal hippocampus or prefrontal cortex was without effect. These results suggest that α7 nAChRs in the ventral hippocampus play a specific role in the retrieval of associative drug memories following a period of extinction, making them potential targets for the prevention of relapse.
Inhibition of alpha7 nicotinic receptors in the ventral hippocampus selectively attenuates reinstatement of morphine-conditioned place preference and associated changes in AMPA receptor binding.

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ABSTRACT

Recurrent relapse is a major problem in treating opiate addiction. Pavlovian conditioning plays a role in recurrent relapse whereby exposure to cues learned during drug intake can precipitate relapse to drug taking. α7 nicotinic acetylcholine receptors (nAChRs) have been implicated in attentional aspects of cognition and mechanisms of learning and memory. In this study we have investigated the role of α7 nAChRs in morphine-conditioned place preference (morphine-CPP). CPP provides a model of associative learning that is pertinent to associative aspects of drug dependence. The α7 nAChR antagonist methyllycaconitine (MLA; 4 mg/kg s.c.) had no effect on the acquisition, maintenance, reconsolidation or extinction of morphine-CPP, but selectively attenuated morphine-primed reinstatement of CPP, in both mice and rats. Reinstatement of morphine-CPP in mice was accompanied by a selective increase in [3H]-AMPA binding (but not in [3H]-MK801 binding) in the ventral hippocampus that was prevented by prior treatment with MLA. Administration of MLA (6.7 µg) directly into the ventral hippocampus of rats prior to a systemic priming dose of morphine abolished reinstatement of morphine-CPP, whereas MLA delivered into the dorsal hippocampus or prefrontal cortex was without effect. These results suggest that α7 nAChRs in the ventral hippocampus play a specific role in the retrieval of associative drug memories following a period of extinction, making them potential targets for the prevention of relapse.

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KEY WORDS

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RUNNING HEAD
alpha7 nAChRs in ventral hippocampus mediate reinstatement of morphine-CPP
INTRODUCTION

Drug addiction is a chronic relapsing brain disorder (Leshner, 1997; Koob & Volkow, 2010), with relapse posing the greatest obstacle to overcoming addiction. For example, almost 3 million people in the USA are estimated to be addicted to prescription opiates or heroin (National Survey on Drug Use and Health, 2013; Lyapustina & Alexander, 2015). Despite the availability of substitution products such as methadone, relapse to heroin use is prevalent. Relapse may be triggered by a variety of stimuli including drug-associated cues, exposure to sub-threshold (priming) doses of drug or stress (Shalev et al., 2002). Understanding the brain mechanisms underlying the different stages of drug addiction may identify novel targets for intervention.

The cholinergic system is intimately engaged with reward circuitry in the brain. Nicotine, acting through nicotinic acetylcholine receptors (nAChRs) within these circuits, sustains tobacco addiction (Wonnacott et al., 2005; Brunzell et al., 2015), which commonly co-exists with the use of other addictive substances. Indeed, tobacco smoking has been regarded as a gateway to illicit drug use (Jessup, 1996; Kandel & Kandel, 2015). Physiologically, nAChRs mediate the actions of endogenous acetylcholine, which is implicated in attention (notably to cues that prompt goal-directed behaviours), motivation, and learning and memory, important facets of addiction (Luchicchi et al., 2014; Sarter et al., 2014). Thus, nAChRs may contribute to general processes underlying drug dependence and nAChRs have been proposed as novel targets for treating addiction (Rahman et al., 2015).

The α7 nAChR, which is highly expressed in the hippocampus and cortex (Yakel, 2014), has gained prominence for its association with attentional aspects of cognition and mechanisms of learning and memory (Levin et al., 2015). In the present experiments we sought to investigate the contribution of α7 nAChRs to opiate reward, using the conditioned place preference (CPP) model of motivational learning, with morphine as the unconditional stimulus. This model is well established (Bardo & Bevins, 2000; Tzschentke 2007; Aguilar et al., 2009) and allows acquisition, maintenance, extinction and reinstatement of drug-seeking behaviour to be interrogated. A previous report (Feng et al., 2011) has implicated α7
nAChRs in morphine-primed reinstatement of CPP in mice but other components of morphine-seeking behaviour were not examined. Therefore, we have used the selective antagonist methyllycaconitine (MLA), delivered systemically and intracerebrally, to block α7 nAChRs at different stages of morphine-CPP. Changes in glutamate receptor density were monitored in parallel, using quantitative autoradiography, in order to shed light into the possible mechanisms underlining these effects. This study has revealed a selective action of α7 nAChR antagonism in the ventral hippocampus that inhibits both morphine-primed CPP and associated increases in AMPA receptor binding in this brain region.
MATERIALS AND METHODS

Animals
Male C57BL/6 mice (6-7 weeks of age at the start of experiments; Charles River, Kent, UK) and Wistar rats (400g, University of Bath breeding colony) were housed in groups of 4, except following surgery when rats were singly housed, in a controlled environment (12:12 hour light-dark cycle (lights on 07:00); constant temperature (24±2°C) and humidity (55±5%)). Food and water were available ad libitum. Animals were handled daily for one week prior to the start of experiments. All experiments were approved by the UK Home Office and performed in accordance with UK Animals (Scientific Procedures) Act of 1986, and conform to the ARRIVE guidelines (Kilkenny et al., 2010).

