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Targeted expression of μ-opioid receptors in a subset of striatal direct-pathway neurons restores opiate reward

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μ-opioid receptors (MORs) are necessary for the analgesic and addictive effects of opioids such as morphine, but the MOR-expressing neuronal populations that mediate the distinct opiate effects remain elusive. Here we devised a new conditional bacterial artificial chromosome rescue strategy to show, in mice, that targeted MOR expression in a subpopulation of striatal direct-pathway neurons enriched in the striosome and nucleus accumbens, in an otherwise MOR-null background, restores opiate reward and opiate-induced striatal dopamine release and partially restores motivation to self administer an opiate. However, these mice lack opiate analgesia or withdrawal. We used Cre-mediated deletion of the rescued *MOR* transgene to establish that expression of the MOR transgene in the striatum, rather than in extrastriatal sites, is needed for the restoration of opiate reward. Our study demonstrates that a subpopulation of striatal direct-pathway neurons is sufficient to support opiate reward-driven behaviors and provides a new intersectional genetic approach to dissecting neurocircuit-specific gene function *in vivo*.

The endogenous opioid system in the brain is crucial for the processing of natural reward stimuli that drive behavioral reinforcement but has also been implicated in the abuse of addictive substances, including opiates, alcohol, nicotine and cannabinoids[1,2]. After binding to endogenous opioid peptides (for example, enkephalin, β-endorphin or dynorphin) or exogenous opiate drugs (for example, morphine), the opioid receptors activate intracellular signaling through inhibitory G proteins, which typically leads to suppression of neuronal activity[2,3]. The study of targeted gene-knockout mice has demonstrated that among the three major opioid receptors, MOR, δ and κ, only the MOR is essential for opiate reward, analgesia and dependence[4]. MORs are broadly expressed throughout the brain, and numerous pharmacological studies using local infusion of agonists or antagonists have provided important insights into the potential sites of MOR-mediated actions in the brain[1,5]. However, the ability of such studies to draw firm conclusions as to which MOR-expressing neuronal populations mediate specific opiate effects are limited because of the mixtures of MOR-expressing neuronal populations in any given brain region and the fact that opioid receptors can be trafficked to distal axonal terminals to modulate presynaptic release[1,5].

The mammalian striatum, consisting of the dorsal striatum and nucleus accumbens (NAc), receives input from dopaminergic (DA) neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) and is a key neuronal substrate for natural and drug rewards[1,3]. Intriguingly, MOR expression in the striatum is enriched in clusters of medium spiny neurons (MSNs) that define the striosome (or patch) compartment, which is surrounded by the matrix compartment[5–7]. The striosome and matrix MSNs can be divided further into two subpopulations: those in the striatal direct pathway that send inhibitory projections to the substantia nigra (including both the substantia nigra pars reticulata and SNc) and those in the striatal indirect pathway that send inhibitory projections to the globus pallidus externa[6,7]. Neuroanatomical tracing studies have suggested that striosome, rather than matrix, MSNs in the direct pathway preferentially form monosynaptic connections with DA neurons in the SNc and VTA[8,9]. However, functional evidence for such inhibitory synaptic connections remains inconsistent[10].

Previous evidence suggests that MORs are expressed in both direct-pathway and indirect-pathway MSNs in the striosome, but there appears to be an overabundance of direct-pathway MSNs in at least some striosomes[8,9]. In this study, we devised a new conditional bacterial artificial chromosome (BAC) transgenic rescue strategy to assess directly the functional importance of MOR expression in striosomal and NAc direct-pathway MSNs in pathological opiate reward and reinforcement.

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RESULTS

Re-expression of MOR in striatal direct-pathway neurons

The MOR-immunoreactive striosome compartment in the mouse is generally considered to contain both direct-pathway and indirect-pathway MSNs[6,7]. We confirmed this previous observation by double fluorescent localization of mouse MOR and GFP in the striata of GENSAT *Drd1-GFP* and *Drd2-GFP* BAC mice, which genetically label striatal direct- and indirect-pathway MSNs, respectively (**Supplementary Fig. 1**)[11]. We found both Drd1-GFP– and Drd2-GFP–labeled MSNs in the striosome (**Supplementary Fig. 1a–f**). Moreover, using high-resolution confocal imaging, we found MOR expression in Drd1-GFP$^+$ direct-pathway MSNs (**Supplementary Fig. 1g–i**), a result that is consistent with the interpretation that endogenous MOR is expressed in direct-pathway MSNs in the striosome. In this study, we sought to address whether MOR expression in the striatal neuronal subpopulation of the direct pathway modulates opiate-driven behavioral effects *in vivo*.

We used a new conditional BAC-mediated transgenic rescue strategy to re-express MOR in specific neuronal populations of *MOR* (also called *Oprm1*) knockout mice[4]. To target MOR expression to a pattern closely resembling that of endogenous MOR in direct-pathway MSNs, we used the GENSAT mouse *Pdyn* (encoding prodynorphin) BAC (RP23-358G23)[11]. This BAC drives *GFP* transgene expression in a relatively restricted pattern in the striatum, with the GFP-labeled striatal axonal projection pattern being consistent with the interpretation that the *Pdyn* BAC transgene drives GFP expression in direct-pathway MSNs[7,11]. By immunostaining for endogenous MOR in the striatum of *Pdyn-GFP* mice, we found that the GFP$^+$ neuronal patches in these mice were indeed colocalized with the MOR$^+$ striosome (**Fig. 1a**). To further establish, at single-cell resolution, that the GFP$^+$ MSNs are indeed direct-pathway MSNs that also coexpress MOR, we performed fluorescence-activated cell sorting (FACS) of adult striatal neurons from either *Pdyn-GFP* or *Drd2-GFP* mice using our established protocol (**Supplementary Fig. 2**)[12], in which we selected single FACS-sorted GFP$^+$ neurons under the microscope and used them to prepare RNA for single-cell gene expression analyses[13]. All the single FACS-sorted neurons from both *Pdyn-GFP* and *Drd2-GFP* mice expressed *Darpp-32* (also called *Ppp1r1b*) (**Fig. 1b**), which is an MSN-specific marker. Notably, RT-PCR analyses with two separate sets of MOR-specific primers showed that 32 out of 33 FACS-sorted single *Pdyn-GFP*$^+$ MSNs also expressed *MOR*. Moreover, these neurons also coexpressed two direct-pathway MSNs markers, *Drd1* and *Pdyn*, but not a marker of indirect-pathway MSNs (*Drd2*). Conversely, single *Drd2-GFP*$^+$ neurons expressed *Drd2* but not *Drd1* or *Pdyn* and were also negative for *MOR* transcripts. These results support our conclusion that the *Pdyn* BAC selectively drives transgene expression in a subpopulation of direct-pathway MSNs that coexpress *MOR* in the striatum. Our result regarding *MOR* expression in the *Drd2-GFP*$^+$ neurons should be interpreted with caution for several reasons: we sampled only a relatively small number of such neurons, striosome MSNs comprise only about 20% of the total striatal MSNs, and there appears to be a higher abundance of direct-pathway MSNs within the striosome[6,8]. Thus, we cannot exclude that a small subset of MOR$^+$ MSNs in the striosome are in the indirect pathway. In summary, our expression analyses support the use of the *Pdyn* BAC to drive the transgenic re-expression of MOR to interrogate the functional role of MOR specifically in this subpopulation of direct-pathway MSNs in opiate-driven behaviors *in vivo*.

To create *Pdyn* BAC transgenic mice re-expressing MOR, we re-engineered the *Pdyn* BAC to first delete an extra gene on the BAC (*Stk35*; **Fig. 1c**) and then inserted a mouse *MOR* cDNA followed by a poly(A) signal upstream of the *Pdyn* open reading frame to ensure that *Pdyn* itself is not overexpressed by the transgene. An innovative aspect of the BAC transgene design is the flanking of the *MOR* sequence with two *loxP* sites (**Fig. 1c**) so that the transgenic expression of MOR can be conditionally switched off in Cre-expressing cells. Pronuclear microinjection of this engineered BAC construct into inbred C57BL/6 embryos resulted in transgenic mice expressing MOR under the control of *Pdyn* genomic regulatory elements on the BAC (*Pdyn-MOR* mice). The *Pdyn-MOR* transgene was subsequently

