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**Abstract:** Identifying non-addictive opioid medications is a high priority in medical sciences, but μ-opioid receptors mediate both the analgesic and addictive effects of opioids. We found a significant pharmacodynamic difference between morphine and methadone that is determined entirely by heteromerization of μ-opioid receptors with galanin Gal₁ receptors, rendering a profound decrease in the potency of methadone. This was explained by methadone’s weaker proficiency to activate the dopaminergic system as compared to morphine and predicted a dissociation of therapeutic versus euphoric effects of methadone, which was corroborated by a significantly lower incidence of self-report of “high” in methadone-maintained patients. These results suggest that μ-opioid-Gal₁ receptor heteromers mediate the dopaminergic effects of opioids that may lead to a lower addictive liability of opioids with selective low potency for the μ-opioid-Gal₁ receptor heteromer, exemplified by methadone.
Introduction

The opioid epidemic represents a severe public health crisis (1). Maintenance treatment with the \( \mu \)-opioid receptor (MOR) agonist methadone is the most highly researched and evidence-based treatment for opioid use disorder (OUD) (2). Yet public perception concerning the “substitution” of illicit drugs (such as heroin) with medication (such as methadone) has led to stigmatized views of maintenance treatment, stalling addiction treatment policy advancement and access to medication-based treatments. MOR agonism also offers the most effective treatment for severe pain, making the search for a non-addictive opioid drug the holy grail of pain research (1).

Opioids also represent an efficacious treatment approach for patients with refractory Restless Legs Syndrome (RLS) (3); but again, their use is tempered by the perceived risk of addiction. MOR is a G protein-coupled receptor (GPCR) that is essential for opioid-induced analgesia, but also responsible for adverse effects, including respiratory depression, reduced gastrointestinal motility and euphoria that can lead to addiction (4, 5). One recent strategy to obtain opioids with lower side effects has been developing ligands with functional selectivity or biased agonists. This rests on the assumption that a MOR agonist promoting preferential G protein-mediated versus \( \beta \)-arrestin-mediated signaling could dissociate desired from undesired effects (5, 6). However, recent preclinical and clinical findings demonstrating abuse liability in animals and euphoric effects in humans challenge the utility of these biased agonists (7, 8).

Another potential approach to harnessing opioids’ therapeutic effects involves targeting GPCR heteromers (9, 10). Different orthosteric ligands for a specific GPCR can demonstrate different properties, affinity or efficacy, upon GPCR heteromerization (9). This could provide differential effects of ligands for GPCRs localized in different areas of the central nervous system (CNS) (9). The neuropeptide galanin acts as a modulator of neurotransmission in the
CNS and the peripheral nervous system (11). It is co-expressed with different neurotransmitters and co-released by the major ascending noradrenergic, serotonergic, histaminergic and cholinergic systems (11). Galanin activates three subtypes of GPCRs, Gal1R, Gal2R and Gal3R. Gal1R and Gal2R are broadly expressed in the CNS, including cortical, thalamic and hypothalamic areas, as well as the spinal cord, while Gal3R has a more restricted CNS expression and is predominantly expressed in peripheral tissues (11). Biochemical and behavioral studies also indicated the functional presence of galanin and galanin receptors in dopaminergic areas, including the ventral tegmental area (VTA) and the nucleus accumbens (NAc) which could mediate an antagonistic effect of galanin on opioid reward (12). We recently reported the existence of functionally significant heteromers of MOR and Gal1R in the VTA which could explain these galanin-opioid antagonistic interactions (13).

MOR-Gal1R heteromerization was first demonstrated in mammalian transfected cells using the biophysical techniques bioluminescent resonance energy transfer (BRET) and bimolecular fluorescence complementation (BiFC), where the two putative interacting receptors are separately fused to two biosensors that only interact when in very close proximity. BiFC has allowed to interrogate about the interface of GPCR heteromers by using synthetic peptides with amino acid sequences of the transmembrane domains (TM) possibly involved in the heteromer intermolecular interactions (13, 14). In transfected cells, a synthetic peptide with the amino acid sequence of TM 5 of the MOR (TM5 peptide) selectively destabilized MOR–Gal1R heteromerization and a negative cross talk between MOR and Gal1R ligands, by which galanin could counteract MAPK activation induced by the endogenous MOR agonist endomorphin-1 (EM1) (13). This heteromer-specific interaction could also be identified in the rat VTA, where TM5 specifically counteracted EM1-induced MAPK activation and dopamine release (13).
These results demonstrated that the modulatory effect of galanin was dependent on the integrity of the heteromer and that MOR-Gal1R heteromers represent a main population of MOR in the VTA that modulate dopamine neuronal function (13).

The present study was initially aimed at answering two main questions that arose from our study on MOR-Gal1R heteromers: (i) what are the mechanisms involved in the interactions between galanin and opioid ligands within the MOR-Gal1R heteromer? and (ii) do these interactions also involve morphine and synthetic opioids, such as methadone or fentanyl, differentially? We could demonstrate the existence of a significant pharmacodynamic difference between methadone versus morphine and fentanyl that is determined entirely by MOR-Gal1R heteromerization, rendering a profound decrease in the potency of methadone. This would explain the significantly weaker ability of methadone, as compared to morphine and fentanyl, to activate the dopaminergic system and would predict a dissociation of therapeutic versus euphoric effects of methadone.
Results

Allosterism within the MOR-Gal1R heteromer. We created cell lines with human embryonic kidney cells (FLP-FRT-HEK cells; see Methods) stably transfected with human MOR alone and with both MOR and Gal1R. Two clones, MU cells and MU-GAL cells, were selected according to their functional response to EM1 and the preferential Gal1R agonist M617 (15). We then measured G protein-dependent signaling with dynamic mass redistribution (DMR, see Methods) assay techniques. The MOR antagonist CTOP (13) and the Gal1R/Gal2R antagonist M40 (13, 15) were used to control agonist specificity. As expected, M40 (0.01-1 μM) dose-dependently counteracted DMR induced by M617 (0.1 μM) in MU-GAL cells (Supplemental Figure 1; one-way ANOVA: $F_{3,8} = 324, P<0.001$; Dunnett’s multiple comparisons, versus M617 alone: $P < 0.001$ for all concentrations of M40). CTOP (1 μM) significantly counteracted signaling induced by EM1 (0.1 μM) in MU and MU-GAL cells and, unexpectedly, M40 (1 μM) also significantly counteracted the effect of EM1 (0.1 μM) in MU-GAL, but not in MU cells (Figure 1A; one-way ANOVA: $F_{5,24} = 820, P < 0.001$; Tukey’s multiple comparisons, versus EM1 in the corresponding cell line: $P < 0.001$ for all significant differences). The same results were reproduced with morphine: DMR induced by morphine (0.1 μM) was significantly counteracted by CTOP (1 μM) in MU and MU-GAL cells, and by M40 (1 μM) in MU-GAL cells (Figure 1B; one-way ANOVA: $F_{5,30} = 96.4, P < 0.001$; Tukey’s multiple comparisons, versus EM1 in the corresponding cell line: $P < 0.001$ for all significant differences). In addition, in MU-GAL cells, EM1 (0.1 μM) produced significant MAPK activation (ERK12 phosphorylation) and DAMGO (0.1 μM) induced significant MOR internalization, which were significantly counteracted by both CTOP (1 μM) and M40 (1 μM) (Figures 1C and 1D; one-way ANOVA: $F_{4,54} = 18.16, P <
0.001 and $F_{4,25} = 17.2$, $P < 0.001$, respectively; Tukey’s multiple comparisons, versus control, EM1 or DAMGO: $P < 0.001$ for all significant differences). M617 (0.1 μM) did not induce MOR internalization in MU-GAL cells (Figure 1D).

