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Journal

Nature Neuroscience, 17(2)

ISSN

1097-6256

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Publication Date

2014-02-01

DOI

10.1038/nn.3622

Peer reviewed

HHS Public Access

Author manuscript

Nat Neurosci. Author manuscript; available in PMC 2014 August 01.

Published in final edited form as:

Nat Neurosci. 2014 February ; 17(2): 254–261. doi:10.1038/nn.3622.

Targeted Expression of Mu-Opioid Receptors in a Subset of Striatal Direct-Pathway Neurons Restores Opiate Reward

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AUTHOR CONTRIBUTIONS

CY and XWY designed the study, interpreted the results and wrote the manuscript. CY performed experiments, analyzed the data, and made Figs. 1, 2, 3, 5, and 6a–6g and Supp. Figures S1–S8. SBO and NTM did experiments for and made Fig. 6h and 6i. AJ, JDJ, WMW did experiments for and made Fig. 4. WG and YS contributes to Fig. 1b. CSP contribute to Fig. 1 and Supp. Fig. S3. KWR, NM, NPM, BLK and CJE contributed to the experimental design, actual experiments and data analyses for Figs. 2, 3, 5, and Supp. Figs. S6, S7. CC and MSL contributed to revision of the MS. XWY and CJE contributed to Supp. Fig. S9.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

SUMMARY

Mu-Opioid Receptors (MOR) 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 novel conditional BAC rescue strategy to show that mice with targeted MOR expression in a subpopulation of striatal direct-pathway neurons enriched in the striosome and nucleus accumbens, in an otherwise MOR-null background, restore opiate reward, opiate-induced striatal dopamine release, and partially restore motivation to self-administer opiates. However, they lack opiate analgesia or withdrawal. Importantly, we used Cre-mediated deletion of the rescued MOR transgene to establish that striatal, rather than a few extrastriatal sites of MOR transgene expression, is needed for the restoration of opiate reward. Together, our study demonstrates that a subpopulation of striatal direct-pathway neurons is sufficient to support opiate reward-driven behaviors and provides a novel intersectional genetic approach to dissect 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, [WY1] but its aberrant activation is also implicated in the abuse of addictive substances including opiates, alcohol, nicotine and cannabinoids[1,2]. Upon binding to endogenous opioid peptides (*e.g.* enkephalin, endorphin, dynorphin) or exogenous opiate drugs (*e.g.* morphine), the opioid receptors activate intracellular signaling via inhibitory G proteins that typically leads to suppression of neuronal activities[2,3]. The study of targeted gene knockout mice has demonstrated that among the three major opioid receptors, Mu, Delta and Kappa, only the Mu-Opioid Receptor (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 potential brain sites of MOR-mediated actions[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 due to 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 (dStr) and nucleus accumbens (NAc), receives input from dopaminergic (DA) neurons in the ventral tegmental areas (VTA) and substantia nigra pars compacta (SNc), and serves as 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[5,6,7]. The striosome and matrix MSNs can be further divided into two sub-populations, those in the striatal direct-pathway sending inhibitory projections to the substantia nigra (including both substantia nigra pars reticulata and SNc), and those in the striatal indirect-pathway sending inhibitory projections to globus pallidus externa (GPe)[6,7]. Neuroanatomical tracing studies suggest that striosome rather than matrix MSNs in the direct-pathway preferentially form monosynaptic input onto the DA neurons in the SNc and VTA[8,9]. However, functional evidence for such inhibitory synaptic connections remains inconsistent[10]. Prior evidence suggests that MOR is expressed in both the direct-pathway and indirect pathway MSNs in the striosome, but at least in some

striosomes there appears to be an overabundance of direct-pathway MSNs[8,9]. In this study, we devised a novel conditional BAC transgenic rescue strategy to directly assess the functional significance of MOR expression in the striosomal and NAc direct-pathway MSNs in pathological opiate reward and reinforcement.

RESULTS

MOR re-expression in the 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 prior observation by double fluorescent localization of murine MOR and green fluorescent protein (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. S1)[11]. We found both Drd1-GFP and Drd2-GFP labeled MSNs in the striosome (Supplementary Fig. S1a–S1f). Moreover, using high-resolution confocal imaging, we saw MOR expression in Drd1-GFP$^+$ direct-pathway MSNs (Supplementary Fig. 1g–1i), consistent with the interpretation that endogenous MOR is expressed in the 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 novel conditional BAC-mediated transgenic rescue strategy to re-express MOR in specific neuronal populations of *MOR* knockout (*MOR-KO*) mice[4]. To target MOR expression to a pattern closely resembling that of the endogenous MOR in the direct-pathway MSNs, we used the GENSAT murine *Prodynorphin* (*Pdyn*) 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 consistent with the interpretation that *Pdyn* BAC transgene drives GFP expression in the 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 are indeed co-localized with the MOR$^+$ striosome (Fig. 1a). To further establish, at single-cell resolution, the *Pdyn-GFP*$^+$ MSNs are indeed direct-pathway MSNs that also co-express 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. S2)[12], with single FACS-sorted GFP$^+$ neurons picked under microscope and used to prepare RNA for single-cell gene expression analyses[13]. As shown in Fig. 1b, all the single FACS-sorted neurons from both *Pdyn-GFP* and *Drd2-GFP* express *Darpp-32*, a MSN specific marker. Importantly, reverse transcriptase-PCR analyses with two separate sets of MOR-specific primers showed that 32 out of 33 FAC-sorted single *Pdyn-GFP*$^+$ MSNs also express *MOR*. Moreover, these neurons also co-express direct-pathway MSNs markers, *Drd1* and *Pdyn*, but not a marker of indirect-pathway MSNs (*i.e. Drd2*). To the contrary, single *Drd2-GFP*$^+$ neurons express *Drd2*, but not *Drd1* and *Pdyn*, and are also negative for *MOR* transcripts. These results support our conclusion that *Pdyn-BAC* selectively drives transgene expression in a subpopulation of direct-pathway MSNs that co-express *MOR* in the striatum. Our result regarding *MOR* expression in the *Drd2-GFP*$^+$ neurons should be interpreted with caution for

several reasons: we only sampled a relatively small number of such neurons, the striosome MSNs is 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 *Pdyn-BAC* to drive the transgenic re-expression of MOR to specifically interrogate the functional role of MOR 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 murine *MOR* cDNA followed by a polyadenylation (PolyA) signal upstream of the *Pdyn* open reading frame to ensure that *Pdyn* itself is not overexpressed by the transgene. Finally, 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 C57/BL6 embryos resulted in transgenic mice expressing MOR under the control of *Pdyn* genomic regulatory elements on the BAC (*Pdyn-MOR*). The *Pdyn-MOR* transgene was subsequently bred onto a *MOR-KO* background[4] to generate *Pdyn-MOR/MOR-KO* ("*Rescue*") mice. Using a highly selective MOR antibody[14], we showed that MOR expression in the *Rescue* mouse brain is largely restricted 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–1g, Supplementary Fig. S3). Since *Pdyn-BAC* drives transgene expression within the striatum and is highly selective for the subpopulation of direct-pathway MSNs in the strisome and NAc (Fig. 1b, Supplementary Fig. S1)[6,11], we concluded that the *Rescue* mice confer re-expression of MOR in a subpopulation of direct-pathway MSNs that endogenously express MOR. Finally, we used quantitative Western blots to determine the levels of MOR expression in the *Rescue* mice and found that these mice express MOR protein at about 2.76 fold of endogenous MOR in Wildtype (WT) mice (Supplementary Fig. S4). Since prior viral-mediated targeted expression of other CNS genes have lead to conclusions on the crucial roles of the re-expressed genes in specific brain regions in mediating various behavioral effects *in vivo*[15,16], we expect that the exquisite neuroanatomical and cellular specificity conferred by the BAC-mediated MOR re-expression in *Rescue* mice should also be informative on the role of direct-pathway MSN subpopulations in opiate-driven behaviors.