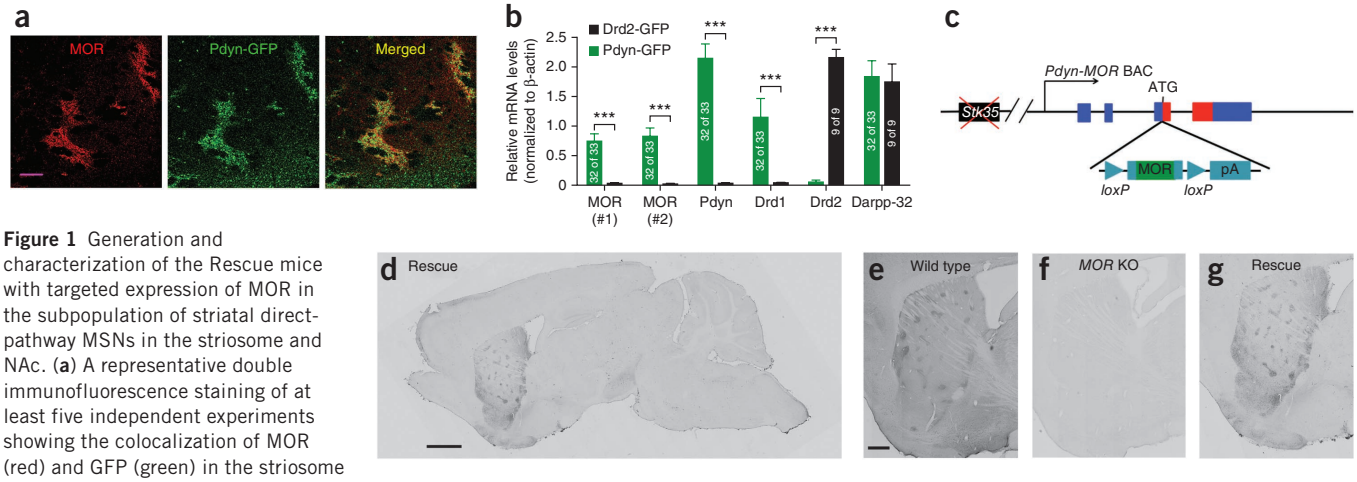

Figure 1 Generation and characterization of the Rescue mice with targeted expression of MOR in the subpopulation of striatal direct-pathway MSNs in the striosome and NAc. (**a**) A representative double immunofluorescence staining of at least five independent experiments showing the colocalization of MOR (red) and GFP (green) in the striosome of the GENSAT *Pdyn-GFP* mouse brain. Scale bar, 100 μm. (**b**) mRNA expression for multiple genes in FACS-sorted single GFP$^+$ cells from *Pdyn-GFP* and *Drd2-GFP* mice as determined by single-cell quantitative PCR analyses. Data were normalized to the reference gene β-actin. Data represent the mean ± s.e.m. The numbers within each bar represent the number of positive cells out of the total number cells tested by real-time PCR. The majority of single neurons from *Pdyn-GFP* mice are positive for *MOR* transcripts (with two independent sets of PCR primers, MOR#1 and MOR#2), whereas all single neurons from *Drd2-GFP* mice are negative for *MOR* ($t_{(41)} = 7.325$, $P < 0.0001$, MOR#1; $t_{(41)} = 6.326$, $P < 0.0001$, MOR#2; *Pdyn-GFP*, $n = 33$; *Drd2-GFP*, $n = 9$). ***$P < 0.0001$. (**c**) A schematic representation of the MOR cDNA in the *Pdyn-MOR* conditional BAC transgenic construct. pA, BGH polyadenylation signal. (**d**) A representative immunohistochemical staining of six mice of MOR in Rescue (*Pdyn-MOR MOR* knockout) mice using an antibody that specifically recognizes the C terminus of MOR. Scale bar, 1 mm. (**e–g**) Expression of MOR in the striatum and cortex in wild-type, *MOR* knockout (KO) and Rescue mice. Scale bar, 200 μm.

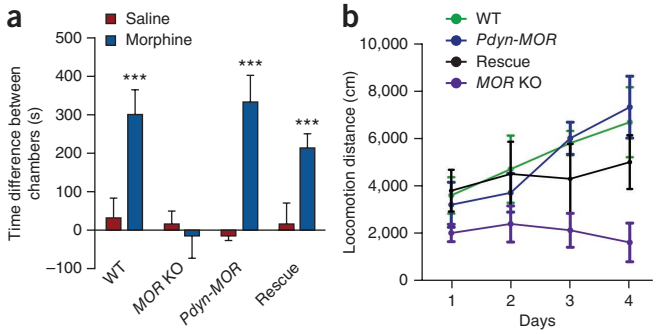

Figure 2 Restoration of the rewarding and locomotor effects of morphine by *Pdyn* BAC–driven expression of *MOR* in Rescue mice. (**a**) Morphine-induced CPP is absent in *MOR* knockout mice but is restored to wild-type levels in Rescue mice. Two-way analysis of variance (ANOVA) revealed a significant CPP deficit in *MOR* knockout mice ($F_{(1, 26)} = 61.14$, $P < 0.001$, wild type compared to *MOR* knockout, two-way ANOVA; wild type, $n = 7$; *MOR* knockout, $n = 8$), which is restored in the Rescue mice ($F_{(1,26)} = 3.619$, $P = 0.0683$, wild type compared to Rescue, two-way ANOVA; wild type, $n = 7$; rescue, $n = 8$). Significant CPP was revealed by *post hoc* test in wild type ($P < 0.001$) and Rescue ($P < 0.001$) but not *MOR* knockout ($P = 0.1642$) mice. The morphine CPP in *Pdyn-MOR* transgenic mice is comparable to that in wild-type controls ($F_{(1,26)} = 4.171$, $P = 0.0514$, two-way ANOVA; wild type, $n = 7$; *Pdyn-MOR*, $n = 8$). (**b**) Deficits in morphine-induced locomotor sensitization in *MOR* knockout mice were restored in Rescue mice to a level that was comparable to those in the wild-type and *Pdyn-MOR* mice. Repeated-measures ANOVA analysis revealed a significant genotype effect and day × genotype interaction ($F_{(3,81)} = 13.284$, $P < 0.001$; wild type, $n = 7$; $n = 8$ for all other genotypes), with *post hoc* test showing a significant sensitization effect of morphine on wild-type, *Pdyn-MOR* and Rescue mice ($P < 0.05$ for all groups) but not *MOR* knockout mice ($P = 0.2411$). Bonferroni's *post hoc* test also showed that Rescue mice are significantly different from *MOR* knockout mice ($P = 0.041$) but not when compared with wild-type mice ($P = 0.677$). All values are shown as the mean ± s.e.m. ***$P < 0.01$.

bred onto a *MOR* knockout background[4] to generate *Pdyn-MOR*/*MOR* knockout (Rescue) mice. Using a highly selective MOR antibody[14], we showed that MOR expression in the Rescue mouse brain was restricted largely to clusters of neurons in the striatum and NAc, with only a few extra-striatal regions, including layer I of the frontal cortex, hippocampus and parabrachial nucleus in the brain stem (**Fig. 1d–g** and **Supplementary Fig. 3**). Because the *Pdyn* BAC drives transgene expression within the striatum and is highly selective for the subpopulation of direct-pathway MSNs in the striosome and NAc (**Fig. 1b** and **Supplementary Fig. 1**)[6,11], we concluded that the Rescue mice confer re-expression of MOR in a subpopulation of direct-pathway MSNs that endogenously express MOR. We then used quantitative western blot analysis to determine the levels of MOR expression in the Rescue mice and found that these mice express MOR protein at about 2.76-fold the level of endogenous MOR in wild-type mice (**Supplementary Fig. 4**). As previous viral-mediated targeted expression of other CNS genes has lead to conclusions regarding the crucial roles of re-expressed genes in specific brain regions in mediating various behavioral effects *in vivo*[15,16], we expect that the neuroanatomical and cellular specificity conferred by BAC-mediated re-expression of MOR in Rescue mice should also be informative regarding the role of direct-pathway MSN subpopulations in opiate-driven behaviors.

Morphine sensitization and place preference in Rescue mice

We next evaluated the phenotypic consequences of *Pdyn* BAC–driven expression of MOR. We first showed that *Pdyn-MOR* and Rescue mice lacked general changes in development, locomotion or body weight despite modest levels of striatal MOR overexpression (**Supplementary Fig. 5**). We next asked whether such targeted re-expression of MOR could restore any key opiate-driven behaviors that are absent in *MOR* knockout mice[4]. We first tested for the rewarding properties of morphine in the conditioned place preference (CPP) paradigm[4]. Using two independent cohorts of mice, we were able to reproducibly show lack of CPP in *MOR* knockout mice and restoration of CPP to wild-type control levels in Rescue mice (**Fig. 2a** and **Supplementary Fig. 6a**). Notably, transgenic overexpression of MOR in *Pdyn-MOR* mice did not elicit excessive levels of CPP compared to wild-type mice (**Fig. 2a**), suggesting a lack of gain-of-function effects on opiate reward by the transgene itself. These results demonstrate that targeted re-expression of MOR driven by the *Pdyn* promotor is sufficient to restore morphine-induced reward *in vivo*.