The effects of M40 indicated the existence of a robust cross-antagonism, by which a Gal1R antagonist counteracts MOR-signaling. This type of cross-antagonism usually implies the existence of allosteric interactions between orthosteric ligands within a GPCR heteromer (9). To demonstrate this possibility, we performed competitive-inhibition experiments with $[^3H]$DAMGO versus DAMGO in the presence and absence of M617 and M40. The two-state dimer model (see Methods) was used to analyze the possible presence of cooperativity of DAMGO, and the presence of allosteric modulations by M617 and M40. The binding of $[^3H]$DAMGO was not cooperative (monophasic competition curves for both cell lines), and the calculated density of $[^3H]$DAMGO binding sites in MU and MU-GAL cells was (in mean ± SEM) 8.7 ± 1.4 (n = 12) and 2.5 ± 0.5 (n = 13) pmol/mg protein, respectively. In MU-GAL cells, both M617 and M40 produced a pronounced decrease of $[^3H]$DAMGO binding (Figures 1E and 1F), due to a significant (seven to nine-fold) reduction in the affinity of DAMGO (increase in $K_{DB1}$ values; see Methods and Supplemental Figure 2; one-way ANOVA: $F_{2,22} = 49.8$, $P < 0.001$; Tukey’s multiple comparisons, versus DAMGO alone: $P < 0.001$ in both cases). On the other hand, both ligands were ineffective in MU cells (no significant change in $K_{DB1}$ values; Supplemental Figure 2; one-way ANOVA: $F_{2,21} = 1.8$, $P < 0.195$). This represents an example of an effective allosteric modulation of the affinity of an orthosteric ligand of one of the protomers in a GPCR heteromer by orthosteric ligands of the other molecularly different GPCR protomer (9). In addition, the complete cross-antagonism by M40 on the signaling and internalization of
different MOR agonists (EM1, morphine and DAMGO) indicates that M40 also exerts a negative allosteric modulation on the efficacy of MOR agonists.

Selective low potency of methadone at the MOR-Gal1R heteromer. Next goal was to explore the possible emergence of different properties of MOR agonists upon MOR-Gal1R heteromerization. BRET experiments can be used to successfully evaluate qualitative differences between different ligands in their ability to induce changes in the interactions between a GPCR and a G-protein subtype (equivalent to ligand-induced G-protein activation) (16). BRET donor Renilla Luciferase 8 (Rluc) was fused to MOR and BRET acceptor yellow fluorescence protein (YFP, Venus variant) was fused to the alpha subunit of the Gi1 protein. These constructs were transiently co-transfected to HEK-293T cells with and without Gal1R, and concentration-response curves of the MOR agonists morphine, EM1, DAMGO, fentanyl and methadone were evaluated in the presence and absence of M40 (Figures 2A to 2E). Gal1R cDNA was transfected in excess of four times the amount of MOR cDNA to insure the highest potential for MORs to form heteromers. In our previous study, MOR-Rluc:GALR1 cDNA transfected ratio was 1:1.6 and M40 did not significantly counteract EM1-induced ERK1/2 phosphorylation (13).

Implementing a 1:4 ratio, both CTOP (1 μM) and M40 (1 μM) significantly counteracted the increase in ERK1/2 phosphorylation induced EM1 (0.1 μM) (Supplemental Figure 3A; one-way ANOVA: $F_{3,16} = 132$, $P < 0.001$; Tukey’s multiple comparisons, versus control or EM1: $P < 0.001$ for all significant differences). In agreement with the predominance of MOR-Gal1R heteromers in the VTA, both CTOP (10 μM) and M40 (10 μM) were also able to significantly antagonize ERK1/2 phosphorylation induced by EM1 (1 μM) in rat VTA slices (Supplemental Figure 3B; one-way ANOVA: $F_{3,16} = 64.2$, $P < 0.001$; Tukey’s multiple comparisons, versus control or EM1: $P < 0.001$ for all significant differences). In the absence of Gal1R, the five
agonists showed a very similar potency and efficacy, properties that were not modified by M40 (Figures 2A to 2E). However, significant differential changes in ligand properties were obtained with co-transfection of Gal1R. Morphine, EM1 and fentanyl showed a significant decrease in efficacy ($E_{\text{max}}$) (Figure 2F; unpaired $t$ test, two tailed: $t_{12} = 4.1$, $P = 0.001$, $t_{16} = 3.8$, $P = 0.002$ and $t_{18} = 5.7$, $P < 0.001$, respectively), while methadone showed a significant and very pronounced decrease in its potency $EC_{50}$ (Figure 2G; Mann Whitney test, two tailed: $U = 2$, $n_1 = n_2 = 6$, $P = 0.002$). The right shift of the concentration-curve of methadone was of two orders of magnitude (Figures 2E to 2G). M40 (1 µM) completely counteracted all opioid agonist effects in cells transfected with Gal1R (Figures 2A to 2E). This effect was not observed in cells not transfected with Gal1R, which substantiated the notion that the pharmacodynamic changes associated with co-transfection of Gal1R depend on heteromerization with MOR. The flattening of the concentration-response curves of the MOR agonists with M40 confirmed its additional ability to exert a significant negative allosteric modulation of their intrinsic efficacy. As an additional control of the dependence of MOR-Gal1R heteromerization, neither the co-expression of Gal2R, which does not heteromerize with MOR (11), or the presence of M40 (1 µM) modified the concentration-response curve of methadone in cells co-transfected with Gal2R (Supplemental Figure 4).

**Weak dopaminergic activation by methadone.** We then used microdialysis techniques in awake freely-moving rats to observe whether results obtained in vitro with morphine and methadone translated in an in vivo preclinical model, with VTA as the target brain structure. As mentioned above, our recent studies indicate that a predominant population of MOR that modulate dopamine cell function in the VTA form heteromers with Gal1R (13). Furthermore, intracranial self-administration experiments indicated that MOR localized in the VTA and
rostromedial tegmental nucleus (tail of the VTA) are involved with the reinforcing effects of opioids (17-19), effect which depends on their ability to stimulate the VTA-NAc dopaminergic system (20-23). Perfusion of morphine (1, 3 and 10 μM) within the VTA through a modified microdialysis probe (that allows the controlled local infusion of large peptides) (13, 24) produced significant and sustained concentration-dependent somato-dendritic dopamine release, with the most effective concentration (10 μM) being significantly counteracted by local infusion of M40 (10 μM; Figure 3A; one-way ANOVA: $F_{3,25} = 6.4, P = 0.002$; Dunnetts’s multiple comparisons, versus morphine 1 μM: $P = 0.046, P < 0.001$ and $P = 0.199$ for morphine 3 μM, morphine 10 μM and morphine 10 μM plus M40, respectively). Methadone, on the other hand, could only produce a significant increase in the extracellular concentration of dopamine in the VTA at a much higher concentration, 300 μM; at 10 or 100 μM, methadone was ineffective, although at 100 μM, co-infusion with the MOR-Gal1R destabilizing peptide TM5 (60 μM), but not the control peptide TM1 (60 μM), did produce a significant dopamine increase (Figure 3B; one-way ANOVA: $F_{4,33} = 6.4, P < 0.001$; Dunnetts’s multiple comparisons, versus methadone 10 μM: $P = 0.872, P = 0.008, P = 0.037$ and $P = 0.998$ for methadone 100 μM, methadone 300 μM and methadone 100 μM plus TM5 or TM1, respectively). Therefore, in complete agreement with the biochemical data, the minimal concentration of methadone required to elicit a significant VTA dopamine release was two orders of magnitude larger than that of morphine (300 μM versus 3 μM, respectively). Furthermore, the selective effect of the MOR-Gal1R heteromer-destabilizing peptide confirmed that the large difference in the potencies of morphine and methadone are determined by the pharmacodynamic changes dependent on MOR-Gal1R heteromerization.
In contrast with the results obtained in the VTA, perfusion of effective intra-VTA concentrations of morphine (10 μM) or methadone (300 μM) in the NAc did not produce local dopamine release (Figure 3C; paired t test, two-tailed: $t_7 = 0.9$, $P = 0.384$, and $t_7 = 1.4$, $P = 0.215$, respectively). Finally, systemic administration of morphine (1 mg/kg, i.p.) produced significant dopamine release both in the VTA and in the NAc (Figure 3D; paired t test, two-tailed: $t_5 = 2.8$, $P = 0.040$, and $t_7 = 2.6$, $P = 0.036$, respectively), while the same dose of methadone (1 mg/kg, i.p.) did not produce significant changes in either brain area (Figure 3D; paired t test, two-tailed: $t_7 = 0.5$, $P = 0.624$, and $t_6 = 1.6$, $P = 0.169$, respectively). Collectively, the results of these microdialysis experiments with morphine and methadone indicate, first, that MOR agonists stimulate the mesolimbic dopaminergic system by activating MOR in the VTA, which induces simultaneous somato-dendritic and terminal release of dopamine—findings that are supported by previous seminal studies (20-23). But more specifically, opioids stimulate the VTA-NAc dopaminergic system by activating VTA MOR-Gal1R heteromers.

