Ketamine blocks the correlation between morphine-induced conditioned place preference and anxiety-like behaviors in mice.

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Running title: Ketamine effects on morphine-induced CPP

Abstract

Patients suffering from opioid use disorder often relapse during periods of abstinence, which is posited to be caused by negative reinforcement driving motivated behaviors. Here, we explored whether opioid-seeking behaviors in mice were correlated with the negative affect of anxiety. To do this, we conditioned mice to associate a specific context with morphine, an opioid with strongly addictive properties, using a three compartment chamber. 24 h following five days of morphine (10 mg/kg, i.p.) conditioning, anxiety levels were tested by measuring time in the open arms of an elevated plus maze. The next day, mice were placed in the three compartment chamber to measure morphine-induced conditioned place preference (CPP). Our results show that following morphine conditioning, mice spent significantly less time in the open arm of the elevated plus maze, which was negatively correlated with the CPP score. Furthermore, we found that an acute treatment with (*R*,*S*)-ketamine (10 mg/kg, i.p.), a medication demonstrating promise for preventing anxiety-related phenotypes, 30 min. prior to testing on post conditioning day 1, increased time spent in the open arm of the elevated plus maze in morphine conditioned mice. Lastly, we found that a second injection of ketamine 30 min. prior to CPP tests on post conditioning day 2 attenuated morphine-induced CPP, which lasted for up to 28 d. Overall, our results suggest that morphine-induced CPP may be driven by negative reinforcement, which can be prevented by acute ketamine administration.

Keywords: negative affect, morphine, conditioned place preference, anxiety, opioid use disorder, ketamine, psychedelics

Introduction

Ketamine is a potent noncompetitive antagonist at the N-methyl-D-aspartate (NMDA) receptor (Ebert et al., 1997) and is approved by the Food and Drug Administration as a general anesthetic in humans. Ketamine also is used off label for analgesia (Quibell et al., 2011), complex regional pain syndrome (Sigtermans et al., 2009), refractory status epilepticus (Gaspard et al., 2013), and most recently for treatment of resistant depression (Iadarola et al., 2015). Additionally, there is growing evidence that ketamine has significant short- and long-term anxiolytic effects (Glue et al., 2018).

The motivation to continually seek and obtain addictive substances during periods of abstinence or recovery is caused, in part, by the necessity to avoid aversive internal states. Evidence for this comes from patients with substance use disorders who self-report urges and intentions to take drugs to avoid drug-withdrawal symptoms (O'Brien, 1975;Baker et al., 2004;Wikler, 2013) or to cope with negative affect (Perkins and Grobe, 1992;Zinser et al., 1992;Wetter et al., 1994;Cooney et al., 1997;Conklin and Perkins, 2005;Fox et al., 2007). For example, abstinence from morphine, a highly addictive opioid, facilitates increases in anxiety (Gold et al., 1978;1979), which is a potential factor in continued drug use (Martins et al., 2012).

In order to better understand the mechanisms mediating drug-craving and subsequent relapse, preclinical models have been developed whereby drug-seeking behaviors are monitored in drug-exposed rodents. One such model of addiction is the conditioned place preference (CPP) paradigm; a drug is paired with a context over a period of days, followed by a test day, when the time spent in the drug-paired or the non-drug paired contexts is measured. Many reports have demonstrated that morphine elicits robust place preference for the morphine-paired context following short- or long-term abstinence at varying doses and conditioning paradigms (Tzschentke, 2007;Koo et al., 2014;Graziane et al., 2016;Hearing et al., 2016). However, evidence suggesting that this “drug context-seeking” behavior is mediated by negative affective states remains lacking. Here, we attempted to correlate the negative affect of anxiety, measured using the elevated plus maze (Pellow et al., 1985), with morphine-induced CPP and investigate whether acute (*R,S*)-ketamine administration prior to testing was sufficient to alter anxiety and/or morphine-induced CPP behaviors.

Methods

Animals

All experiments were done in accordance with procedures approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee. Male C57BL/6J mice aged 5-8 weeks were purchased from Jackson Labs (stock #000664) (Bar Harbor, ME), singly-housed, and maintained on a regular 12 hour light/dark cycle (lights on 07:00, lights off 19:00) with *ad libitum* food and water. In total, 7 mice treated with morphine were removed from the study due to one of the following reasons: 1) a missed injection of morphine during conditioning evidenced by the lack of Straub tail, and a significant decrease in locomotor time compared to the previous days or 2) placed in the wrong context during drug pairing, or 3) the home cage was flooded during conditioning.