Morphine administration increases locomotor activity in mice, and repeated treatment progressively increases this locomotor response (sensitization), an effect that is absent in *MOR* knockout mice[4]. We next tested whether Rescue mice also express morphine-induced locomotion and sensitization. We found that morphine-induced locomotor stimulation and sensitization were absent in *MOR* knockout mice but were restored to wild-type levels in Rescue mice (**Fig. 2b**). This result suggests that targeted MOR expression in striatal direct-pathway MSNs within the striosome and NAc is sufficient to not only mediate the locomotor stimulatory effects of morphine but also trigger a similar adaptive response to repeated morphine injections as that seen in wild-type mice.

Remifentanil self administration in Rescue mice

The CPP procedure tests the degree to which the rewarding effects of opioids, delivered noncontingently, can be conditioned to a context through repeated pairings. However, reward is only one dimension of the psychoactive properties of opiates that supports voluntary drug seeking and taking. The ability of a drug to act as a reinforcer of seeking and taking behavior is measured using self-administration procedures in which subjects engage in an action (for example, a lever press) to elicit the effects of the drug[17]. Such procedures provide a more direct and sensitive measure of the motivation to seek out the rewarding effects of the drug and arguably demonstrate closer construct and face validity as a model of human drug abuse behaviors[18]. Opioid receptors in a given neuronal population that are able to sustain opiate reward as measured by CPP may not necessarily sustain aspects of opioid reinforcement that are indexed by self administration[1].

To address the role of MORs in striosomal and NAc direct-pathway MSNs in opiate-induced reinforcement, we trained rescue, *MOR* knockout and wild-type mice to self administer remifentanil, which is a potent short-acting synthetic opioid with a half-life of about 4 min (ref. 19). In contrast to *MOR* knockout mice, which lacked any motivation to self administer remifentanil, both wild-type and Rescue mice obtained a stable level of remifentanil self administration across the last 3 d at the fixed-ratio 1 (FR1) reinforcement schedule (one lever press results in one infusion; **Fig. 3a**). Further within-session analysis of the rate of infusions earned showed that both wild-type and Rescue mice obtained more infusions than *MOR* knockout mice during the 2-h session once stable self administration at FR1 had been obtained (**Fig. 3b**). Moreover, there was no effect of genotype on inactive lever responding (**Fig. 3c**). This experiment demonstrates that Rescue mice, unlike *MOR* knockout mice, can acquire opioid self

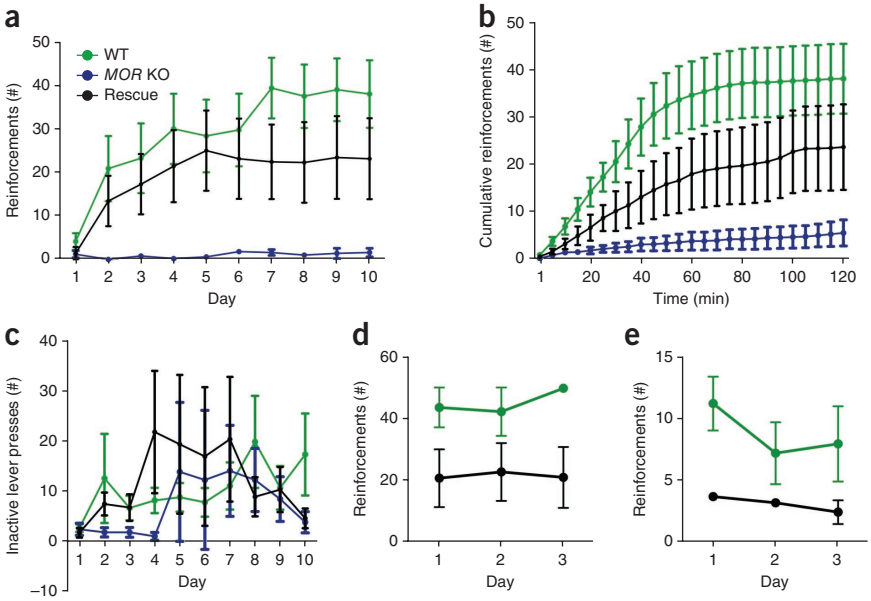

Figure 3 Selective MOR expression in striosome and NAc direct-pathway MSNs partially rescues opiate self administration. (**a**) Wild-type and Rescue mice, but not *MOR* knockout mice, self administered remifentanil (0.10 mg per kg body weight intravenously) under the FR1 reinforcement schedule. Repeated-measures ANOVA analysis revealed a significant day × genotype interaction ($F_{(18,17)} = 2.974$, $P = 0.015$). Wild-type mice self administered more drug across days than *MOR* knockout mice (genotype × day: $F_{(9,11)} = 21.873$, $P < 0.001$) and Rescue mice ($F_{(9,13)} = 3.485$, $P = 0.021$), and Rescue mice self administered more drug than *MOR* knockout mice ($F_{(9,10.072)} = 4.920$, $P = 0.010$). (**b**) The cumulative number of infusions earned during the 2-h session averaged over the last 3 d of FR1 showed that not only did wild-type and Rescue mice earn more infusions than *MOR* knockout mice, but wild-type mice also earned these infusions at earlier time points within the session than Rescue mice (wild type compared to *MOR* knockout, $F_{(24,264)} = 10.367$, $P < 0.001$; Rescue compared to *MOR* knockout, $F_{(24,240)} = 2.506$, $P < 0.001$; wild type compared to rescue, $F_{(12,156)} = 2.660$, $P < 0.01$, repeated-measures ANOVA). (**c**) The number of inactive lever presses across the 10 d of FR1 did not differ (genotype × day: $F_{(18,17)} = 1.578$, $P = 0.176$), showing that wild-type, *MOR* knockout and Rescue mice are not significantly different with respect to non–drug directed responding. Data from wild-type ($n = 8$), *MOR* knockout ($n = 5$) and Rescue ($n = 7$) mice were analyzed in **a**–**c**. (**d**) Responding in the FR3 reinforcement schedule demonstrated that wild-type mice obtained more remifentanil infusions than Rescue mice (wild type compared to rescue, $F_{(1,7)} = 5.672$, $P = 0.049$, repeated-measures two-way ANOVA; wild type, $n = 5$; rescue, $n = 4$). (**e**) Progressive ratio reinforcement schedules demonstrated that the wild-type mice obtained more infusions than Rescue mice before reaching the break point (wild type compared to rescue, $F_{(1,6)} = 7.107$, $P = 0.037$, repeated-measures two-way ANOVA; wild type, $n = 4$; rescue, $n = 4$). The data shown and analyzed are from the last 3 d at FR3 (**d**) and the progressive ratio task (**e**), when stable responding was achieved. All values are shown as the mean ± s.e.m.

administration. However, further analyses revealed that the motivation to obtain remifentanil was only partially restored in these mice. First, Rescue mice earned infusions at a slower pace during FR1 testing (**Fig. 3a**,**b**) and gained fewer infusions overall during FR3 testing (three consecutive lever presses are required to trigger one infusion) (**Fig. 3d**) than wild-type mice. In addition, Rescue mice exhibited lower infusion 'break points' during a progressive ratio task, indicating that they were willing to exert less effort than wild-type mice to obtain the opiate drug (**Fig. 3e**). These results demonstrate that re-expression of MOR driven by the *Pdyn* promoter restores opioid-seeking and opioid-taking behaviors that are lacking in the *MOR* knockout mice. Moreover, our results also suggest that full restoration of opiate reinforcement behavior may require MOR expression in other opiate-sensitive neuronal populations beyond the subpopulation of striatal direct-pathway MSNs.

Lack of opiate analgesia and withdrawal in Rescue mice

Chronic use of opiates leads to physical dependence that contributes to drug craving and relapse and is a major factor in the addiction cycle[20]. Physical withdrawal symptoms can be precipitated in wild-type but not *MOR* knockout mice by injecting the opiate antagonist naloxone in chronically morphine-treated animals[4]. Neither *MOR* knockout mice nor Rescue mice exhibited any of the classical signs of morphine withdrawal that were present in wild-type littermates after the chronic morphine regimen (**Fig. 4a** and **Supplementary Fig. 7**). This finding is consistent with the previous suggestion that MORs in other brain areas are the primary neuronal substrates for morphine physical withdrawal[21].

A major clinical utility of opiates is analgesia, which is thought to be mediated by both spinal and supra-spinal activation of MOR[22]. A role for the striatum in modulating opiate analgesia has been suggested[23]. To test whether MOR re-expression in the striatal direct-pathway MSN subpopulations can mediate opiate analgesia, we used two well-characterized tests. In both the hot-plate and tail-flick tests, morphine-induced analgesia was present in wild-type controls but was absent in both Rescue and *MOR* knockout mice (**Fig. 4b**,**c**), suggesting that MOR expression outside the striosome and NAc direct-pathway MSNs is necessary for the analgesic effects of morphine in acute pain.

