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Characterization of kappa opioid receptor mediated, dynorphin-stimulated [^{35}S]GTPγS binding in mouse striatum for the evaluation of selective KOR ligands in an endogenous setting

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Abstract

Differential modulation of kappa opioid receptor (KOR) signaling has been a proposed strategy for developing therapies for drug addiction and depression by either activating or blocking this receptor. Hence, there have been significant efforts to generate ligands with diverse pharmacological properties including partial agonists, antagonists, allosteric modulators as well as ligands that selectively activate some pathways while not engaging others (biased agonists). It is becoming increasingly evident that G protein coupled receptor signaling events are context dependent and that what may occur in cell based assays may not be fully indicative of signaling events that occur in the naturally occurring environment. As new ligands are developed, it is important to assess their signaling capacity in relevant endogenous systems in comparison to the performance of endogenous agonists. Since KOR is considered the cognate receptor for dynorphin peptides we have evaluated the selectivity profiles of dynorphin peptides in wild-type (WT), KOR knockout (KOR-KO), and mu opioid receptor knockout (MOR-KO) mice using [^{35}S]GTPγS binding assay in striatal membrane preparations. We find that while the small molecule KOR agonist U69,593, is very selective for KOR, dynorphin peptides promiscuously stimulate G protein signaling in striatum. Furthermore, our studies demonstrate that norBNI and 5′GNTI are highly nonselective antagonists as they maintain full potency and efficacy against dynorphin signaling in the absence of KOR. Characterization of a new KOR antagonist, which may be more selective than NorBNI and 5′GNTI, is presented using this approach.

Keywords

Dynorphin; kappa opioid receptor; [^{35}S]GTPγS binding; mouse striatum; selectivity; drug discovery; KOR antagonist

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1. Introduction

The endogenous opioid system, comprised of kappa-, mu-, delta-receptors, ORL1 (opioid receptor like 1), and opioid peptides, is an important mediator system of emotional and behavioral responses to stress (Herz, 1983; Shippenberg et al., 2007; Snyder and Childers, 1979; Van't Veer and Carlezon, 2013). Upon stress exposure, dynorphin peptides are released in the brain and activate kappa opioid receptors (KOR) to produce analgesia (Dykstra et al., 1987; Millan, 1989), and dysphoria (Land et al., 2008; Pfeiffer et al., 1986). The disruption of the ability to respond to stress properly can lead to elevated risks of developing anxiety-like responses (Van't Veer and Carlezon, 2013), depression-like states (Knoll and Carlezon, 2010; Lutz et al., 2014), and increased drug-seeking behaviors (Bruchas et al., 2010; Lutz and Kieffer, 2013a; Wee and Koob, 2010). Thus, the dynorphin/KOR system has been a drug target for treating pain, addiction and affective disorders.

The endogenous dynorphin family of neuropeptides arises from cleavage of prodynorphin by protein convertase and consists of seven major peptides: big dynorphin (DynA(1-32)), DynorphinA(1-17), DynorphinA(1-8), leumorphin(DynB(1-29)), DynorphinB(1-13), α-neo-endorphin, and β-neo-endorphin as well as several minor peptides (Chavkin, 2013; Schwarzer, 2009). Prodynorphin mRNA is distributed throughout the CNS, with highest levels detected in areas including hippocampus, striatum, and spinal cord (Morris et al., 1986). Prodynorphin-derived peptides have been detected using immunohistochemistry methods and have been found to be widely distributed throughout different brain regions (Hollt et al., 1980; Khachaturian et al., 1982; Zamir et al., 1983) for review see (Schwarzer, 2009). Such a wide distribution of this neuropeptide family is indicative of its potential overall importance in diverse functions of the CNS.

Dynorphins are considered as cognate agonists for KOR for which they display high affinity (~0.1 nM in KOR-transfected CHO cells), however, they also display low nM affinity for MOR and DOR as well as ORL1 expressed in CHO cells as well as in brain (Merg et al., 2006; Quirion and Pert, 1981; Toll et al., 1998; Zhang et al., 1998).

Functionally, dynorphins are promiscuous agonists. While [^{35}S]GTPγS binding assay confirm that dynorphins can potently activate G protein coupling of recombinant KORs expressed in CHO or C6 cells (Alt et al., 1998; Childers et al., 1998; Merg et al., 2006; Remmers et al., 1999) studies have also shown that dynorphins can activate other opioid receptors. For example, DynA(1-17) activates expressed KOR, MOR, delta opioid receptors (DOR), and opioid-like receptors (ORL1) in K^+ current assays using xenopus oocytes (Zhang et al., 1998). Moreover, in rat hippocampal cells, DynA(1-17) and dynorphinB were shown to be agonists at MOR as shown evoke electro-physiological responses in pyramidal neurons (Chavkin et al., 1985) DynA(1-17) stimulation of [^{35}S]GTPγS binding in guinea pig striatum produces a non-saturating linear dose-response curve that exceeds the E_{MAX} observed with KOR selective agonists U50,488 and bremazocine (Childers et al., 1998) suggesting that other receptors may be contributing to the observed maximal responses. To further complicate matters, DynA(1-17) has also been shown to activate bradykinin receptors (Lai et al., 2006).

There is considerable interest in modulating the KOR opioid receptor in the pursuit of pharmaceutical therapies for pain, addiction and depression. Since cellular signaling assays may not always be representative of the endogenous environment, and since the interplay of cellular signaling scaffolds will direct agonist signaling, it is important to assess receptor-mediated signaling in an endogenous setting. Moreover, since current drug development strategies involve the generation of allosteric and functionally selective agonists at the KOR, we felt that it was important to characterize a KOR-dependent signaling cascade in a more endogenous setting in order to validate and evaluate newly developed KOR ligands for potency and efficacy. In the present study, we investigate the pharmacological properties of dynorphin peptides using [^{35}S]GTPγS binding as a functional assay in the mouse striatum, a region that is important for regulating dopaminergic tone and is enriched with presynaptic KOR expression on 5-HT and dopamine neuronal projections (Chefer et al., 2013; Land et al., 2009; Schwarzer, 2009). While G protein coupling assays on membrane preparations from striata may not recapitulate all of the intricacies involved in receptor signaling, it is a useful model to compare ligand properties in a manner that is comparable to cell based screening assays but using receptors expressed in brain. To determine the specificity of the agonists, we utilize both KOR-knockout (KO) and MOR-KO mice, as well as antagonists to each receptor. We confirm that the KOR agonist U69,593 selectively activates KOR. However, we demonstrate that dynorphins are generally nonselective, and they induce considerable activity via MOR. Moreover, we find that the “selective” KOR antagonists, Nor-BNI and 5′GNTI, are potent and fully efficacious for inhibiting dynorphin-stimulated G protein signaling even in the absence of KOR. These findings emphasize the need for more selective antagonists for studying the contribution of KOR to dynorphin-mediated effects in vivo.