Drugs

(−)-morphine sulfate pentahydrate was provided by the National Institute on Drug Abuse Drug Supply Program. Ketamine hydrochloride (racemic mixture of 50% *R*-ketamine and *S*-ketamine) (Dechra Pharmaceuticals, Northwich, United Kingdom) was purchased from the Comparative Medicine Department at the Pennsylvania State University College of Medicine.

Conditioned Place Preference

Conditioned place preference (CPP) chambers (Med Associates) were located in the mouse housing room and consisted of three distinct compartments separated by manual guillotine-style doors. Each compartment had distinct contextual characteristics: the middle (neutral) compartment (2.85″ × 5″ × 5″) had grey walls and grey plastic floor, while the choice compartments (6.6″ × 5″ × 5″ each) had either white walls and stainless steel mesh floor or black walls and stainless steel grid floor. All compartments were illuminated with a dim light during use. Immediately following use the entire preference chamber was cleaned thoroughly with a scent-free soap solution. Mouse locations, activity counts, and time spent in each compartment were collected via automated data-collection software (Med Associates) via infrared photobeam strips lining each compartment. Morphine administration was verified with the Straub tail response and enhanced locomotor activity.

Habituation. Mice were placed in the center compartment with free access to all three compartments for 20 min once a day for two days. Time spent (seconds) in each compartment was recorded.

Conditioning. 24 h after habituation, mice received 5 d conditioning training. Morphine-paired compartments were assigned based on the least preferred side (a biased approach) (Tzschentke, 2007) calculated by averaging time spent in each compartment over the 2 habituation days. Similar to conditioning studies with alcohol (Gremel et al., 2006), we find that C57BL/6J mice will reliably develop morphine CPP using a biased approach. During conditioning, mice received an injection of saline and were placed into the most preferred compartment for 40 min. 6 h later, mice received an injection of saline (control group) or morphine (10 mg/kg, i.p.) and were placed into their least preferred compartment for 40 min. (Koo et al., 2014;Graziane et al., 2016).

Post conditioning. 48 h or 28 d after the last conditioning day, mice were placed in the 3-compartment chamber and allowed to move freely for 20 min. Our post-conditioning took place at a time point corresponding to 3 h prior to drug conditioning (e.g., morphine conditioning took place at 3 P.M., post-conditioning tests took place 2 or 28 days later at 12 P.M.). CPP scores were calculated as time spent in the drug-paired side minus the average time spent on the same side during preconditioning (Bohn et al., 2003). Activity counts are defined as any beam break within a current zone. This is inclusive of grooming, rearing, and lateral movements. Mice were treated with 0.9% saline (0.1 ml, i.p.) or with ketamine (10 mg/kg, i.p.) 30 min. prior to the first CPP test.

Elevated Plus Maze

The elevated plus maze for mice (Stoelting, Item #60140) was raised approximately 50 cm from the ground. The floor of the elevated portion of the maze was gray. Two opposite arms (35 × 5 cm each) of the maze were enclosed by a 15 cm high wall and the remaining two arms were "open." A center space (5 cm^2) between these four arms was also not enclosed. The elevated portion of the apparatus was cleaned thoroughly with a scent-free soap solution after each trial. Behavioral tests were performed in the animal housing room under ambient light of the light cycle.

24 h after the last conditioning day in the CPP apparatus, mice were placed in the center space facing the open arm and allowed to explore the apparatus for 5 minutes prior to being placed back into their home cage (Grisel et al., 2008). Each trial was video recorded using a GoPro camera (Hero7 white) and analyzed by researchers blinded to treatment condition of the mice. Time in the open arm was measured when the body of the mouse cleared the center space. Mice were treated with 0.9% saline (0.1 ml, i.p.) or ketamine (10 mg/kg, i.p.) 30 min. prior to the elevated plus maze test.

Statistical Analysis

Statistical significance was assessed in GraphPad Prism software using a Student's t-test, one- or two-way ANOVA with Bonferroni's correction for multiple comparisons as specified. F values for two-way ANOVA statistical comparisons represent interactions between variables unless stated otherwise. Two-tailed tests were performed for all studies. For correlation analysis, the Pearson's correlation coefficient, and subsequent linear regression, were determined. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of morphine conditioned place preference is correlated with anxiety-like behaviors