Drugs
Morphine hydrochloride was purchased from MacFarlan Smith, Edinburgh, UK; methyllycaconitine (MLA) was purchased from Tocris Cookson, Avonmouth, UK. For peripheral administration, drugs were dissolved in sterile saline and injected in a volume of 10 ml/kg. Control animals received vehicle injections (sodium chloride 0.9% w/v, Hameln pharmaceuticals, Gloucester, UK). For intracerebral injections, MLA (2.8 mg/ml) was dissolved in saline. [3H]-(+)-MK801 (22.5 Ci/mmol) and [3H]-AMPA (58.1 Ci/mmol) were from Perkin Elmer Life Sciences, USA. CNQX, (+)-MK801 and all other reagents were from Sigma, Poole, Dorset, UK.

CPP procedure
CPP was carried out essentially as previously described (Ribeiro Do Couto et al, 2003; Cordery et al., 2014). Mouse CPP was in two-compartment shuttle boxes (Ugo Basile, Gemonio, Italy). Compartments were 15cm square; one compartment consisted of grey walls and a metal floor with circular holes, the other had striped walls and square holes. Rat CPP was in three-compartment shuttle boxes (Tracksys, Nottingham, UK). Compartments were 40cm square, linked via a 10cm square central area; one compartment consisted of
horizontal black and white stripes and a metal floor with circular holes, the other had vertical black and white stripes and a metal floor with square holes.

Experiments were performed between 08:00 and 17:00 under dim white light (light intensity approximately 15 lux). During all preference test sessions (typically 15 minutes) the animal had free access to both compartments; the time each animal spent in each compartment and their locomotor activity (distance travelled) was recorded using EthoVision XT version 8.0 (Tracksys) tracking software.

Acquisition of morphine-CPP (Figure 1A). Following drug-free habituation trials (1x15 minute session/day for 2 days, with free access to both compartments) to reveal any innate preference for either of the compartments, animals were pseudorandomly assigned to treatment groups that were organised so that mean baseline preferences for a particular compartment were close to zero. Morphine conditioning consisted of 1x40 minute trial/day for 4 days, starting 2 days after the habituation test. Each animal was confined to one compartment following injection of morphine (mice: 10 mg/kg i.p.; rats 5 mg/kg s.c.) or saline. The drug/saline pairing and compartment were reversed on consecutive days, so that each animal received 2 morphine injections and 2 saline injections. A counterbalanced design was employed such that within a treatment group, half the animals were drug-paired with one compartment type and the other half were drug-paired with the other compartment type; the order of morphine or saline presentation was also counterbalanced. A drug-free post-conditioning preference test was carried out 24 h after the last drug treatment. Animals were placed in the CPP box with free access to both compartments for 15 min and time spent in each compartment was recorded, to determine their preferences. Saline-only controls were included in a preliminary experiment to validate the established protocol (Supplementary Figure S1). Thereafter, all subjects received morphine in the counterbalanced design described, to test the effects of α7 nAChR antagonism.

To examine the effect of systemic MLA on morphine-CPP acquisition, animals received MLA (4 mg/kg s.c.) or saline 20 minutes before each conditioning dose of morphine.
**Maintenance of morphine-CPP (Figure 1B).** To assess whether MLA affected the maintenance of morphine-CPP, animals were subjected to morphine conditioning as described above. Following the post conditioning preference test to establish that they had acquired morphine-CPP, they were subjected to additional preference tests over the following 4 days and one week later. MLA (4mg/kg) or saline was given 20 minutes before each test.

**Reconsolidation of morphine-CPP (Figure 1D).** Animals acquired morphine-CPP as described above. Three days after the post-conditioning preference test, all animals received morphine and were immediately confined to their drug-paired side of the CPP box for 40 minutes (reconsolidation step). MLA (4 mg/kg) or saline was administered at the end of this session, prior to return to the home cage. A preference test was carried out 1 day and 7 days later.

**Extinction and reinstatement of morphine-CPP (Figure 1E, 4 & 5).** Animals acquired morphine-CPP, as described above, followed by extinction training. Animals received saline injections only, paired with alternate compartments of the CPP box, over 4 consecutive days. On the following day the animals were subjected to another preference test. Only those that met criterion (less than 70% of time spent in the previously drug-paired compartment) were considered to show extinction and proceeded to the reinstatement phase. Less than 5% of subjects were excluded.

Three days later, for the reinstatement of morphine-CPP, animals received a priming dose of morphine (mice: 5mg/kg i.p.; rats 2.5 mg/kg s.c.) prior to being given an extended preference test (30 min, based on a preliminary experiment), with free access to both compartments of the CPP box; the time spent in each compartment was monitored over two 15 min bins.