Morphine sensitization and CPP in *Rescue* mice

We next evaluated the phenotypic consequences of *Pdyn-BAC* driven MOR expression. We first showed that the *Pdyn-MOR* and *Rescue* mice lack general changes in development, locomotion or body weight, despite the modest levels of striatal MOR overexpression in these mice (Supplementary Fig. S5). We next asked whether such targeted MOR re-expression could restore any key opiate-driven behaviors that are absent in *MOR-KO* mice[4]. We first tested for the rewarding properties of morphine in the CPP paradigm[4]. Using two independent cohorts of mice, we were able to reproducibly show the lack of CPP in *MOR-KO* mice and the restoration of CPP to WT control levels in the *Rescue* mice (Fig. 2a **and** Supplementary Fig. S6a). Interestingly, transgenic overexpression of MOR in *Pdyn-MOR*

mice did not elicit excessive levels of CPP compared to WT mice (Fig. 2a), suggesting the lack of gain-of-function effects on opiate reward by the transgene itself. These results demonstrated that targeted re-expression of MOR in the striatal direct-pathway MSN subpopulation 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-KO* mice[4]. We next tested whether *Rescue* mice would also express morphine-induced locomotion and sensitization. As shown in Fig. 2b, morphine-induced locomotor stimulation and sensitization was absent in the *MOR-KO* mice but was restored to the WT levels in the *Rescue* mice. This result demonstrates that targeted MOR expression in striatal direct-pathway MSNs within the striosome and NAc is sufficient not only to mediate the locomotor stimulatory effects of morphine, but also to trigger a similar adaptive response to repeated morphine injections seen in WT mice.

Remifentanil self-administration in *Rescue* mice

The CPP procedure tests the degree to which the rewarding effects of opioids, delivered non-contingently, can be conditioned to a context through repeated pairings. Reward, however, is only one dimension of the psychoactive properties of opiates that supports voluntary drug-seeking and drug-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 (*e.g.*, lever press) to elicit the drug's effects[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 able to sustain opiate reward as measured by CPP may not necessarily sustain aspects of opioid reinforcement indexed by self-administration[1].

To address the role of MORs in striosomal and NAc direct-pathway MSNs in opiate-induced reinforcement, *Rescue*, *MOR-KO* and WT mice were trained to self-administer remifentanil, a potent short-acting synthetic opioid with a half-life of about 4 minutes[19]. In contrast to *MOR-KO* mice which lacked any motivation to self-administer remifentanil, both WT and *Rescue* mice obtained a stable level of remifentanil self-administration across the last 3 days 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 WT and *Rescue* mice obtained more infusions than *MOR-KO* during the 2-hour 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 demonstrates that the *Rescue* mice, unlike *MOR-KO* mice, can acquire opioid self-administration. However, further analyses revealed that the motivation to obtain remifentanil is only partially restored in these mice. Firstly, *Rescue* mice earned infusions at a slower pace during FR1 testing (Fig. 3a and 3b) and gained fewer infusions overall during FR3 testing (Fig. 3d) than the WT mice. In addition, *Rescue* mice exhibited lower infusion "break points" during a progressive ratio (PR) task, indicating that they were willing to exert less effort than WT mice to obtain the opiate drug (Fig. 3e). These results demonstrate that MOR re-expression in the

subpopulation of striatal direct-pathway MSNs partially restores opioid-seeking and taking behaviors that are lacking in the *MOR-KO* 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 main factor in the addiction cycle[20]. Physical withdrawal symptoms can be precipitated in WT, but not *MOR-KO* mice, by injecting the opiate antagonist naloxone in chronically morphine treated animals[4]. Neither *MOR-KO* mice nor *Rescue* mice exhibited any of the classical signs of morphine withdrawal that are present in WT littermates, after the chronic morphine regimen (Fig. 4a; Supplementary Fig. S7). This finding is consistent with the prior suggestion that MORs in other brain areas are the likely 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 MOR activation[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 WT controls but was absent in both *Rescue* and *MOR-KO* mice (Fig. 4b and 4c), suggesting that MOR expression outside the striosome and NAc direct-pathway MSNs is necessary for opiate analgesia.

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 the *Rescue* mice, we performed an intersectional genetic approach taking advantage of the conditional BAC transgene design (Fig. 1c). Since the *Pdyn-MOR* transgene drives the re-expression of MOR in a few sites outside of the striatum, we decided to cross onto the *Rescue* mouse background the *Rgs9-Cre* transgene, which drives selective Cre expression in all the striatal MSNs (including both direct-pathway and indirect-pathway MSNs; Fig. 5a)[24]. The resulting *Pdyn-MOR/MOR-KO/Rgs9-Cre* (*Rescue/Cre*) mice have nearly complete elimination of *MOR* transgene expression in the striatal direct-pathway MSN subpopulation, the neuronal cell type residing at the intersection between the *Pdyn-MOR* rescue transgene and *Rgs9-Cre* expression domains, leaving the expression of MOR rescue transgene in the extrastriatal sites intact (Fig. 5a; Supplementary Fig. S8). Importantly, the *Rescue/Cre* mice no longer exhibit morphine CPP under conditions where their *Rescue* and WT littermates exhibit robust morphine CPP (Fig. 5b **and** Supplementary Fig. S6b).

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

Morphine-induced striatal dopamine release in *Rescue* mice

Similar to other drugs of abuse, morphine administration elicits dopamine release from the terminals of VTA and SNc neurons in the NAc and dStr, which is thought to be contributing to the reinforcing and locomotor-activating effects[3,25]. Since VTA/SNc GABAergic interneurons express MORs[5,25] and tonically inhibit DA neurons[26], morphine's inhibitory action 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 DA release in reward processing remain to be clarified[1,28].