Methadone is also pharmacodynamically different from morphine in its greater ability to promote MOR internalization and consequent recycling of non-desensitized MOR (25, 26). This difference may explain methadone’s reduced tolerance relative to morphine (morphine characteristically induces little MOR internalization [25, 26]). But this difference would predict weaker dopaminergic activation by morphine, the opposite of what was observed in the present study. Nonetheless, to discard a possible role of differential internalizing properties on VTA-NAc dopaminergic activation, we evaluated the effect of the intra-VTA infusion of DAMGO and fentanyl on somato-dendritic dopamine release. It should be noted that in terms of chemical structure, morphine, methadone and fentanyl belong to different chemical classes of opioids (27). Similar to methadone and in contrast to morphine, DAMGO and fentanyl promote pronounced
internalization (28). As with morphine and in contrast to methadone, these drugs’ potencies for MOR are not modified upon heteromerization with Gal1R (Figure 2G). Yet both DAMGO and fentanyl produced a significant somato-dendritic dopamine release when perfused in the VTA at a much lower concentration (10 μM) than the minimal effective concentration of methadone (300 μM) (Figure 3E; paired t test, two-tailed: \( t_6 = 3.7, P = 0.009 \), and \( t_9 = 4.1, P = 0.003 \), respectively). In agreement with the well-known lipophilicity-dependent pharmacokinetic profile of fentanyl (29), the low dose of 0.03 mg/kg (i.p.) produced a similar response to that of 1 mg/kg of morphine, with a significant dopamine release both in the VTA and in the NAc (Figure 3F; paired t test, two-tailed: \( t_6 = 4.0, P = 0.007 \), and \( t_6 = 3.8, P = 0.009 \), respectively).

Methadone has substantially higher brain penetrability than morphine (30). It follows that at sufficiently high doses, methadone concentration levels in the VTA should surmount its selective low potency for the MOR-GalR1 heteromer. Indeed, doses higher than 1 mg/kg (i.p.) of methadone have been reported to produce significant dopamine release in the NAc (31, 32). Nonetheless, lower doses should still be sufficient to activate MOR localized in other areas of the CNS, which might mediate its therapeutic effects (see Discussion). In fact, methadone is more potent than morphine at producing analgesic effects in rodents upon acute systemic administration (33, 34). We should then be able to demonstrate that methadone, at the dose of 1 mg/kg (i.p.), although it does not activate the VTA-NAc dopaminergic system, is able to influence MOR localized in areas of the CNS other than the VTA.

To substantiate this hypothesis, a series of in vivo PET imaging experiments in the same rat strain were conducted. The uptake of \(^{18}\text{F}\)2-fluoro-2-deoxy-D-glucose (\(^{18}\text{F}\)FDG) was measured to monitor the whole brain metabolic activity changes induced by methadone and morphine (1 mg/kg for each, i.p.), during the interval with maximal morphine-induced dopamine
release (between 30 and 80 min after drug administration) (Figure 4). A predominant increase in
the baseline metabolic activity in the frontal cortex, dorsal striatum, thalamus, inferior colliculus
and deep cerebellar nuclei was observed when analyzing the average standardized uptake value
ratio (SUVR) calculated using the whole brain as a reference region (Figure 4A). Both morphine
and (more visibly) methadone induced an apparent decrease in the cortical and striatal metabolic
activity (Figure 4A). In agreement with the microdialysis experiments, voxel-based statistical
parametric mapping revealed that morphine, but not methadone, significantly modified the
metabolic activity in the ventral striatum in two separate analyses. First, morphine, but not
methadone administration, significant decreased metabolic activity in a basal forebrain region
that comprises NAc and its projecting areas ventral pallidum, lateral preoptic area, lateral
hypothalamus and lateral septum (35, 36), as compared with baseline values (Figure 4C;
statistical parametric maps of significant decreases of [18F]FDG uptake, according to paired t
tests, P < 0.05, filtered by clusters of more than 100 contiguous voxels). Second, volume of
interest (VOI) analysis of selected regions demonstrated a remarkable differential pattern of
[18F]FDG uptake: morphine significantly decreased metabolic activity in the basal forebrain but
did not significantly modify metabolic activity in the frontal cortex and dorsal striatum (Figure
4D; Wilcoxon matched-pairs test, two-tailed: number of pairs = 7, P = 0.016, P = 0.078 and P =
0.469, respectively). On the other hand, methadone did not significantly modify metabolic
activity in the basal forebrain, but it significantly decreased [18F]FDG uptake both in the frontal
cortex and dorsal striatum (Figure 4D; Wilcoxon matched-pairs test, two-tailed: number of pairs
= 7, P = 0.218, P = 0.016 and P = 0.031, respectively). Although this method could not provide
sufficient resolution to visualize changes in the VTA, the results support our conclusions from
the biochemical and microdialysis experiments regarding the pharmacodynamic-dependent weak
influence of methadone on the VTA-NAc dopaminergic system. In addition, as expected from its higher brain penetrability, methadone was able to produce a more significant effect than morphine in brain areas other than the NAc.

*Lower incidence of euphoric effects with methadone.* The MOR-Gal1R heteromer-dependent weak dopaminergic activation by methadone would predict that methadone should not promote self-administration in animals. Counter to this prediction, several early studies showed that methadone is self-administered in rodents and monkeys (37-39). However, an important, little-acknowledged factor in studies of methadone self-administration is previous exposure to other opioids (37) and methadone, as an efficacious MOR ligand, could promote self-administration by attenuating aversive effects associated with morphine or heroin withdrawal. Remarkably, a more recent study in rats showed that methadone is weakly reinforcing even with a previous history of heroin self-administration (32). Thus, methadone only partially maintained self-administration, and in sharp contrast to equivalent doses of heroin, failed to induce reinstatement of drug-seeking behavior in rodents (32). It is conceivable that in the previous experiments, the doses, route and schedule of administration of methadone favored its capacity to activate the VTA-NAc dopaminergic system. In fact, methadone was administered at doses very similar to those of morphine (37-39). Yet our results from in vivo microdialysis and PET experiments would predict that lower doses of methadone would dissociate the effects of the dopaminergic system from other CNS effects.

An additional extension of our findings is that methadone should be associated with a significantly lower incidence of euphoria in humans, since the subjective feelings of “high” associated with drugs of abuse correlates with the activation of the canonical VTA-NAc dopamine/addiction circuit (40, 41). At a minimum, we would expect a dissociation of the
euphoric effects of methadone from its therapeutic effects. The study of methadone-associated euphoria has received little attention in the literature. Outside of the context of addiction (and thus in opioid-naïve patients), there is little evidence to suggest that methadone produces euphoria (42, 43). And in the context of OUD, the few relevant references we found that explore methadone-associated euphoria either do not take into account previous opioid exposure (44, 45), or do not disambiguate off-label isolated methadone use from off-label methadone taken in combination with other drugs (46, 47). Nevertheless, careful analysis of some of these studies (44, 45) finds support for subjective feelings of euphoria at relatively high doses, dependent on the subject’s level of tolerance, as well as for their dissociation from other effects of methadone (see Discussion).