Opiate reward in Rescue mice requires striatal MOR

To further strengthen the conclusion that the striatal direct-pathway MSN subpopulation is sufficient for opiate reward in Rescue mice, we performed an intersectional genetic approach, taking advantage of the conditional BAC transgene design (**Fig. 1c**). As the *Pdyn-MOR* transgene drives the re-expression of MOR in a few sites outside of the striatum, we crossed the *Rgs9-cre* transgene, which drives selective expression of Cre in all striatal MSNs (including both direct-pathway and indirect-pathway MSNs), onto the Rescue mouse background (**Fig. 5a**)[24]. The resulting *Pdyn-MOR MOR* knockout *Rgs9-cre* (Rescue-*cre*) mice had nearly complete elimination of *MOR* transgene expression in the striatal direct-pathway MSN subpopulation, which is the neuronal cell type that resides at the intersection between the *Pdyn-MOR* rescue transgene and the *Rgs9-cre* expression domains, leaving the expression of the *MOR* rescue transgene in the extrastriatal sites intact (**Fig. 5a** and **Supplementary Fig. 8**). Notably, the Rescue-*cre* mice no longer exhibited morphine CPP under conditions in which their Rescue and wild-type littermates exhibited robust morphine CPP (**Fig. 5b** and **Supplementary Fig. 6b**).

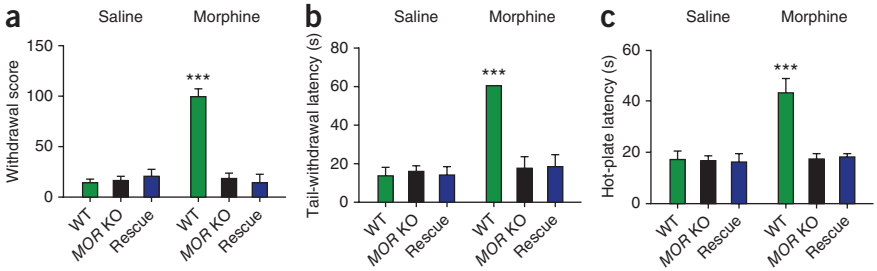

Figure 4 *Pdyn* BAC–driven expression of MOR in an otherwise *MOR* knockout background did not restore morphine analgesia or naloxone-precipitated withdrawal. (**a**) Naloxone withdrawal, as quantified by the total withdrawal score, is not manifested in the Rescue mice. Naloxone precipitated significant withdrawal symptoms in morphine-treated wild-type mice but not Rescue mice or *MOR* knockout mice ($t_{(14)} = 10.07$, $P < 0.001$, $n = 8$, wild type; $t_{(12)} = 0.2598$, $P = 0.7714$, $n = 7$, *MOR* knockout; $t_{(12)} = 0.6430$, $P = 0.5811$, $n = 7$, rescue; Student's *t* test, two tailed). Naloxone-precipitated withdrawal symptoms are not rescued in Rescue mice ($F_{(1,24)} = 0.5187$, $P = 0.4783$, *MOR* knockout compared to rescue, two-way ANOVA). (**b**) Morphine analgesia as measured by tail withdrawal is absent in Rescue mice compared to wild-type controls ($Q_{(1)} = 11.127$, $P = 0.003$ for the genotype × treatment interaction, nonparametric two-way ANOVA for ranking; wild type, $n = 8$ per group; rescue, $n = 7$ per group). The deficits of the analgesia effect of morphine in *MOR* knockout mice measured by tail withdraw were not restored in Rescue mice ($Q_{(1)} = 0.191$, $P = 0.666$ for the genotype × treatment interaction, nonparametric two-way ANOVA for ranking; $n = 7$ for all groups). (**c**) Morphine analgesia as measured by hot plate is absent in Rescue mice compared to wild-type controls ($Q_{(1)} = 8.808$, $P = 0.006$ for the genotype × treatment interaction, nonparametric two-way ANOVA for ranking; wild type, $n = 8$ per group; rescue, $n = 8$ per group). The deficits of the analgesia effect of morphine in *MOR* knockout mice measured by tail withdraw were not restored in Rescue mice ($Q_{(1)} = 0.261$, $P = 0.613$ for the genotype × treatment interaction, nonparametric two-way ANOVA for ranking; $n = 8$ for all groups). All values are shown as the mean ± s.e.m. $^{***}P < 0.01$.

Thus, our new BAC-mediated intersection genetic approach provides conclusive evidence that MOR expression in the direct-pathway MSNs, but not in extrastriatal sites, is responsible for restoring opiate reward in Rescue mice.

Morphine-induced striatal dopamine release in Rescue mice

Similarly to other drugs of abuse, morphine administration elicits dopamine release from the terminals of VTA and SNc neurons in the NAc and dorsal striatum, which is thought to contribute to the reinforcing and locomotor-activating effects[3,25]. As VTA and SNc GABAergic interneurons express MORs[5,25] and tonically inhibit DA neurons[26], the inhibitory action of morphine on these neurons has been postulated to mediate disinhibition of DA neurons[26]. Additionally, MOR agonists are also known to elicit somatodendritic release of dopamine from DA neurons in VTA[27], which may contribute to the reinforcing effects of these drugs. The precise cellular mechanisms and roles of opioid-induced dopamine release in reward processing remain to be clarified[1,28].

To further probe the basal ganglia neurocircuitry mechanisms that are correlated with the rescue of opiate reward and reinforcement in our new model, we examined MOR expression in the VTA and SNc of wild-type, *MOR* knockout and Rescue mice. Unlike wild-type mice, our Rescue mice did not exhibit MOR staining of neuronal soma in VTA and SNc (**Fig. 6a–d**), which is consistent with the lack of GFP staining in VTA and SNc interneurons of *Pdyn-GFP* mice[11]. Moreover, Rescue mice showed neuropil MOR staining in the VTA and SNc that was abolished in Rescue-*cre* mice (**Supplementary Fig. 8f,g**), suggesting that this reflects axonal terminal staining of MOR expressed in the striatal direct-pathway MSNs. Furthermore, double immunofluorescent staining showed that Rescue mice exhibited MOR terminal

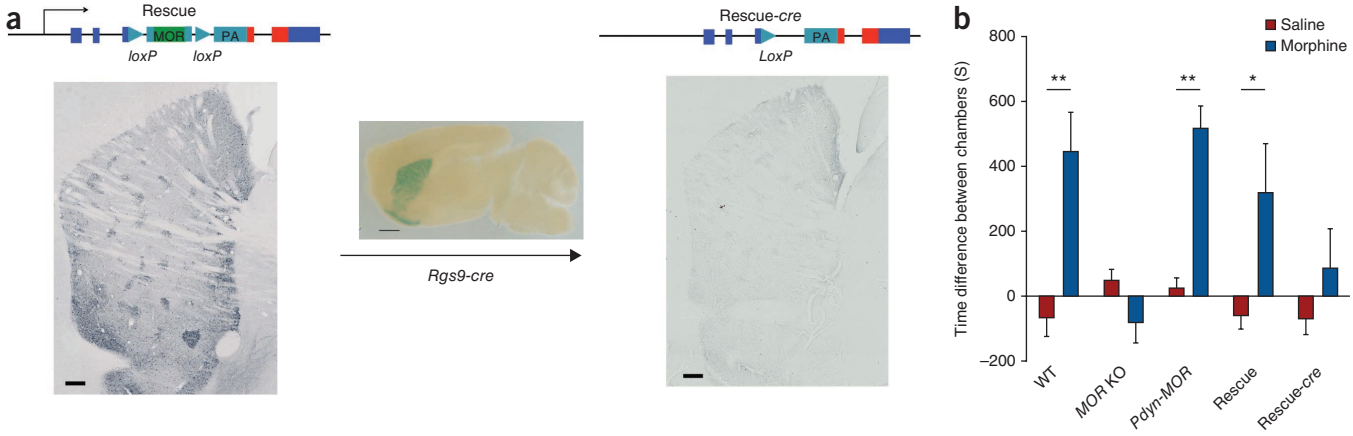

Figure 5 *Rgs9-cre*–mediated deletion of the *Pdyn-MOR* transgene in the striatum of Rescue-*cre* mice abolishes morphine reward effects in the CPP assay. (**a**) Representative MOR immunohistochemistry staining of the brains of five Rescue (left) and three Rescue-*cre* (right) mice. The specificity of *Rgs9-cre* to the striatum was confirmed by crossing to a *lacZ* reporter mouse (middle)[40]. Scale bars, 200 µm (left and right); 1 mm (center). (**b**) CPP is restored in Rescue mouse littermates, but such a rescue effect is eliminated by the genetic removal of the *MOR* transgene in striatal MSNs in Rescue-*cre* mice ($F_{(4,\ 68)} = 5.186$, $P < 0.01$ for genotype × significant CPP in the wild-type ($n = 7$ in the saline group, and $n = 8$ in the morphine group), *Pdyn-MOR* ($n = 8$ in the saline group, and $n = 9$ in the morhpne group) and Rescue groups ($n = 8$ in the saline group, and $n = 7$ in the morphine group) but not in the *MOR* knockout ($n = 8$ in the saline group, and $n = 9$ in the morphine group) and Rescue-*cre* groups ($n = 7$ per group) when comparing morphine-treated groups to saline-treated groups ($P < 0.001$, wild type; $P < 0.001$, *Pdyn-MOR*; $P = 0.0266$, rescue; $P = 0.1271$, *MOR* knockout; $P = 0.2774$, Rescue-*cre*)). The CPP deficits in the *MOR* knockout mice were not restored in the Rescue-*cre* mice ($F_{(1,27)} = 3.351$, $P = 0.0718$ for the genotype × treatment interaction, two-way ANOVA). All values are shown as the mean ± s.e.m. $^{*}P = 0.0266$, $^{**}P < 0.01$.

