

# Morphine-induced mu opioid receptor trafficking enhances reward yet prevents compulsive drug use

Amy Chang Berger, Jennifer L. Whistler\*

**Keywords:** addiction; compulsivity; endocytosis; mouse self-administration; mu opioid receptor

DOI 10.1002/emmm.201100144

Received January 04, 2011

Revised March 29, 2011

Accepted March 31, 2011

Morphine, heroin and other commonly abused opioids induce little mu opioid receptor (MOR) trafficking compared to endogenous opioids. We utilized knock-in mice expressing a mutant recycling MOR (RMOR) that desensitizes and is internalized in response to morphine to show that facilitating MOR trafficking not only enhances morphine reward but, despite this, reduces the development of addiction-like behaviours. To demonstrate this, we developed a novel model of the transition from controlled to compulsive drug use that recapitulates many features of human addiction, including persistent drug seeking despite adverse consequences and a decreased preference for alternative rewards. These behaviours emerged spontaneously in wild-type but not RMOR mice, and their intensity predicted the reinstatement of morphine seeking after extended abstinence, while prior morphine intake did not. These results confirm previous findings in the rat that addiction can be dissociated from both reward and consumption. Most importantly, these results demonstrate that one can simultaneously reduce the 'addictiveness' of morphine and enhance its desirable effects by promoting agonist-induced MOR trafficking.

## INTRODUCTION

For decades it has been a priority of opioid pharmaceutical research to develop an analgesic that can be used for extended periods of time without causing tolerance, dependence and addiction. No such drug has yet been developed; however, currently available opioids, which are primarily agonists of the mu opioid receptor (MOR), a  $G_{i/o}$ -coupled receptor, induce varying degrees of tolerance and dependence when they are used at acutely equi-antinociceptive doses (Duttaroy & Yoburn, 1995; Grecksch et al, 2006; Kim et al, 2008; Walker & Young, 2001). This is not surprising when one considers that the potency and efficacy with which a drug stimulates  $G_{i/o}$  signalling is not necessarily related to the potency and efficacy with which it activates regulatory processes, such as receptor phosphorylation and G protein-independent signalling (Groer et al, 2007; Madhavan et al, 2010; Rajagopal et al, 2010).

For example, following activation by endogenous opioid peptides and the small molecule drug, methadone, the MOR is rapidly phosphorylated by GPCR kinases (GRKs) and bound by arrestins (for review, see von Zastrow, 2010). These events uncouple the receptor from G protein, resulting in the desensitization of G protein-dependent signal transduction. In addition, arrestins recruit components of the endocytic machinery, and internalized MORs are subsequently resensitized by recycling back to the plasma membrane (von Zastrow, 2010).

In contrast, morphine and other commonly abused opioids, including heroin and oxycodone, induce substantially less GRK phosphorylation and arrestin recruitment (Kovoor et al, 1998; Whistler & von Zastrow, 1998; Zhang et al, 1998). The functional consequences of this vary depending on the cell type or brain region examined, because the endogenous complement of GRKs and arrestins varies, and because under certain circumstances, morphine may induce MOR desensitization by other kinases, including protein kinase C (Bohn et al, 2002; Chu et al, 2010; Kelly et al, 2008). Consequently, in some parts of the brain, morphine induces little MOR desensitization or endocytosis compared to endogenous opioids and methadone, and this results in relatively persistent MOR activation (Alvarez et al, 2002; Ingram et al, 1998; Keith et al, 1998; Trafton

Neuroscience Program, University of California, San Francisco, Ernest Gallo Clinic and Research Center, 5858 Horton Street, Suite 200, Emeryville, CA 94608, USA

\*Corresponding author: Tel: +1 510 985 3127, Fax: +1 510 985 3101; E-mail: jennifer.whistler@ucsf.edu

et al, 2000); while in other parts of the brain, morphine induces desensitization but little endocytosis, resulting, instead, in comparatively persistent MOR silencing (Bagley et al, 2005; Dang & Williams, 2004; Sim et al, 1996; Ueda et al, 2001).

Importantly, both scenarios have been implicated in morphine tolerance and dependence. For example, in cells where morphine induces neither desensitization nor endocytosis, the cell compensates for persistent MOR activation by the upregulation of pathways that oppose receptor signalling. Specifically, whereas acute MOR activation inhibits cAMP production by adenylyl cyclase, chronic morphine treatment leads to an increase in basal cAMP levels (Bonci & Williams, 1997; Hack et al, 2003; Nestler, 1996). This superactivation of the cAMP pathway contributes to tolerance in the presence of morphine and withdrawal upon its removal. Indeed, inhibitors of this pathway attenuate withdrawal signs in morphine dependent animals (Lane-Ladd et al, 1997; Punch et al, 1997).

On the other hand, in cells where morphine induces MOR desensitization but little endocytosis, the failure to resensitize receptors leads to tolerance caused by a loss of signalling capacity. Consistent with this, beta-arrestin 2 knock-out mice have enhanced morphine analgesia and reduced analgesic tolerance (Bohn et al, 2000; Bohn et al, 1999). However, they still develop dependence to a similar degree as wild-type (WT) animals, likely because compensatory adaptations to persistent MOR signalling have not been prevented.

We hypothesized that facilitating the combination of MOR desensitization and endocytosis in response to morphine would reduce both persistent MOR activation and persistent desensitization and, therefore, reduce both tolerance and dependence. To test this hypothesis, we generated a mutant recycling MOR (RMOR) that desensitizes, internalizes and recycles in response to morphine (Finn & Whistler, 2001).

As we predicted, RMOR mice show increased analgesia and develop reduced tolerance and dependence selectively in response to morphine. These findings suggest that opioid drugs that promote MOR trafficking should induce less tolerance and dependence than existing drugs; however, they do not address whether such drugs would have an increased or decreased liability for abuse.

The relationship between addiction and either analgesia, which is increased in the RMOR mice, or tolerance and dependence, which are decreased, is far from clear. Since opioid analgesia and reward are mediated by overlapping brain circuits (Altier & Stewart, 1998; Olmstead & Franklin, 1997), morphine would be expected to be a more potent reinforcer in RMOR mice, potentially putting them at higher risk for addiction. However, while reward is a necessary prerequisite for recreational drug use, the vast majority of opioid users do not become addicts.

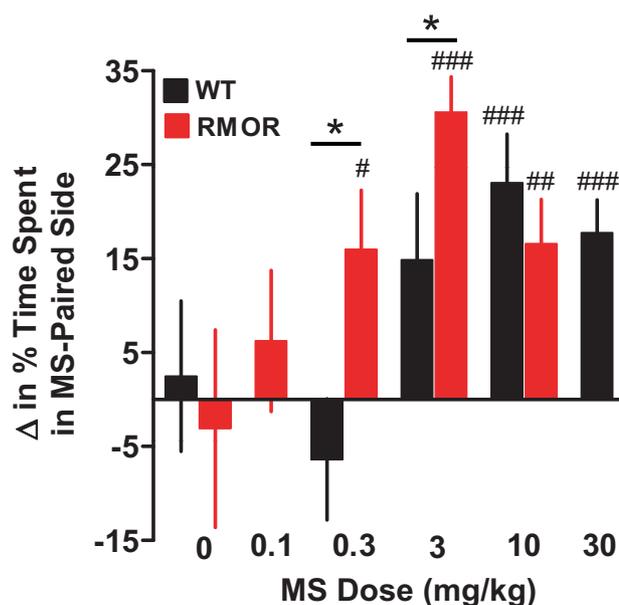
Similarly, while most opioid addicts endorse dependence, there is no consistent relationship between the severity of withdrawal at the initiation of treatment and worse clinical outcomes (Chakrabarti et al, 2010; Ziedonis et al, 2009). In addition, the escalation of drug use in human addicts is primarily due to an increase in the frequency of intoxication events rather than an increase in the amount of drug taken during each event, suggesting that tolerance also is not a predominant driver of drug use (Zernig et al, 2007).