Anecdotally, patients being treated with daily methadone for OUD rarely report a methadone-associated “high”. To explore this more deeply, we took advantage of data being collected as part of two separate ongoing clinical studies (see table S1 for the characteristics of study participants in clinical trial studies). These unique patient populations were being treated with daily regimens of methadone and include: (i) data from a large registry of patients with RLS, and (ii) patients diagnosed with OUD enrolled in an urban medication-assisted treatment program. The rationale for approaching the question of methadone-associated euphoria in two separate clinical settings was that methadone represents a very effective therapeutic treatment for the two conditions (2, 3), each of which possesses a different etiology, symptomatology and accepted treatment course.

For the first patient population (RLS; Supplemental Table 1), we compared self-report of past-two-week drug treatment-associated euphoria between patients prescribed methadone (n = 122) and patients prescribed other MOR agonist (n = 101; which included morphine, codeine,
oxycodone, hydrocodone and hydromorphone). A significantly lower proportion of RLS patients receiving methadone (0.8%) endorsed subjective feelings of a methadone-associated “high”, compared to patients receiving other MOR agonist treatment (8%; Figure 5A; \( \chi^2 = 6.9 \), two-sided, \( P = 0.008 \)). This finding was mirrored in a separate methadone-prescribed population of patients diagnosed with Opioid Use Disorder (OUD) (n = 30), who despite significant increases in methadone dose (table S1), reported no perceived methadone-associated euphoria at either two weeks or three months post-entry into medication-assisted treatment (Figure 5B, Supplemental Table 1). Importantly, scores on a measure of withdrawal (Subjective Opioid Withdrawal Scale, SOWS) for these same patients were significantly decreased (Figure 5B; Wilcoxon matched-pairs test, two-tailed: number of pairs = 30, \( P = 0.009 \)), indicating a dissociation between the therapeutic and euphoric effects of methadone.

The ongoing clinical trial of patients with OUD afforded another, separate measure of methadone-associated euphoria, in the form of self-report of motivation for seeking first-time off-label use of a comprehensive list of drugs, including methadone (Supplemental Table 1). We found that a significantly lower proportion of patients with OUD endorsed having sought methadone non-medically for the express intent of achieving a “high” (3.8%; n = 26), relative to initial non-medical use of other MOR agonists (35.4%; n = 48, Figure 5C; \( \chi^2 = 9.1 \), two-sided, \( P = 0.002 \)). Indeed, in our study, the largest reason why these patients sought off-label methadone was for “control of withdrawal symptoms.” This finding is in accord with a now-classic literature demonstrating that although opioid-addicted individuals use methadone illicitly, they do so, not for the purposes of achieving a “high” (as they do with other prescription opioids), but for purposes of self-medication (48, 49). Collectively, these data support our hypothesis that
methadone may be less euphorigenic than other opioids due to its weak ability to activate the VTA-NAc dopaminergic system.
Discussion

Morphine and methadone long have been known to display very similar pharmacological properties, both being selective MOR agonists with similar affinities and with high efficacy for the human MOR (50-53). Indeed, methadone has been reported to be a more efficacious MOR ligand than morphine (52, 53). Taking also into account the higher brain penetrability of methadone (30), it could be predicted that methadone is a more efficacious analgesic than morphine. In fact, methadone has been reported to be more potent analgesic and to produce significantly less antinociceptive tolerance than morphine in experimental animals (25, 34). Its reduced tolerance has been attributed to methadone’s superior ability to induce MOR internalization and consequent recycling of non-desensitized MOR (25, 26). Yet, methadone is almost never given as a first-line analgesic in human clinical settings. Here we add new basis for promoting methadone as a prescription opioid of choice, especially for those patients that may be more vulnerable to OUD: a specific pharmacodynamic property that endows methadone with a higher therapeutic index as compared with other prescription opioids, with a higher ratio of therapeutic versus euphoric effects.

This pharmacodynamic property of methadone is determined by heteromerization of MOR with Gal1R in the VTA, which renders methadone significantly less potent at activating the VTA-NAc dopaminergic system than other MOR agonists. Thus, a much higher concentration of methadone was needed to directly activate MOR in the VTA, relative to morphine, fentanyl and DAMGO. In agreement with the local effective dose differences between methadone and morphine, when systemically administered at a relatively high dose, only morphine was effective at activating the VTA-NAc dopaminergic system, which was associated with significant metabolic changes in the NAc and main output areas, as demonstrated with PET
experiments. On the other hand, methadone, at the same dose, produced a significantly different qualitative pattern than morphine and was only effective at producing significant metabolic changes in the cortex and dorsal striatum. This dissociation of the potential dopaminergic effects of methadone from other central effects also implies that MOR do not form heteromers with Gal1R outside of the VTA. In fact, in other areas of the CNS, including the spinal cord, MOR form heteromers with δ-opioid receptors (DOR), which seem to be significantly involved in the antinociceptive effects of opioids (54, 55), including methadone (56).

Due to its high brain penetrability, depending on the dose, route of administration and the subject’s level of tolerance, methadone could still surmount its selective decrease in potency for the MOR-Gal1R heteromer and activate the VTA-NAc dopaminergic system. In fact, previous studies reported mild euphoric symptoms upon oral methadone in methadone-maintained subjects, which were dose- and tolerance-dependent (45). This could also explain the results of the present study, with the presence of a small proportion of methadone-treated patients that reported a “high”. In addition, methadone was also reported to produce euphoria with similar intensity to that obtained by heroin or morphine upon intravenous administration (44). In this study, a slightly lower potency and efficacy of methadone was found at eliciting euphoria and positive symptoms as compared with morphine. This implies that, due to its higher brain penetrability, higher concentrations of methadone in the brain may be necessary to induce euphoria as compared to morphine. In the same study, the miotic effects of morphine and heroin paralleled the observer-rated liking, while methadone produced very long-lasting miosis, which was still present when the subjective effects were markedly attenuated or absent. The authors concluded that “these findings may indicate that there are differences in mechanisms of actions between the various effects of methadone” (44). The present results provide an explanation for
those apparently different mechanisms, by the existence of different pharmacodynamic properties of methadone for MOR-Gal1R heteromers, localized in the VTA, versus MOR localized in other areas of the CNS, including those involved in pupillary constriction (57).