**Preliminary data showed development of reinstatement over the 30 minute test session (as in Mueller et al., 2002), therefore the second 15 min bin was taken for reinstatement values.** MLA (4 mg/kg s.c.) or saline was administered 20 min before the priming dose of morphine. In mice, saline-only controls were included in a preliminary experiment to validate the
established protocol (Supplementary Figure S1). Reinstatement produced more variable data sets, requiring a larger sample size for statistical power.

**Intracerebral cannula implantation and drug delivery**

Bilateral cannulae were implanted into the medial PFC (mPFC), ventral or dorsal hippocampus of 52 rats following acquisition and extinction of morphine-CPP, but prior to the reinstatement test. Rats were anaesthetised with isoflurane (induction 4%, maintenance, 2-3%, Baxter, UK), placed into a stereotaxic frame and guide cannulae were implanted into the mPFC (coordinates relative to bregma: anterior-posterior +3.2, medial-lateral ±0.75, dorsal-ventral -2.8), the dorsal hippocampus (anterior-posterior -3.2, medial-lateral ±2.5, dorsal-ventral -1.8) or ventral hippocampus (anterior-posterior -5.3, medial-lateral ±5.2, dorsal-ventral -4.5). Dummy cannulae were inserted and secured with a dust cap to prevent post-surgical infection. The rats were rehydrated with 0.9% saline solution (10 ml/kg s.c.), and given an antibiotic (0.2 ml clamoxyl LA, 150 mg/ml s.c.) and an analgesic (Caprieve, 5mg/kg, s.c). Animals were allowed to recover for 7 days.

Infusions were performed in conscious, gently restrained animals 15 min before the reinstatement trial. MLA or saline (2.4 µl/hemisphere) was delivered over 4 min (0.6 µl/min) via a 33 gauge infusion cannula, using an infusion pump (Harvard apparatus). The cannulae were left in place for a further 4 min. Infusions were conducted in pairs of animals.

To verify cannula placement, at the end of the experiment rats were killed by rising CO₂ asphyxiation and 0.5 ml brilliant blue dye was infused via each cannula, using the infusion pump. Brains were frozen in isopentane and stored at -80°C. Coronal sections were made using a cryostat at -21°C. Sections containing the dye marks were compared with a brain atlas (Paxinos and Watson, 2007; Figure S2). Three animals were culled due to adverse reaction to surgery; no animals were excluded following verification of cannula placement.

**Quantitative autoradiography**

Twenty-four mice underwent morphine-CPP acquisition followed by extinction training (Figure 1C), and were then randomly assigned to one of 4 groups:

- Saline pretreatment / saline reinstatement (6)
• MLA pretreatment / saline reinstatement (6)
• Saline pretreatment / morphine-primed reinstatement (6)
• MLA pretreatment / morphine-primed reinstatement (6)

Animals were killed by cervical dislocation immediately after the reinstatement test, brains were frozen in isopentane and stored at -80°C. Adjacent 20 µm coronal sections were collected containing the prefrontal cortex (bregma +1.94 mm), striatum (bregma +1.42 mm), dorsal hippocampus (bregma -1.22 mm) and the mid-brain/ventral hippocampus (bregma -3.08 mm; Franklin and Paxinos, 1997). Consecutive sections were freeze-thaw mounted onto separate gelatin-subbed glass slides for total and non-specific labelling and processed for autoradiography as described previously (Kitchen et al. 1997).

**NMDA receptor quantitative autoradiography.** Slides were preincubated (20 min, 20ºC) in 50 mM Tris-HCl, pH 7.4, containing 50 µM glutamate, 50 µM glycine and 50 µM spermidine. Subsequently slides were incubated (1 h, 4ºC) in the same buffer containing 70 nM [³H](+)-MK801 to determine total binding. Non-specific binding was determined in the presence of 1 mM (+)-MK801 (Reynolds, 2001). Slides were rinsed twice for 30 s in ice-cold 50mM Tris-HCl, pH7.4, followed by distilled water and rapidly dried with a stream of cool air and placed in a sealed container with anhydrous calcium sulphate for 1 week.

**AMPA receptor quantitative autoradiography** (Duncan et al., 2002). Slides were preincubated (20 min, 20ºC) in 50mM Tris-HCl, pH7.4, containing 50 mM sodium thiocyanate. Total binding was determined by incubating sections (45 min, 20ºC) in the same buffer containing 10nM [³H]-AMPA. Non-specific binding was determined in adjacent sections incubated in the additional presence of 0.1mM CNQX. Slides were rinsed three times for 20 s in ice-cold 50mM Tris-HCl, pH7.4, followed by distilled water and rapidly dried with a stream of cool air and stored with anhydrous calcium sulphate as for [³H](+)-MK801 binding.