Thus, it was not possible to predict whether facilitating morphine-induced MOR trafficking in the RMOR mice would increase or decrease their likelihood of developing addiction. To answer this question, it was necessary to model not just reward, but the transition from controlled to compulsive drug taking in animals voluntarily self-administering morphine. Here, we report a novel mouse model of oral morphine self-administration and the spontaneous emergence of addiction-like behaviours. We used this model to show that RMOR mice are less likely than WT mice to become addicted to morphine, despite the fact that they are initially more sensitive to its rewarding effects. Moreover, we provide evidence that facilitating morphine-induced MOR trafficking prevents increases in the reinforcing strength of morphine that occur during withdrawal and abstinence.

## RESULTS

### Opioid reinforcement in naive mice

Morphine reward was measured using a conditioned place preference (CPP) paradigm (see Materials and Methods section). RMOR mice exhibited greater sensitivity to the rewarding effects of morphine, as indicated by a leftward shift in the dose response curve (Fig 1, genotype  $\times$  dose interaction for doses 0, 0.3, 3 and 10 mg/kg:  $F[3,90] = 2.8$ ,  $p < 0.05$ ). Thus, a low dose of morphine (0.3 mg/kg) that was insufficient to produce detectable CPP in WT mice (black,  $p = 0.34$ ) produced robust CPP in RMOR mice (red,  $p < 0.05$ ).



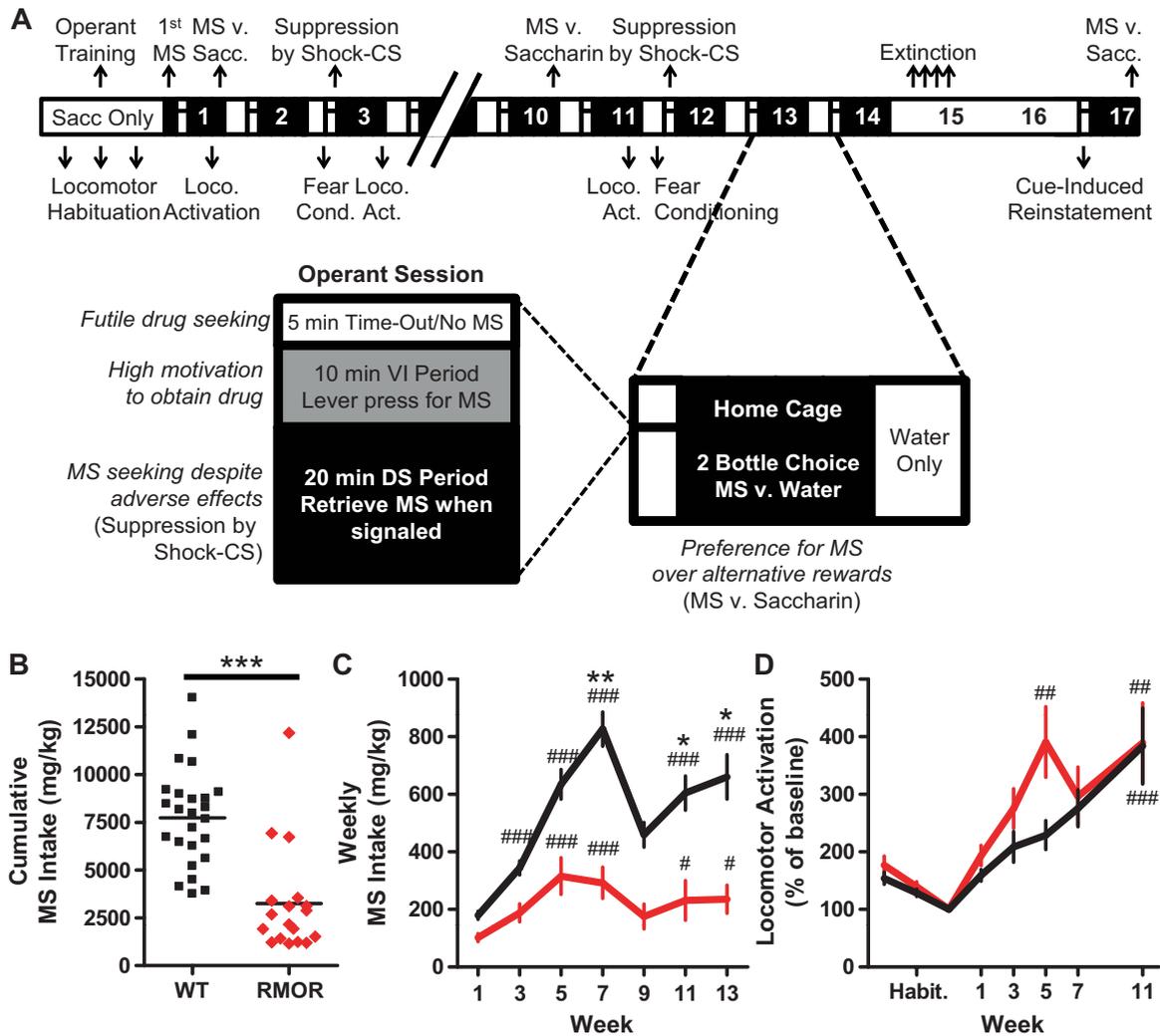
**Figure 1. Morphine reward in opioid-naive WT and RMOR mice.** RMOR (red) and WT (black) mice were treated with morphine (MS) and saline in chambers with distinctive environmental cues. Their preference for the MS-paired context was later assessed during a drug-free state. Data are presented as the mean  $\pm$  SEM. \* $p < 0.05$  WT v. RMOR. # $p < 0.05$ , ### $p < 0.01$  and #### $p < 0.001$  v. baseline preference.

In contrast, the CPP produced by methadone was equivalent in the two genotypes (Supporting Information Fig S1A). This demonstrates that the mutant RMOR functions equivalently to the WT receptor in response to ligands, like methadone, that promote MOR trafficking, and indicates that the potentiation of morphine reward in RMOR mice is due to a selective enhancement of morphine-induced trafficking and not some other non-specific gain-of-function. Similar results were obtained with operant self-administration of subcutaneous

opioids (Supporting Information Fig S1B). Specifically, a lower dose of morphine but not methadone was required to sustain operant responding in RMOR mice compared to WT mice.

**Chronic self-administration of oral morphine**

We combined elements of operant self-administration and the two bottle choice test to track the development of addiction-like behaviours in mice drinking a sweetened morphine solution (Fig 2A). Repeated measures of drug seeking behaviour were



**Figure 2. Chronic oral morphine self-administration in WT and RMOR mice.**

- A.** Schematic of the longitudinal study of morphine (MS) consumption and drug seeking behaviour. Mice were first trained in the operant task, using saccharin reinforcement. Mice then consumed MS over a 14 weeks period—in an operant session on the first day of each week and in their home cages on days 2–5. During this time, periodic operant and two bottle choice tests were conducted to probe their motivation and compulsivity. Specifically, motivation was assessed by measuring lever pressing for MS both during a ‘no drug’ time-out and during a variable interval (VI) task. Persistent drug seeking at the expense of alternative activities and in the face of adverse consequences was modelled by measuring the animals’ preference for MS versus a highly palatable saccharin solution and the suppression of MS seeking by a shock-paired tone (Shock-CS) during a discriminative stimulus (DS) task, respectively. At the end of the 14 weeks period, lever pressing for MS was extinguished, and cue-induced reinstatement was measured following a 15 days drug-free period.
- B.** Cumulative MS consumption of individual WT (black) and RMOR (red) mice.
- C.** Weekly MS consumption.
- D.** Locomotor activity induced by voluntary MS intake. Data are presented as the mean ± SEM. Significance of *post hoc* (Bonferroni) tests: \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001 WT v. RMOR; #*p* < 0.05, ###*p* < 0.01 and ####*p* < 0.001 v. earliest time point.

taken for each subject to determine whether that individual's motivation to consume morphine increased over time.