Repeated exposure to morphine increases levels of anxiety both in humans and in animal models of substance use disorders (Gold et al., 1978;1979;Becker et al., 2017). Additionally, it is posited that relapse to opioids in abstinent patients is caused by negative affective states driving negative reinforcement (Solomon and Corbit, 1978;Koob and Le Moal, 2008;Evans and Cahill, 2016). In an attempt to provide evidence that morphine-induced CPP, a preclinical model of addiction-like behavior (Bardo and Bevins, 2000), is mediated, in part, by negative reinforcement, 24 h following the last morphine conditioning session (**Fig. 1A**), we measured anxiety levels using an elevated plus maze (Pellow et al., 1985). We found that morphine-treated animals, who showed robust locomotor sensitization by conditioning day 5 (**Fig. 1B**), expressed a significant decrease in the percent time spent in the open arm of the elevated plus maze compared to saline-treated controls ($t_{(18)}=3.59$, $p=0.002$, Student's t-test) (**Fig. 1C**). To correlate anxiety levels with CPP scores, mice underwent CPP tests 24 h following anxiety tests (**Fig. 1A**). We found that 5 d morphine conditioning elicited significant increases in place preference ($t_{(18)}=3.70$, $p=0.002$, Student's t-test) (**Fig. 1D**), which was negatively correlated with the percent time spent in the

open arms of the elevated plus maze (Pearson's correlation coefficient = -0.788). This correlation was not observed in saline-treated animals (Pearson's correlation coefficient = -0.354). A simple linear regression was carried out to investigate the relationship between anxiety levels and morphine-induced CPP. A significant regression equation was found in morphine-treated mice ($F_{(1,5)}$=8.21, p=0.04, R^2=0.62), while saline-treated animals did not display a significant relationship between anxiety levels and saline-induced place conditioning ($F_{(1,11)}$=1.57, p=0.24, R^2=0.13) (**Figs. 1E and F**). Overall, these results suggest that morphine conditioning in a CPP paradigm is sufficient to facilitate anxiety-like behaviors during 24 h abstinence and these anxiety-like behaviors are correlated with morphine-induced CPP.

Ketamine blocks morphine-induced anxiety-like behaviors and morphine CPP

Evidence suggests that (*R,S*)-ketamine, a noncompetitive NMDA receptor antagonist (Lodge et al., 1982;Kohrs and Durieux, 1998), is an effective treatment for anxiety and substance use disorders (Krupitsky et al., 2002a;Ivan Ezquerra-Romano et al., 2018;Taylor et al., 2018). Because of this, we investigated whether an acute injection of (*R,S*)-ketamine (30 min. prior to testing) would be sufficient to block anxiety-like behaviors and/or morphine-induced CPP (**Fig. 2A**). Following conditioning with morphine, which produced robust locomotor sensitization (**Fig. 2B**), we found that (*R,S*)-ketamine treatment significantly increased the percent time in the open arms of the elevated plus maze ($F_{(3, 32)}$=17.36, p<0.0001, one-way ANOVA, Bonferroni post hoc test) (**Fig. 2C**) and was sufficient to prevent morphine-induced CPP ($F_{(3, 32)}$=5.64, p=0.003, one-way ANOVA, Bonferroni post hoc test) (**Fig. 2D**) without altering locomotor activity on test day (during morphine abstinence) ($F_{(3,32)}$=0.602, p=0.618, two-way repeated measures ANOVA, Bonferroni post hoc test) (**Fig. 2E**). In addition, we observed that an acute (*R,S*)-ketamine injection blocked the negative correlation between the percent time spent in the open arms of the elevated plus maze and morphine-induced CPP (Pearson's correlation coefficient = 0.3036) (**Fig. 2F and G**). There was no correlation observed in saline-conditioned mice treated with (*R,S*)-ketamine (Pearson's correlation coefficient = -0.1868). A simple linear regression revealed no linear relationship between anxiety and CPP in morphine conditioned mice ($F_{(1,4)}$=0.406, p=0.56, R^2=0.092) or saline-conditioned mice ($F_{(1,8)}$=0.289, p=0.605, R^2=0.035).

Acute ketamine treatment blocks the long-term expression of morphine CPP

Lastly, we have previously shown that morphine-induced CPP, using the described paradigm, is sufficient to elicit long-lasting CPP for up to 28 d post conditioning (Graziane et al., 2016). To test whether ketamine was sufficient to prevent the prolonged expression of morphine-induced CPP, animals treated with ketamine at 24 and 48 h post conditioning were re-exposed to the CPP test 28 d post-conditioning. We found that acute injection of (*R,S*)-ketamine on post conditioning days 1 (prior to elevated arm maze tests) and 2 (prior to CPP tests) was sufficient to prevent the prolonged expression of morphine-induced CPP on post-conditioning day 28 ($F_{(3, 35)}$=7.39, p=0.0006, one-way repeated measures ANOVA, Bonferroni post hoc test) (**Fig. 3**).