2. Methods and Materials

2.1 Animals

All mice, including wild-type C57BL/6J, MOR-KO (strain: B6.129S2-Oprm1tm1Kff/J), KOR-KO (strain: B6.129S2-Oprk1tm1Kff/J), were purchased from The Jackson Laboratory (Bar Harbor, Maine). In all studies, male mice were used between the ages of 4–7 months to allow for habituation prior to procuring striata. Studies were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and with approval by The Scripps Research Institute Animal Care and Use Committee; the Scripps vivarium is fully AAALAC accredited.

2.2 Compounds and Reagents

DynA(1-17), DAMGO, and naloxone were purchased from Tocris Bioscience (Ellisville, MO). DynA(1-13), DynB(1-13), DynB(1-9) obtained from American Peptide Company (Sunnyvale, CA). (+)-(5α,7α,8β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide (U69,593), norBNI, 5′-GNTI were purchased from Sigma (St. Louis, MO), and was prepared in ethanol or DMSO as 10 mM stock. [^{35}S]GTPγS was purchased from PerkinElmer (Waltham, MA). All reagents were then diluted to working concentrations in vehicle containing equal concentrations of DMSO and ethanol not exceeding 0.4 % of either in any assay.

2.3 [^{35}S]GTPγS binding assay in Mouse Striatum

Striata (inclusive of the dorsal and ventral regions) from adult male mice were dissected and homogenized by tissue tearer and glass homogenizer in homogenization buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 1mM DTT), passed through a 26 gauge needle 8 times, centrifuged twice at 20,000 *g* for 30 minutes at 4°C, and resuspended in assay buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA and 20 μM GDP, 1mM DTT). For each reaction, 2.5 μg of membrane protein were incubated in assay buffer containing ~ 0.1 nM of [^{35}S]GTPγS and increasing concentrations of compounds in a total volume of 200 μL for 2 hours at room temperature. The reactions were terminated by separating membrane bound and free [^{35}S]GTPγS through filtration with GF/B filters using a 96-well plate harvester (Brandel Inc., Gaithersburg, MD). Filters were dried overnight and radioactivity was determined with a TopCount NXT HTS microplate scintillation and luminescence counter (PerkinElmer). Mice were used between the ages of 4–7 months; no apparent changes in receptor coupling were noted in mice from different ages within this range.

2.4 Radioligand binding in Mouse Striatum

Striatal tissues from both wide-type and KOR knockout mice was prepared using same procedure as described above for [^{35}S]GTPγS binding. After centrifugation, homogenized tissue was resuspended in binding buffer (50 mM Tris-HCl pH 7.4). For each reaction, 100 μg of membrane protein were incubated in binding buffer containing ~2.2 – 2.5 nM of [^{3}H]U69,593 (43.6 Ci/mmol, PerkinElmer) in a total volume of 200 μL. Total binding was determined by the binding in the presence of vehicle and non-specific binding was determined in the presence of 10 μM cold U69,593. Reactions were incubated at room temperature for 2 hours. GF/B filters were pre-soaked with 0.1% polyethyleneimine to limit nonspecific binding. The reactions were terminated by separating bound and free [^{3}H]U69,593 through filtration with GF/B filters using a Millipore manifold 12-well vacuum harvester (Millipore) with cold 10 mM Tris buffer. Filters were washed 6 times with cold Tris buffer and allowed to dry overnight. Radioactivity was determined by adding 5 ml scintillation fluid and counting using a scintillation counter (Beckman Coulter LS6500).

2.5 Data analysis and Statistics

Fold stimulations by agonists were calculated by dividing raw radioactivity counts in agonist treated condition by the raw counts of vehicle treated condition. Antagonist responses are normalized to the % stimulation achieved with dose of the indicated agonist. Sigmoidal dose response curves were generated using three-parameter non-linear regression analysis in GraphPad Prism 6.01 software (GraphPad, La Jolla, CA). The EC_{50} or IC_{50} values and maximal responses (E_{Max}) of drugs were obtained from the average of each individual experiment following nonlinear regression analyses and are reported as the mean ± standard error of the mean (S.E.M.). All compounds were run in parallel assays in 2–3 replicates per *n* (1 n per mouse). All assays were done for $n \geq 3$ independent experiments.

3. Results

3.1 Dynorphins are potent and efficacious agonists in wild-type mouse striatum

To determine the agonist properties of select dynorphin peptides DynA(1-17), DynA(1-13), DynB(1-13), DynB(1-9), [^{35}S]GTPγS binding assays were performed using C57Bl/6 adult male mouse striatal membrane preparations (Figure 1). We chose these four peptides to compare between A and B types, as well as to represent different peptide lengths. The KOR-selective agonist, U69,593, was run in parallel and is shown for comparison. U69,593 produces a 30% increase in stimulation over vehicle (E_{MAX}) and a potency (EC_{50}) of 0.47 μM (Table 1). Interestingly, the dynorphin peptides stimulate [^{35}S]GTPγS binding in a nearly linear fashion that does not plateau. This poor hyperbolic fit of the curve leads to exaggerated estimations of maximal stimulation which precludes the ability to calculate a reliable E_{MAX} value (Figure 1). In some curves, 100 μM was used to test whether the curves would saturate, yet the linear trend continued (data not shown). Since the dynorphin dose response curves do not reach saturable maximal plateaus, we used the dynorphin concentration of 20 μM as an imposed maximum response to estimate dynorphin potencies (reported as EC_{50} value in table 1). As shown in table 1, the dynorphin peptides produce estimated potencies around 1 μM, with DynB(1-9) below 1 μM. Together, these data suggest that the dynorphin peptides tested here are potent and efficacious agonists for stimulating G protein signaling in the brain, however, their effects greatly exceed the stimulation produced by U69,593.