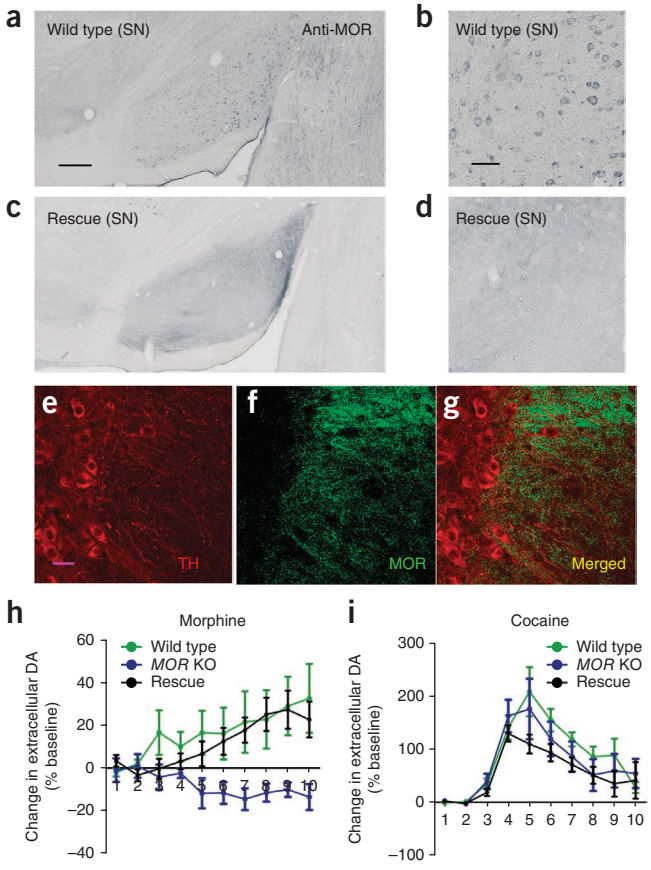

Figure 6 Selective MOR expression in the axonal terminals of direct-pathway MSNs in the VTA and SNc but not midbrain interneurons can restore morphine-induced striatal dopamine release in Rescue mice. (**a**–**d**) Representative immunohistochemistry staining of sagittal brain sections showing the expression of MOR in the substantia nigra (SN) of wild-type (**a**,**b**) and Rescue (**c**,**d**) mice. Data are representative of experiments containing three mice per group. Scale bars, 200 µm (**a**,**c**); 50 µm (**b**,**d**). (**e**–**g**) A representative double immunofluorescence staining of Rescue mouse brain sections showing the presence of MOR (green) in the direct-pathway MSN axonal terminals in VTA, which partially overlap with the cell body and dendrites of DA neurons that are immunostained with antibody to tyrosine hydroxylase (TH; red). Data are representative of experiments containing three mice per group. Scale bar, 20 µm (**e**–**g**). (**h**) Morphine-elicited striatal dopamine efflux was abolished in *MOR* knockout mice compared to wild-type mice but was restored in Rescue mice (genotype effect: $H_{(2)} = 10.81$; $P < 0.01$, Kruskal-Wallis test followed by Bonferroni-adjusted Mann-Whitney *U* pairwise comparisons; $n = 7$ for all genotypes). The numbers on the *x* axes in **h** and **i** are consecutive 15-min samples. The first two samples are the pre-injection baseline samples. (**i**) Wild-type, *MOR* knockout and Rescue mice showed similar dopamine responses to cocaine (genotype effect: $H_{(2)} = 3.47$; $P = 0.18$, Kruskal-Wallis test followed by Bonferroni-adjusted Mann-Whitney *U* pairwise comparisons; $n = 7$ for all genotypes). All values are shown as the mean ± s.e.m.

staining in the VTA and SNc that partially overlapped with DA neuronal cell bodies and more fully overlapped with their dendrites (**Fig. 6e**–**g**). This result is consistent with studies demonstrating that striosomal direct-pathway MSNs may form monosynaptic contacts with DA neurons in the SNc and VTA[9]. To test whether MOR expression in striatal direct-pathway MSNs, but not midbrain interneurons, could restore *in vivo* morphine-induced dopamine efflux in the striatum, we performed dopamine microdialysis. Although the *MOR* knockout mice lacked any morphine-induced elevation in extracellular dopamine in the striatum, the Rescue mice exhibited full restoration of this effect to a level comparable with wild-type mice (**Fig. 6h**). Saline injections had negligible effects (data not shown), and blockade of dopamine reuptake with cocaine elevated extracellular dopamine levels to similar extents across wild-type, *MOR* knockout and Rescue mice (**Fig. 6i**). Together, these results suggest that restoration of MOR expression in the direct-pathway MSNs in the striosome and NAc is sufficient to rescue morphine-induced elevation of extracellular dopamine in the striatum (**Supplementary Fig. 9**), an *in vivo* neurochemical correlate that is elicited by a variety of natural and drug rewards[29].

DISCUSSION

In this study, we used a new conditional BAC rescue strategy in mice to demonstrate that targeted MOR expression in a subpopulation of striatal direct-pathway MSNs in the striosome and NAc restores opiate-induced CPP and sensitization and partially restores opiate reinforcement but does not rescue opiate analgesia and withdrawal. Because the *MOR* transgene is expressed in a few extrastriatal sites, we used Cre-mediated striatal-specific deletion of the rescue transgene to establish that MORs in the striatal direct-pathway MSNs are necessary for opiate reward in Rescue mice. Moreover, we also showed that Rescue mice exhibit morphine-induced elevation of extracellular dopamine levels in the striatum, providing evidence that MOR-expressing striatal direct-pathway MSNs can functionally regulate nigrostriatal dopamine release. Thus, our study provides *in vivo* evidence that MORs in striosomal and NAc direct-pathway neurons are sufficient to mediate opiate reward and reinforcement.

The anatomical basis of opiate reward processing is a neurobiologically and clinically important issue[1]. Previous knowledge of the neuronal substrate for opiate drug reward has been based largely on the influence of opioid receptor agonist or antagonist infusion into local brain regions on opiate reinforcement or place conditioning[1]. These studies provide strong evidence for VTA and NAc as key brain regions in the rewarding and reinforcing properties of opiates[1]. However, the mixture of multiple cell types expressing MORs in these brain regions precluded the identification of cell types that mediate the effects of the infused drugs. As previous genetic studies have demonstrated that the MOR is the only opioid receptor that is necessary for opiate reward, analgesia and dependence[4], we reasoned that distinct MOR-expressing neuronal populations can be interrogated genetically for their roles in mediating opiate-induced behavioral effects *in vivo*. Our BAC-mediated targeted re-expression of MOR permitted us to address whether expression of MORs in a specific neuronal population is sufficient for opiate reward-related behaviors. The anatomical and cellular specificity of transgene expression driven by the *Pdyn* BAC was confirmed by our expression analyses of FACS-sorted single MSNs from *Pdyn-GFP* mice (**Fig. 1b**).

Our study provides insights on a subpopulation of MOR-expressing direct-pathway MSNs, which normally reside in the striosome and NAc, in reward and reinforced behaviors. In demonstrating that Rescue mice can restore opiate-induced dopamine release in the striatum, as well as that such enhancement of striatal and NAc dopamine transmission is associated with opiate reinforcement, it is tempting to speculate that MOR function in this subset of direct-pathway MSNs can influence reinforced behaviors by regulating the activity of midbrain DA neurons. This hypothesis is based on the rationale that striosome direct-pathway MSNs are known to form monosynaptic GABAergic inputs onto the DA neurons in VTA and SNc[9] and that our Rescue mice had restored MOR expression in the striatonigral

axonal terminals in VTA and SNc (**Supplementary Fig. 8**) and opiate-induced striatal dopamine release. Future *in vitro* and *in vivo* electrophysiological studies will be needed to address how MORs in the direct-pathway MSN neuronal subpopulations may regulate DA neuron activities in both Rescue and wild-type mice.