Discussion

Our results show that the percent time spent in the open arms of the elevated plus maze is decreased in animals conditioned with morphine. Additionally, we show a significant correlation between anxiety-like behaviors and morphine CPP such that decreases in the time spent in the open arms of the elevated plus maze (increased anxiety-like behavior) is correlated with increases in the time spent in the morphine-paired context. Lastly, we show that an acute injection of (*R,S*)-ketamine 30 min prior to the elevated plus maze and CPP tests is sufficient to block morphine-induced anxiety-like behaviors and morphine-induced CPP on post-conditioning day 1 and up to 28 d post conditioning.

Anxiety-like behaviors during morphine abstinence

Morphine possesses anxiolytic-like properties during initial exposure (Koks et al., 1999;Sasaki et al., 2002;Shin et al., 2003). However, during opioid abstinence, symptoms of anxiety (Gold et al., 1978;1979;Li et al., 2009;Shi et al., 2009) or anxiety-like behaviors are observed (Cabral et al., 2009;Becker et al., 2017). Here, we show that 24 h following repeated morphine injections (once a day for 5 days), mice display anxiety-like behaviors in the elevated plus maze (**Fig. 1C**). These results are similar to previous studies showing escalating doses of morphine over a 6 day period induce anxiety-like behaviors in the marble burying task (Becker et al., 2017). Additionally, our observed morphine-induced anxiety-like behavior is timed with anxiogenic neurobiological responses that occur during acute opioid abstinence including, increases in norepinephrine release in the extended amygdala (Fuentealba et al., 2000), norepinephrine-induced modulation of the extended amygdala (Aston-Jones et al., 1999;Delfs et al., 2000;Smith and Aston-Jones, 2008), activation of the amygdalar corticotrophin-releasing factor (CRF) system (Heinrichs et al., 1995;Maj et al., 2003), and decreases in dopamine transmission (Diana et al., 1995).

The observed correlation between anxiety-like behaviors and drug-seeking behaviors following morphine conditioning in a three chamber apparatus (**Fig. 1F**) may suggest that animals seek the drug-paired chamber as a consequence of negative reinforcement to alleviate aversive affective states facilitated by opioid abstinence. Importantly, our injection regimen of morphine 10 mg/kg once a day for 5 consecutive days does not induce signs of somatic withdrawal in mice including jumping, wet dog shakes, teeth chattering, rearing, tremor, diarrhea, or mastication (Gallego et al., 2010), coinciding with the lack of observed somatic withdrawal symptoms following a more prolonged injection regimen of 5 daily morphine (10 mg/kg, i.p.) injections over 4 weeks (Robinson and Kolb, 1999). Although, more studies are required, it is plausible that specific opioid dosing regimens may be implemented in a preclinical setting in order to separate opioid-induced negative affective states (e.g., anxiety) from confounds induced by somatic signs of opioid withdrawal. This idea has been demonstrated previously with doses of naloxone (used to precipitate opioid withdrawal) that were sub-threshold for somatic signs of opioid withdrawal (Gracy et al., 2001).

Ketamine's effects on anxiety-like behaviors

Ketamine has recently been shown to be a potential effective treatment for anxiety disorders (Glue et al., 2018;Shadli et al., 2018;Taylor et al., 2018). In humans, ketamine displays a biphasic dose effect on anxiety, with low doses decreasing anxiety and higher doses increasing

anxiety (Jansen, 1989;Krystal et al., 1994). Likewise, in rodents, ketamine induces anxiolytic-like behaviors (Engin et al., 2009;Zhang et al., 2015;Fraga et al., 2018) as well as anxiogenic-like phenotypes likely dependent upon the dose, temporal relationship between ketamine injection and test onset, and rodent species (Silvestre et al., 1997;da Silva et al., 2010). Here, we demonstrate that in C57BL/6J mice, acute injection of ketamine at 10 mg/kg, i.p. 30 min prior to testing is sufficient to block morphine-induced anxiety-like behaviors during a 24 h abstinence time period (**Fig. 2C**). Additionally, we find that ketamine significantly increases percent time in the open arm of the elevated plus maze in mice conditioned with saline. This significant change observed in saline conditioned animals suggests that ketamine, at the dose and temporal relationship of ketamine injection and test onset, is sufficient to overcome baseline anxiety-like behaviors in animals exposed to a novel environment (i.e., elevated plus maze). Together, these findings suggest that ketamine is sufficient to reduce anxiety-like behaviors and morphine-seeking behaviors, further providing evidence that negative affective states drive morphine-induced place preference in the CPP model.