3.2 Dynorphin peptides activate G protein signaling in KOR-KO and MOR-KO striatal membranes

To evaluate the contribution of KOR in dynorphin-stimulated [^{35}S]GTPγS binding, we assessed G protein activation in striatum from KOR-KO mice purchased from Jackson Labs (Bar Harbor, ME, described by (Simonin et al., 1998). Basal [^{35}S]GTPγS binding in the KOR-KO striatum is ~30% lower compared to that observed in C57Bl6 (WT) striatal membranes tested in parallel (Mean ± S.E.M = 72.6 ± 2.3% in KOR-KO), suggesting that KOR constitutive activity may contribute significantly to the basal tone of G protein signaling observed in striatum (Figure 2A). When compared for the ability to stimulate G protein, U69,593-induced signaling is absent in KOR-KO striatum (Figure 2B). However, even in the absence of KOR, dynorphins still stimulate G protein coupling to nearly the same extent as that observed in WT mice (Figure 2C–F). The maximum responses induced by 20 μM of the dynorphins are only slightly reduced in the KOR-KO mice, and the curves generally maintain a linear trend of stimulation with increasing doses. Again, to estimate potencies, maximum effects were imposed at the stimulation seen at 20 μM and these estimated EC_{50}'s are reported in Table 1 for comparison.

The KOR-KO mice were first reported 15 years ago and were obtained for this study from a commercial vendor (Jackson Labs, Bar Harbor, ME). This KOR-KO mouse strain was shown to lack KOR radioligand [^{3}H]CI-977 binding in total brain homogenates by saturation [^{3}H]CI-977 binding (Simonin et al., 1998). In our hands, genotyping of the mice using the recommended primers from Jackson Labs confirmed the genetic ablation of KOR

(data not shown). Moreover, we also confirmed a lack of KOR expression by assessing radioligand binding using [^{3}H]-U69,593 (Figure 3A).

Since dynorphins are known to also act on MOR, we investigated whether MOR signaling was enhanced in the KOR-KO mice. The selective MOR enkephalin analog agonist DAMGO ([D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin) displays similar potency and maximal response in KOR-KO striatum as in WT tissue, suggesting that MOR signaling in this assay is not altered in absence of KOR (Figure 3b, Table 1).

To gain further insight as to the role of MOR in dynorphin actions in striatum, we examined G protein signaling in mice lacking MOR. In the MOR-KO mouse brain, the KOR and DOR receptor densities have been reported to remain unchanged and KOR-mediated G protein coupling was previously shown to be preserved (Matthes et al., 1996; Park et al., 2000). Compared to the WT striatum membranes, basal G protein signaling is not altered in the MOR-KO mice (Mean ± S.E.M = 92.0 ± 7.5%)(Figure 4A). In the [^{35}S]GTPγS binding assays, U69,593 has an EC_{50} value of 0.32 μM in MOR-KO striatum with the maximal responses reaching 1.25 fold stimulation at E_{MAX} (Figure 4B, Table 1). Dynorphin peptides also stimulate G protein signaling in a nearly linear fashion in the absence of MOR; maximal responses produced by 20 μM dynorphins are slightly lower than that observed in WT striatum (Figure 4C–F, Table 1) which is similar to what is seen when KOR is ablated. These data suggest that dynorphin peptides act at both KOR and MOR in mouse striatum.

3.3 Use of antagonists to assess KOR contribution to dynorphin-mediated signaling in striatum

To further refine the contributions of MOR and KOR to dynorphin-stimulated G protein signaling, classic MOR and KOR antagonists were used. First, a saturating concentration of the putatively selective KOR antagonist, norBNI (10 μM) was applied in the presence of increasing concentrations of agonists in the [^{35}S]GTPγS binding assay. NorBNI fully blocks U69,593 stimulated G protein activation (Figure 5A) at this concentration. However, while it significantly shifts the potencies for dynorphin stimulation, a full blockade could not be not achieved at this high concentration of norBNI. However, these studies do provide some insight as to the range of dynorphin stimulation that may be mostly attributable to KOR stimulation (< 1 μM dynorphin), if NorBNI is acting to selectively antagonize KOR.

3.4 Competitive [^{35}S]GTPγS binding assays using antagonists

To further analyze the opioid receptor components mediating the responses produced by 1 μM dynorphin, we performed competitive [^{35}S]GTPγS binding assays using increasing concentrations of NorBNI as well as other opioid antagonists that are conventionally used as “selective” antagonists (5′-GNTI (KOR); CTOP (MOR); and the non-selective opioid receptor antagonist naloxone) in the presence of 1 μM DynA(1-17) or 1 μM DynB(1-9) in either WT, KOR-KO, or MOR-KO striatum (Figure 6). Since 1 μM dynorphin produces different maximal responses in each genotype), data are presented as the percentage of the average responses of 1 μM dynorphin in each genotype as 100% to allow for comparison between genotypes. Antagonist potencies (IC_{50} values) and maximal percentages of inhibition are summarized in Table 2. As one might expect, we find that in WT tissue, the

conventional KOR antagonists, norBNI, 5′-GNTI, and the nonselective opioid antagonist, naloxone, fully block 1 μM DynA(1-17) stimulation with potencies between 10 nM to 50 nM. The role of MOR in dynorphin-stimulated G protein signaling is supported by studies with CTOP, a selective MOR antagonist, wherein CTOP partially blocks dynorphin-stimulated G protein signaling (53%–58%) in WT striatal membranes (Figure 6 A,B). The observation that CTOP only partially blocks dynorphin-induced G protein signaling while NorBNI and 5′GNTI act as efficaciously as the nonselective antagonist, naloxone, suggesting that these KOR antagonists may not be highly selective for KOR. On the other hand, CTOP may be selective for MOR as only part of the response is blocked, presumably permitting KOR or other receptor contributions to signaling to persist. The selectivity of these responses was further tested in KOR- and MOR-KO mice.