Our findings are also consistent with the notion that MORs in multiple neuronal populations inside and outside the striatum could have a role in opiate reward-related behaviors. Although morphine CPP was restored in Rescue mice, remifentanil self administration in Rescue mice was only partially restored as compared to their wild-type littermates. This latter finding, in this arguably more sensitive assay of opioid drug reward, suggests the likely contribution of other MOR-expressing neuronal populations in regulating opiate reward and reinforcement. Indeed, MOR agonists have been shown to disinhibit DA neuron activity through suppression of presynaptic VTA interneurons[26] and GABAergic neurons in the rostromedial tegmental nucleus[29]. Moreover, other neuronal cell types such as the striatal cholinergic interneurons and neurons in the nucleus tractus solitarius have also been implicated in opiate reward[26,30,31]. Future MOR-based genetic studies should allow for the systematic interrogation of the roles of MOR in these opiate-sensitive neuronal cell populations in opiate reward and reinforcement.

Our study is an initial step in the dissection of the neuronal populations in which MOR synthesis may contribute to opiate reward. We are cognizant of several potential limitations of our approach based on the transgenic re-expression of MOR in *MOR* knockout mice. First, our genetic experiment was designed to address a sufficiency role of MOR in striatal direct-pathway neuronal subpopulations in opiate reward *in vivo*, and it does not address the related but independent question of whether MOR expression in the striatal direct-pathway MSNs is necessary for opiate reward and reinforcement. Complementary genetic studies with selective deletion of endogenous *MOR* in specific neuronal populations will be needed to address whether MOR expression in a given neuronal population is necessary for opiate-induced behaviors. Second, the transgenic re-expression of MOR based on the *Pdyn* BAC leads to MOR expression that is higher than the levels of endogenous MOR in the striatum, and the expression pattern of the *MOR* transgene may also have subtle differences (for example, at extrastriatal sites of expression) from endogenous MOR (**Supplementary Fig. 3**). However, our results should still be interpretable, as we showed the colocalization of a *Pdyn* BAC–driven transgene with endogenous MOR in the striosomal direct-pathway MSNs at single-neuron resolution. Moreover, the *Pdyn-MOR* transgenic mice themselves did not appear to have overt basal or opiate-induced behavioral deficits, and the Rescue mice restored a subset of opiate-driven behaviors that are normally present in wild-type mice but are absent in *MOR* knockout mice. Hence, our results are consistent with the transgenic rescue of a loss-of-function mutant rather than new gain-of-function phenotypes elicited by the transgene. Such a classic transgenic rescue approach, with overexpression of the rescuing transgene by non-native promoters, has been used routinely in invertebrate genetic systems. Moreover, conceptually similar viral-mediated re-expression studies in mice have been highly informative on the circuit-specific roles of several CNS genes in behavioral control[15,16]. To further strengthen the current observations, future genetic experiments should aim at conditional re-expression of MOR from the endogenous MOR genomic locus to assess whether the precise endogenous level of MOR in a given neuronal population can mediate certain opiate-driven behaviors. Our study also reveals a correlate, but not yet causal, role between restoration of opiate-induced striatal dopamine release and opiate reward in the Rescue mice. The precise mechanism through which the rescuing *MOR* transgene modulates DA neuron function remains to be defined. This is an important issue to resolve, as opiates are known to mediate reward through both dopamine-dependent and dopamine-independent mechanisms[32–34], and striosomal direct-pathway MSNs also project to both the midbrain DA neurons and, to some extent, GABAergic neurons[8], which may have distinct roles in opiate reward[33,34]. Such limitations notwithstanding, our study is an important step forward toward a MOR-based genetic dissection of the roles of distinct neuronal populations in opiate-driven behaviors *in vivo*.

Our study provides some insight into the functional organization of striatal projection neuron circuitry and suggests a possible explanation for the restricted, striosome-specific pattern of MOR expression in the striatum. Striatal MSNs can be categorized on the basis of two distinct systems: the direct and indirect pathways, and the striosome and matrix compartments[6,7]. These two-layered MSN categorization systems result in four distinct subtypes of MSNs based on their afferent and efferent connectivities and molecular marker expression[6,7]. The availability of genetic access to the striatal direct- and indirect-pathway MSNs, provided by GENSAT BAC transgenic mice[11], enables rapid progress toward understanding the differential physiological functions of striatal direct- and indirect-pathway MSNs, including their roles in natural and drug rewards[35,36]. However, these studies do not distinguish the direct- or indirect-pathway MSNs in striosome as compared to matrix compartments, even though such compartmentalization is thought to have an important role in basal ganglia function[37]. Our study shows that the *Pdyn* BAC confers some specificity to the striosomal direct-pathway MSNs, as expression analyses of FACS-purified single *Pdyn-GFP* neurons showed selective expression of direct-pathway markers (*Drd1* and *Pdyn*) and a striosomal marker (*MOR*) but not the indirect-pathway marker (*Drd2*). The predominant, but not absolute, expression of MOR in the striosomal direct-pathway MSNs relative to the indirect-pathway MSNs has been noted previously in single-neuron tracing experiments[6,8]. This observation is also consistent with the finding that genetic ablation of D1 MSNs (but preserving D2 MSNs) leads to the loss of the majority of MOR staining in the striatum[38]. Future studies will be needed to address the functional distinction of MOR expressed in the striatal direct- and indirect-pathway MSNs. Our study also offers some functional insight into the restricted, patchy pattern of MOR expression in the striatum[5,6] and suggests that MORs in the striosomal direct-pathway MSNs may contribute to opiate reward. In addition, our *in vivo* striatal microdialysis experiment showed that MOR in the striosome and NAc direct-pathway MSNs can regulate striatal dopamine release. This finding is consistent with the interpretation that MOR^+ striatal direct-pathway MSNs are part of reciprocal neural circuits between the striatum and VTA and SNc DA neurons that are postulated to have important roles in reward and reinforcement behaviors[9,37].

Our study demonstrates a new intersectional genetic strategy to assess a single gene in a specific neuronal population for its physiological or pathological roles in a mammalian model. Previous mouse genetic tools, such as *cre*- and *loxP*-mediated conditional gene inactivation[39], often cannot interrogate the role of a single neuronal population because the majority of available Cre mouse lines express the recombinase in multiple cell types in the brain. An intersectional genetic approach based on sequential use of Flp and Cre recombinases offers a solution for genetic access to a single cell type that is defined by the overlapping expression domains of the recombinases[40]. Our current study demonstrates an alternative strategy based on the conditional BAC rescue approach. We showed that the conditional BAC transgene design, with strategically placed *loxP* sites flanking

MOR on the BAC, allows efficient and selective removal of the rescue *MOR* transgene only in a single neuronal cell type at the intersection of the BAC and Cre expression domains. Given the rich repertoire of publicly available knockout and Cre mouse lines, as well as well-characterized BAC transgenes[11], we envision that the BAC-mediated intersectional genetic approach may help elucidate not only the cellular targets for opiate-induced effects, including reward, analgesia and dependence, but also the cell type–specific function of many other important genes in the mammalian brain.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.C. and X.W.Y. designed the study, interpreted the results and wrote the manuscript. Y.C. performed experiments, analyzed the data and made **Figures 1–3**, **5** and **6a–g** and **Supplementary Figures 1–8**. S.B.O. and N.T.M. designed and did experiments for and made **Figure 6h,i**. A.S.J., J.D.J. and W.M.W. did experiments for and made **Figure 4**. W.G. and Y.E.S. contributed to **Figure 1b**. C.S.P. contributed to **Figure 1** and **Supplementary Figure 3**. K.W.R., N.M., N.P.M., N.T.M., B.L.K. and C.J.E. contributed to the experimental design, actual experiments and data analyses for **Figures 2**, **3** and **5** and **Supplementary Figures 6** and **7**. C.C. and M.S.L. contributed to revision of the manuscript. X.W.Y. and C.J.E. contributed to **Supplementary Figure 9**.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. To generate *Pdyn-MOR* mice, we modified the GENSAT mouse *Pdyn* BAC (RP23-358G23), which contains two genes, *Pdyn* and *Stk35*. The *Stk35* gene was deleted by the RecA-mediated BAC modification method using the pLD53 shuttle vector[41,42]. The mouse MOR cDNA followed by a poly(A) was inserted into exon 3 of the *Pdyn* gene on the BAC in front of the *Pdyn* translation intiation codon. We also included a partial *GFP* sequence in the 3′ untranslated region of the MOR cDNA, which did not interfere with the expression of intact MOR protein but may facilitate the detection of the transgene transcripts. BAC DNA was prepared as previously described[41] and microinjected into C57BL/6 fertilized eggs to generate *Pdyn-MOR* mice. Germline transmission and genotyping of the transgenic offspring were tracked by PCR amplification of the transgene sequence from the genomic DNA. The *Pdyn-MOR* mice were bred onto a *MOR* knockout background[4] to generate the Rescue mice. GENSAT *Pdyn-GFP* mice[11] were obtained from the Mutant Mouse Regional Resource Center. All mice (60–120 days old, both sexes) in this study were bred and maintained on an inbred C57BL/6 background under standard conditions that are consistent with the US National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of UCLA. A 12-h light, 12-h dark cycle is used in the UCLA vivarium. Mice of the same sex were group housed with up to five mice per cage. All behavioral tests were performed during the light phase.