At a more general level, the present study provides compelling evidence for the role of GPCR heteromers as targets for drug development (9, 10). Heteromerization represents a biological mechanism that allows allosteric modulations between endogenous ligands (such as the ability of galanin to modulate the potency and efficacy of endogenous opioids to activate the VTA-NAc dopaminergic system) (11). But it also determines the potential emergence of new properties of exogenous compounds, such as the cross-antagonism of M40, which could be used as a new therapeutic strategy to counteract the dopaminergic effects of opioids. Selective Gal1R agonists, which also counteract MOR signaling in the MOR-Gal1R heteromer (13), could have a more important application in analgesia. This is because Gal1R are also co-localized in the spinal dorsal horn with MOR and have been shown to play a significant role in the well-demonstrated antinociceptive role of galanin (58). In addition, there is clear preclinical evidence for a synergistic spinal antinociceptive effect with opioids (59), which indicates that co-localization of spinal MOR and Gal1R does not imply heteromerization (which would predict an antagonistic interaction between MOR and Gal1R ligands). Therefore, a new clinical strategy for analgesia could be co-administration of Gal1R agonists with opioids, which should allow us to decrease the effective analgesic doses of opioids (synergistic spinal effect) while counteracting their euphorigenic effects (antagonistic VTA effect). Unfortunately, although some attempts have been made, such as with the non-selective galanin receptor agonist galnon (12, 60), the discovery of selective, potent, non-peptidic small molecules targeting Gal1R suitable for clinical use may be a promising therapeutic strategy for the future.
At a more general level as well, but of more relevance from the results obtained in the present preclinical and clinical studies, heteromerization determines potential pharmacodynamic differences between exogenous compounds, such as between morphine and methadone, which endows methadone with a less addictive opioid profile. Taken together, the current findings provide powerful rationale for the development of potentially safer methadone-like compounds that preferentially target MOR not forming heteromers with Gal1R. Additionally, and most importantly from an OUD perspective, these findings argue against the misconception that methadone maintenance treatment is simply the substitution of a licit opioid for an illicit one.
Methods

**Stable cell lines.** The cDNAs for human MOR and Gal1R were obtained from Missouri S&T cDNA Resource Center and were modified N-terminally with in-frame fusion of a signal peptide for enhanced cell surface expression (61) followed by a Flag epitope tag and subcloned to pcDNA™5/FRT plasmid. All constructs were confirmed by sequencing analysis. These plasmids were co-transfected to Flp-In™293 cells with the Flp recombinase expression vector pOG44 (1 µg/9 µg) to obtain FLP-FRT-HEK stable cell lines expressing MOR or Gal1R. Transfection was performed using lipofectamine method following the instructions of the supplier (Invitrogen). Clones resistant to hygromicin B (50 µg/ml) were isolated and a cell line expressing MOR (MU cells) and another expressing Gal1R (GAL cells) selected according to a significant functional response to the MOR agonist EM1 and the Gal1R agonist M617, respectively, measured by dynamic mass redistribution (DMR) assay (see below). A stable cell line expressing both MOR and Gal1R (MU-GAL cells) was then generated using GAL cells. The cDNA of MOR cloned in pcDNA3.1 vector was fused at its N-terminus with a signal peptide followed by a Myc epitope tag and transfected to GAL cells. Clones resistant to Geneticin (400 µg/ml) were isolated and a cell line expressing MOR and Gal1R (MU-GAL cells) was selected according to a significant functional response to both EM1 and M617 (see Results).

**Dynamic mass redistribution (DMR).** A global cell signaling profile or DMR was measured using an EnSpire Multimode Plate Reader (PerkinElmer) (62). This label-free approach uses refractive waveguide grating optical biosensors, integrated into 96-well microplates. Changes in local optical density are measured in a detection zone up to 150 nm above the surface of the sensor. Cellular mass movements induced upon receptor activation are
detected by illuminating the underside of the biosensor with polychromatic light and measured as changes in the wavelength of the reflected monochromatic light. These changes are a function of the refraction index. The magnitude of this wavelength shift (in picometers) is directly proportional to the amount of DMR. All DMR assays were performed using EnSpire Plate Reader 2300 (PerkinElmer). MU cells or MU-GAL cells were directly seeded in EnSpire-LFC 96 well plates and cultured overnight to form a confluent monolayer in the cell culture medium. The cell seeding density was 30,000 cells per well/100 µl. After being washed four times using in Hank’s balanced salt solution (HBSS, Invitrogen), the cells were maintained with 60 µl HBSS and further incubated inside the plate reader for 2 hours. Agonists (EM1, morphine or M617) were added after a 10-minutes baseline reading and DMR was measured for 1 hour. The antagonists (CTOP or M40) were added 10 minutes before the baseline reading. All data reported were calculated based on the amplitudes of DMR signals at 10 minutes after agonist-induced stimulation and background. Statistical analysis was performed with Prism 7 (GraphPad Software).

ERK1/2 phosphorylation in cells in culture. MU-GAL cells or HEK-293T cells (with or without transient transfection with MOR and Gal1R) were maintained in culture with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (Atlanta), 2 mM L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco) and kept in an incubator at 37°C and 5% CO2 with selection antibiotics (hygromycin B and Geneticin). Cells were seeded to 12-well plates (0.25x10⁶/well) in full growth medium. The day before the assay, the medium was changed to DMEM without no FBS for approximately 16 hours before the addition of ligands. Then, cells were incubated or not with the antagonists (CTOP or M40) in HBSS (1 µM final concentration) or the same volume of Hank’s balanced salt solution for 15 minutes and then with EM1 (0.1 µM)
for 7 minutes. Cells were rinsed with ice-cold phosphate-buffered saline and lysed by the addition of 100 μl of ice-cold lysis buffer (provided by the Cell Signaling kit). Determination of pMAPK levels was performed using an enzyme-linked sandwich ELISA kit (Cell Signaling, Danvers) following the protocol suggested by the provider. Statistical analysis was performed with Prism 7.

**Fluorescence activated cell sorting (FACS).** MOR agonist-induced internalization in MU-GAL cells, which express a Myc-tagged MOR, was performed using FACS with FACSCanto™ II system (BD Biosciences). Briefly, MU-GAL cells were harvested and incubated with the MOR agonist DAMGO at room temperature. The antagonists CTOP or M40 were added 10 minutes before the agonist. After drug treatment at room temperature, cells were chilled to 4°C and washed using FACS buffer (DPBS with 1% bovine serum albumin and 0.1% sodium azide). The anti-Myc monoclonal antibody 1:500 (Cell Signaling Technology) was added, following by the anti-mouse antibody conjugated with Alexa Fluor 647 (Invitrogen). After and additional wash, cell surface mean fluorescence of 10,000 live cells was analyzed on a FACSCanto™ II system (BD Biosciences). Statistical analysis was performed with Prism 7.

**Radioligand-binding experiments.** Upon reaching 80-90% confluence, MU and MU-GAL cells were harvested using pre-mixed Earle’s Balanced Salt Solution (EBSS) with 5 mM EDTA (Life Technologies) and centrifuged at 3,000 rpm for 10 minutes at 21°C. The supernatant was removed, and the pellet was resuspended in 10 ml hypotonic lysis buffer (5 mM MgCl₂, 5 mM Tris, pH 7.4 at 4°C) and centrifuged at 14,500 rpm for 30 minutes at 4°C. The pellet was then resuspended in fresh binding buffer. A Bradford protein assay (Bio-Rad, Hercules) was used to determine the protein concentration, and membranes aliquots were frozen in fresh binding buffer at -80°C for future use. The binding buffer was made of 50 mM Tris and 5 mM MgCl₂ at pH 7.4.
On test day, the test compound was diluted into half-log serial dilutions using 30% DMSO vehicle. Membranes were diluted in fresh binding buffer at a stock concentration of 300-500 µg/ml. Radioligand competition experiments were conducted in 96-well plates containing 300 µl fresh binding buffer, 50 µl of diluted test compound, 100 µl of membranes (final amount of 30 µg/well for MOR cells and 30-50 µg/well for MOR-GAL cells, respectively), and 50 µl of radioligand diluted in binding buffer ([³H]DAMGO: 3 nM final concentration). Aliquots of [³H]DAMGO solution were also quantified accurately to determine the added radioactivity. Non-specific binding was determined using 10 µM CTOP, and total binding was determined with 30% DMSO vehicle in the presence or absence of 1 µM M40 or M617. All dilutions were tested in triplicate and the reactions incubated for 60 minutes at room temperature. The reaction was terminated by filtration through Perkin Elmer Uni-Filter-96 GF/B, presoaked for 60 minutes in 0.5% polyethylenimine, using a Brandel 96-Well Plates Harvester Manifold (Brandel Instruments). The filters were washed 3 times with 3 ml (3x1 ml/well) of ice-cold binding buffer and water, 65 µl Perkin Elmer MicroScint20 Scintillation Cocktail was added to each well and filters were counted using a Perkin Elmer MicroBeta Microplate Counter.