In KOR-KO striatal membranes, 1 μM DynA(1-17)- or 1 μM DynB(1-9) stimulates G protein signaling to nearly the same extent as seen in WT mouse striatum (Table 1, Figure 2); however, G protein signaling is potently and fully antagonized by norBNI and 5′GNTI even in the absence of KOR, suggesting that norBNI and 5′-GNTI are not selective for KOR in vivo (Table 2, Figure 6 A,B). CTOP competitively antagonizes 75–80% of 1 μM dynorphin-induced responses (Figure 6 C,D). This gain in efficacy (~20%) implies that MOR is a major contributor to the dynorphin-stimulated G protein signaling observed in mouse striatal membranes, assuming that CTOP is indeed selective for MOR. This assumption is supported by the complete loss of CTOP efficacy in the MOR-KO striatum (Figure 6 E,F). These data, summarized in Table 2, indicate that both KOR and MOR are major components of the dynorphin-induced responses in mouse striatum and that norBNI and 5′-GNTI are not selective for KOR.

3.5 Evaluation of a novel KOR antagonist in mice striatum

We have previously reported on a novel series of KOR antagonists based on a sulfonamide scaffold (Frankowski et al., 2014). Compound 1 (Figure 7 A) has higher affinity for KOR over DOR and MOR when evaluated in CHO cells overexpressing the human MOR, DOR or KOR (Figure 7B). Compound 1 also displaces ^{3}H-U69,593 radioligand binding in mouse striatum with a calculated affinity of ~3 fold less than NorBNI (Figure 7A) and yet antagonizes 10 μM U69,593-stimulated [^{35}S]GTPγS binding in WT striatum to a similar extent at NorBNI (Compound 1: 15.8 ± 3.9 nM IC_{50}, 99 ± 11% competition; NorBNI: 6.0 ± 1.6 nM IC_{50}, 99 ± 7 % competition; n=3). When tested for its ability to compete against dynorphin B-stimulated G protein signaling, norBNI fully antagonizes G protein signaling in MOR and KOR-KO mice while Compound 1 only partially blocks the effects of 1 μM DynB(1-9) (Figure 4D and E). In MOR-KO striatum, Compound 1 blocked 49 ± 5 % with an IC_{50} of 20.6 ± 5 nM (n= 12) while NorBNI fully antagonized (I_{MAX} 98 ± 5 %) DynB(1-9) with an IC_{50} of 9.1 ± 2.3 nM (n=11). In the absence of KOR, Compound 1 antagonizes DynB(1-9) stimulation to 28 ± 2% with an IC50 of 143 ± 66 nM (n=9) while NorBNI fully antagonized DynB(1-9) (IMAX= 93.0 ± 10.7 %) with a potency of 45.3 ± 10.1 nM (n=6). While there is still an effect of the sulfonamide compound on antagonizing DynB(1-9) stimulation in the absence of KOR, it is apparent that this compound is more selective than the canonical KOR antagonist, NorBNI, in suppressing the activation of KOR in an endogenous setting.

4. Discussion

In our study, we found the selective KOR full agonist U69,593 stimulates [^{35}S]GTPγS binding to membranes of mouse striatum to a saturatable extent, whereas dynorphin peptides stimulate signaling in a linear fashion, suggesting the contributions of additional opioid receptors. Deletion of either KOR or MOR does not abolish dynorphin-mediated responses although U69,593 signaling in KOR-KO and DAMGO signaling in MOR-KO are lost. Interestingly, antagonist competition studies show that norBNI and 5′GNTI, as well as naloxone, robustly inhibit dynorphin- stimulated G protein signaling in the absence of KOR, demonstrating that these antagonists, like dynorphins, are non-selective for KOR.

While it is clear from this study as well as earlier studies (Alt et al., 1998; Childers et al., 1998; Merg et al., 2006; Remmers et al., 1999), that dynorphin peptides are not very selective for KOR, the selectivity of U69,593 for KOR is reinforced by the current results. While most of the dynorphin peptides used in this study produced nonsaturating linear dose response curves in G protein signaling, DynB(1-9) dose-response curves approached a saturatable plateau more so than the others and the maximum stimulation obtained was also the lowest of the peptides. These data suggest that DynB(1-9) may be more selective for KOR in these experimental conditions. DynB(1-9) contains the first 9 amino acid sequence in the N-terminus of DynB(1-13), which is a prevalent endogenous peptide. Pervious structural analysis of the dynorphin sequence suggested that the charged basic residues in the C-terminal domain of dynorphin-A (arginine-7, lysine-11, and lysine-13) and comparable positions in DynB (arginine-7, lysine-10; a-neo: arginine-7, lysine-10) are important for kappa receptor selectivity (Chavkin et al., 1985; Chen et al., 2007; James et al., 1984). In contrast, shorter dynorphins such as DynA(1-8) and β-neo-endorphin, which lack the C-terminal lysines and have lower kappa receptor potencies and selectivity (Chavkin and Goldstein, 1981; James et al., 1984). In the current study, DynB(1-9) emerges as a potential tool for studying KOR activation in mouse striatal membranes as its effects may be more selectively attributed to KOR.

NorBNI is considered the gold-standard antagonist for defining KOR-mediated signaling events in vivo although prior studies have demonstrated its nonselectivity in overexpression opioid receptor binding and signaling assays (Merg et al., 2006; Toll et al., 1998; Zhang et al., 1998). The current study makes use of KOR-KO mice to demonstrate that NorBNI maintains potentcy and efficacy in blocking dynorphin-stimualted G protein signaling in the absence of KOR. Radioligand binding experiments against trititaed agonists for each opioid receptors reveal that norBNI has high binding affinities to kappa, mu, and delta-opioid receptors overexpressed in CHO cells (0.2 nM at KOR vs. [^{3}H]U69,593; 21 nM at MOR vs. [^{3}H]DAMGO, 5.7nM at DOR vs. [^{3}H]DPDPE)(Narita et al., 1999; Toll et al., 1998). The differences of binding affinity between different receptors are even smaller in the brain tissue (0.3 nM to KOR, 8.3 nM to MOR, 6.3 nM to DOR, (Toll et al., 1998). Another commonly used KOR antagonist, 5′-GNTI, seems to be more selective for KOR in CHO cells (0.1 nM to KOR, 100 nM to MOR, 25 nM to DOR, (Jones and Portoghese, 2000). However, in this study, since 5′-GNTI potently inhibits dynorphin stimulation of G protein signaling in KOR-KO striatum, behaving similarly as norBNI, it is very likely that 5′-GNTI also lacks selectivity to opioid receptors in the brain.