To further probe the basal ganglia (BG) neurocircuitry mechanisms correlated with the rescue of opiate reward and reinforcement in our new model, we examined MOR expression in the VTA/SNc of WT, *MOR-KO* and *Rescue* mice. Unlike WT mice, our *Rescue* mice do not exhibit MOR staining of neuronal soma in VTA and SNc (Fig. 6a–6d), consistent with the lack of GFP staining in the VTA and SNc interneurons of *Pdyn-GFP* mice[11]. Moreover, *Rescue* mice show neuropil MOR staining in the VTA and SNc that is abolished in *Rescue/Cre* mice (Supplementary Fig. S8f–g), suggesting that it reflects axonal terminal staining of MOR expressed in the striatal direct-pathway MSNs. Furthermore, double immunofluorescent staining showed that *Rescue* mice exhibit MOR terminal staining in the VTA and SNc that partially overlaps with DA neuronal cell bodies and more fully overlaps with their dendrites (Fig. 6e–6g), consistent with studies demonstrating that striosomal direct-pathway MSNs may form monosynaptic contacts with DA neurons in the SNc/VTA[9]. To test whether MOR expression in striatal direct-pathway MSNs, but not in midbrain interneurons, could restore *in vivo* morphine-induced dopamine efflux in the striatum, we performed dopamine microdialysis. While the *MOR-KO* mice lacked any morphine-induced elevation in extracellular dopamine in the striatum, the *Rescue* mice exhibited full restoration of this effect, to a level that was comparable to WT mice (Fig. 6h). Saline injections had no significant effects (not shown) and blockade of dopamine reuptake with cocaine elevated extracellular dopamine to a similar extent across WT, *MOR-KO* 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. S9), an *in vivo* neurochemical correlate elicited by a variety of natural and drug rewards[29] .

DISCUSSION

In this study, we utilized a novel conditional BAC rescue strategy in mice to demonstrate that targeted MOR expression in a subpopulation of the striatal direct-pathway MSNs in the striosome and NAc restores opiate-induced CPP and sensitization, partially restores opiate reinforcement, but does not rescue opiate analgesia and withdrawal. Since the MOR transgene expresses in a few extrastriatal sites, we used Cre-mediated striatal-specific deletion of the rescue transgene to establish that MOR in the striatal direct-pathway MSNs is necessary for opiate reward in the *Rescue* mice. Moreover, our study also showed that *Rescue* mice exhibit morphine-induced elevation of extracellular dopamine in the striatum,

providing evidence that MOR-expressing striatal direct-pathway MSNs can functionally regulate nigrostriatal dopamine release. Together, our study provides *in vivo* evidence that MOR in the 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 question[1]. Previous knowledge of the neuronal substrate for opiate drug reward is largely based 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 mediating the effects of the infused drugs. Since prior genetic studies have demonstrated that 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-induce behavioral effects *in vivo*. Our BAC-mediated targeted re-expression of MOR permits us to address whether MOR expression in a specific neuronal population is sufficient for opiate reward-related behaviors. The anatomical and cellular specificity of the transgene expression driven by *Pdyn-BAC* is 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, normally residing in the striosome and NAc, in reward and reinforced behaviors. By demonstrating that the *Rescue* mice can restore opiate induced dopamine release in the striatum, and 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 rationales that striosome direct-pathway MSNs are known to form monosynaptic GABAergic inputs onto the DA neurons in VTA and SNc[9], and our *Rescue* mice restore MOR expression in the striatonigral axonal terminals in VTA and SNc (Supplementary Fig. S8) and opiate-induced striatal dopamine release. Future *in vitro* and *in vivo* electrophysiological studies are needed to address how the MOR in the direct-pathway MSN neuronal subpopulations may regulate DA neuron activities in both *Rescue* and wildtype mice.

Our findings are also consistent with the notion that MORs in multiple neuronal populations in and outside the striatum could play a role in opiate reward-related behaviors. While morphine CPP was restored in the *Rescue* mice, remifentanil self-administration in the *Rescue* mice was partially restored compared to their WT littermates. This latter finding, in this arguably more sensitive assay of opioid drug reward, suggests the likely contribution of other MOR-expression neuronal populations in regulating opiate reward and reinforcement. Indeed, MOR agonists have been shown to disinhibit DA neuron activity via suppression of presynaptic VTA interneurons[26] and GABAergic neurons in the rostromedial tegmental nucleus (RMTg)[39]. Moreover, other neuronal cell types such as the striatal cholinergic interneurons and neurons in the nucleus tractus solitarius are also implicated in opiate reward[26,30,31]. Future MOR-based genetic studies should allow systematic interrogation of

the roles of MOR in these opiate-sensitive neuronal cell populations in opiate reward and reinforcement.

Our study represents 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 the MOR-KO mice. First, our genetic experiment is 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 *Pdyn-BAC* leads to MOR expression that is higher than the endogenous MOR levels in the striatum, and the expression pattern of the MOR transgene may also have subtle differences (*e.g.* extrastriatal sites of expression) from endogenous MOR (Supplementary Fig. S3). However, our results should still be interpretable since we have shown the co-localization of *Pdyn-BAC*-driven transgene with endogenous MOR in the striosomal direct-pathway MSNs at single-neuron resolution. Moreover, the *Pdyn-MOR* transgenic mice themselves do not appear to have overt basal or opiate-induced behavioral deficits, and the *Rescue* mice restoring a subset of opiate-driven behaviors normally present in the WT mice that are absent in the *MOR-KO* mice. Hence, our results are consistent with the transgenic rescue of a loss-of-function mutant, rather than novel 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 routinely been used 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. Finally, our study reveals a correlate, but not yet a 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 question to resolve since 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 dopaminergic neurons and to some extent the GABAergic neurons[8], which may play distinct roles in opiate reward [33,34]. Such limitations notwithstanding, our study represents an important step forward towards 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 based on 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 connectivity 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 towards understanding the differential physiological function 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-pathways MSNs in striosome vs. matrix compartments, even though such compartmentalization is thought to play an important role in basal ganglia function[37]. Our study shows that *Pdyn-BAC* confers some specificity to the striosomal direct-pathway MSNs, since[WY2] 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 before in single-neuron tracing experiments[6,8]. It is also consistent with the finding that genetic ablation of D1-MSNs (but preserving D2-MSNs) leads to the loss of majority of MOR staining in the striatum[38]. Future studies are 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]. Our study suggests that MOR in the striosomal direct-pathway MSNs may contribute to opiate reward. Finally, our *in vivo* striatal microdialysis experiment showed that MOR in the striosome/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 dopaminergic neurons that are postulated to play important roles in reward and reinforcement behaviors[9,37].

Finally, our study demonstrates a novel intersectional genetic strategy to assess a single gene in a specific neuronal population for its physiological or pathological roles in a mammalian model. Prior mouse genetic tools, such as Cre/loxP mediated conditional gene inactivation[39], often cannot interrogate the role of a single neuronal population since 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 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 not only help to elucidate the cellular targets for opiate-induced effects including reward, analgesia and dependence, but also could help to elucidate the cell-type-specific function of many other important genes in the mammalian brain.