Despite the evidence suggesting that the antagonistic effects of ketamine on NMDA receptors in the bed nucleus of the stria terminalis attenuate negative affective states (Louderback et al., 2013), the mechanisms mediating the observed anxiolytic-like effects are unknown. In addition to acting as a non-competitive antagonist to NMDA receptors in the extended amygdala, evidence suggests that ketamine interacts with hyperpolarization-activated cyclic nucleotide-gated (HCN) channels as well as dopamine, serotonin, sigma, opioid, and cholinergic receptors (Scheller et al., 1996;Cai et al., 1997;Kubota et al., 1999;Lydic and Baghdoyan, 2002;Wang et al., 2012;Zanos et al., 2018). Additionally, ketamine metabolites are biologically active as antagonists to NMDA receptors (Ebert et al., 1997) and α7 nicotinic acetylcholine receptors (Moaddel et al., 2013), while also possessing agonistic activity for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Zanos et al., 2016;Tyler et al., 2017). Because of the promiscuous activity of ketamine and its metabolites, it has been difficult to pinpoint how ketamine influences anxiety states both in humans and in preclinical models.

Ketamine's effects on morphine-induced conditioned place preference

Using a paradigm known to induce robust CPP for up to 28 d post conditioning (Graziane et al., 2016), we show that an acute injection of ketamine 30 min prior to the CPP test on abstinence day 2 is sufficient to block morphine-induced CPP. These results are not likely caused by changes in locomotor activity as activity counts during habituation (baseline) were not significantly different from activity counts measured following ketamine administration (**Fig. 2E**). Our results are in line with previous publications demonstrating that ketamine blocks morphine-induced CPP in mice (Suzuki et al., 2000). However, the effects on locomotor activity are conflicting. Whereas, our results and those from previous publications show that ketamine does not influence locomotor activity (Lindholm et al., 2012), others have found that locomotor activity is increased (Filibeck and Castellano, 1980) or decreased following ketamine administration (Akillioglu et al., 2012). These discrepancies are likely due to the temporal relationship between ketamine treatment and test onset. Here, we performed our tests 30 min following ketamine injection similar to previous studies (Lindholm et al., 2012), while tests performed 5 min or 15 min following ketamine administration appear to increase or decrease locomotor activity, respectively (Filibeck and Castellano, 1980;Akillioglu et al., 2012). Given

that the half-life of ketamine is ~13-25 min. in mice following i.p. administration (Maxwell et al., 2006;Zanos et al., 2016;Ganguly et al., 2018), it is possible that the locomotor effects observed are due to ketamine action prior to metabolism, while the effects on negative affect are potentially attributed to ketamine metabolites including hydroxynorketamine (Li et al., 2015;Zanos et al., 2016). This hypothesis will need to be tested in future experiments.

Ketamine abolishes the correlation between anxiety-like behaviors and morphine-induced CPP

Based on our findings that ketamine elicited anxiolytic-like behaviors following an acute injection, it is possible that the acute administration of ketamine was sufficient to prevent a negative affective state during 24 h morphine abstinence, thus facilitating the lack of motivation to seek a context paired with a drug reward (i.e., morphine-induced CPP). It is also plausible that the block of morphine-induced CPP by ketamine may be mediated by its effects on cognition and memory, thus blocking the recall of morphine-context associations (Ghoneim et al., 1985;Newcomer et al., 1999;Morgan et al., 2004) (Malhotra et al., 1996;Pfenninger et al., 2002). However, based on previous findings (Honey et al., 2005;Zhai et al., 2008;Goulart et al., 2010), these ketamine-induced deficits in cognitive functioning and memory are not likely to influence already encoded morphine-context associations. This does not rule out that ketamine's effects on morphine-induced CPP may be mediated by other mechanisms of action as ketamine has proven effective for treating pain (Weisman, 1971;Laskowski et al., 2011;Jonkman et al., 2017), depression (Khorramzadeh and Lotfy, 1973;Sofia and Harakal, 1975), and inflammation (Roytblat et al., 1998;Beilin et al., 2007;Loix et al., 2011).

Ketamine as a treatment option for substance use disorders

There is growing clinical and preclinical evidence that ketamine may be a potential treatment option for substance use disorders (Ivan Ezquerra-Romano et al., 2018;Jones et al., 2018). Through the use of ketamine psychotherapy (Ivan Ezquerra-Romano et al., 2018), alcohol-dependent patients (Krupitsky and Grinenko, 1997;Kolp et al., 2006), heroin-dependent patients (Krupitsky et al., 2002b;Krupitsky et al., 2007), and cocaine-dependent patients (Dakwar et al., 2017) showed greater rates of abstinence and reductions in drug craving. These results have been echoed in preclinical models of substance use disorders as acute ketamine injections significantly attenuate alcohol self-administration (Sabino et al., 2013) and prevent the reconsolidation of morphine-induced CPP (Zhai et al., 2008). Here, we discovered a novel and unexpected loss of long-term expression of morphine-induced CPP in animals injected with (*R,S*)-ketamine at time points corresponding to 24 and 48 h post CPP conditioning. These results demonstrate the profound effect that (*R,S*)-ketamine has on reward-related behaviors and opens up many avenues including, investigating temporal effects of ketamine treatment at later time points following conditioning, the neurocircuit mechanisms mediating this prolonged ketamine effect on morphine-induced CPP, and the specificity for drug-context associations versus other forms of memory. With the ever increasing use of ketamine as an antidepressant in major depressive disorder (Berman et al., 2000;Diazgranados et al., 2010;Ibrahim et al., 2011;Zarate et al., 2012;Murrough et al., 2013b), applying its therapeutic use to patients suffering from substance use disorders holds potential value as an alternative treatment option.