Mu and kappa opioid receptors are not the only receptors that signal via dynorphins. In addition to MOR, DOR and KOR, other receptors such as bradykinin receptors (Lai et al., 2006), ORL1(Zhang et al., 1998) as well as non-GPCR targets such as acid-sensing inward rectifying channels (ASICs) (Vick and Askwith, 2015). However, the dynorphins, as well as the antagonists used here have not been systematically tested against every possible GPCR target and therefore, the degree of promiscuity is impossible to gauge. In this study, we chose to focus on MOR and KOR as the primary targets for dynorphins, although we could have extended our studies to characterize another very prominent target, DOR. Dynorphin peptides, as well as NorBNI have significant affinity for DOR (Toll et al., 1998). In a pilot study assessing the effects of naltrindole, the commonly used DOR antagonist, we find that it potently and fully antagonizes 1 μM dynorphin-stimulated G protein signaling to a similar extent as NorBNI in the WT striata (for DynA(1-17): IC_{50} = 20 $_{\pm}$ 7 nM n=2; and for DynB(1-9): 16 ± 9 nM, n=3) (data not shown). Since the DOR-KO mice were not tested here, nor other potential GPCR targeted deletion mice, it is difficult to say if these data demonstrate DOR mediated dynorphin signaling or a lack of naltrindole selectivity.

Interestingly, basal activity in the KOR-KO striatal membranes was consistently and significantly decreased when compared to striatal membranes from C57BL/6 and MOR-KO mice in studies performed in parallel. These observations suggest that KOR may be significantly contributing to a high basal tone of G protein-mediated signaling in vivo. Opioid receptors have been shown to display basal signaling activity in the absence of agonists (Liu and Prather, 2001; Sadee et al., 2005; Wang et al., 2004). For example, the MOR displays a basal signaling activity in brain homogenates that can be suppressed by inverse agonists β-CNA and BNTX (Wang et al., 2004) by less than 10% (which correlates with the data presented in figure). Basal activity of KOR was first revealed in a ligand screening for KOR receptors wherein a peptide ligand behave as an inverse agonist (Becker et al., 1999). Later [^{35}S]-GTPγS binding experiments using HEK cells expressing hKOR also showed that norBNI and 5′-GNTI decrease basal activity level of KOR suggesting a constitutive activation for recombinant KOR receptors. Since the KORs are coupled to inhibitory $G\alpha_{i/o}$ proteins and decrease synaptic transmission, the constitutive activity of KOR may allow a bi-directional regulation at synaptic transmission where decreased KOR activity release inhibition from KOR signaling.

The current study suggests that high concentrations of dynorphin (>1 μM) will activate more than KOR in striatum. However, such high concentrations, are likely biologically relevant. Radio-immunoreactivity assays have detected concentrations of dynorphin A (1-13) as high as ~ 1200 pmol/g in the pituitary, and as low as 1 pmol/g in the cerebellum (Hollt et al., 1980). Other studies report DynB levels at ~ 1100 pmol/g in the substantia nigra in rat brain (Zamir et al., 1983). Moreover, since dynorphins and opioid receptors show overlapping but distinct anatomical distributions in the brain (Lutz and Kieffer, 2013b; Mansour et al., 1994), it is possible that this differential distribution may be a mechanism of fine tune on the regulation by dynorphin/opioid receptor system. Furthermore, the high levels of endogenous dynorphins may contribute to the elevated basal tone of [^{35}S]-GTPγS binding in the striata. However, it is evident that the loss of KOR does little to hamper dynorphin-mediated signaling to G proteins in striatum of mouse. Overall, this study reiterates prior conclusions that dynorphin peptides mediate their effects at more targets than KOR.

KOR “selective” antagonists have been used to define KOR in neurological systems and as inspiring pharmaceutical agents for antidepressants and drug addiction therapeutics development. However, our studies question whether the effects of dynorphin and the “selective antagonists” are exclusively or even primarily due to actions at KOR. There is confidence that KOR does indeed play a significant role in behavioral effects seen in mouse models of depression, aversion and stress responses and substantial evidence supports KOR involvement in NorBNI antagonist actions as studies in KOR-KO mice recapitulate the effects of antagonism of KOR agonists (Jamot et al., 2003; Kieffer and Gaveriaux-Ruff, 2002; Kovacs et al., 2005; McLaughlin et al., 2006; Redila and Chavkin, 2008; (Bruchas et al., 2010; Land et al., 2008; Land et al., 2009; Van’t Veer et al., 2013; Van’t Veer and Carlezon, 2013). Moreover, despite the fact that NorBNI isn’t selective for KOR, doesn’t mean that it does not exert efficacy by preventing KOR responses in vivo. However, it is difficult to use a knockout mouse to test the effects of an antagonist when the target is not there to be stimulated and antagonists are defined by their ability to block an activated receptor. In a study by Bruchas et al., NorBNI was used to induce responses downstream of KOR (acting as an agonist) leading to JNK activation; this effect was lost in the KOR-KO mice (Bruchas et al., 2007). However, this report should serve as a reminder that so-called selective antagonists such as NorBNI cannot be simply seen as an “inert blocker” of only KOR nor can it be exclusively be used to define neurological circuitry mediated by dynorphin peptides and the KOR.

As our pharmacological toolbox grows for different G protein coupled receptors, it should be recognized that GPCRs can be driven by their interacting ligands to assume different conformations allowing them to engage more or less with available signaling scaffolds within a receptor microdomain or “receptosome.” Given the contribution of KOR to basal G protein signaling tone, the performance of an “antagonist” at KOR versus an “inverse agonist” or “partial agonist” may become of interest as there may be more selective ways to harness dynorphin-mediated signaling without blocking the receptor. In conclusion, while it has been previously recognized that dynorphins are not selectively activating KOR, this study helps to realize how much other systems can contribute to the responses. Furthermore, these studies help to characterize an endogenous system wherein the ability to modulate KOR activation by endogenous peptides may be used to assess selectivity of KOR modulators, including agonists, antagonists, partial agonists, inverse agonists, and allosteric modulators for more selectively assessing the contribution of this important receptor in neurobiology.