**Radioligand-binding data analysis.** Radioligand competition curves were analyzed by nonlinear regression using Grafit curve-fitting software (Erithacus), by fitting the binding data to the mechanistic two-state dimer receptor model, as described in detail elsewhere (63). To calculate the macroscopic equilibrium dissociation constants from competition experiments, the following general equation must be applied:

$$
A_{\text{bound}} = \frac{\left( K_{DA2} A + 2 A^2 + \frac{K_{DA2} A B}{K_{DB}} \right) R_T}{K_{DA1} K_{DA2} + K_{DA2} A + A^2 + \frac{K_{DA2} A B}{K_{DB}} + \frac{K_{DA1} K_{DA2} B}{K_{DB1}} + \frac{K_{DA1} K_{DA2} B^2}{K_{DB1} K_{DB2}}} 
$$
where A and B represent the assayed radioligand and competitor concentration, respectively. $K_{D1}$ and $K_{D2}$ are, respectively, the equilibrium dissociation constants of the first and second binding of A or B to the receptor homodimer and $K_{DAB}$ is the dissociation constant of B binding to a receptor dimer semi-occupied by A and shows the allosteric modulation between A and B.

Because A and B are the same non-cooperative compound, we have considered the experiment as an auto-competition and the general equation has been simplified due to the fact that $K_{DA2} = 4K_{DA1}$, $K_{DB2} = 4K_{DB1}$, $K_{DA1} = K_{DB1}$, $K_{DA2} = K_{DB2}$ and $K_{DAB} = K_{DB2}$, to provide $K_{DB1}$ values as the measure of affinity of DAMGO, in the presence or absence of Gal1R ligands:

$$A_{bound} = \frac{(4K_{DB1}A + 2A^2 + A B) R_T}{4K_{DB1}^2 + 4K_{DB1}A + A^2 + A B + 4K_{DB1}B + B^2}$$

Moreover, for a more accurate fit, when adjusting the MU-GAL cell data in the presence of Gal1R ligands, we have fixed the number of receptors obtained in the absence of these ligands. This is because, despite that the curves in the presence of Gal1R compounds start from below, the number of receptors must be the same each day. Statistical analysis was performed with Prism 7.

**Transient transfections.** The cDNAs for human MOR and Gal1R were modified N-terminally with in-frame fusion of a signal peptide for enhanced cell surface expression (61), followed by a Flag or Myc epitope tag. The cDNA encoding full-length Renilla Luciferase 8 (Rluc) (64) was fused in frame to the C terminus of MOR using the pcDNA3.1 vector. For Bioluminescent Resonance Energy Transfer (BRET) assays, plasmid cDNAs with MOR-Rluc, GALR1 and G protein subunits ($G_{\alpha i}$-Venus, $G_{\beta 1}$ and $G_{\gamma 2}$) were co-transfected into human embryonic kidney 293T (HEK-293T) cells using polyethylenimine (PEI; Sigma) in a 1:2 weight ratio in 10 cm plates. The ratio of MOR-Rluc and Gal1R cDNA plasmid was 1:4 and the total amount of plasmid cDNA was 15.75 µg (see Results). All constructs were confirmed by
sequencing analysis. Cells were maintained in culture with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Atlanta biologicals), 2 mM L-glutamine (Gibco), and 1% penicillin streptomycin (Gibco) and kept in an incubator at 37 °C and 5% CO₂.

**Bioluminescence resonance energy transfer (BRET).** BRET assay was performed to detect ligand-induced changes in the interaction between MOR (fused to Rluc) and the Gαi subunit (fused to Venus, a variant of the yellow fluorescence protein, YFP) in the presence and absence of Gal1R. Experiments were performed approximately 48 hours after transfection. The transient transfected cells were collected, washed, and resuspended in Dulbecco’s phosphate-buffered saline (DPBS) with 0.1% glucose and 200 µM sodium bisulfite. Approximately 200,000 cells/well were distributed in 96-well plates, and 5 µM coelenterazine H (Nanolight Technology) was added. Two minutes after addition of coelenterazine, increasing concentrations of different MOR agonists were added to different wells in the presence and absence of M40 (added 10 minutes before the agonist). Read the plate after agonist addition using a Mithras LB940 microplate reader (Berthold Technologies, Bad Wildbad, Germany). BRET signal from cells was calculated as the ratio of the light emitted by Rluc8 at 485 nm and Venus at 530 nm. BRET change was defined as BRET ratio for the corresponding drug minus BRET ratio in the absence of the drug. Eₘₐₓ and EC₅₀ values are expressed as the basal subtracted BRET change and in the dose-response graphs. Non-linear fitting to obtain Eₘₐₓ and EC₅₀ values and statistical analysis were performed with Prism 7.

**ERK1/2 phosphorylation in VTA slices.** Male Sprague-Dawley rats (2 months old; animal facility of the Faculty of Biology, University of Barcelona) were used. The animals were housed two per cage and kept on a 12-hours dark/light cycle with food and water available ad libitum,
and experiments were performed during the light cycle. All procedures were approved by the Catalan Ethical Committee for Animal Use (CEAA/DMAH 4049 and 5664). Animals were killed by decapitation under 4% isoflurane anesthesia, and brains were rapidly removed, placed in ice-cold oxygenated (O₂/CO₂, 95%/5%) Krebs-HCO₃⁻ buffer (in mM: 124 NaCl, 4 KCl, 1.25 KH₂PO₄, 1.5 MgCl₂, 1.5 CaCl₂, 10 glucose, and 26 NaHCO₃, pH 7.4), and sliced at 4°C using a brain matrix (Zivic Instruments). VTA slices (500 µm thick) were dissected at 4°C in Krebs-HCO₃⁻ buffer; each slice was transferred into a 12-well plate with Corning Netwell inserts containing 2 ml of ice-cold Krebs-HCO₃⁻ buffer. The temperature was raised to 23°C, and after 30 minutes, the medium was replaced by 2 ml of fresh buffer (23°C). Slices were incubated under constant oxygenation (O₂/CO₂, 95%/5%) at 30°C for 4 hours in an Eppendorf Thermomixer (5 PRIME), and the medium was replaced by fresh buffer and incubated for 30 minutes before the addition of any agent. After incubation, the solution was discarded, and slices were frozen on dry ice and stored at -80°C until ERK1/2 phosphorylation was determined. VTA slices were incubated for 20 minutes with medium, CTOP or M40 (10 µM) and treated for 12 minutes with medium or EM1 (1 µM). Then they were lysed by the addition of 300 µl of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaF, 150 mM NaCl, 45 mM β-glycerophosphate, 1% Triton X-100, 20 µM phenyl-arsine oxide, 0.4 mM NaVO₄, and protease inhibitor mixture). Cellular debris was removed by centrifugation at 13,000 X g for 5 minutes at 4°C, and the protein was quantified by the bicinchoninic acid method using BSA dilutions as standard. Phosphorylated proteins were then determined by Western blot, using a mouse anti-phospho-ERK1/2 antibody (1:2500; Sigma) and rabbit anti-total-ERK1/2 antibody (1:40000; Sigma). Bands were visualized by the addition of a mixture of IRDye 800 (anti-mouse) antibody (1:10,000; LI-COR) and IRDye 680 (anti-rabbit) antibody (1:10000; LI-COR) and scanned by an Odyssey infrared scanner (LI-
Cor). Band densities were quantified using the scanner software exported to Excel (Microsoft). The level of phosphorylated proteins was normalized for differences in loading using the total (phosphorylated plus non-phosphorylated) protein band intensities. Statistical analysis was performed with Prism 7.