General experimental design. The sample sizes used for our experiments were selected on the basis of results from previous studies to ensure detection of statistically significant morphine-elicited behavior differences in the wild-type and *MOR* knockout groups[4,43–46]. No individual data point was excluded from the statistical analyses, except for in the operant intravenous opiate self administration and striatal dopamine microdialysis tests. Mice were randomly selected and assigned to control and opioid treatment groups for behavioral studies, with the genotype, gender, age and litter factor controlled. All experiments were carried out with the investigators blind to the experimental groups.

Immunohistochemical staining. To internalize MOR to facilitate its immunohistochemical detection, animals received an intraperitoneal injection of etorphine (0.1 mg per kg body weight) and were euthanized 20 min later. Then mice were perfused with 4% paraformaldehyde (PFA) and post-fixed overnight. Floating sections (40 µm) were incubated with rabbit anti-MOR antibodies (1:1,000 dilution)[14] at 4 °C for 36 h. Then the sections were incubated with biotinylated goat anti-rabbit IgG antibody (ab64256, Abcam) at 1:200 dilution at room temperature for 2 h and then in ABC kit (Vector, PK-4005) and developed using diaminobenzidine. Histochemical staining of MOR was done using brain slices from three to four mice per genotype for each experiment. Sections were imaged using an Olympus VS110 microscope.

Double immunofluorescence staining with MOR antibodies. PFA-fixed brain sections of 40 µm were prepared for immunohistochemical staining. For double immunofluorescence studies, floating sections were blocked for 30 min with 3% normal goat serum and 3% bovine serum albumin. After incubation with anti-MOR rabbit monoclonal antibodies (Abcam, ab134054, 1:500 dilution) and sheep polyclonal anti-TH antibody [UMB3] (Millipore, AB-1542, 1:1,000 dilution) overnight at 4 °C, Alexa Fluor 488–conjugated goat anti-rabbit IgG antibodies (Molecular Probes, A11008) and Alexa Fluor 568–conjugated goat anti-sheep IgG antibodies (Molecular Probes, A21099) were applied to the sections at 1:200 dilution and incubated for 2 h in the dark at room temperature. Immunofluorescence staining of MOR was performed using brain slices from three to four mice per genotype for each experiment. Sections were mounted in Vectashield with 4′,6-diamidino-2-phenylindole (Vector), and images were taken using a Leica TCS-SP confocal microscope (Leica, Heidelberg, Germany) and a VS120 WSI scanning system (Olympus America Inc., Center Valley, PA).

To detect the colocalization of MOR with GFP in the *Drd1-GFP* and *Drd2-GFP* mice with confocal microscopy, MOR was internalized with etorphine. Mouse brains were sliced (400 µm) with a vibratome in artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4 and 25 glucose bubbled with 95% O_2, 5% CO_2, pH 7.4. The brain slices were then incubated in ACSF with 500 nM etorphine hydrochloride for 30 min at room temperature. Then the brain slices were transferred to 4% PFA in PBS and fixed in 4% PFA at 4 °C overnight.

FACS and single-cell real-time PCR. FACS of GFP-labeled MSNs was modified from our established protocol[12]. Mice were euthanized, and their dorsal striata were extracted immediately. The striatal tissue was placed in 1 ml of Hibernate A (HA-LF; Brain Bits) and minced. Tissue was incubated with papain and DNase (PAP2 and D2, respectively; Worthington) with gentle rotation at 31–32 °C for 30 min. Tissue pieces were transferred into 2 ml room-temperature HABG containing Hibernate A (HA, Brainbits) with 2% B27 supplement (17504044, Invitrogen), 0.25% Glutamax (35050-061, Invitrogen) and 1% penicillin-streptomycin (15140122, Invitrogen), incubated for 5 min and triturated with a Pasteur pipette. Filtered (70 µm) tissue was centrifuged for 3 min at 430*g* through a three-step density gradient of Percoll (P1644, Sigma). Cells in the bottom layer were resuspended in 1 ml of Hibernate A. Propidium iodide (PI; 20 µg ml^{-1}) was used to label dead cells. Subsequently, FACS-based purification of GFP^+PI^- neurons was performed on a FacsARIA cell sorter (Becton Dickson) at the UCLA Flow Cytometry Core Facility[12].

Single FACS-sorted neurons were picked at random under microscope based on a published protocol[47]. The single-cell cDNA preparation was performed following a previously described protocol[48]. Briefly, single *Pdyn-GFP*–positive cells were manually picked under microscope and then seeded to 4.45 µl freshly prepared cell lysis buffer. No cell negative control was prepared. After single-cell lysis, reverse transcription was performed, followed by free primer removal and poly(A) tailing. Second-strand synthesis was then performed. Afterwards, cDNA was amplified with 20 cycles of PCR. Expression of Gapdh was checked by TaqMan PCR at this point in a 10-µl reaction system with 1 µl cDNA product. Those samples with threshold cycle (Ct) around 22–23 were purified by QIAquick PCR purification kit (28104, Qiagen) and eluted with 40 µl EB buffer.

The relative abundance of target genes was determined by real-time quantitative PCR (qPCR) analysis using a KAPA Biosystems SYBR FAST qPCR kit (KK4611, KAPA Biosystems, Woburn, MA) on a Roche LightCycler 480 PCR system (Roche, Roche Applied Science, USA). Each 10 µl of reaction mixture contained 5 µl 2× SYBR FAST Master Mix, 1 µM of template cDNA (100–200 ng μl^{-1}), 0.2 µl of forward and reverse primer (100 mM) and RNase-free water. Real-time cycling conditions included an initial incubation step at 95 °C for 4 min followed by 40 cycles each of 95 °C, 25 s; 57 °C, 25 s; and 72 °C, 1 s. The melt-curve data were collected using a gradual temperature increase from 40 to 95 °C at a rate of 0.3 °C every 10 s. A nontemplate control was run with every assay. Reactions were set up in triplicates. The crossing point (Cp) of each reaction was recorded, and the difference between the Cp values (ΔCp) was determined. The relative abundance was calculated using the formula $2^{-\Delta\Delta Cp}$ (ref. 13). Student's *t* test was used to determine the difference between neurons from two groups of mice. For all statistical tests, significance was accepted at $P < 0.05$. The sequences of the PCR primers used in this experiment are listed in **Supplementary Table 1**.

Western blotting. Lysates were prepared by homogenizing brain tissue in a modified RIPA buffer followed by centrifugation at 4 °C for 15 min at 16,100*g*. The pellet fraction was further dissolved in 10% SDS and heated to 70 °C for 10 min. The mixture was then spun at 2,000*g* for 30 s, and the supernatant was used for western blotting. Protein samples were prepared from the dorsal striatum of three to six mice per genotype for loading in NuPAGE LDS buffer (Invitrogen) and heated for 10 min at 70 °C. Proteins were resolved Bis-Tris NuPAGE gels and transferred onto polyvinylidene difluoride membranes. Immunoblots were probed with an anti-MOR rabbit monoclonal antibody (Abcam, ab134054, 1:1,000) and an anti–β-actin rabbit monoclonal antibody (Abcam, Ab8227, 1:10,000) overnight at 4 °C. The blot was then incubated with goat anti-rabbit IgG secondary antibody (7074, Cell Signaling) and horse anti-rabbit IgG secondary antibody (7076, Cell Signaling) at a dilution of 1:200 for 1 h at room temperature. Chemiluminescent detection was accomplished using ECL Plus Western Blotting Detection reagents (GE Healthcare). Densitometric values from scanned western blot films were obtained using ImageQuantTL software (GE Healthcare). All data are expressed as the mean ± s.e.m. Because of non-normality of distributions in some genotypes, differences across

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genotype were tested by nonparametric Kruskal-Wallis one-way ANOVA followed by Bonferroni-adjusted Mann-Whitney *U* tests for *post hoc* pairwise comparisons, with significance accepted at $P < 0.05$ (two sided).