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Abbreviations

CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2
DAMGO	[D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin

Dyn	dynorphin
DOR	delta opioid receptor
[^{35}S]GTPγS	guanosine 5′-O-(3-[^{35}S]thio)triphosphate
KOR	kappa opioid receptor
MOR	mu opioid receptor
5′-GNTI	5′-guanidinonaltrindole
GPCR	G protein coupled receptor
KO	knock-out
norBNI	Norbinaltorphimine
WT	wild-type

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Highlights

- Dynorphin peptides stimulate [35S]GTPγS binding in KOR- and MOR-KO mouse striata.
- NorBNI and 5′GNTI block dynorphin-induced G protein coupling in KOR-KO striata.
- A novel sulfonamide KOR antagonist is more selective than NorBNI in mouse striatum.

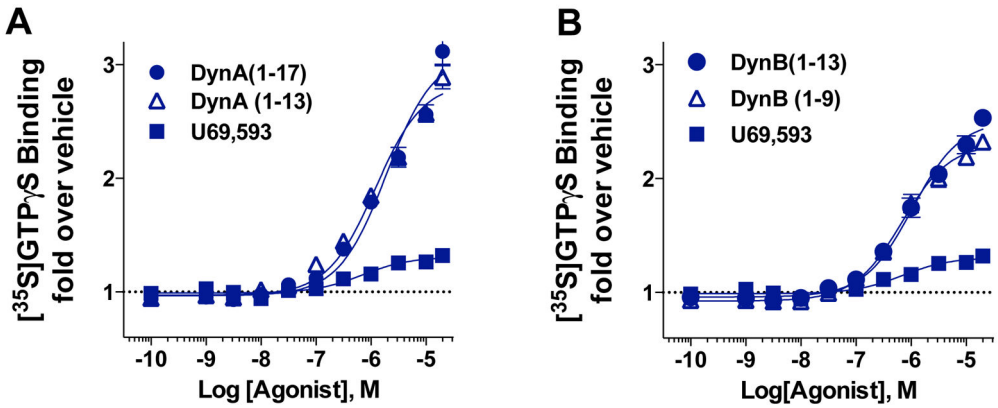


Figure 1. Dynorphin in C57 WT brain

Dynorphin peptides stimulate [^{35}S]GTPγS binding in C57Bl/6 WT mouse striatal membranes in a concentration dependent manner. (A)Dynorphin A peptides [DynA(1-17), DynA(1-13)] and (B) and Dynorphin B [DynB(1-13), DynB(1-9)] are shown in comparison to the stimulation achieved with the selective KOR agonist, U69,593. Data are presented as fold of [^{35}S]GTPγS binding over vehicle-treated conditions. Pharmacological parameters can be found in Table 1 (n = 4–9 individual assays).

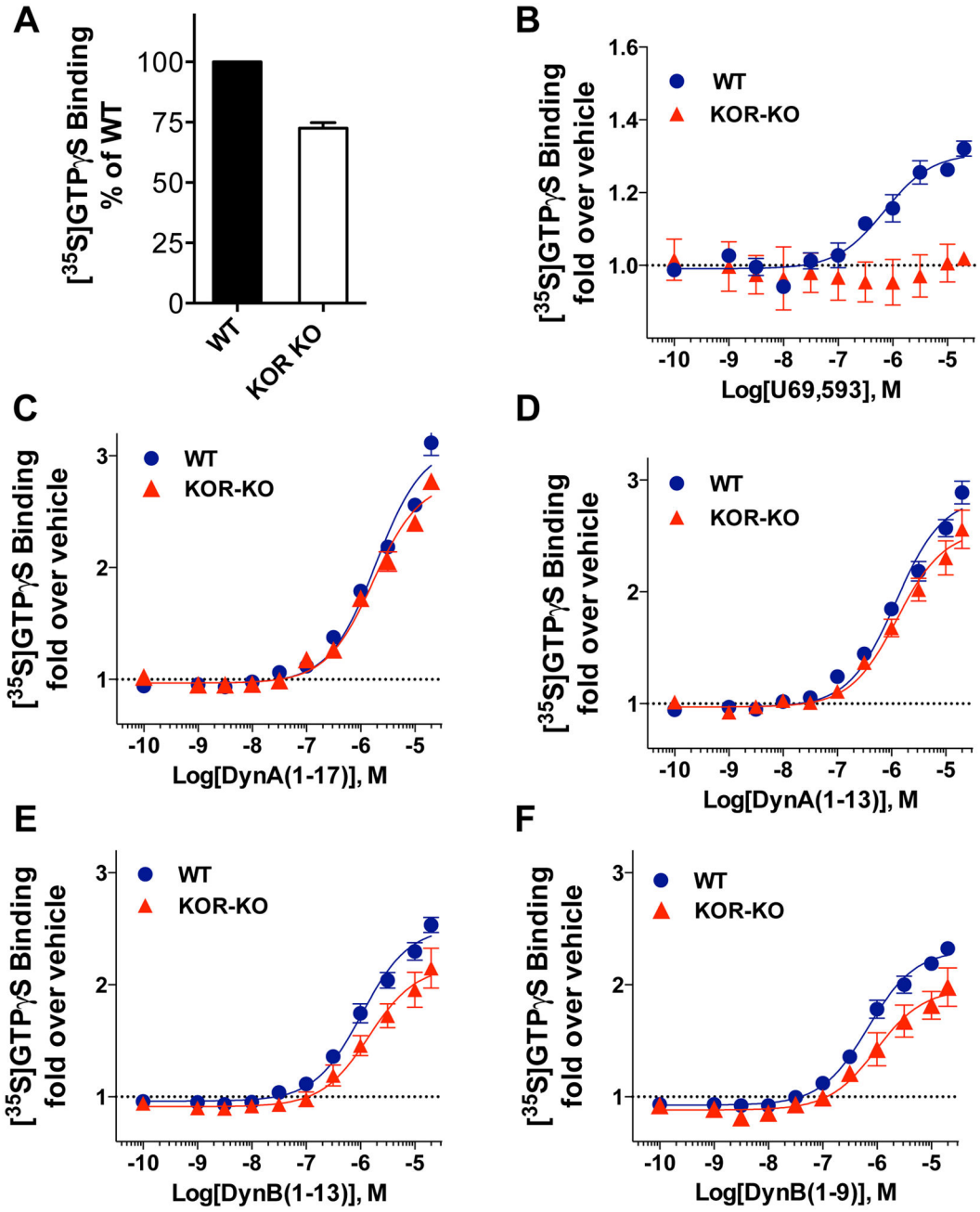


Figure 2. Dynorphin in KOR-KO brain

Dynorphin peptides robustly stimulate [^{35}S]GTPγS binding in the absence of KOR. (A). KOR-KO mouse striatal membranes reveal a decrease in basal [^{35}S]GTPγS binding (Mean ± S.E.M = 72.6 ± 2.3% in KOR-KO versus 100% in WT, $p < 0.0001$, *t*-test). (B). U69,593 no longer stimulates [^{35}S]GTPγS binding in KOR-KO striatal membranes. (C–F) Dynorphin peptides (each tested is indicated in the abscissa) retain significant potency and efficacy in the absence of KOR. The data plotted in 2B is the same as plotted in figure 1 for U69,593 stimulation but on a different scale. Pharmacological parameters can be found in Table 1 (*n* = 4–9 individual assays); the data are plotted at the mean ± SEM.