METHODS

Animals

To generate *Pdyn-MOR* mice, we modified the GENSAT murine *Prodynorphin* (*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 murine MOR cDNA followed by a polyadenylation signal (PolyA) was inserted into the 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 C57B6 fertilized eggs to generate *Pdyn-MOR* mice. Germline transmission and genotyping of the transgenic offspring were tracked by PCR amplification of transgene sequence from the genomic DNA. The *Pdyn-MOR* mice were bred onto a *MOR-KO* background[4] to generate the *Rescue* mice. GENSAT *Pdyn-GFP* mice[11] were obtained from Mutant Mouse Regional Resource Center. All mice (60–120 days old, both sexes) in this study were bred and maintained on an inbred C57B6 background, and under standard conditions that are consistent with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of University of California, Los Angeles. A 12-hour light /12-hour dark cycle is used in the UCLA vivarium. Mice of the same sex were group housed with up to 5 mice per cage. All behavioral tests were performed during the light phase.

General experimental design

The sample sizes used for our experiments were selected based on results from previous studies to ensure detection of statistically significant morphine elicited behavior difference in WT and *MOR-KO* groups[4,43–46]. No individual data points were excluded from the statistical analyses except for the operant intravenous opiate self-administration and the striatal dopamine microdialysis tests (see below). 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 the MOR to facilitate its immunohistochemical detection, animals received an intraperitoneal (i.p.) injection of etorphine (0.1 mg/kg), and euthanized 20 minutes (min) later. Then mice were perfused with 4% PFA then post-fixed overnight. Floating sections (40 μm) were incubated with rabbit anti-MOR antibodies (1:1000 dilution)[14] at 4°C for 36 hours (hr). Then the sections were incubated with biotinylated goat anti-rabbit IgG antibody at 1:200 dilution at room temperature for 2 hr and then in ABC kit (Vector, PK-4005), and developed using diaminobenzidine (DAB). Histochemical staining of MOR has been done using brain slices from 3–4 mice per genotype for each experiment. Sections were imaged using an Olympus VS110 microscope.

Double immunofluorescence staining with the MOR antibodies

PFA fixed brain sections of 40 μm were prepared for immunohistochemical staining. For double immunofluorescence studies, floating sections were blocked 30 min with 3% normal goat serum and 3% bovine serum albumin (BSA). After incubation with anti-MOR rabbit monoclonal antibodies (Abcam, ab134054; 1:500 dilution) and sheep polyclonal anti-TH antibody [UMB3] (Millipore, AB-1542, 1:1000 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 then applied to the sections at 1:200 dilution and incubated for 2 hr in the dark at room temperature. Immunofluorescence staining of MOR has been performed using brain slices from 3–4 mice per genotype for each experiment. Sections were mounted in Vectashield with DAPI (Vector), and images were taken using a Leica TCS-SP confocal microscope (Leica, Heidelberg, Germany) and VS120 WSI scanning system (Olympus America Inc., Center Valley, PA).

In order to detect the co-localization of the 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: in mM), 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 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 sorting and single cell real time PCR

FAC-sorting of GFP-labeled MSNs was modified from our established protocol[12]. Mice were euthanized and their dorsal striata extracted immediately. Striatal tissue was placed in 1 ml of Hibernate A (HA-LF; Brain Bits) and minced. Tissue was incubated with papain/DNase (PAP2 and D2, respectively, Worthington) with gentle rotation at 31–32°C for 30 mins. Tissue pieces were transferred into 2 mL room temperature HABG, incubated for 5 mins 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 (20 μg/ml) 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 published protocol[49]. Single cell cDNA preparation was performed following a previously described protocol[50]. Briefly, single *Pdyn-GFP* positive were manually picked under microscope, and then seeded to 4.45ul 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 10ul reaction system with 1 ul cDNA product. Those

samples with Ct around 22–23 were purified by QIAquick PCR purification kit and eluted with 40μl EB buffer.

The relative abundance of target genes was determined by Real-Time qPCR analysis using 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 μ reaction mixture contained 5μL 2X SYBR FAST Master Mix, 1 μM of template cDNA (100–200 ng/μL), 0.2 μ, of forward and reverse primer (100 mM) and RNase-free water. Real-time cycling conditions included initial incubation step at 95°C for 4 min followed by 40 cycles each of 95°C, 25 sec; 57°C, 25 sec; and 72°C, 1 sec respectively. The melt-curve data was collected using gradual temperature increase from 40°C to 95°C at the rate of 0.3°C/10 sec. A non-template 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 (ΔCp) was determined. The relative abundance was calculated using the formula $2^{-\Delta\Delta Cp13}$. 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 in Supplementary Table S1.

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 2000 × g for 30 s, and the supernatants was used for western blotting. Protein samples were prepared from dorsal striatum of 3–6 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; 1:1000) and an anti-β-actin rabbit monoclonal antibody (Abcam, Ab8227; 1:10000) overnight at 4°C. The blot was then incubated with goat anti-Rabbit IgG secondary antibody at a dilution of 1:200 for 1 hour at room temperature. Chemiluminescent detection was accomplished using ECL Plus Western Blotting Detection reagents (GE Healthcare). Densitometric values from scanned Western blot film were obtained using ImageQuantTL software (GE Healthcare). Statistical analysis: Data are expressed as mean ± SEM. Due to non-normality of distributions in some genotypes, difference across genotype was tested by non-parametric 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 conditioned placement preference (CPP) and locomotor sensitization

Place conditioning was conducted in a two-compartment apparatus that recorded position and locomotion of the mice were recorded, as previously described[43–46]. A 20 min "pre-test" was recorded under drug-free conditions whereby animals were given free access to both compartments. The following 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 following day, each mouse received an s.c. injection of vehicle or 10 mg/kg morphine before being placed for 40 min in the chamber opposite to that of the previous