Mice were allowed to self-administer morphine in an operant session on the first day of each week, and they were given unlimited access to both morphine and water in their home cages on days 2–5 (Fig 2A). On the last 2 days of the week, only water was provided in the home cage. Thus, operant sessions were always conducted in an otherwise drug-free state.

Each operant session consisted of three components to measure three different aspects of compulsivity (Fig 2A):

- (1) *High motivation to obtain drug.* We measured the rate of lever pressing to earn a morphine reward. Lever presses were reinforced on a variable interval (VI; 25 s) schedule, meaning that morphine was delivered for the first response after an unpredictable amount of time, averaging 25 s, had passed. In a VI schedule, the total amount of drug available is held constant, and there is no direct relationship between the number of responses emitted and the number of reinforcers received. VI schedules are thus designed to produce steady rates of lever pressing that reflect how hard a subject is willing to work for a given amount of drug.
- (2) *Futile drug seeking.* Addicts expend a great deal of time and energy to obtain drug, even when the likelihood of success is extremely low. We measured lever pressing during a time-out period when it was signalled to the animals that morphine was not available.
- (3) *Persistent drug seeking in the face of adverse consequences.* Addicts continue to use drug despite its directly harmful effects. They also expose themselves to harmful situations in order to obtain drug. We modelled the latter by measuring conditioned suppression of drug seeking (Vanderschuren & Everitt, 2004). Mice learned that they could retrieve a morphine reinforcer if they entered the reward port during the presentation of a light discriminative stimulus (DS). During separate fear conditioning sessions, they also learned that an auditory cue predicted the delivery of a footshock. This auditory cue was then played during presentation of the DS to determine whether it suppressed morphine retrieval. All fear conditioning sessions were conducted in the drug-free state.

A fourth feature of addiction was periodically measured during home cage drinking sessions:

- (4) *Reduced preference for alternative rewards.* Mice were normally presented with the choice between sweetened morphine and water; however, they were occasionally given the choice between morphine plus saccharin and saccharin alone (0.2%). This concentration of saccharin is highly palatable to mice, and they will normally drink it to the exclusion of water (Supporting Information Fig S2A and B).

In addition to these longitudinal measures to detect compulsive changes in drug taking behaviour, we also measured daily morphine consumption and the reinstatement of drug seeking following extinction and 15 days of abstinence.

#### *Absolute morphine consumption*

RMOR mice ( $n = 18$ ; red diamonds) consumed significantly less morphine than WT mice ( $n = 25$ ; black squares) over the course of the experiment (Fig 2B;  $p < 0.0001$ ). Indeed, all but three (17%) RMOR mice consumed less than every WT mouse in the study.

An analysis of the pattern of consumption across time revealed main effects of genotype and time and a significant interaction of the two (Fig 2C;  $F[1,246] = 30.0$ ,  $p < 0.0001$ ;  $F[6,246] = 31.2$ ,  $p < 0.0001$ ; and  $F[6,246] = 9.4$ ,  $p < 0.0001$ ). On average, both genotypes increased their intake over time (week 7 v. week 1: WT  $p < 0.001$ , RMOR  $p < 0.001$ ); however, the rate and magnitude of escalation were both greater in WT mice (weeks 3 and 9 v. week 1, WT  $p < 0.001$ , RMOR  $p > 0.05$ ).

The difference in morphine consumption between RMOR and WT mice could not be explained by a genotype difference in sweet or bitter taste discrimination or preference, as both saccharin and quinine solutions were equally consumed and preferred (saccharin) or avoided (quinine) by RMOR and WT mice (Supporting Information Fig S2A–D).

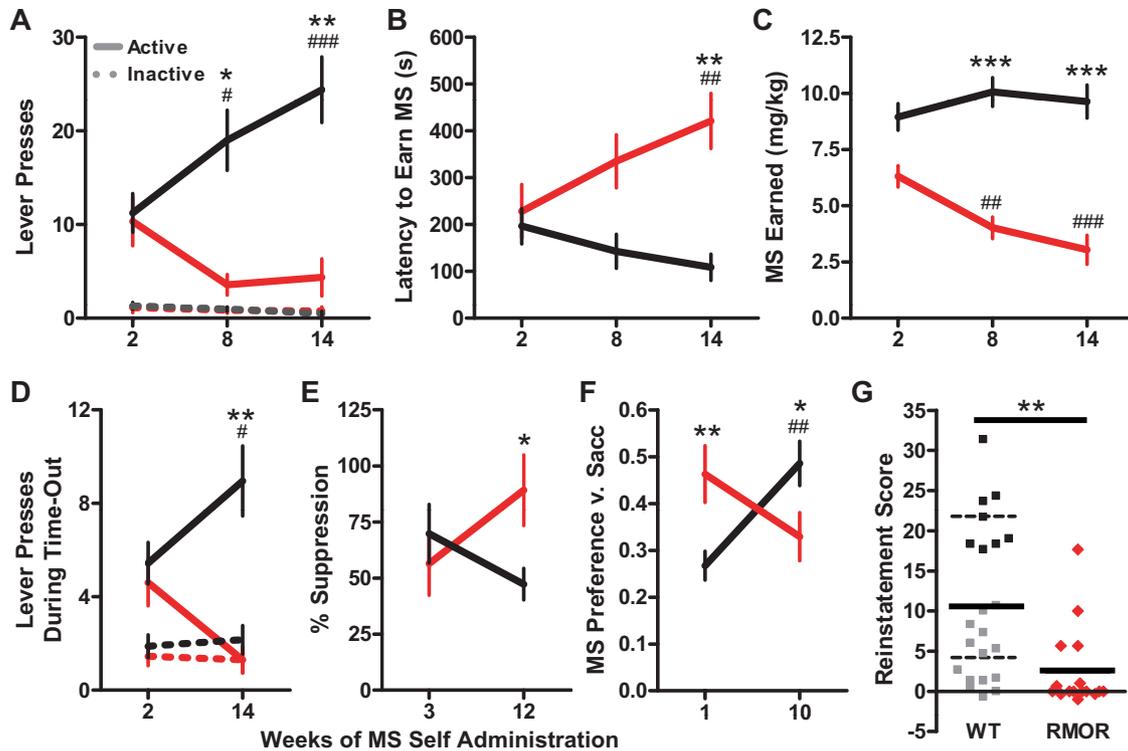
#### *Effective morphine consumption*

RMOR mice require less morphine than WT mice to achieve the same level of analgesia and reward (Fig 1 and (Kim et al, 2008)). They would, therefore, be expected to require less drug to attain the subjective effects that govern voluntary morphine intake. Despite the striking difference in the absolute amounts of morphine consumed, the behavioural activation induced by morphine self-administration was similar in WT and RMOR mice across time (Fig 2D, time effect only:  $F[4,88] = 7.6$ ,  $p < 0.0001$ ). Thus, by at least one measure, WT and RMOR mice appeared to titrate their consumption to achieve comparable physiological effects. There was a trend towards a significant genotype  $\times$  time interaction before week 7 ( $F[2,44] = 2.9$ ,  $p = 0.066$ ), suggesting that RMOR mice may even have initially consumed higher effective doses of morphine. This is unlikely to be due to greater behavioural sensitization in the RMOR mice, as passive administration of either morphine or cocaine produced similar degrees of locomotor sensitization in both genotypes (Supporting Information Fig S2E–H).

#### *High motivation to obtain drug and futile drug seeking*

The rate of lever pressing was measured in both a VI and a time-out task, in which it was signalled to the mice that morphine was not available (Fig 2A). Statistical analysis revealed a significant genotype  $\times$  time interaction and a main effect of genotype on lever pressing in both tasks (Fig 3A, VI task:  $F[2,82] = 11.2$ ,  $p < 0.0001$  and  $F[1,82] = 16.1$ ,  $p < 0.001$ ; Fig 3D, time-out task:  $F[1,41] = 10.6$ ,  $p < 0.01$  and  $F[1,41] = 12.4$ ,  $p < 0.01$ ), the latency to earn morphine (Fig 3B  $F[2,82] = 6.2$ ,  $p < 0.01$  and  $F[1,82] = 13.6$ ,  $p < 0.001$ ) and the amount of morphine earned (Fig 3C,  $F[2,82] = 8.2$ ,  $p < 0.001$  and  $F[1,82] = 57.1$ ,  $p < 0.0001$ ).