Slides were apposed to photographic film (Kodak Biomax MR-1, Sigma-Aldrich, UK) along with autoradiographic [³H] microscale standards (GE Healthcare, Amersham, U.K.) for 3
weeks ([3H]-AMPA) or 4 weeks ([3H]-(+)-MK801). Sections for all treatment groups were processed in parallel and apposed to the same film at the same time; slides were developed and analysed in parallel (Kitchen et al. 1997); structures were identified by reference to the mouse brain atlas of Franklin & Paxinos (2001) and analysed using an image analyzer (MCID; Image Research, Linton, UK). Specific binding was determined by subtracting non-specific binding from total binding: for [3H]-AMPA labelling, non-specific binding was homogeneous therefore a representative non-specific binding area was subtracted from all total values; for [3H]-(+)-MK801 labelling, non-specific binding was taken from the corresponding area for each brain region analysed.

**Data analysis** All data are presented as mean ± standard error of the mean (S.E.M). CPP data are presented as Preference Scores for the time (s) spent in morphine-paired compartment; these are calculated as time spent in morphine-paired compartment – 450 (half the maximum time, in seconds). All behavioural analyses were done using in vivo stat with a one-way ANOVA with repeated measures, with post hoc analysis with Benjamini-Hochberg test for multiple comparisons when significant interaction effects were observed (P<0.05). For analysis of the effects of intracerebral MLA a one-way ANOVA was performed. For analysis of autoradiographic binding, a 2-way ANOVA with factors ‘treatment’ (saline versus MLA) and ‘behaviour’ (saline- versus morphine-primed reinstatement) was performed for each region analysed.
RESULTS

Effect of the α7 nAChR antagonist MLA on morphine-CPP in mice

Mice readily acquired morphine-CPP and displayed a significant preference for the compartment associated with the morphine dose (10 mg/kg i.p.) after four days of conditioning (Figure S1). This could be extinguished by pairing saline treatments with both compartments. Morphine-CPP could be reinstated by a priming dose of morphine (5mg/kg i.p.) in mice that had acquired morphine-CPP prior to extinction (Figure S1). The conditioning and priming doses of morphine were based on previously published studies in mice (Feng et al., 2011; Tzschentke, 2007) and pilot studies to optimise priming dose.

The involvement of α7 nAChRs in morphine-CPP was examined by systemic administration of MLA (4 mg/kg s.c.). This dose of MLA was based on literature evidence for its selectivity and efficacy in mice (Feng et al., 2011). MLA itself did not induce any conditioned place preference or aversion (MLA + saline: habituation 3.7±28.6s, post conditioning 33.1±40.7s versus saline + saline: habituation 18.3±52.9s, post conditioning 16.2±41.0s, no effect of treatment p=0.75 or test p=0.45, n=7 / treatment group; data not shown).

MLA was administered at different stages of morphine-CPP (either prior to morphine administration, prior to testing, or immediately after a morphine-conditioned reconsolidation trial; Figure 1A, 1B, 1C respectively). MLA given prior to morphine or saline before each conditioning trial did not affect acquisition of morphine-CPP (Figure 1A). There was no significant effect of treatment (F(1,22)=0.15, p=0.699) but a significant effect of test (F(1,30)=24.09, p=<0.001). Post-hoc pairwise comparisons revealed significant morphine-CPP in saline + morphine group (preference score during habituation: 10.8±39.0s; post-conditioning: 164.1±34.5s, n=16, p=0.012), and MLA + morphine group (preference score during habituation: -3.2±36.7s; post-conditioning: 179.8±45.8s, n=16, p=0.003). There was no significant difference between the MLA pretreated and the saline pretreated groups (n=16, p=0.69).
To assess if α7 nAChRs have any effect on the maintenance and expression of morphine-CPP, mice underwent morphine conditioning followed by a post conditioning preference test, and were again tested for their preference scores on the following 4 days and 1 week later. MLA was given 20 min before each preference test (Figure 1B). CPP was significantly learned in both treatment groups: there was a significant effect of test ($F_{(6,78)}=4.37, p=0.001, n=12/treatment$) but not of treatment ($F_{(1,13)}=0.78, p=0.394$). Post hoc pairwise comparisons indicated significant expression of morphine-CPP on preference test day 1 (saline: $p=0.004$, MLA: $p=0.024$, n=12/treatment). Preference scores declined only slowly over time with repeated testing; importantly, there was no significant difference at any time-point between saline and MLA-treated animals, showing that MLA did not affect the expression and maintenance of morphine-CPP.

To determine if α7 nAChRs play any role in the reconsolidation of morphine-CPP, MLA was given immediately after a reconsolidation trial (in which morphine, 10 mg/kg, was paired with the previously drug-paired compartment; Figure 1C). Mice were tested for their compartment preference 24 h and 7 days later (Figure 1D). There was no significant effect of MLA treatment ($F_{(1,17)} = 0, p=0.961$) but a significant effect of test ($F_{(2,44)} = 14.25, p<0.001, n=12/treatment group$). Post hoc analysis revealed no significant difference between treatment groups after 24 h or one week later indicating that MLA had no effect on reconsolidation of morphine-induced CPP.