day. This process was repeated 3 times. Locomotion during the drug-conditioning sessions were recorded simultaneously and analyzed for the locomotor sensitization. The day following the last drug-conditioning session, the expression of place conditioning to the drug-paired compartment was assessed under the same conditions as the pre-test. Statistical analysis: data are expressed as mean ± SEM. For the CPP Test, equal variance and covariance could be assumed, treatment x genotype interactions were determined by two-way ANOVA using Sigmaplot (v12) followed by Bonferroni tests for tests for *post hoc* pairwise comparisons. For statistical analysis of the locomotor sensitization tests, locomotion distance during the habituation days were averaged and used as 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 assay 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/lick the hindpaw or jump was recorded with a stopwatch to the nearest 0.1 seconds (s). Thirty minutes later, mice were injected with morphine (10 mg/kg, s.c.) and post-injection latency was tested after an additional 30 min. A cut-off 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/kg, s.c.) and tested for post-injection latency after a further 30 min. A 15s cutoff latency was employed. Separate naive mice were used for different pain assays. Statistical analysis: Data are expressed as mean ± SEM. Treatment x genotype interactions were determined by non-parametric two-way ANOVA on ranking followed by Mann-Whiteney U test with Bonferroni correction for *post hoc* all 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 (i.p.) injection of morphine at 12h intervals for 6 days[6]. Mice were treated with escalating doses of morphine as follows: day 1: 20 mg/kg, day 2: 40 mg/kg, day 3: 60 mg/kg, day 4: 80 mg/kg, day 5: 100 mg/kg; day 6, only one injection in the morning, 100 mg/kg. Withdrawal was precipitated by injecting naloxone (1 mg/kg, s.c.) 2h 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]. Statistical analysis: Data are expressed as mean ± SEM and analyzed by two-way ANOVA using Sigmaplot (v12) followed by Bonferroni tests for 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 i.d., 0.4 mm o.d, Cathcams, Oxoford, UK.) was implanted in the right jugular vein and flushed daily with saline throughout the experiment[47]. Post surgery animals were singly housed. After 7 days, and on a daily basis thereafter, mice were trained to self-administer remifentanil (0.1 mg/kg/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 the inactive, lever, resulted in an intravenous drug infusion (0.67 μl/g body weight) and presentation of a 20s visual light cue, during which no further drug could be obtained. The total amount of drug that could be obtained during the 2h session was limited to 50 infusions. Self-administration behavior was considered established if a minimum of 10 infusions/session were obtained with no more than 20% variation in the number of infusions earned over the preceding 3 days. Mice underwent a minimum of 10 days 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 3 lever presses resulted in one infusion and were trained at this level for a minimum of three days until stable rates of infusion were obtained. Thereafter the mice underwent PR training for 3 days, in which a within session multiplicative increase in the number of lever presses was required to obtain each subsequent infusion[59]. Catheter patency was tested by an infusion of propofol (20 μl of 1% w/v) after 10 days on FR1 and again after the FR3 and PR any mice failing the patency test were removed from the study. Statistical analysis: Data are expressed as mean ± SEM. Differences between groups across training days were analyzed using 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, e.g., stable responding FR3 and PR data), two way ANOVAs (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[48]. Naïve mice (*WT*, *MOR-KO*, 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, AP: +0.65, ML: +2, DV: −3). During each of the last 2 d of the 5-day recovery period, mice were handled and injected with saline (10 ml/kg, s.c.). At the microdialysis test day, mice were briefly anesthetized with isoflurane and implanted with a CMA 11 microdialysis probe (length, 2mm; outer diameter, 0.24mm; 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 artificial CSF (125 mM NaCl, 2.5 mM KCl, 0.9 mM NaH2PO4, 1.2 mM CaCl2, 1mM MgCl2, pH 7.4) at a flow rate of 1 μl/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 ul of 12.5 mM perchloric acid/250 μM EDTA. After a 1 h baseline period, mice received an injection of sterile saline (10 mg/ml; s.c.) as a control for the effects of handling on striatal dopamine efflux. Forty-five min later, mice were injected with morphine sulfate (10 mg/kg; 10 mg/ml; s.c.). After an additional 2 h, the mice received an injection of cocaine hydrochloride (10 mg/kg; 10 mg/ml; 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 HPLC with electrochemical detection (Antec Leyden), as previously described[48]. A total of 23 mice were assigned to this study. Two mice were excluded based on criteria established before the study: one due to sample contamination and another due to probe malfunction. Subjects were run in 2–4 mouse squads. Drug- and saline-induced changes in striatal DA concentrations were computed as a percentage of baseline concentrations (± SEM), averaging across the two samples collected before immediately before each injection to compute a local baseline. Timing of samples was adjusted for dead volume in output from probe. For statistical analysis, post-injection DA concentration changes were averaged over 8 samples (2h) for morphine and cocaine and 3 samples (45 min) for saline. Variance was similar between groups, but due to non-normality of DA concentration change distributions in some conditions, genotype effects were assessed using non-parametric Kruskal-Wallis one-way analysis of variance 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The research is supported by the University of California Los Angeles Center for Opioid Receptors and Drugs of Abuse funded by National Institute on Drug Abuse at National Institutes of Health (P50 DA005010). X.W.Y. is also supported in part by the David Weil Fund to the Semel Institute at UCLA, and Neuroscience of Brain Disorders Award from The McKnight Endowment Fund for Neuroscience. The *Pdyn-MOR* BAC transgenic mice were generated at the UCLA Transgenic Core Facility. Flow cytometry was performed in the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility (funded by National Institutes of Health awards CA-16042 and AI-28697, and by the JCCC, the UCLA AIDS Institute, and the David Geffen School of Medicine at UCLA). W.G. and Y.S. are supported by the Transcriptome and Epigenetics Core (P50 DA005010) and by Intellectual and Developmental Disabilities Research Center (IDDRC center grant (NIH-P30HD004612).

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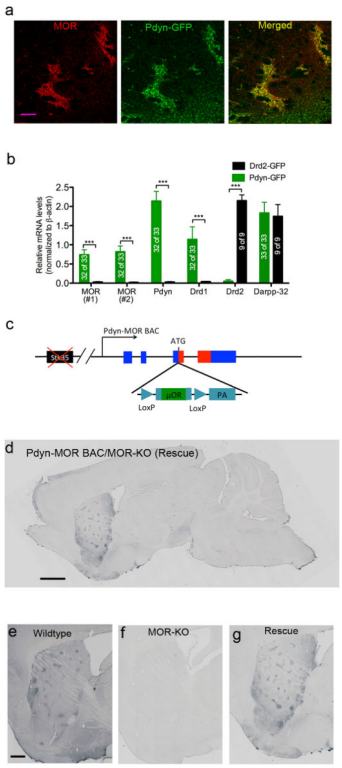

Figure 1.

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 shows the co-localization of MOR (red) and GFP (green) in the striosome of the GENSAT *Pdyn-GFP* mouse brain. Scale bar = 100 μm. **(b)** Single-cell quantitative PCR analyses were performed to assess mRNA expression for multiple genes in FACS-sorted single GFP^+ cells from *Pdyn-GFP* and *Drd2-GFP* mice. Data were normalized to the reference gene β-actin. Data represent 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. Majority of single neurons from *Pdyn-GFP* mice are positive for *MOR* transcripts (with two independent sets of PCR primers) while all single neuron 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). Triple asterisks indicate $p < 0.0001$. **(c)** A schematic representation of the MOR cDNA in the *Pdyn-MOR* conditional BAC transgenic construct. **(d)** A representative immunohistochemical staining of MOR in *Rescue* (*Pdyn-MOR*/*MOR-KO*) 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 WT, *MOR-KO* and *Rescue* mice. Scale bar = 200 μm.