*In vivo microdialysis.* Male Sprague Dawley rats (3 months-old; Charles River Laboratories) were used. Animals were housed two per cage and kept on a 12-hours dark/light cycle with food and water available ad libitum. Experiments were performed during the light cycle. Rats were deeply anesthetized with 3 ml/kg Equithesin (4.44 g of choral hydrate, 0.972 g of Na pentobarbital, 2.124 g of MgSO₄, 44.4 ml of propylene glycol, 12 ml of ethanol, and distilled H₂O up to 100 ml of the final solution; NIDA Pharmacy) and implanted unilaterally in the ventral tegmental area (coordinates in millimeters from bregma with a 10° angle in the coronal plane: anterior, -5.6; lateral, 2.4; vertical, -9) or in the shell of the NAc (anterior, 1.6; lateral, 0.5; vertical, -5.1) with a regular microdialysis probe or with a specially designed microdialysis probe that allows the direct infusion of large peptides within the sampling area (24). Some animals were implanted simultaneously with two regular probes, one in the VTA and the second one in the contralateral NAc (same coordinates as above). After surgery, rats were allowed to recover in hemispherical CMA-120 cages (CMA Microdialysis) equipped with two-channel overhead fluid swivels (Instech) connected to a sample collector (CMA 470, CMA). Twenty-four hours after implanting the probes, experiments were performed on freely moving rats in the same hemispherical home cages in which they recovered overnight from surgery. An artificial cerebrospinal solution containing (in mM) 144 NaCl, 4.8 KCl, 1.7 CaCl₂, and 1.2 MgCl₂ was pumped through the probe at a constant rate of 1 µl/min. After a washout period of 90 min, dialysate samples were collected at 20 min intervals. For peptide infusion, M40 was
dissolved in ACSF to a final concentration of 10 µM, whereas TM peptides were dissolved in 0.1% DMSO in ACSF to a final concentration of 60 µM. All peptides were injected with a 1 µl syringe (Hamilton) driven by an infusion pump and coupled with silica tubing (73 µm inner diameter; Polymicro) to the microdialysis probe infusion cannula (dead volume, 40 nl), which was primed with ACSF and plugged during implantation. All peptides were delivered at a rate of 16.6 nl/min. Some animals received an i.p. administration of either morphine or methadone (1 mg/kg). At the end of the experiment, rats were given an overdose of Equithesin, the brains were extracted and fixed in formaldehyde, and probe placement was verified using cresyl violet staining. Dopamine content was measured by HPLC coupled with a coulometric detector (5200a Coullochem III; ESA). Statistical analysis was performed with Prism 7.

\[^{18}F\]FDG PET imaging. The rats (same strain, sex and age than those used in the microdialysis experiments) were fasted overnight. The next day, the rats received an i.p. injection of saline (1 ml/kg) and were placed in their home cages. Thirty minutes later, the animals were injected (i.p.) with 11 MBq of 2-deoxy-2-[^{18}F]fluoro-D-glucose (FDG, Cardinal Health) and placed back in their home cages. After 30 minutes, the rats were anesthetized with 1.5% isoflurane, placed on a custom-made bed of a nanoScan small animal PET/CT scanner (Mediso Medical Imaging Systems) and scanned during 20 minutes on a static acquisition protocol. A CT scan was acquired at the end of the PET scan and the rats were returned to their home cage. Two days later the animals were fasted overnight, the next day received an i.p. injection of morphine (1 mg/kg) or methadone (1 mg/kg) and the FDG-PET procedure was conducted as described above. In all cases, the PET data were reconstructed using the nanoScan’s built-in algorithm (Teratomo-3D) correcting for attenuation and radioactive decay with a voxel size of 0.4 mm. Images were co-registered to an MRI template using PMOD.
software (PMOD Technologies) and then analyzed using a Matlab R2016 (Mathworks) and SPM12 (University College). Voxel-based repeated measures Student’s t-test were performed, and the resulting parametric images were filtered for statistically significant ($P < 0.05$) clusters larger than 100 contiguous voxels. Additionally, volumes of interest (VOI) corresponding to the frontal cortex, dorsal striatum and the basal forebrain were drawn using PMOD. The VOI values (kBq/cc) were extracted, standardized uptake value ratios (SUV$_R$) were calculated using the whole brain as a reference region and statistical analysis was performed with Prism 7.

Clinical assessment of methadone-associated “high” and symptoms of craving and withdrawal. For the RLS patient population, patients treated with methadone or other MOR agonists are first confirmed not to have a prior history or diagnosis of substance use disorder and are largely opioid naïve when treatment is commenced. Additionally, the treatment course of RLS involves low-to-moderate doses of methadone, with the median dose being 10 mg/d (p.o.), or other opioids. On the other hand, OUD patients treated with methadone often present with a long history of having been exposed to opioids (and thus likely have tolerance to narcotics), and usual treatment course involves daily treatment of much higher doses of methadone, of around 90 mg/d (also p.o.). A commonality between these two very different clinical studies is that for both, albeit with distinct methods, patient-participants give subjective responses to whether they feel a “high” from the methadone—a report that can be assessed quantitatively. The National RLS Opioid Registry is a multi-site registry of patients who are prescribed opioid drugs for the treatment of symptoms of RLS. 226 patients’ data were included in this report, 122 of whom were prescribed methadone, and the other 104 were prescribed other MOR agonists, which included morphine, codeine, oxycodone, hydrocodone and hydromorphone. As part of the battery of assessments participants were asked, “In an average week, do you ever feel high from
the opioid you are taking; for example, feelings of floating, warmth, intense relaxation, or giddiness?”.

The second population of individuals represented in this study receive daily methadone as part of a medication-assisted treatment regimen for OUD. As participants in a three-month clinical trial designed to test the efficacy of a behavioral intervention, these patients respond to a series of questionnaires that allowed us to assess the potential for methadone to produce feelings of “high.” In the first subset of patients, we measured responses on the Subjective Opioid Withdrawal Scale (SOWS), a 16-item self-report instrument to assess common subjective symptoms of craving and withdrawal (65). Additionally, we assessed participant responses to a question included in The Methadone Side Effects Checklist (66) of the perceived severity of problems of methadone-associated symptoms. Symptom severity is rated on a 5-point scale with a one equaling not at all to a 5 equaling very severe. One of these questions asks patients to identify how severe the symptom of feeling “high” or “loaded” on methadone has been for the participant in the past week.

An additional questionnaire implemented in the context of the OUD clinical trial is a tool that allows for a comprehensive assessment of substance use history and treatment, environmental and psychosocial risk factors, and recent use of 34 commonly-used licit and illicit drugs. Self-report of off-label use and initial motivation to use drugs is assessed individually for all 34 drugs, five of which are MOR agonists: heroin, prescription opioids/narcotics (e.g. Vicodin, OxyContin, Percocet, Oxy, Percs, etc.), methadone, prescription fentanyl (e.g. Actiq, Fentora, Duragesic, etc.) and street fentanyl (distinguished from prescription fentanyl due to differences in mode of access: prescription fentanyl from a medicine cabinet, versus fentanyl from a street dealer).
Statistics. Unpaired $t$ test or the non-parametric Mann Whitney test were used when comparing results of two independently treated groups of subjects (cells, rats or patients). Paired $t$ test or the non-parametric Wilcoxon matched-pairs test were used when comparing results of the same subjects before and after a specific treatment. Mann Whitney and Wilcoxon tests were chosen when data did not show a Gaussian distribution (Shapiro-Wilk normality test: $p > 0.05$). One-way ANOVA followed by Tukey’s or Dunnett’s multiple comparisons were used when comparing more than two independently treated groups of subjects. Dunnett’s test was preferred when comparing statistical differences of each of a number of differently treated groups with a single control group. Chi-squared ($\chi^2$) test was used to analyze categorical clinical data from patients with RLS or OUD. All statistical analysis between two groups of non-categorical data were two-tailed and, for all the analyses, the level of statistical significance was set at $p < 0.05$.