Next, MLA was tested on reinstatement of morphine-CPP: mice acquired morphine-CPP which was then extinguished by repeated saline administration in association with both compartments of the CPP apparatus; MLA or saline was given prior to a priming dose of morphine (5 mg/kg) to reinstate morphine-CPP (Figure 1C). Immediately after receiving the priming dose mice were tested in the CPP apparatus for any compartment preference. The priming dose of morphine produced a robust reinstatement of morphine-CPP whereas prior treatment with MLA significantly attenuated reinstatement (Figure 1E). There was no significance in the effect of treatment ($F_{(1,52)} = 1.15, p=0.288$) but a significant effect of test ($F_{(3,156)} = 13.48, p=<0.001, n=20/treatment group$). Post-hoc analysis for multiple
comparisons revealed that only animals that received saline prior to the priming dose of morphine showed significant reinstatement (saline preference score at extinction: 42.2±14.0 s vs 143.1±33.2 s at reinstatement; p=0.003; MLA preference score at extinction: 37.3±13.8 s vs 67.3±41.7 s at reinstatement, p=0.135, n=20/treatment group). The time spent in the drug paired side after morphine-primed reinstatement was significantly different between the two treatments (p=0.0016).

**Glutamate receptor binding: effect of reinstatement of morphine-CPP and MLA pretreatment**

Quantitative autoradiography was used to determine the levels of [³H]-(+)-MK801 and [³H]-AMPA binding in brain sections prepared from the brains of mice that had undergone reinstatement of morphine-CPP, or saline-primed controls, with or without MLA pretreatment. Sections were taken at 4 levels from Bregma. Representative autoradiographs of [³H]-(+)-MK-801 binding are shown in Figure 2A. There was differential labelling of brain regions, with highest levels in the hippocampus, followed by cortical areas, in agreement with previous reports (Bowery et al., 1988). There was no difference in specific [³H]-(+)-MK-801 binding between treatment groups, in any of the brain regions analysed (Figure 2B; supplementary Table S1).

Representative autoradiographs of [³H]-AMPA binding are shown in Figure 3A. Labelling was particularly high in cortical areas and hippocampus, but very low in thalamic areas, as previously reported (Monaghan et al., 1984). Quantitation of specific [³H]-AMPA binding (Figure 3B; supplementary Table S2) revealed a significant increase in [³H]AMPA binding density after morphine reinstatement in the CA1/CA2 sub regions of the ventral hippocampus: (17.1±3.3%; p<0.05; n=4-6/treatment group). There was no significant difference in vCA3 or any other region analysed.

Pretreatment with MLA before morphine-primed reinstatement resulted in significantly lower binding in the CA1/CA2 sub-region of the ventral hippocampus compared with values from mice that received saline before the morphine priming dose (Treatment x Behaviour
interaction \( F(1,15)=5.4, p<0.05; \) Figure 3B; supplementary Table S2). MLA pretreatment of saline controls did not affect \( [\text{H}]\)-AMPA binding and there were no significant differences between treatment groups in any other region examined.

Effect of intracerebral administration of MLA on reinstatement of morphine-CPP in rats

To evaluate the central locus of action of MLA in attenuating reinstatement of morphine-CPP, local administration of MLA was undertaken. Because this approach is more appropriate for use in rats, it was necessary to first replicate the inhibition by systemic MLA of morphine-primed reinstatement of CPP in this species. Male Wistar rats underwent conditioning (morphine 5mg/kg, s.c), extinction and then received either saline or a priming dose of morphine (2.5mg/kg, s.c) immediately before the reinstatement trial. MLA (4mg/kg, s.c) or saline was administered 20 min prior to the morphine-priming dose. Drug doses were based on previous experience (Cordery et al; 2012) and literature precedent (Markou & Paterson, 2001; Tzschentke, 2007; Aguilar et al., 2009; Liu, 2014).

There was a significant acquisition of morphine-CPP that was extinguished and reinstated in response to morphine \( (p=0.007) \) but not saline \( (p=0.34; \) Figure 4A). Systemic MLA significantly inhibited the reinstatement of morphine-primed CPP (Figure 4B). There was no significant effect of treatment \( (F_{1,49} = 0.70, p=0.408) \) but a significant effect of test \( (F_{4,176} = 3.96, p=0.004, n=26/treatment \text{ group}) \). Post-hoc pairwise comparisons revealed that only animals pre-treated with saline prior to the morphine priming dose significantly reinstated (saline preference score at extinction: 25±23 s versus 112±46 s at reinstatement \( (p=0.007) \); MLA preference score at extinction: 18±23s vs 35±46 s at reinstatement, \( p=0.667, n=26/treatment \text{ group}) \). The time spent in the drug paired side was significantly different between the two treatments \( (p=0.024) \).