After only 2 weeks of drinking experience, RMOR and WT mice did not differ in their drug seeking behaviour. However, over time, WT mice progressively increased their response on the active lever during both drug and no drug periods (Fig 3A,  $p < 0.001$ ; Fig 3D  $p < 0.05$ ), whereas RMOR mice appeared to decrease their response, although this change was not



**Figure 3. Longitudinal measures of motivation and compulsivity and the propensity to relapse in WT and RMOR mice.**

- A. Lever pressing during the VI period. WT (black) and RMOR (red) mice expended similar amounts of effort to obtain MS after 2 weeks of drinking; however, over time, only WT mice exhibited a progressive increase in responding on the active lever (solid line). There was no change in responding on the inactive lever (dashed line) in either genotype.
- B. Latency to earn MS during the operant session.
- C. Amount of MS earned during the operant session.
- D. Lever pressing during the time-out.
- E. Suppression of MS seeking by a shock-predictive conditioned stimulus (CS).
- F. Preference for MS + saccharin v. saccharin alone.
- G. Reinstatement of lever pressing. Reinstatement was induced by the non-contingent delivery of a single MS reinforcer and the contingent presentation of a MS-paired CS. WT mice segregated into a high reinstating group (WT-HR, black,  $n = 8$ ) and a low reinstating group (WT-LR, grey,  $n = 14$ ) that was not statistically different from RMOR mice. Data are presented as the mean  $\pm$  SEM. Significance of *post hoc* (Bonferroni) tests: \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  WT v. RMOR; # $p < 0.05$ , ## $p < 0.01$  and ### $p < 0.001$  v. earliest time point.

significant. These changes were specific to the active lever, indicating that they were related to drug seeking and not due to non-specific changes in motor activity (lever  $\times$  time interaction in VI task, WT:  $F[2,96] = 10.1$ ,  $p < 0.001$  and RMOR:  $F[2,68] = 3.7$ ,  $p < 0.05$ ). Importantly, the increase in lever pressing in WT mice was not associated with any increase in the amount of morphine delivered, suggesting that WT mice were willing to work harder to obtain the same amount of drug (Fig 3C). This was not due to a ceiling effect as WT mice were only earning about half of possible reinforcers.

#### Persistent drug seeking in the face of adverse consequences

We next assessed whether morphine seeking could be suppressed by an auditory cue that had previously been paired with a footshock. RMOR and WT mice showed similar conditioned suppression of drug seeking after only 2 weeks of morphine drinking experience. However, following pro-

longed experience, drug seeking became more resistant to suppression in the WT mice than in the RMOR mice (Fig 3E,  $p < 0.05$ ). Statistical analysis revealed a significant genotype  $\times$  time interaction ( $F[1,37] = 4.3$ ,  $p < 0.05$ ). This cannot be explained by a difference in the learning of the cue-shock association as the two genotypes showed equivalent increases in cue-induced freezing following fear conditioning (Supporting Information Fig S3).

#### Reduced preference for alternative rewards

We measured shifts in the preference for sweetened morphine versus a highly palatable saccharin solution. Again, there was a significant genotype  $\times$  time interaction (Fig 3F,  $F[1,41] = 12.5$ ,  $p < 0.01$ ). RMOR mice initially favoured morphine more than WT mice ( $p < 0.01$ ), and a substantial fraction, 40%, showed an outright preference for morphine over saccharin compared to only 12% of WTs. After extended drinking experience, however,

WT mice favoured morphine more than RMOR mice ( $p < 0.05$ ), and 44% showed an outright preference for morphine over saccharin compared to only 22% of RMOR mice.

#### Propensity to relapse

In addition to compulsive drug use, relapse is one of the most consistently observed features of addiction, occurring in up to 90% of patients (Hunt et al, 1971). We measured the reinstatement of morphine seeking following extinction and 15 days of abstinence (Fig 2A). Morphine seeking was provoked by the presentation of a drug-associated conditioned stimulus and the delivery of a single, non-contingent morphine reinforcer.

On average, the level of reinstatement was significantly greater in WT than in RMOR mice (Fig 3G,  $p < 0.01$ ); however, WT mice segregated into two groups. One group (WT-LR, grey squares,  $n = 14$ ) was statistically indistinguishable from RMOR mice ( $p = 0.29$ ), while the other group (WT-HR, black squares,  $n = 8$ ) showed significantly higher reinstatement than RMOR mice ( $p < 0.001$ ).

#### The relationship between compulsivity and relapse

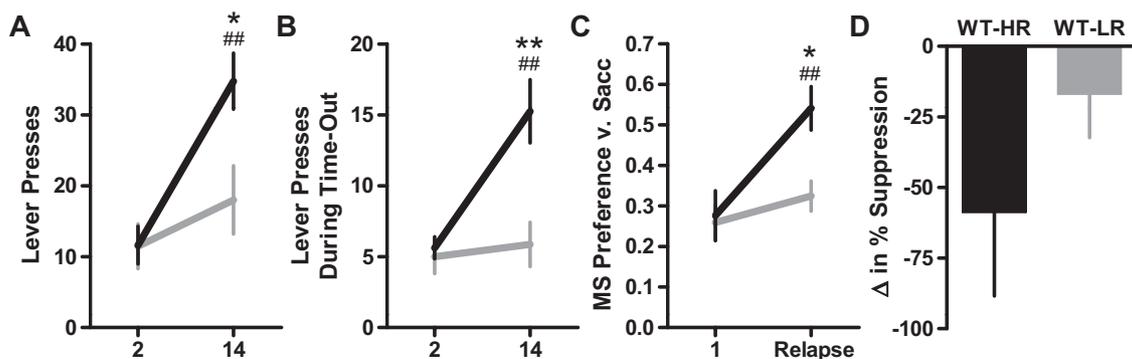
This variability in the WT population provided an opportunity to examine whether the longitudinal measures above (Fig 3) were valid behavioural markers for relapse probability. We hypothesized that if the severity of addiction were related to the intensity of these markers, then WT-HR mice should have shown more compulsive changes over time than WT-LR mice. Indeed, although both groups behaved similarly at the start of drinking, only WT-HR mice exhibited a significant increase in their motivation to obtain morphine in the VI (Fig 4A, group  $\times$  time interaction:  $F[1,21] = 4.6$ ,  $p < 0.05$ ) and time-out tasks (Fig 4B, group effect:  $F[1,21] = 9.2$ ,  $p < 0.01$  and group  $\times$  time interaction:  $F[1,21] = 7.8$ ,  $p < 0.05$ ), and they were the only group to retain a reduced preference for saccharin *versus* morphine after

abstinence (Fig 4C, group effect,  $F[1,20] = 4.5$ ,  $p < 0.05$  and group  $\times$  time interaction,  $F[1,20] = 4.9$ ,  $p < 0.05$ ). The ability of a shock-paired cue to suppress morphine seeking also declined more in WT-HR than in WT-LR mice after extended drinking experience; however, this did not reach statistical significance (Fig 4D).

To determine whether individual differences in these addiction-like behaviours were predictive of the propensity to relapse across all WT mice, we quantified the relationship between reinstatement and a compulsivity score that incorporated all four measures (see Materials and Methods section). There was a significant positive correlation between the compulsivity score and reinstatement (Fig 5A,  $p < 0.01$ ,  $r[20] = 0.62$ ). Interestingly, no single addiction-like behaviour was predictive of reinstatement when taken in isolation, except for one—lever pressing during the time-out period, which correlated with reinstatement more weakly than the composite measure (Supporting Information Fig S4,  $p < 0.05$ ,  $r[20] = 0.48$ ).