Study Approval

All animals used in the study were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the animal research conducted to perform this study was reviewed and approved by the NIDA Intramural Research Program Animal Care and Use Committee (protocol numbers 18-MTMD-13 and 18-NRB-43). Clinical data were obtained from The National RLS Opioid Registry (a multi-site registry of patients who are prescribed opioid drugs for the treatment of symptoms of RLS) and from patients diagnosed with OUD enrolled in an urban medication-assisted treatment program which were participants in a three-month clinical trial designed to test the efficacy of a behavioral intervention (ClinicalTrials.gov Identifier: NCT02941809).
Author contributions

SF and AMB designed the project and wrote the manuscript with inputs from all authors; EDW, ADG, AEJ, KCR, VC, AHN, JWW, MM, EW, NDV, AMB and SF, designed or supervised the experiments and the analysis of the clinical data; NSC, CQ, JB, AB, XG, WR, SL, EM and VCA performed experiments; NSC, CQ, JB, AB, TOC, JP, ASB, EM, XG, WR, EM, MW and VCA analyzed data.

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References


Figure 1. Gal1R-dependent allosteric modulation of MOR agonists. (A and B) Effect of MOR antagonist CTOP and Gal1R/Gal2R antagonist M40 on DMR induced by MOR agonists EM1 (A) and morphine (B) in MU and MU-Gal1R cells; values are shown as dots and means ± SEM, n = 5-6 triplicates/group; ###: $P < 0.001$, versus EM1; one-way ANOVA with Tukey’s multiple comparisons. (C) Effect of CTOP and M40 on MAPK activation induced by EM1 in MU-GAL cells; values are shown as dots and means ± SEM, n = 6-15 triplicates/group; *** and ###: $P < 0.001$ versus control and versus EM1, respectively; one-way ANOVA with Tukey’s multiple comparisons. (D) Effect of CTOP and M40 on internalization of MOR induced by MOR agonist DAMGO and lack of MOR-induced internalization by Gal1R agonist M617 in MU-GAL cells; values are shown as dots and means ± SEM, n = 6 triplicates/group; *** and ###: $P < 0.001$ versus control and versus DAMGO, respectively; one-way ANOVA with Tukey’s multiple comparisons. (E and F), Representative competitive-inhibition experiments of [$^3$H]DAMGO versus DAMGO in membrane preparations from MU (E) and MU-GAL cells (F) with or without M617 or M40; values are expressed in means ± SEM of triplicates. See Results and Supplemental Figure 2 for total number of experiments and statistical comparisons. Concentrations of agonists and antagonists were always 0.1 μM and 1 μM, respectively.
Figure 2. Gal1R-dependent pharmacodynamic differences of MOR agonists. (A-E)
Representative concentration-response experiments of ligand induced BRET changes in HEK-293T cells transfected with MOR fused to Rluc and the alpha subunit of the Gi1 protein fused to the YFP; values are in means ± SEM of triplicates; the effect of increasing concentrations of MOR agonists morphine (A), EM1 (B), DAMGO (C), fentanyl (D) and methadone (E), are evaluated either without co-transfection with Gal1R, in the absence or presence of Gal1R/Gal2R antagonist M40 (red and green curves respectively), or with co-transfection with Gal1R, with or without M40 (blue and purple curves, respectively). (F and G), Comparison of the $E_{\text{max}}$ and $E_{50}$
values obtained with and without co-transfection with Gal1R. In (F), **: $P < 0.01$ versus transfection with MOR alone; unpaired t test, two tailed. In (G), ** $P < 0.01$ versus transfection with MOR alone; Mann Whitney test, two tailed. $E_{\text{max}}$ and $E_{\text{C50}}$ values are shown as dots and in means ± SEM and in medians with interquartile ranges, respectively; n = 5-10 triplicates/group.
Figure 3. Weaker ability of methadone to stimulate the VTA-NAc dopaminergic system as compared with morphine, fentanyl and DAMGO. Microdialysis experiments in rats; values represent dopamine concentrations in means + SEM of percentage of baseline (average of five samples before MOR agonist administration); lined and white rectangles in x-axis: period of MOR agonist perfusion and M40 infusion, respectively; arrow: time of systemic administration. (A and B) Effect of intra-VTA morphine (1-10 μM) or methadone (10-300 μM) on VTA dopamine; in (A), * and ***: P < 0.05 and P < 0.001 versus morphine 1 μM; in (B), * and **: P < 0.05 and P < 0.01 versus methadone 10 μM; n = 7-8 animals/group; one-way ANOVA with Dunnetts’s multiple comparisons, comparing the average of eight samples after MOR agonist administration. (C) Effect of intra-NAc morphine (10 μM) or methadone (300 μM) on NAc dopamine; n = 8 animals/group; non-significant in both cases; paired t test, two-tailed, comparing the average of eight samples after MOR agonist administration versus baseline values. (D) Effect of systemic administration (1 mg/kg, i.p.) of morphine or methadone on VTA and NAc dopamine in VTA and contralateral NAc; *: P < 0.05, n = 6-7 animals/group; paired t test, two-tailed, comparing the average of five samples after MOR administration versus baseline values. (E) Effect of intra-VTA DAMGO (10 μM) or fentanyl (10 μM) on VTA dopamine; n = 7 and 9 animals/group, respectively; **: P < 0.01; paired t test, two-tailed, comparing the average of eight samples after MOR agonist administration versus baseline values. F) Effect of systemic administration (0.03 mg/kg, i.p.) of fentanyl on VTA and NAc dopamine in VTA and contralateral NAc; **: P < 0.01, n = 7 animals/group; paired t test, two-tailed, comparing the average of eight samples after MOR administration versus baseline values.
Figure 4. Differential ability of morphine and methadone to influence metabolic activity in the basal forebrain. Metabolic mapping using [18F]FDG PET in rats. (A) Timeline of the experiment. (B) [18F]FDG uptake after administration of saline (baseline, n = 14), morphine (1 mg/kg, n = 7) or methadone (1 mg/kg, n = 7); coronal and sagittal images (1.5 mm anterior to bregma and 1.4 mm lateral from midline, respectively) represent the average standardized uptake value ratio (SUVr) calculated using the whole brain as a reference region. (C) Voxel-based parametric mapping analyses revealing a significant decreased metabolic activity from baseline values in a basal forebrain region that includes the NAc and its projecting areas after morphine, but not methadone treatment; statistical parametric maps of significant decreases of [18F]FDG uptake (P < 0.05; paired t test). (D and E) Volume of interest (VOI) analyses of the frontal cortex (FCx), the dorsal striatum (DS) and the basal forebrain region (BF), showing a significant differential pattern of [18F]FDG uptake after administration of morphine (D) or methadone (E); values are shown as dots and in medians with interquartile ranges; *: P < 0.05 versus corresponding baseline, n = 7 animals/group; Wilcoxon matched-pairs test, two-tailed.
Figure 5. Very low report of “high” by methadone-treated subjects. (A) Total number and proportions of patients with RLS reporting “high” upon treatment with methadone or with other MOR agonists (other opioids); **: P < 0.01; significantly different proportion of subjects versus patients treated with methadone; χ², two-sided. (B) Assessment of symptoms of craving and withdrawal (SOWS) and perceived severity of methadone-associated euphoria (“high”). Both measures (SOWS and “high”) were obtained after 14 and 84 days of methadone treatment; values are shown as dots and in medians with interquartile ranges; **: P < 0.01 versus 14 days of treatment; Wilcoxon matched-pairs test, two-tailed (n = 30). (C) Total number and proportions of patients with OUD reporting first-time non-medical use of methadone or other MOR agonists (other opioids) with the express intent of achieving a “high” (and not for alleviating withdrawal symptoms or other purpose; see on-line Methods) **: P < 0.01; significantly different proportion of subjects seeking “high” with other opioids versus with methadone; χ², two-sided.