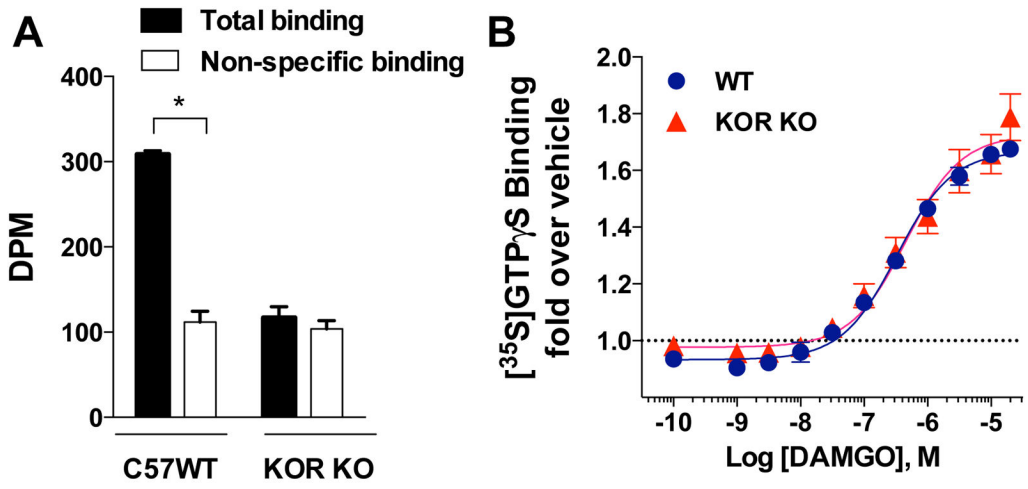


Figure 3. KOR KO

KOR-KO striatal tissue lacks binding sites for U69,593 and MOR activity is maintained. (A) [^{3}H]U69,593 binding assay of both wide-type and KOR knockout mice striatal tissues. Total binding was determined by the binding in the presence of vehicle and non-specific binding was determined in the presence of 10 μM cold U69,593. In the present of 2.2 – 2.5 nM [^{3}H]U69,593, the wild-type striatal tissue show 2.7 ± 0.04 fold binding over the non-specific binding determined by addition of 10 μM U69,593 (* $p < 0.001$, unpaired t test). The calculated receptor density bound to [^{3}H]U69,593 is 19.1 ± 1.4 fmol/mg. In KOR-KO, striatal homogenate lacks [^{3}H]U69,593 binding supporting that the KO mice lack KOR. (B) The MOR-selective enkephalin analog (DAMGO) retains activity KOR-KO as in WT striatal membranes implicating another potential site of action for dynorphin actions in striatum. (n = 3, data are presented as the mean ± SEM).

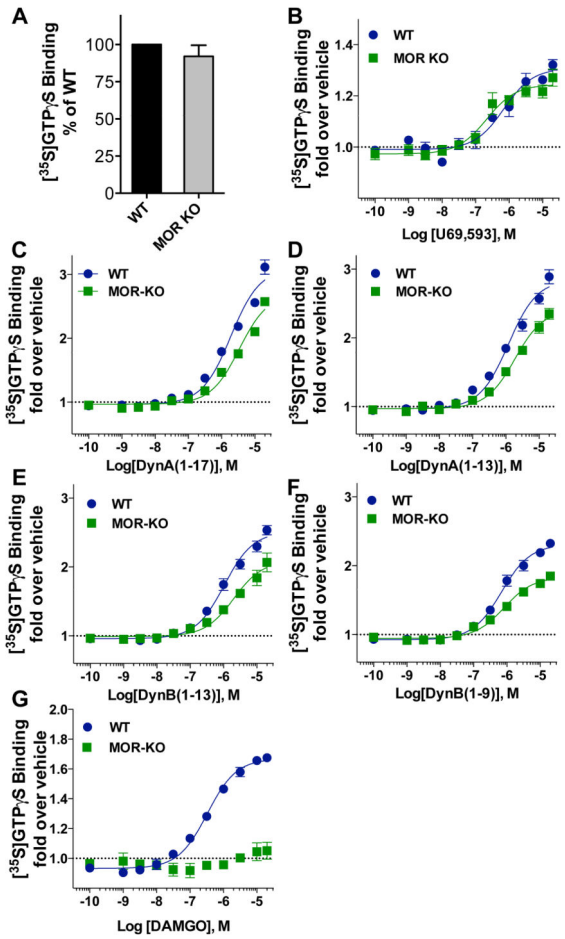


Figure 4. Dynorphins in MOR-KO brain

Dynorphin peptides remain potent and efficacious in MOR-KO mouse striatum. (A) Basal [^{35}S]GTPγS binding is similar between MOR-KO (Mean ± S.E.M = 92 ± 7.5%) and WT C57Bl/6 mice (100%). (B) U69,593 stimulation of [^{35}S]GTPγS binding is unaffected by the deletion of MOR. (C–F) Dynorphin peptides retain significant potency and efficacy in MOR-KO. The U69,593 stiumlation curve in WT for Figure B is the same as in Figure 1. (G) DAMGO stimulation is fully diminished in absence of MOR (curve doesn't converge). Pharmacological parameters can be found in Table 1 (n=4–9 individual assays); the data are plotted at the mean ± SEM.