Limitations to the use of ketamine as a treatment option for substance use disorders

Despite its therapeutic value, ketamine has undesirable side effects including drowsiness, confusion, dizziness, and dissociative psychiatric side effects (Zarate et al., 2006;Diazgranados et al., 2010;Ibrahim et al., 2011;Murrough et al., 2013a). Additionally, evidence suggests that ketamine impairs cognition and memory (Harris et al., 1975;Ghoneim et al., 1985;Malhotra et al., 1996;Newcomer et al., 1999;Pfenninger et al., 2002;Morgan et al., 2004;Honey et al., 2005;Mathew et al., 2010;Driesen et al., 2013) and may cause urological effects (Middela and Pearce, 2011). A limitation of ketamine use as a treatment option for substance use disorders is its abuse potential (Liu et al., 2016). However, controlled studies in patients addressing the abuse potential of low-dose ketamine are lacking and if the long-lasting ketamine effects shown here in mice translate to human patients, the abuse liability can be mitigated by monthly physician-administered injections.

Conclusions

Here, we found that morphine conditioning in a three-compartment apparatus was sufficient to elicit anxiety-like behaviors in mice, which were correlated with morphine-induced CPP. Additionally, we provided evidence that acute ketamine pretreatment produces anxiolytic-like behaviors and blocks morphine-induced CPP for a prolonged time period, suggesting that ketamine is a potential option for attenuating negative reinforcement implicated in substance use disorders.

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Author Contributions Statement

H.E.J., G.M., H.G., D.S.M., S.S. Y.S., and N.M.G. designed the experiments and analyses and wrote the manuscript. H.E.J., G.M., H.G., D.S.M., and S.S performed behavioral training and testing.