Morphine-induced CPP and locomotor sensitization. Place conditioning was conducted in a two-compartment apparatus that recorded the position and locomotion of the mice, as previously described[43–46]. A 20-min pretest was recorded under drug-free conditions in which animals were given free access to both compartments. The next day, each mouse was subcutaneously (s.c.) injected with vehicle immediately before being randomly placed into one of the two compartments for 40 min. The next day, each mouse received an s.c. injection of vehicle or 10 mg per kg body weight morphine before being placed for 40 min in the chamber opposite to that of the previous day. This process was repeated three times. Locomotion during the drug-conditioning sessions was recorded simultaneously and analyzed for locomotor sensitization. The day after the last drug-conditioning session, the expression of place conditioning to the drug-paired compartment was assessed under the same conditions as the pretest. All data are expressed as the mean ± s.e.m. For the CPP test, equal variance and covariance could be assumed, and treatment × genotype interactions were determined by two-way ANOVA using Sigmaplot (v12) followed by Bonferroni tests for *post hoc* pairwise comparisons. For statistical analysis of the locomotor sensitization tests, locomotion distances during the habituation days were averaged and used as the baseline. Locomotion data from the drug-pairing days were normalized to baseline. Differences between groups across time were determined by two-way ANOVA with repeated measures followed by Bonferroni *post hoc* pairwise multiple comparison procedures using Sigmaplot (v12). For all tests, significance was accepted at $P < 0.05$.

Pain assays and assessment of morphine analgesia. Hot-plate and tail-withdrawal assays were used to examine the analgesic effect of morphine[46]. For the hot-plate assay (AccuScan Instruments, San Diego, CA), mice were placed on a warm (62 °C) metal surface inside an acrylic cylinder (7.5-cm diameter × 13-cm height), and the baseline latency to flick or lick the hindpaw or jump was recorded with a stopwatch to the nearest 0.1 s. Thirty minutes later, mice were injected with morphine (10 mg per kg body weight s.c.), and post-injection latency was tested after an additional 30 min. A cutoff latency of 60 s was employed as the endpoint of analgesia. For the tail-withdrawal assay, mice were placed momentarily in a cotton restraint immediately before the distal half of the tail was dipped in water maintained at 49.0 °C by electronic water bath (Lauda) accurate to the nearest 0.1 °C. The baseline latency for the mouse to flick the tail was recorded with a stopwatch to the nearest 0.1 s. Thirty minutes later, mice were injected with morphine (10 mg per kg body weight s.c.) and tested for post-injection latency after a further 30 min. A 15-s cutoff latency was employed. Separate naive mice were used for different pain assays. All data are expressed as the mean ± s.e.m. Treatment × genotype interactions were determined by nonparametric two-way ANOVA on ranking followed by Mann-Whiteney *U* test with Bonferroni correction for all *post hoc* pairwise multiple comparison procedures using Sigmaplot (v12). For all tests, significance was accepted at $P < 0.05$.

Naloxone-precipitated physical withdrawal. Opioid dependence was induced in mice by repeated intraperitoneal injection of morphine at 12-h intervals for 6 d (ref. 6). Mice were treated with escalating doses of morphine as follows: day 1, 20 mg per kg body weight; day 2, 40 mg per kg body weight; day 3, 60 mg per kg body weight; day 4, 80 mg per kg body weight; day 5, 100 mg per kg body weight; day 6, only one injection in the morning, 100 mg per kg body weight. Withdrawal was precipitated by injecting naloxone (1 mg per kg body weight s.c.) 2 h after the last administration. Somatic signs of withdrawal were evaluated immediately for 30 min. The following somatic signs were monitored: jumps, wet-dog shakes, paw tremor bouts, sniffs, teeth chattering and ptosis. A global score was calculated by using a coefficient for each sign (0.8; 1; 0.35; 0.5; 7.5; 4.5; and 1.5, respectively)[6]. All data are expressed as the mean ± s.e.m. and were analyzed by two-way ANOVA using Sigmaplot (v12) followed by Bonferroni tests for *post hoc* pairwise comparisons. For all tests, significance was accepted at $P < 0.05$.

Operant intravenous opiate self administration. A catheter (0.2-mm inner diameter, 0.4-mm outer diameter; Cathcams, Oxoford, UK) was implanted in the right jugular vein and flushed daily with saline throughout the experiment[49]. After 7 d and on a daily basis thereafter, these mice, who had been single housed since the surgery, were trained to self administer remifentanil (0.1 mg per kg body weight infusion) in operant chambers in which the active and inactive levers were randomly assigned to each mouse (Med-Associates Georgia, VT). A response on the designated active, but not inactive, lever resulted in an intravenous drug infusion (0.67 µl per g body weight) and presentation of a 20-s visual light cue, during which no further drug could be obtained. A maximum of 50 infusions were allowed during the 2-h sessions. Self-administration behavior was considered to be established if a minimum of ten infusions per session were obtained with no more than 20% variation in the number of infusions earned over the preceding 3 d. Mice underwent a minimum of 10 d of acquisition training at FR1, during which one lever press delivered one drug infusion, at which point mice that had not achieved the minimal infusion number or stable rates of infusion were removed from the study. The remaining mice proceeded to FR3, where three lever presses resulted in one infusion, and were trained at this level for a minimum of 3 d until stable rates of infusion were obtained. Thereafter the mice underwent progressive ratio training for 3 d, in which a within-session multiplicative increase in the number of lever presses was required to obtain each subsequent infusion[49]. Catheter patency was tested by an infusion of propofol (20 µl of 1% (wt/vol)) after 10 d on FR1 and again after FR3 and progressive ratio training, and any mice failing the patency test were removed from the study. All data are expressed as the mean ± s.e.m. Differences between groups across training days were analyzed using a linear mixed model, which can be used to maintain test validity by directly modeling unequal covariance across repeated measures (training days), which is intrinsic to a learning experiment, where responding starts off low and increases in variance (and mean) as days progress (SPSS 21). These models included fixed effects for genotype, day and their interaction, along with an unstructured residual covariance matrix. For tests where equal variance and covariance could be assumed, as determined by Mauchley's test of sphericity (because learning had already occurred, for example, stable responding in FR3 and progressive ratio data), two way ANOVA (day and genotype) with repeated measures and factorial analysis were used. For all tests, significance was accepted at $P < 0.05$.

Striatal dopamine microdialysis. The study is based on published protocol[50]. Naive mice (wild type, *MOR* knockout and rescue; $n = 7$ per genotype) underwent surgery for implantation of a unilateral guide cannula (CMA 11) targeting the right striatum (coordinates in mm relative to bregma: anteroposterior, +0.65; mediolateral, +2; dorsoventral, −3). During each of the last 2 d of the 5-d recovery period, mice were handled and injected with saline (10 ml per kg body weight s.c.). On the microdialysis test day, mice were briefly anesthetized with isoflurane and implanted with a CMA 11 microdialysis probe (length, 2 mm; outer diameter, 0.24 mm; molecular cutoff, 6 kDa). The probe was attached to a dual-channel liquid swivel with FEP tubing (inner diameter, 0.12 mm; CMA) and was continuously perfused with ACSF (125 mM NaCl, 2.5 mM KCl, 0.9 mM NaH_2PO_4, 1.2 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4) at a flow rate of 1 µl per min. Approximately 4 h later, dialysate sampling was initiated at 15-min intervals (15 µl per sample). Dialysate was collected into refrigerated (8 °C) tubes containing 1.5 µl of 12.5 mM perchloric acid and 250 µM ethylenediaminetetraacetic acid. After a 1-h baseline period, mice received an injection of sterile saline (10 ml per kg body weight s.c.) as a control for the effects of handling on striatal dopamine efflux. Forty-five minutes later, mice were injected with morphine sulfate (10 mg per kg body weight s.c.). After an additional 2 h, the mice received an injection of cocaine hydrochloride (10 mg per kg body weight s.c.). Sampling continued for a further 2 h. Drug treatment order was fixed for all subjects (i.e., not randomized) to minimize carryover effects and facilitate data interpretation. Probe placements were confirmed as being within the striatum. Dialysate dopamine levels were quantified by high-performance liquid chromatography with electrochemical detection (Antec Leyden), as previously described[48]. A total of 23 mice were assigned to this study. Two mice were excluded on the basis of criteria established before the study, one because of sample contamination and another because of probe malfunction. Subjects were run in squads of two to four mice. Drug- and saline-induced changes in striatal dopamine concentrations were computed as a percentage of baseline concentrations (±s.e.m.), averaging

across the two samples collected immediately before each injection to compute a local baseline. Timing of the samples was adjusted for dead volume in output from the probe. For statistical analysis, post-injection dopamine concentration changes were averaged over eight samples (2 h) for morphine and cocaine and over three samples (45 min) for saline. Variance was similar between groups, but because of non-normality of the dopamine concentration change distributions in some conditions, genotype effects were assessed using nonparametric Kruskal-Wallis one-way ANOVA tests followed by Bonferroni-adjusted Mann-Whitney *U* tests for *post hoc* pairwise comparisons, with significance accepted at $P < 0.05$ (two sided) for all analyses.

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