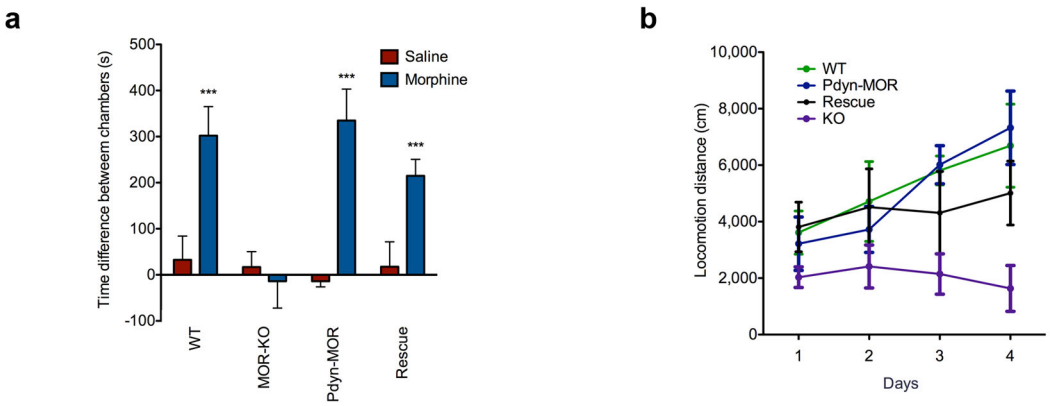

Figure 2.

Restoration of the rewarding and locomotor effects of morphine by *Pdyn-BAC*-driven expression of the *MOR* in *rescue* mice. (**a**) Morphine-induced CPP is absent in the *MOR-KO* mice, but it is restored to the WT level in the *Rescue* mice. Two-way ANOVA analysis revealed significant CPP deficit in *MOR-KO* mice ($F_{(1, 26)}$=61.14, p<0.001, WT vs. *MOR-KO*, Two way ANOVA; WT, n=7; *MOR-KO* n=8), which is restored in the *Rescue* mice ($F_{(1,26)}$=3.619, p=0.0683, WT vs. *Rescue* two way ANOVA; WT, n=7; *Rescue* n=8). Significant CPP was revealed by *post doc* test in WT ($p < 0.001$), *Rescue (p < 0.001)* but not *MOR-KO* (*p > 0.05)* mice. The morphine CPP in *Pdyn-MOR* transgenic mice is comparable to that in WT controls ($F_{(1,26)}$=4.171, $p = 0.0514$, two way ANOVA; WT, n=7; n=8, *Pdyn-MOR*). (**b**) Deficits in morphine-induced locomotor sensitization in *MOR-KO* mice were restored in the *Rescue* mice to a level that is comparable to the WT and *Pdyn-MOR* mice. Repeated-measures ANOVA analysis revealed a significant genotype and day x genotype interaction ($F_{(3,81)} = 13.284$, $p < 0.001$; WT, n = 7; n = 8 for other genotypes), with *post hoc* test showing significant sensitization effect of morphine on WT, *Pdyn-MOR* and *Rescue* mice ($p < 0.05$ for all groups), but not *MOR-KO* mice ($p > 0.05$). Bonferroni's *post hoc* test also showed that *Rescue* mice are significantly different from *MOR-KO* mice ($p = 0.041$), but not when compared with WT mice ($p = 0.677$). Values are mean ± SEM. Triple asterisks indicate $p < 0.01$.

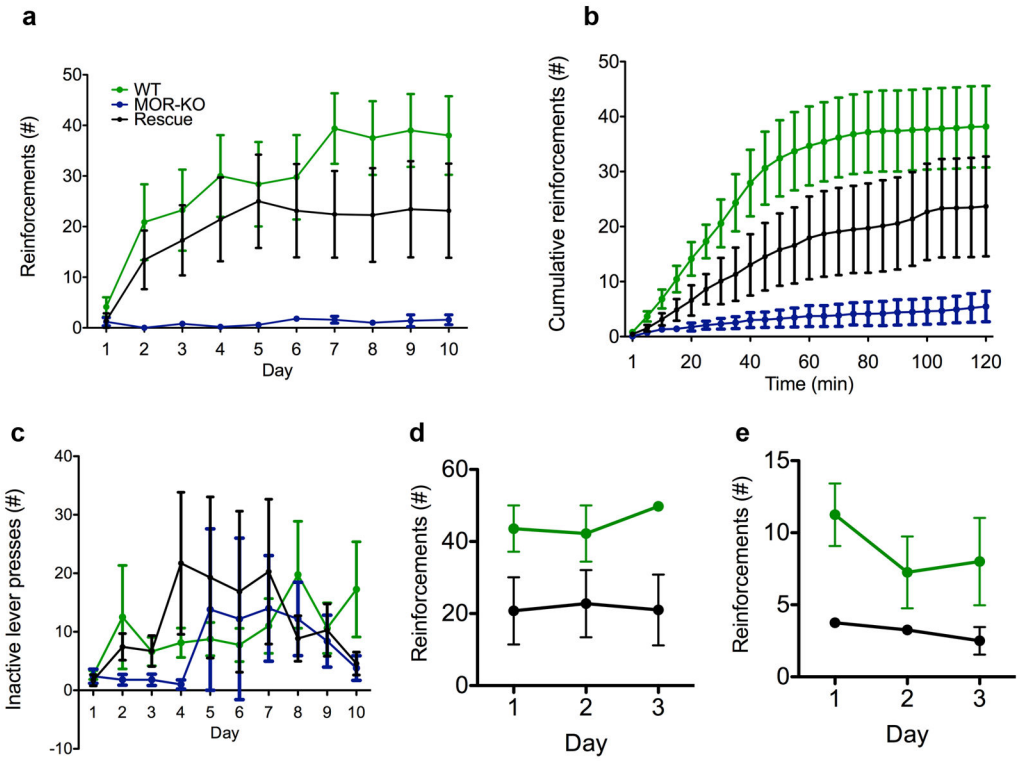

Figure 3.

Selective MOR expression in striosome and NAc direct-pathway MSNs partially rescues opiate self-administration. (**a**) WT and *Rescue* mice, but not *MOR-KO* mice, self-administered remifentanil (0.10 mg/kg i.v.) under the FR1 reinforcement schedule. Repeated-measures ANOVA analysis revealed a significant day x genotype interaction $F_{(18, 17)}$=2.974, p = 0.015. WT mice self-administered more drug across days than *MOR-KO* mice (genotype x 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 than *MOR-KO* mice ($F_{(9,10.072)}$ = 4.920, p = 0.010. (**b**) The cumulative number of infusions earned during the 2-hour session, averaged over the last three days of FR1, show that not only did WT and *Rescue* mice earn more infusions than *MOR-KO* mice, WT mice also earned these infusions earlier time points within the session than *Rescue* mice (WT vs *MOR-KO*, $F_{(24,264)}$ = 10.367, $p < 0.001$; *Rescue* vs *MOR-KO*, $F_{(24,240)}$ = 2.506, $p < 0.001$; WT vs *Rescue*, $F_{(12,156)}$ = 2.660, $p < 0.01$, Repeated-measures ANOVA). (**c**)The number of inactive lever presses across the 10 days of FR1 did not differ (genotype x day: $F_{(18,17)}$=17.000=1.578, p = 0.176), showing that WT, *MOR-KO* and *Rescue* mice are not significantly different with respect to non-drug-directed responding. Data from WT (n=8) and *MOR-KO* (n=5) and *Rescue* (n=7) mice were analyzed in (a, b) and (c). (**d**) FR3 reinforcement schedule responding demonstrated that the WT mice obtained more remifentanil infusions than *Rescue* mice (WT vs *Rescue*, $F_{(1,7)}$ = 5.672, p = 0.049; Repeated-measures two way ANOVA, n=5, WT; n=4, *Rescue*). (**e**) PR reinforcement schedules demonstrated that the WT mice obtained more infusions than *Rescue* mice before reaching breakpoint (WT vs *Rescue*, $F_{(1,6)}$ = 7.107, p = 0.037, Repeated-measures two way

ANOVA, n=4, WT; n=4, *Rescue*). The data shown and analyzed are the last 3 days at FR3 (**d**) and PR **(e**), when stable responding was achieved). Values are mean ± SEM.