Declaration of Interest

Declarations of interest: none

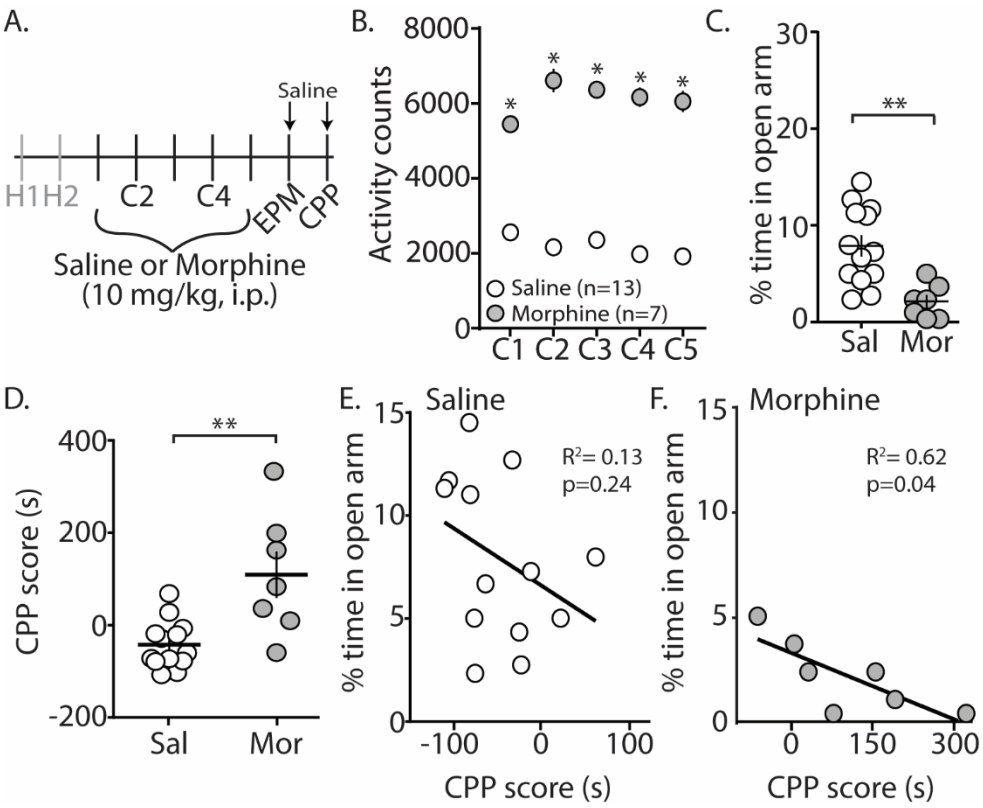

Figure 1. Morphine conditioning in a CPP paradigm elicits anxiety-like behaviors during 24 h abstinence. (A) Time line and drug regimen of the behavioral procedure. Animals underwent two days of habituation (H), followed by five days of saline or morphine (10 mg/kg, i.p.) conditioning (C), before being subjected to tests measuring anxiety-like behaviors using an elevated plus maze (EPM) 24 h post conditioning. 24 h post EPM tests, CPP tests were performed. Animals were injected with saline 30 min. prior to EPM and CPP tests. (B) Summary showing that morphine conditioning over 5 days produces robust locomotor sensitization ($F_{(4, 72)}$= 9.24, p<0.0001, two-way ANOVA, Bonferroni post hoc test). (C) Summary showing that morphine (Mor)-conditioned mice spent significantly less time in the open arms of the elevated plus maze compared to saline (Sal)-conditioned mice 24 h following the last conditioning day ($t_{(18)}$=3.59, p=0.002, Student's t-test). (D) Summary showing that morphine produced reliable CPP ($t_{(18)}$=3.70, p=0.002, Student's t-test). (E) Correlation of the % time in the open arm of the elevated plus maze and CPP score in saline- or (F) morphine-conditioned animal. *p<0.05, **p<0.01.