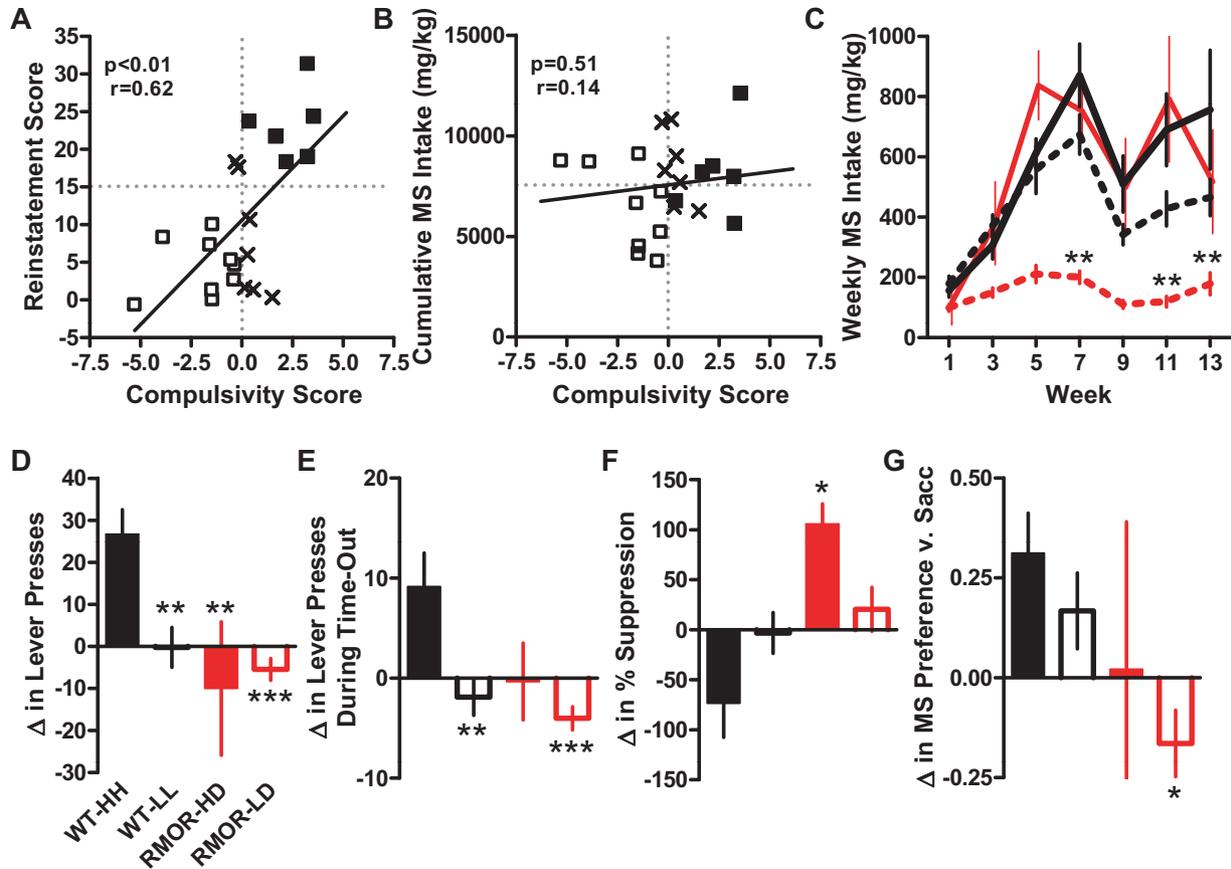
#### The relationship between consumption and compulsive drug seeking

Given the wide disparity in morphine consumption between WT and RMOR mice, we next examined whether differences in addiction-like behaviours or reinstatement could be explained by differences in consumption. In sharp contrast to the relationship between the compulsivity score and reinstatement, there was no significant relationship between cumulative morphine intake and either reinstatement ( $p = 0.27$ ) or the compulsivity score in WT mice (Fig 5B,  $p = 0.51$ ). In addition, we compared the pattern of intake over time in WT mice that demonstrated both high reinstatement and high compulsivity (WT-HH,  $n = 6$ , solid black) and WT mice that exhibited both low reinstatement and low compulsivity (WT-LL,  $n = 9$ , open/dashed black; Fig 5C). Both WT-HH and WT-LL mice showed an initial escalation in drinking (time effect:  $F[6,78] = 27.1$ ,



**Figure 4. Longitudinal measures of motivation and compulsivity in WT-HR and WT-LR mice.** WT-HR (black) and WT-LR (grey) mice initially showed similar levels of the four addiction-like behaviours in Fig 3; however, over time, only WT-HR mice exhibited a progressive increase in:

- Lever pressing during the VI period.
- Lever pressing during the time-out.
- Preference for MS *v.* saccharin.
- Similarly, only WT-HR mice showed a trend towards reduced inhibition of MS seeking by a shock-predictive after extended drinking experience ( $p = 0.09$ ). Data are presented as the mean  $\pm$  SEM. Significance of *post hoc* (Bonferroni) tests: \* $p < 0.05$  and \*\* $p < 0.01$  WT *v.* RMOR; ### $p < 0.01$  *v.* earliest time point.



**Figure 5. The relationship of compulsivity to reinstatement and consumption in WT and RMOR mice.**

- A.** Compulsivity v. reinstatement in WT mice. A compulsivity score was calculated for each WT mouse that quantified how much its behaviour deviated from the mean for all WT mice across the four longitudinal measures in Figs 3 and 4. Note that since the average WT mouse showed an increase in compulsivity, a negative score does not necessarily indicate a decline in compulsivity. The mice in the upper right quadrant exhibited both high reinstatement and higher than average compulsivity (WT-HH, solid black,  $n = 6$ ); whereas, the mice in the lower left quadrant exhibited both low reinstatement and lower than average compulsivity (WT-LL, open/dashed black,  $n = 9$ ).
- B.** Compulsivity v. consumption in WT mice.
- C.** Weekly MS consumption of WT-HH, WT-LL, high drinking RMOR (RMOR-HD, solid red,  $n = 3$ ) and low drinking RMOR (RMOR-LD, open/dashed red,  $n = 15$ ) mice.
- D.–G.** Changes in addiction-like behaviours. Unlike WT-HH mice, RMOR-HD mice did not differ from either WT-LL or RMOR-LD mice in:
- D.** Lever pressing during the VI period (week 14–week 2).
- E.** Lever pressing during the time-out period (week 14–week 2).
- F.** Suppression of MS seeking by a shock-predictive CS (week 12–week 3).
- G.** Preference for MS v. saccharin (week 10–week 1). Data are presented as the mean  $\pm$  SEM. Significance of *post hoc* (Bonferroni) tests: \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  v. WT-HH mice.

$p < 0.001$ ; week 7 v. week 1: WT-HH and WT-LL  $p < 0.001$ ). This escalation was sustained to a greater extent in WT-HH than in WT-LL mice (group  $\times$  time interaction,  $F[6,78] = 3.3$ ,  $p < 0.01$ ); however, this was only significant at late time points (weeks 11 and 13 v. week 7, WT-HH  $p > 0.05$ , WT-LL  $p < 0.01$ ), when addiction-like behaviours had already developed. Furthermore, the absolute amount of morphine consumed was not statistically different between WT-HH and WT-LL mice at any time point.

Finally, we examined whether the small proportion of high drinking RMOR mice (RMOR-HD,  $n = 3$ , solid red) exhibited

addiction-like changes that were obscured in the aggregate analysis of RMORs. RMOR-HD mice consumed up to 10 $\times$  as much morphine as their low drinking counterparts (RMOR-LD,  $n = 15$ , dashed/open red; Fig 2B), and their overall intake and pattern of escalation were indistinguishable from WTs (Fig 5C). However, despite their high consumption, RMOR-HD mice did not show more compulsive changes in any of the four addiction-like behaviours than either RMOR-LD or WT-LL mice (Fig 5D–G). Thus, high morphine intake was not associated with increased compulsivity or reinstatement in either WT or RMOR mice.