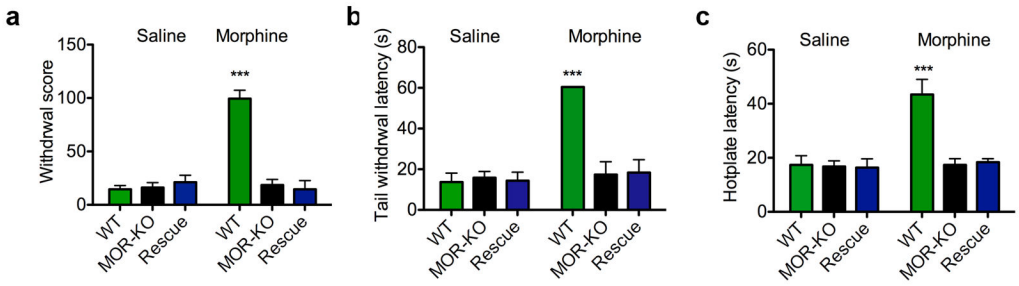

Figure 4.

Pdyn-BAC-driven expression of the MOR in an otherwise *MOR-KO* background did not restore morphine analgesia and naloxone-precipitated withdrawal. (**a**) Naloxone withdrawal, as quantified by the total withdrawal score, is not manifested at all in the *Rescue* mice. Naloxone precipitated significant withdrawal symptom in morphine treated WT mice, but not *Rescue* mice and *MOR-KO* mice ($t_{(14)}$ =10.07, $p < 0.001$, n=8, WT; $t_{(12)} = 0.2598$, p=0.7714, n=7, *MOR-KO*; $t_{(12)} = 0.6430$, p = 0.5811, n=7, *Rescue*; S*tudent's t*-test, two-tailed). Naloxone-precipitated withdrawal symptom is not rescued in *Rescue* mice ($F_{(1,24)}$=0.5187, p = 0.4783, *MOR-KO* vs Rescue, two-way ANOVA). (**b**) Morphine analgesia as measured by tail withdrawal is absent in *Rescue* mice compared to WT controls ($Q_{(1)} = 11.127$, $p = 0.003$ for genotype x treatment interaction, non-parametric two-way ANOVA for ranking; n=8, WT groups; N=7, *Rescue* groups). The deficits of analgesia effect of morphine in *MOR-KO* measured by tail withdraw was not restored in *Rescue* mice ($Q_{(1)} = 0.191$; p=0.666, for genotype x treatment interaction, non-parametric two-way ANOVA for ranking, n=7 for all groups). **(c)** Morphine analgesia as measured by hotplate is absent in *Rescue* mice compared to WT controls ($Q_{(1)} = 8.808$, $p = 0.006$ for genotype x treatment interaction, non-parametric two-way ANOVA for ranking; n=8, WT groups; n=8, *Rescue* groups). The deficits of analgesia effect of morphine in *MOR-KO* measured by tail withdraw was not restored in *Rescue* mice ($Q_{(1)} = 0.261$; $p = 0.613$, for genotype x treatment interaction, non-parametric two-way ANOVA for ranking, n=8 for all groups). Double asterisks indicate $p < 0.01$

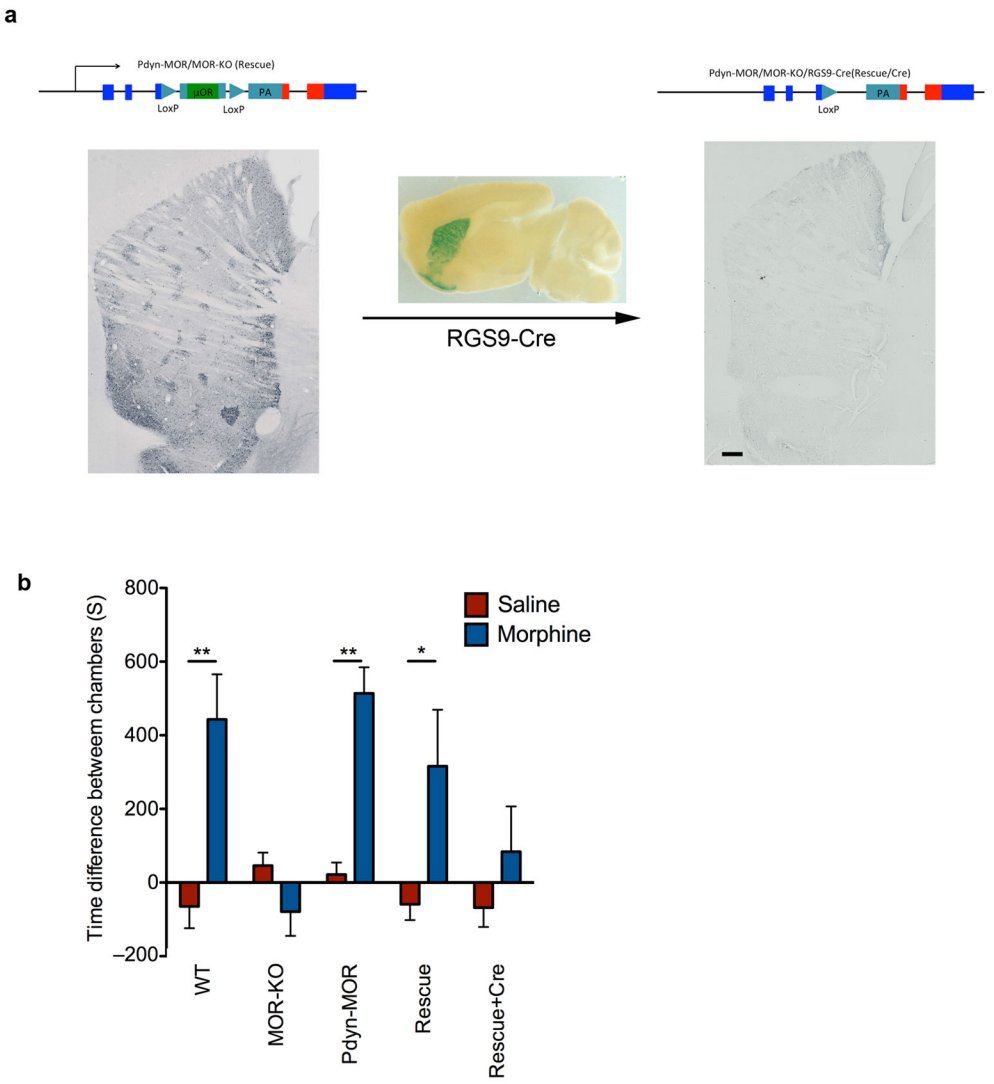

Figure 5.