To test the effect of local delivery of MLA on morphine-primed reinstatement of CPP, rats underwent acquisition and extinction of morphine-CPP. Bilateral cannulae were then implanted in the dorsal hippocampus, ventral hippocampus, or mPFC. One week post
surgery, animals received a bilateral infusion of saline or MLA (6.75 µg/hemisphere) 15 min prior to a morphine priming dose (2.5 mg/kg, s.c) and free access to the CPP compartments to determine any preference. This dose of MLA delivered intracerebrally has been reported to be effective in other behavioural models (Addy et al., 2003; Nott & Levin, 2006). Prior to surgery there was a significant effect of morphine-CPP (F(2,94) = 3.38, p=0.0381) but no significant effect of treatment group (F(1,47) = 0.03, p=0.8657, n=25/treatment group), indicating acquisition and extinction of morphine-CPP with no difference between the groups that would subsequently receive local MLA or saline infusions (Figure 5). Animals that received saline intracerebrally prior to the priming dose of morphine showed robust reinstatement of morphine-CPP (Figure 5). Reinstatement was not affected by prior infusion of MLA into the dorsal hippocampus (p=0.82, n=8, Figure 5A) or mPFC (p=0.70, n=8, Figure 5C). However, MLA infused into the ventral hippocampus abolished morphine-primed reinstatement of CPP (p=0.012, n=8, saline preference score at reinstatement: 164±47s versus MLA preference score at reinstatement: -70±67s; Figure 5B). Thus, MLA delivered into the ventral hippocampus, but not the dorsal hippocampus or mPFC, completely blocks morphine-primed reinstatement of morphine-CPP.
DISCUSSION

This study corroborates and extends a previous report (Feng et al., 2011) that blockade of α7 nAChRs attenuates reinstatement of morphine-primed CPP in mice. Here we have reproduced this finding in a different strain of mouse (C57BL/6, compared with BalbC used by Feng et al. (2011)) and extended it to Wistar rats. Further, we have shown that α7 nAChR blockade by systemic delivery of MLA is specific in only inhibiting the reinstatement phase of the CPP paradigm, and that this action is reproduced by MLA delivered into the ventral hippocampus (but not the dorsal hippocampus or mPFC). This locus of action of MLA accords with increases in AMPA receptor binding in CA1/CA2 regions of the ventral hippocampus in response to morphine-primed reinstatement that are also attenuated by systemic MLA.

CPP provides a model of Pavlovian associative learning, in which the unconditional stimulus (drug) is paired with a conditional stimulus (environmental context) (Bardo & Bevins, 2000; Tzschentke, 2007; Aguilar et al., 2009). The ability of rodents to rapidly learn to positively associate a distinctive chamber of the CPP apparatus with the unconditioned stimulus, measured as the drug-free preference score, reflects the incentive salience of the drug. This associative learning has face validity for human drug addiction in which strong associations are formed with the context of drug taking (Napier et al., 2013). Other aspects of drug dependence: maintenance, reconsolidation and extinction, are also modelled in the CPP paradigm. The endurance of the associative learning beyond extinction is demonstrated by the ready reinstatement of CPP in response to a trigger, in this case a priming drug dose (Aguilar et al., 2009). Reinstatement provides a useful model relevant to relapse in abstinent addicts (Napier et al., 2013). Relapse is a major social problem and interventions that target this aspect of drug dependency would be advantageous.

The selective effect of MLA on morphine-primed reinstatement is robust, as demonstrated in both mice and rats and via two modes of delivery (systemic and intracerebral). The data suggest that α7 nAChRs contribute specifically to this phase of the addictive drug cycle.
Interestingly, a selective effect for α7 nAChRs in cue-induced reinstatement of nicotine self-administration has also been reported (Liu, 2014). More recently, increasing kynurenic acid (a negative modulator of α7 nAChRs) was found to attenuate both drug- and cue-induced reinstatement of nicotine and cocaine self-administration in squirrel monkeys (Secci et al., 2017). Evidence that this effect was mediated by α7 nAChRs was provided by the opposing action of a positive allosteric modulator. Together, these studies support a role for α7 nAChRs more generally in circuitry responsible for the retrieval of drug and context associated memories. Basal forebrain cholinergic activity appears necessary for cue-induced relapse (Pitchers et al., 2017), and α7 nAChRs may mediate, at least in part, this cholinergic signalling.

The hippocampus is a major target of cholinergic projection neurons from the basal forebrain. α7 nAChRs are highly expressed in the hippocampus where they can modulate glutamate-mediated synaptic plasticity (Yakel, 2014). Synaptic strengthening is correlated with an increase in the number of synaptic AMPA (but not NMDA) receptors (Kessels & Malinow, 2009) and this may be reflected in the significant increase in [3H]-AMPA binding localised to the CA1/CA2 area of the ventral hippocampus after morphine-primed reinstatement. Inhibition of the increased [3H]-AMPA binding by prior administration of MLA (Fig 3) suggests that α7 nAChRs play a critical role in facilitating such synaptic changes. The ability of MLA delivered into the ventral hippocampus (but not other brain areas examined) to abolish morphine-primed reinstatement links the behavioural response to α7 nAChR-mediated mechanisms in this brain region. The ventral hippocampus is connected to circuitry related to emotion, stress and affect; connections to the nucleus accumbens shell, prefrontal cortex and amygdala give it a central role in motivational learning (Fanselow and Dong, 2010).