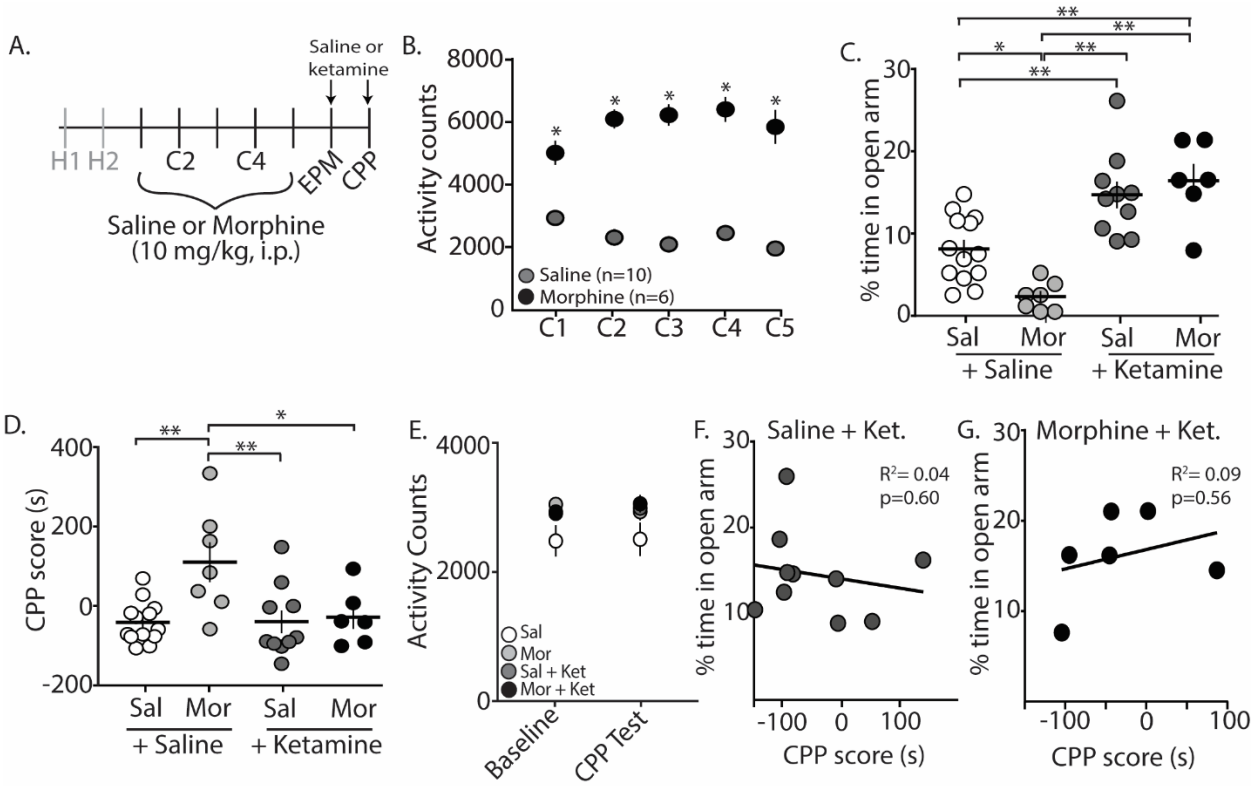

Figure 2. Acute (*R*,*S*)-ketamine injection produces anxiolytic-like behaviors in mice 24 h after conditioning and blocks morphine-induced CPP. (A) Time line and drug regimen of the behavioral procedure. Saline or (*R*,*S*)-ketamine (10 mg/kg, i.p.) was injected 30 min. prior to elevated plus maze (EPM) test and the first conditioned place preference (CPP) test. (B) Summary showing that morphine conditioning over 5 days (C1-C5) produces robust locomotor sensitization ($F_{(4, 56)}$= 12.55, p<0.0001, two-way ANOVA, Bonferroni post hoc test). (C) Summary showing that (*R*,*S*)-ketamine significantly increased the time spent in the open arms of the elevated plus maze in both saline (Sal)- and morphine (Mor)-conditioned mice ($F_{(3, 32)}$=17.36, p<0.0001, one-way ANOVA, Bonferroni post hoc test) (animals not receiving (*R*,*S*)-ketamine are the same data as shown in Fig. 1C). (D) Summary showing that morphine produced reliable CPP at post conditioning day 2, which was blocked by (*R*,*S*)-ketamine 30 min prior to testing ($F_{(3, 32)}$=5.64, p=0.003, one-way ANOVA, Bonferroni post hoc test) (saline and morphine groups are the same animals as shown in Fig. 1D). (E) Summary showing the activity counts in the CPP chamber during habituation (baseline) and during the CPP test in saline (Sal)- or morphine (Mor)-conditioned mice treated with saline or (*R*,*S*)-ketamine 30 min prior to testing ($F_{(3,32)}$=0.602, p=0.618, two-way repeated measures ANOVA, Bonferroni post hoc test). (F) Correlation of the % time in the open arm of the EPM and CPP score in saline- or (G) morphine-conditioned animals treated with (*R*,*S*)-ketamine 30 min. prior to testing. *p<0.05, **p<0.01.

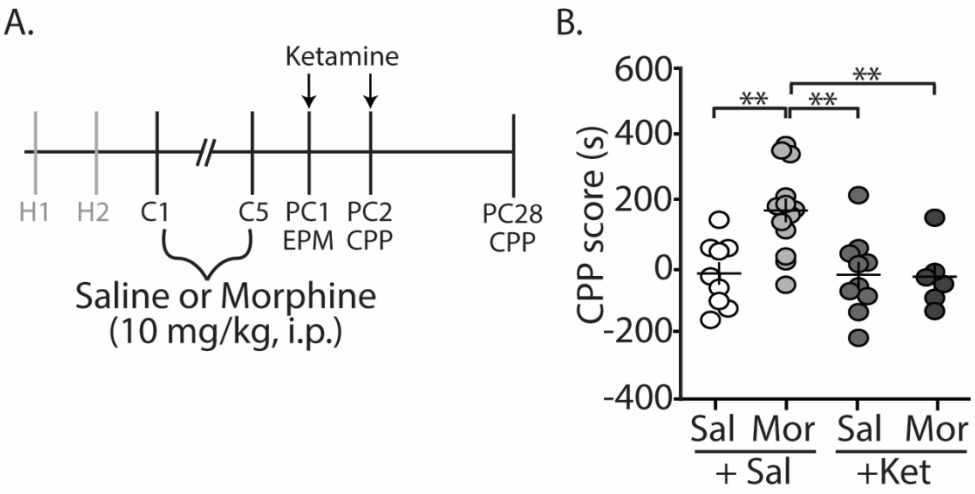

Figure 3. (*R*,*S*)-ketamine is sufficient to prevent the prolonged retention of morphine-induced CPP at post conditioning day 28. (A) Time line and drug regimen of the behavioral procedure. (*R*,*S*)-ketamine (10 mg/kg, i.p.) was injected 30 min. prior to the EPM test on post-conditioning day 1 (PC1) and the first CPP test on post conditioning day 2 (PC2). The second CPP test was run 28 d post conditioning (PC28). Saline and morphine conditioned animals, not treated with (*R*,*S*)-ketamine, did not undergo an EPM test. Their first CPP test score on PC1 was published (see (McDevitt and Graziane, 2019)). (B) Summary showing that morphine produced reliable CPP 28 d post conditioning, which was blocked by (*R*,*S*)-ketamine ($F_{(3, 35)}$=7.39, p=0.0006, one-way repeated measures ANOVA, Bonferroni post hoc test). Abbrev.: EPM=elevated plus maze; CPP=conditioned place preference. **p<0.01.

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