## Opioid reinforcement in mice with a history of chronic morphine experience

### Reinforcement during acute withdrawal

We have previously shown that, following chronic morphine treatment and withdrawal, RMOR mice exhibit a less intense physical withdrawal syndrome than WT mice (Kim et al, 2008). To determine whether the affective symptoms of dependence are also reduced, we measured the place aversion induced by naloxone (NX)-precipitated withdrawal.

RMOR and WT mice were treated with twice daily injections of morphine (10 mg/kg) or saline for 5 days. This regimen produces a higher degree of analgesic tolerance and physical dependence in WT than in RMOR mice, even though the effective dose is much greater in RMOR mice (Supporting Information Fig S5). Thirty minutes after their final injection, NX-induced aversion was measured using a place conditioning paradigm (see Materials and Methods section).

Morphine produced a positive place preference in both WT ( $p < 0.001$ ) and RMOR mice ( $p < 0.01$ ; Fig 6A, 0 mg/kg NX). Notably, the magnitude of this preference was similar to that measured in naive mice (Fig 1), despite the fact that the WT mice were highly tolerant to the analgesic effects of this dose of morphine, suggesting in itself either a lack of tolerance or sensitization to morphine reward.

A low dose of NX (0.1 mg/kg) reversed morphine CPP in WT ( $p = 0.15$ ) but not RMOR mice ( $p < 0.01$ ). Importantly, a high dose of NX (1 mg/kg) not only reversed morphine reward in WT mice but also revealed a strong aversive effect of withdrawal ( $p < 0.05$ ). In sharp contrast, the same dose in RMOR mice blocked morphine CPP but had no negative reinforcing effect ( $p = 0.9$ ). This dose of NX (1 mg/kg) had no motivational effects in opioid-naive mice of either genotype (Fig 6A, open bars). These data suggest that chronic morphine induces affective dependence in WT but not RMOR mice; thus, the aversive effects of withdrawal could provide a strong incentive to maintain morphine consumption in WT mice.

### Reinforcement during abstinence

To determine whether chronic morphine exposure induced a lasting change in morphine reward, we measured CPP during protracted abstinence. RMOR and WT mice were treated with twice daily injections of morphine (10 mg/kg) or saline for 5 days. Two weeks later, morphine-induced place preference was measured as in Fig 1.

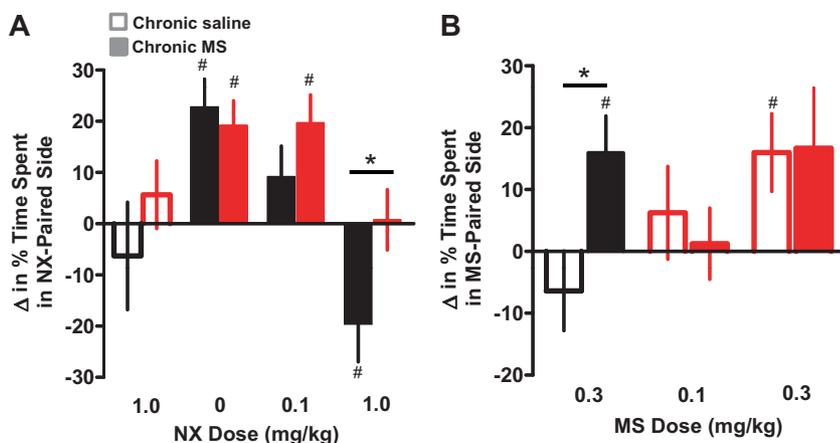
In this paradigm, a low dose of morphine (0.3 mg/kg), that was insufficient to produce CPP in opioid-naive WT mice ( $p = 0.34$ ), produced robust CPP in morphine-treated WT mice that was equivalent to that generated by a 10-fold higher dose in naive mice ( $p < 0.05$ ; Fig 6B). In contrast, repeated morphine administration did not induce any shift in the threshold dose of morphine required to elicit CPP in RMOR mice. Thus, a relatively short course of morphine treatment caused a long lasting reward sensitization in WT but not RMOR mice.

## DISCUSSION

### A mouse model of opioid addiction

The diagnosis of addiction in human subjects is a clinical diagnosis. There is no set of laboratory findings or cutoff values for drug intake that defines addiction. Rather, the determination is made by identifying the presence of a constellation of symptoms that, taken together, indicate a loss of control over drug use. According to the diagnostic criteria set forth in the DSM-IV (Diagnostic and Statistical Manual of Mental Disorders), this loss of control may be manifested by difficulty limiting the amount of drug consumption, excessive time and effort devoted to obtaining and using drug, abandonment of important alternative activities and continued drug use despite harmful consequences.

In contrast, most animal studies related to addiction only take into account the acute reinforcing effects of drug or the amount of drug use over relatively short periods of time. Unfortunately, neither of these factors may be truly indicative of the long-term risk of addiction. Realizing the limitations of these approaches, several groups have developed multidimensional models that capture the spontaneous emergence of addiction in rats self-



**Figure 6. Morphine reward in WT and RMOR mice following chronic morphine treatment.**

**A.** Negative reinforcing effect of MS withdrawal. Mice that had been treated chronically with MS (solid bars) or saline (open bars) were injected with naloxone (NX) in a chamber with distinctive environmental cues. Their preference or avoidance of the NX-paired side was measured 1 week later.

**B.** Sensitization of MS reward during protracted abstinence. Mice were treated chronically with MS or saline. CPP was measured as in Fig 1 2 weeks after spontaneous withdrawal. Data are presented as the mean  $\pm$  SEM. \* $p < 0.05$  WT v. RMOR. # $p < 0.05$  v. baseline preference.

administering intravenous cocaine or heroin for extended periods of time (Ahmed et al, 2000; Chen et al, 2006; Deroche-Gamonet et al, 2004). The power of these models comes from their ability to differentiate compulsive drug use from controlled drug use, which represents the majority of drug use in our society and is not a clinical problem. While these models have clear advantages, they have not yet been applied to mice, due in large part to the technical difficulty of maintaining intravenous access for prolonged periods (Thomsen & Caine, 2007).

Here, we have presented a mouse model of opioid addiction using chronic self-administration of oral morphine. Like the multidimensional rat models described above, this model recapitulates many features of human addiction, including price inelasticity or a willingness to work harder for the same amount of drug, punishment-resistant drug seeking and an increased preference for drug over alternative 'natural' rewards. Importantly, like the DSM-IV criteria for human addiction, these behaviours have prognostic value, and their presence predicted a higher propensity to relapse. WT mice that scored higher on a composite of four addiction-like behaviours showed higher levels of reinstatement than mice that scored lower (Fig 5A). Furthermore, just as in human addiction, no single behaviour had a similar predictive value to the composite measure, illustrating the necessity of a multidimensional approach (Supporting Information Fig S4).

Using this model, we found that extensive self-administration experience was necessary for the emergence of compulsive morphine seeking, as has been demonstrated for other substances in the rat (Deroche-Gamonet et al, 2004; Vanderschuren & Everitt, 2004; Wolfgramm & Heyne, 1995). However, while a long duration of drug use was critical, surprisingly, a high amount of drug consumption was neither necessary nor sufficient. WT mice with high and low levels of both reinstatement and addiction-like behaviours did not differ in either their cumulative morphine intake or their early pattern of escalation, and there were heavy and light drinkers in both groups (Fig 5B and C).

A similar dissociation between consumption and the development of compulsivity has been described for cocaine and ethanol self-administration and is clinically evidenced by the emergence of addiction in only a minor subset of prescription opioid users (Deroche-Gamonet et al, 2004; Vengeliene et al, 2009). This dissociation is generally attributed to individual differences in susceptibility traits, such as impulsivity and anxiety (Everitt et al, 2008; Heilig et al, 2010). Another possibility is that the intensity of drug taking may be more important than the absolute amount taken (Belin et al, 2009).