Rgs9-Cre-mediated deletion of *Pdyn-MOR* transgene in the striatum of *Rescue/Cre* mice abolishes morphine reward effects in the conditioned placement preference assay. (**a**) Representative MOR immunohistochemistry staining of the brain from *Rescue* (left panel) and *Rescue/Cre* (right panel) mice. The specificity of *Rgs9-Cre* to the striatum was confirmed by crossing to a LacZ reporter mouse (middle panel)[40]. (**b**) CPP is restored in *Rescue* mouse littermates, but such rescue effect is eliminated by the genetic removal of MOR transgene in the striatal MSNs in *Rescue/Cre* mice (n=7–9 in each group; $F_{(4, 68)}$ = 5.186, $p < 0.01$ for genotype x significant CPP in WT, *Pdyn-MOR* and Rescue groups but not *MOR-KO* and *Rescue-Cre* when compare to saline treated groups ($p < 0.001$, WT; $p < 0.001$, *Pdyn-MOR; p* < 0.05, *Rescue; p* > 0.05, *MOR-KO; p* > 0.05, *Rescue-Cre*, respectively). The CPP deficits in the *MOR-KO* mice were not restored in the *Rescue-Cre* mice ($F_{(1,27)}$ = 3.351, p = 0.0718 for genotype x treatment interaction, two way ANOVA, n = 7–9 per groups). Values are mean ± SEM. Double asterisks indicate $p < 0.01$, and single asterisk indicates $p < 0.05$.

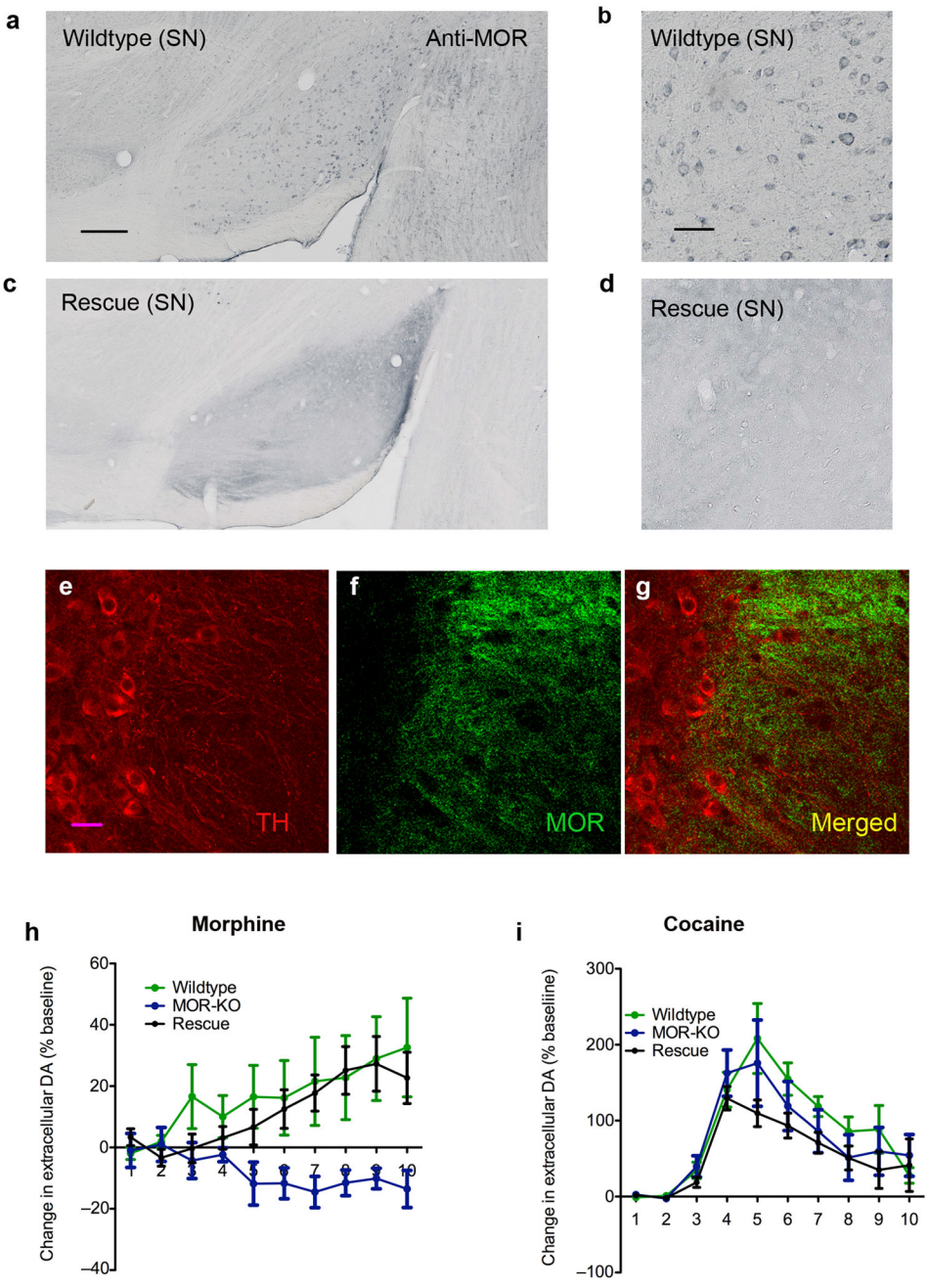

Figure 6.

Selective MOR expression in the axonal terminals of direct-pathway MSNs in the VTA/SNc, but not in the midbrain interneurons, can restore morphine-induced striatal dopamine release in *Rescue* mice. **(a–d)** Representative immunohistochemistry staining of sagittal brain sections shows the expression of the MOR in the substantia nigra of W*T* (**a** and **b**) and *Rescue* mice (**c** and **d**). Scale bar = 500 μm in **a** and **c**; Scale bar = 100 μm in **b** and **d**. (**e**–**g**) A representative double immunofluorescence staining of *Rescue* mouse brain sections shows 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 against tyrosine hydroxylase (TH; in Red). (**h**) Morphine elicited striatal dopamine efflux was abolished in *MOR-KO* mice compared to WT 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). (**i**) WT, *MOR-KO* 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). Values are mean ± SEM.