With respect to nAChRs in addiction, most attention has focussed on the roles of nAChR subtypes in nicotine dependence. α4β2 nAChRs are critical in mediating the rewarding properties of nicotine (Picciotto et al., 1998; Pons et al., 2008) and the α4β2 nAChR-selective antagonist dihydroβerythroidine (DHβE) reduces self-administration (Watkins et al.,
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1999; Grottick et al., 2000), with a locus of action in the VTA (Corrigal et al., 1994). In contrast, pharmacological antagonism or genetic deletion of α7 nAChRs has been reported to have no effect on nicotine self-administration (Grottick et al., 2000; Pons et al., 2008; Liu, 2014) or acquisition of nicotine-CPP (Walters et al., 2006), although other studies have shown an attenuation of intravenous (Markou & Paterson 2001) or intracranial nicotine self-administration (Besson et al., 2012), without affecting the somatic signs of nicotine withdrawal (Markou & Paterson 2001). However, Liu (2014) found that systemic MLA (but not DHβE) selectively attenuated cue-induced reinstatement of nicotine seeking in rats, without affecting the development of nicotine self-administration. These findings suggest distinct roles for α4β2 and α7 nAChRs in nicotine reward and relapse, respectively, and resonate with the selective role for α7 nAChRs in the current study, in which acquisition, maintenance and reconsolidation of morphine-CPP were unaffected by MLA.

In the present work, systemically administered MLA substantially attenuated morphine-primed reinstatement of CPP whereas delivery of MLA into the ventral hippocampus completely abolished the response. This could reflect a bigger local dose effect intracerebrally that fully antagonised α7 nAChRs, whereas the systemic dose of 4 mg/kg might be sub-optimal. Indeed Liu (2014) found a significant dose-effect of MLA at 2.5 and 10 mg/kg for inhibition of reinstatement of cue-induced nicotine self-administration in rats. However, high doses of MLA run the risk of also antagonising other nAChR subtypes (notably α6β2 nAChRs) because this antagonist is selective rather than specific for α7 nAChRs (Mogg et al., 2002). The negligible level of α6-containing nAChRs in the hippocampus supports a specific action of MLA at α7 nAChRs in the ventral hippocampus. An alternative explanation of the greater efficacy of locally administered MLA is that α7 nAChRs may exert opposing influences in other brain regions such as the PFC (Bloem et al., 2014; Udakis et al., 2016) that temper the profound antagonism mediated by the ventral hippocampus.

This study provides evidence for a selective role for α7 nAChRs in the ventral hippocampus in the reinstatement of morphine-primed CPP, which encourages further investigation of α7
α7 nAChRs appear to play a similar role in nicotine reinstatement (Liu, 2014) and have been implicated in cocaine reinstatement (Secci et al., 2017). Therefore, an α7 nAChR-mediated mechanism may contribute to the reinstatement of drug seeking more generally in abstinent addicts.
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Authors’ contributions

VLW, CPB, DH and SW were responsible for the study concept and design, and interpretation of results. AB and VLW were responsible for the design and analysis of the autoradiography experiments. VLW carried out the experiments and analysed the data. PG contributed to the acquisition of autoradiographic binding data. SW drafted the manuscript and CPB provided critical revision of the text and figures. All authors critically reviewed the manuscript and approved the final version for publication.
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Figure legends

**Figure 1 Effect of MLA on stages of morphine-CPP in C57BL/6 mice.** A. Acquisition.

Mice were tested for any innate preference for a particular chamber (Habituation) and then pseudo-randomly allocated to two groups with comparable mean preference scores. The MLA group received MLA (4 mg/kg) 20 minutes prior to a conditioning dose of morphine (10 mg/kg) or saline, paired with alternative compartments, on 4 consecutive days. The control group received saline instead of MLA. A preference test was conducted on the next day, giving the animals free access to both compartments of the CPP apparatus for 15 minutes. Preference scores indicate the time spent in the morphine-paired compartment in seconds minus 450 (half of the total time). Data are expressed as the mean ± SEM. Both groups showed significant acquisition of morphine-CPP (*saline: p<0.05, **MLA: p<0.005, n=16 per group) indicating that MLA treatment had no effect on the acquisition of morphine-CPP.

B. Maintenance. Mice were allocated to two groups and both groups were treated identically, to acquire morphine-CPP as in A. They were tested for expression of morphine-CPP on 5 consecutive days and 1 week later. One group received MLA (4 mg/kg) 20 minutes prior to each preference test, the control group received saline instead of MLA. Both groups acquired morphine-CPP (**p<0.005, n=12 per group). MLA treatment had no effect on the expression of morphine-CPP during the maintenance phase.