We observed that several mice with high levels of compulsivity and/or reinstatement had previously engaged in binge-like episodes, during which individual mice occasionally drank up to 800 mg/kg or 30 ml of morphine in a single 24 h session. Another mouse that exhibited this behaviour actually died during a home cage drinking session, possibly due to morphine overdose. However, from the present study, it is difficult to say whether intense drinking bouts were a cause or

consequence of increased compulsivity. It will be an important area for future study to discover the determinants of resistance and susceptibility to morphine addiction in WT mice.

### Morphine-induced MOR trafficking reduces the risk of morphine addiction

Morphine, like other drugs of abuse, produces both positive reinforcement, which is necessary for the acquisition of self-administration, and chronic changes in myriad brain functions, which are necessary for the maintenance of harmful drug use. We found that facilitating morphine-induced MOR desensitization, endocytosis and recycling enhanced positive reinforcement. At first glance, this might have been expected to increase the risk of morphine abuse, as the initial euphoric effects of prescription opioids have been hypothesized to be more intense in patients who go on to develop addiction (Bieber et al, 2008; Haertzen et al, 1983). However, we found striking evidence to the contrary. In fact, improved morphine-induced MOR trafficking reduced the long-term risk of progressing to addiction despite the fact that morphine was more acutely rewarding.

In every measure of addictive behaviour, WT mice showed a progressive increase over time, whereas RMOR mice showed no significant change (Fig 3). Following a period of abstinence, WT mice also demonstrated significantly greater reinstatement of morphine seeking. Although the majority of RMOR mice drank considerably less than WT mice, high drinking RMOR mice did not differ from either low drinking RMOR mice or WT mice with low reinstatement in their motivation to obtain morphine (Fig 5D–G). Furthermore, WT and RMOR mice appeared to titrate their consumption to achieve similar levels of net behavioural activation when morphine was freely available (Fig 2D). When obtaining morphine was associated with a cost, however, WT mice became increasingly motivated to maintain their level of intake, whereas RMOR mice became less motivated and consequently consumed less (Fig 3).

Chronic morphine self-administration induces changes in many brain functions, including executive and cognitive functions, reward processing and stress reactivity and coping (Koob & Volkow, 2010). While a constellation of changes is likely necessary to stimulate and sustain compulsive drug use, resistance to change in one or a few arenas might be sufficient to confer a significant protective advantage. We examined alterations in morphine reward caused by chronic morphine administration, because these changes are most likely to be mediated directly by MOR-expressing cells. We found that the reinforcing effect of morphine was intensified both during withdrawal and during protracted abstinence in WT mice. In contrast, RMOR mice exhibited resistance to developing both affective dependence (Fig 6A) and reward sensitization (Fig 6B) when they received the same morphine regimen as WT mice.

One possible mechanism by which MOR trafficking could prevent these consequences of chronic morphine is the prevention of a cAMP-dependent increase in GABA release onto dopamine neurons of the ventral tegmental area (VTA; Madhavan et al, 2010). In opioid-naïve WT mice, morphine both suppresses the cAMP pathway and, via a cAMP-independent mechanism, promotes dopamine release by inhibiting GABA release from

MOR-expressing terminals (Johnson & North, 1992; Vaughan et al 1997). However, the failure of morphine to induce robust MOR internalization and the subsequent persistent activation of MORs ultimately results in the upregulation of the cAMP pathway and an increased probability of GABA release in the absence of drug (Bonci & Williams, 1997; Hack et al, 2003).

This single change in synaptic function likely has multiple consequences for the functioning of the VTA to nucleus accumbens reward axis. First, the increase in basal inhibitory tone may produce a dysphoric, hypodopaminergic state—in other words, the state of affective dependence. Second, since GABA release is coupled to the cAMP pathway in dependent but not naive animals, it may actually be more sensitive to inhibition by morphine in the dependent state, thereby providing a molecular basis for reward sensitization.

We have previously shown that both cAMP superactivation and the cAMP-dependent increase in GABA release are prevented in RMOR mice that have been treated chronically with morphine (He et al, 2009; Madhavan et al, 2010). In addition, we have shown that chronic morphine induces brain region-specific increases in NMDA receptor and glucocorticoid receptor expression in WT but not RMOR mice (He et al, 2009). These changes have been hypothesized to play roles in withdrawal-related hyperalgesia and stress-induced craving, respectively, and may also contribute to the reduced susceptibility of RMOR mice to harmful morphine use (Ambroggi et al, 2009; Dunbar & Pulai, 1998).

In conclusion, the results presented here demonstrate that there is no simple relationship between addiction and either drug consumption or reward and underscore the necessity of long-term studies that directly examine the transition from controlled to compulsive drug seeking in order to model addiction. Taken together with our previous findings, they also demonstrate that enhancing agonist-induced MOR trafficking reduces the development of tolerance, dependence and addiction, while preserving analgesia and the desirable subjective effects of opioids, and suggest a strategy for identifying novel opioid drugs with increased utility for treating chronic pain and possibly anxiety.

## MATERIALS AND METHODS

### Animals

The generation of RMOR knock-in mice was as described previously (Kim et al, 2008) and was based on the receptor mutation first described by Finn and Whistler (2001) in which the sequence EFCIPSTSTIEQQNSARIRQTRHPSTAN contained entirely within exon 3 was replaced with the corresponding sequence QLCRTPCGRQEPGSLRRPRQATTRERVACTPS from the delta opioid receptor.

CPP/CPA and self-administration experiments were performed using mice with different genetic backgrounds. CPP and CPA were assessed in WT and RMOR mice on a mixed C57Bl/6 × 129.SvEv background, whereas, chronic self-administration was examined in a congenic strain that had been backcrossed for 11 generations with C57Bl/6 mice. Although it would have been preferable to use the congenic strain for all studies, the CPP/CPA experiments were completed before the congenic animals were available. Nevertheless, this difference

should have little impact on the overall direction or interpretation of the presented results, as each experiment was internally controlled for genotype and answered an independent research question. In addition, we have previously shown that RMOR mice have a similar reduction in analgesic tolerance and physical dependence compared to their WT littermates, regardless of their background composition (Kim et al, 2008; Madhavan et al, 2010).

Unless otherwise noted, all subjects were male and between 2 and 4 months of age. All animal experiments were performed in accordance with the Ernest Gallo Clinic & Research Center Institutional Animal Care and Use Committee guidelines.

### Chronic oral morphine self-administration

A single large cohort of WT ( $n = 25$ ) and RMOR ( $n = 18$ ) mice was used for this experiment. Mice were singly housed on a reverse light cycle (lights off from 10AM to 10PM). All mice were between 6 and 8 weeks of age at the start of training.

### Operant training

Training took place in Med Associates operant chambers. All sessions were conducted during the animals' dark cycle. Training was conducted in five stages: (1) acquisition of lever pressing on a fixed ratio (FR) schedule of reinforcement, (2) transition to a VI schedule, (3) introduction of a time-out period, (4) acquisition of DS rules and (5) introduction of the inactive lever. Two sessions were conducted at each stage, except for Stage 1.

**Stage 1:** Mice were deprived of water for 18 h prior to the first session. Only the active lever was extended to facilitate learning. A single response on this lever triggered the delivery of 15  $\mu$ l of 0.2% saccharin sodium (w/v) and the illumination of a cue light located above the reward port for 2.5 s. While the cue light was on, additional lever presses were recorded but had no scheduled consequences. After 20 reinforcers had been earned, the FR requirement was increased by 1. This continued for 6 h or until the animal had earned 20 reinforcers at an FR level of 4. Most mice acquired lever pressing during their first training session. If they did not, they were given free access to water for at least 24 h before a second round of water deprivation and training.

**Stage 2:** Mice were required to press the active lever once for the first reinforcer, twice for the second, three times for the third and four times for the fourth. Subsequent lever presses were reinforced on a VI(25 s) schedule. Time intervals were randomly selected from a 12 element Fleshler–Hoffman series so that the probability of reinforcement remained constant across time. Interval length varied from 1 to 90 s. Sessions lasted for 15 min.