C. Schematic for reconsolidation and reinstatement experiments.

D. Reconsolidation. Two groups of mice were treated identically, to acquire morphine-CPP as in A. Three days later mice received a further dose of morphine (10 mg/kg) in the drug-paired compartment. Immediately afterwards one group received MLA (4 mg/kg) while the control group received saline. The mice underwent a preference test the next day and one week later. Both groups acquired morphine-CPP (**p<0.005, n=12 per group). Their preference for the drug-paired compartment was consolidated by morphine, and unaffected by MLA.
E. Reinstatement. Two groups of mice were treated identically, to acquire morphine-CPP as in A, followed by 4 days of extinction, (saline injections only, paired with alternative compartments) on separate days. Both groups acquired morphine-CPP (**p<0.005, n=20 per group) and this was attenuated following extinction training. On the following day, mice received a priming dose of morphine (5 mg/kg) prior to an extended preference test (30 min); one group received MLA (4 mg/kg) 20 minutes before morphine; the control group received saline instead of MLA. Only the control group of mice showed significant reinstatement of morphine-CPP in contrast to MLA-treated mice (saline: **p<0.005). The time spent in the morphine-paired compartment was significantly different between MLA and saline treatments ("p<0.005"). MLA significantly inhibited reinstatement of morphine-CPP.

Figure 2 Effect of morphine-CPP reinstatement, in the presence and absence of MLA, on NMDA receptor binding in mouse brain. Animals underwent morphine-CPP acquisition and extinction (Fig1 C). A parallel set of animals followed the same procedure but received saline instead of morphine. Each group was then randomly divided into two sets that received either saline or MLA (4 mg/kg) 20 minutes before saline or a priming dose of morphine (5 mg/kg), and were then tested for reinstatement of morphine-CPP. Immediately afterwards, mice were killed and brains were prepared for autoradiography as described in the Methods. Sections were labelled with 70 nM [3H]-(+)-MK801, in the absence and presence of 1 µM (+)-MK801 to determine non-specific binding.

A. Representative computer-enhanced autoradiograms of [3H]-(+)-MK801 binding in coronal brain sections of mice taken at 4 levels corresponding to prefrontal cortex (bregma 1.94 mm), striatum (bregma 1.42 mm), dorsal hippocampus (bregma -1.22 mm) and the midbrain/ventral hippocampus (bregma -3.08 mm; Franklin and Paxinos, 1997). Binding levels are represented using a pseudocolour interpretation of black and white film images in fmol/mg tissue equivalent. Representative autoradiograms for non-specific binding (NSB) are shown (far right column).
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B. Quantitative NMDA receptor binding. Levels of [³H]-(+)-MK801 binding (fmol/mg tissue equivalent) are shown for prelimbic cortex (PrL), infralimbic cortex (IL), CA1 and CA2 regions of the ventral hippocampus combined (vCA1+CA2), ventral hippocampus CA3 (vCA3), dorsal hippocampus CA1, CA2 and CA3 separately (dCA1, dCA2, dCA3), for each of the treatment groups (saline + saline control, saline + morphine-primed reinstatement, MLA + saline control, MLA + morphine-primed reinstatement). Data are presented as mean ± S.E.M. (n=4-7). There were no statistically significant differences between any treatment groups in any of the brain regions quantified.

Figure 3 Effect of morphine-CPP reinstatement, in the presence and absence of MLA, on AMPA receptor binding in mouse brain. Animals underwent morphine-CPP acquisition and reinstatement, and their brains were prepared for autoradiography as described in the legend to Figure 2. Sections were labelled with 10nM [³H]-AMPA, in the absence and presence of 0.1µM CNQX to determine non-specific binding.

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**Figure 4 Morphine-CPP in Wistar rats and effect of MLA on reinstatement.**

A. Rats were divided into two groups, both groups underwent acquisition and extinction of morphine-CPP (5 mg/kg morphine) as described in the Methods. Following extinction, one group received a priming dose of morphine (2.5 mg/kg) while the other group received saline (arrow). They were tested for reinstatement of morphine-CPP. Data are presented as mean ± S.E.M. preference scores (n=26). Morphine-primed group was significantly different from saline controls, **p<0.01 (one-way ANOVA and Benjamini-Hochberg test for multiple comparisons).

B. Rats were divided into two groups, both groups underwent acquisition and extinction of morphine-CPP as above. Following extinction, both groups received a priming dose of morphine (2.5 mg/kg), but one group received MLA (4 mg/kg) prior to the priming dose while the other group received saline (arrow). They were tested for reinstatement of morphine-CPP. Data are presented as mean ± S.E.M. preference scores (n=26). Saline-pretreated morphine-primed group was significantly different from morphine-primed group that received MLA, *p<0.05 (one-way ANOVA and Benjamini-Hochberg test for multiple comparisons).

**Figure 5 Effect of intracerebral administration of MLA on reinstatement of morphine-CPP in rats.**

Rats acquired morphine-CPP that was extinguished prior to the implantation of an intracerebral cannula in the dorsal hippocampus A., ventral hippocampus B., or prefrontal cortex C. Animals then received either saline or MLA (6.75 µg/hemisphere) prior to a priming dose of morphine (2.5 mg/kg) (arrows) and testing for reinstatement of morphine-CPP. Data are presented as mean ± S.E.M. preference scores (n=8). In ventral hippocampus, saline-pretreated morphine-primed group was significantly different from morphine-primed group.
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