**Stage 3:** A 5 min time-out was introduced at the beginning of the session, during which the active lever was extended, but responses had no scheduled consequences. The time-out was signalled by a flashing cue light located above the lever. The time-out was followed by a 10 min VI period. During this period, the first lever press was always reinforced, and subsequent presses were reinforced on a VI (25 s) schedule.

**Stage 4:** The first 15 min of the session were identical to Stage 3. At the end of the VI period, the active lever was retracted, marking the start of a 20 min DS period. During this period, a DS was presented on a VI (30 s) schedule. The DS consisted of illumination of the cue light that had already been paired with saccharin. If the animal entered the reward port during DS presentation, a reinforcer was delivered. The cue light remained on for 10 s or until 2.5 s after port entry.

## The paper explained

### PROBLEM:

Opioids, like morphine, are extremely effective for the treatment of acute pain. However, their utility for chronic pain, a much greater clinical need, is sorely limited by the development of long-term side effects, including addiction. Unlike endogenous opioids, morphine, heroin and other commonly abused opioids fail to induce robust MOR internalization. This leads to an aberrant pattern of signal transduction, which may contribute to the long-term adverse effects of these drugs.

### RESULTS:

We generated a mutant RMOR, which is internalized in response to morphine. Knock-in mice, which express the mutant RMOR in place of the WT receptor, were more sensitive to the rewarding effects of morphine. Consistent with this, when RMOR and WT

mice were allowed to voluntarily consume morphine, RMOR mice were initially equally or more motivated to obtain the drug. Over the course of several months, however, only WT mice became increasingly motivated and continued to seek morphine despite harm and at the expense of alternative rewards.

### IMPACT:

Facilitating MOR trafficking appears to enhance the desirable, therapeutic actions of opioids while reducing their negative side effects. This work suggests a strategy to develop novel analgesics that have a reduced propensity to cause tolerance, physical dependence and addiction. In addition, this study is the first to demonstrate compulsive drug seeking in the mouse, the only genetically tractable mammal.

*Stage 5:* This stage was identical to Stage 4 except that the inactive lever was extended during the time-out and VI period. Inactive lever presses were recorded but were not reinforced.

### *Morphine drinking schedule*

Following the completion of operant training, mice began to self-administer morphine (MS, for morphine sulphate) both in their home cages and in the operant chambers. The MS solution was sweetened with 0.2% saccharin, and the concentration of MS that mice drank in their home cages was gradually increased from 0.3 mg/ml during the 1st week to 0.5 mg/ml during the 2nd and 3rd weeks to 0.75 mg/ml for the remainder of the experiment. The concentration that mice self-administered in the operant chambers was not increased beyond 0.5 mg/ml. MS was introduced in the home cage at the beginning of the dark cycle on day 1 of the 1st week. The 1st operant session was conducted at the beginning of the dark cycle on the next day. On all subsequent weeks, operant sessions were conducted on day 1. On days 2–5, mice had unlimited access to MS and water in their home cages, and on days 6 and 7, they had access to water only. This basic weekly schedule was repeated for 14 weeks. MS operant sessions were identical to saccharin operant sessions except that the volume of MS delivered was set to 15  $\mu$ l per 20 g of body weight. Mice were weighed once a week before the operant session. Home cage drinking bottles were weighed and the positions of MS and water bottles were rotated daily at the end of the light cycle.

### *Behavioural activation*

Locomotor activity was recorded in an open field chamber (20 cm  $\times$  20 cm  $\times$  20 cm) equipped with high-density infrared beam arrays (AccuScan Instruments, Columbus, OH). Motion was detected by consecutive beam breaks, and total distance travelled was determined using DigiPro software. Animals were habituated to the chambers during three sessions prior to the start of MS drinking. Subsequently, MS-stimulated activity was measured on days 3 or 4 of weeks 1, 3, 5, 7 and 11 of drinking. Activity was recorded for 30 min beginning 4 h after the start of the dark cycle.

### *Conditioned suppression of MS seeking*

Fear conditioning was conducted on day 7 of weeks 2 and 11, when animals were in a drug-free state. During conditioning sessions, mice were exposed to eight footshock + auditory cue (CS) pairings on a VI (60 s) schedule. Shock intensity and duration were set to 0.4 mA and 1 s, respectively. The CS lasted 10 s and consisted of either a continuous tone (2900 Hz, 65 dB) or a series of tones or clicks. The shock was delivered during the last 1 s of the CS. Animals were trained with a different CS on weeks 2 and 11. The order of CS presentation was counterbalanced.

Mice were exposed to the CS during the operant session preceding fear conditioning in order to measure the baseline suppression caused by the CS alone. During the DS portion of this session, 25% of DS presentations occurred during simultaneous presentation of the CS. The CS was turned on 3 s before the DS. The % suppression was calculated as  $1 - (\% \text{ of DS} + \text{CS presentations resulting in MS delivery} / \% \text{ of DS only presentations resulting in MS delivery})$ . Conditioned suppression of morphine seeking was measured in the same way during the operant session following fear conditioning. Data are presented as suppression during the test—baseline suppression.

### *Preference for MS v. saccharin alone*

On day 5 of weeks 1, 8 and 10, mice were provided with MS (0.3 mg/ml in 0.2% saccharin) and saccharin alone (0.2%) instead of MS and water. This was repeated one more time after reinstatement.

### *Extinction of lever pressing*

Extinction began on day 1 of week 15. Sessions consisted of two periods that were identical to the time-out and VI periods of routine operant sessions, except that neither active nor inactive lever presses had scheduled consequences. Animals underwent three extinction sessions per day for 4 days. By the end of this time, all animals had met the extinction criteria of  $<3$  (the average baseline number of inactive lever presses) or 20% of baseline active lever presses during the 'VI period' for three consecutive sessions.

### Cue-induced reinstatement

A single reinstatement session was conducted on day 1 of week 17. The reinstatement session was identical to the time-out and VI periods of routine operant sessions, except that a single, non-contingent MS reinforcer was delivered at the beginning of the VI period and lever presses were reinforced with illumination of the MS-associated cue light only.

### Statistical analysis

For self-administration experiments, the statistical significance of the effects of group and time were tested using two-way analysis of variance (ANOVA) with repeated measures, followed by the Bonferroni *post hoc* test. Unless otherwise noted, remaining comparisons were made using two-tailed, unpaired *t*-tests (for two groups) or one-way ANOVA (for more than two groups).

A compulsivity score was calculated for each WT mouse. For every addiction-like behaviour, each animal was given a sub-score equal to how much its behaviour changed between the earliest and latest time points measured before reinstatement (*i.e.* the difference in the number of lever presses between weeks 2 and 14) minus the average amount of change for all WT mice and then divided by the standard deviation for all WT mice. For example, an animal that showed an increase in lever pressing that was equal to the average increase observed for all WT mice would receive a sub-score of 0. On the other hand, an animal that showed an increase in lever pressing that was one standard deviation below the average increase would receive a sub-score of  $-1$ . The sub-scores for all four behaviours were summed to yield the overall compulsivity score.

For more detailed Materials and Methods see Supporting Information.

### Author contributions

ACB conceived experiments, performed experiments, analysed data and wrote the paper. JLW conceived experiments and wrote the paper.

### Acknowledgements

The authors thank M. Ferwerda and S. Taylor for assistance with animal husbandry and J. Kim, L. He, R. Van Rijn, J. Enquist, R. Li and J. Simms for technical assistance. We are grateful to H. L. Fields, P. H. Janak and A. J. Roberts for thoughtful reading of the manuscript. This work was supported by NIDA grants, DA015232 and DA019958, and funds provided by the state of California for substance abuse research through the University of California, San Francisco.

Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

### For more information

Author's homepage  
<http://www.galloresearch.org/jennifer-whistler.htm>

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