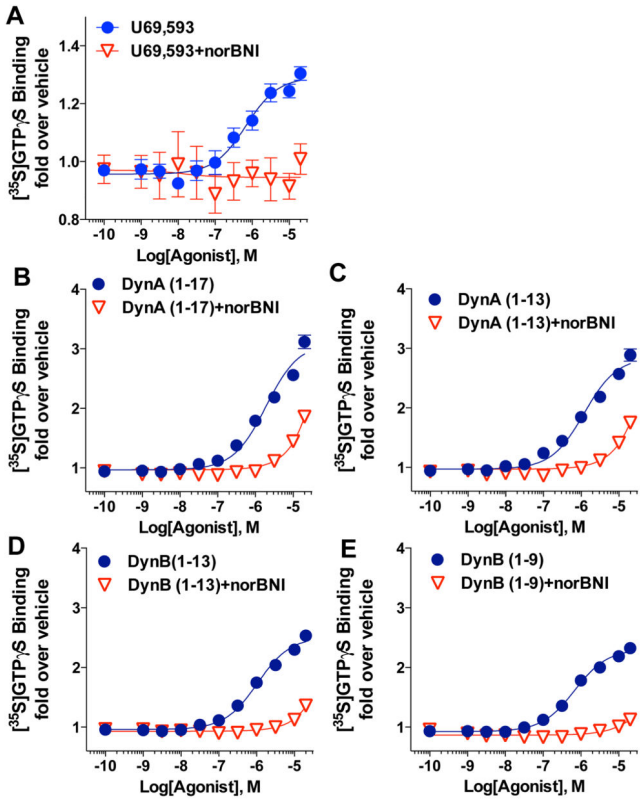


Figure 5. norBNI blocks low concentration of dynorphin signals

Dynorphin or U69,593 stimulation of [^{35}S]GTPγS binding is antagonized by 10 μM NorBNI in striatum. (A). U69,593 stimulation is fully antagonized by 10 μM norBNI. (B–E) (B–C), NorBNI does not fully block dynorphin A peptides at this concentration, (D–E) yet more efficiently competes with dynorphin B peptides. Pharmacological parameters can be found in Table 2 (*n* = 3–9 individual assays); the data are plotted at the mean ± SEM.

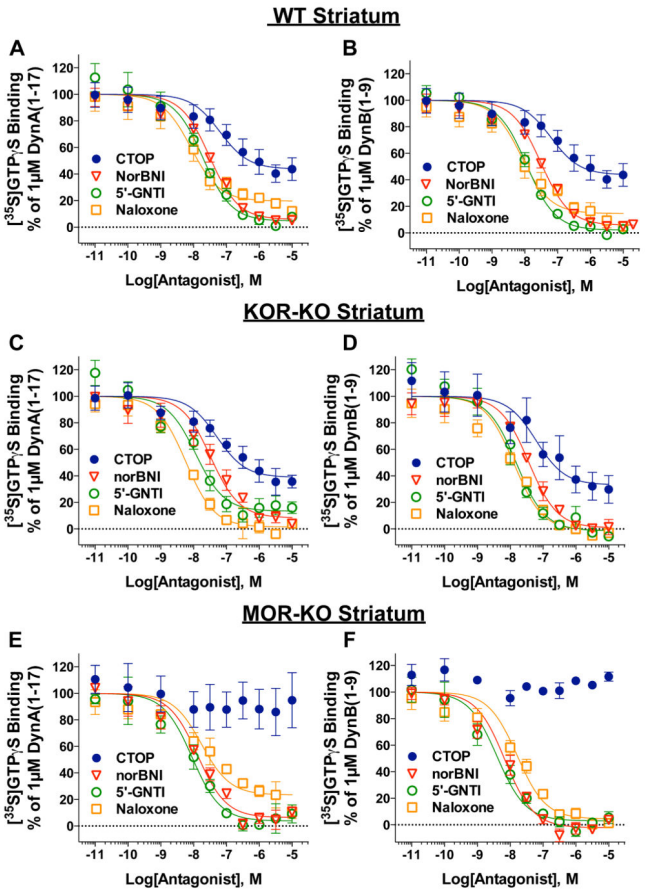
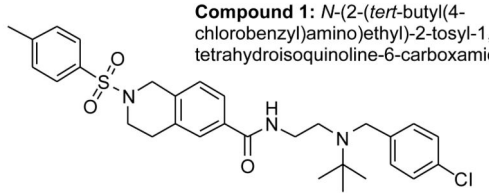


Figure 6. Antagonist competition in C57WT/KOR-KO/MOR-KO

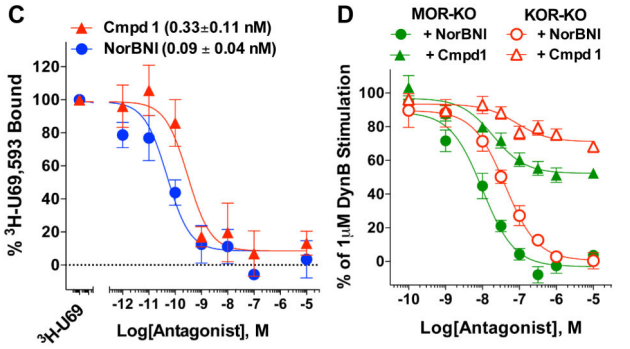
Antagonist competition assays against 1 μM DynA(1-17) or DynB(1-9). Agonist induced responses (1 μM DynA(1-17) or DynB(1-9)) were inhibited with increasing concentrations of indicated antagonists in WT (A,B), KOR-KO (C,D), or MOR-KO (E,F) striatal preparations. All the inhibition curves are reported as the percentage of the response obtained in the absence of antagonist. Antagonist potencies (IC_{50} values) and maximal percentages of inhibition are summarized in Table 2; (*n* = 3–9 individual assays); the data are plotted at the mean ± SEM.

A



B Compound 1 Binding affinities in CHO Cells Expressing Kappa, Mu or Delta Opioid Receptors

KOR	MOR	DOR	MOR/KOR	DOR/KOR
K_i nM	K_i nM	K_i nM	K_i ratio	K_i ratio
0.49 ± 0.043	821 ± 324	>10000	1663	>20276



E Compound 1 inhibition of 1 μM DynB(1-9) stimulation in MOR- or KOR-KO mouse striatum

MOR-KO		KOR-KO	
IC_{50}, nM	I_{MAX} %	IC_{50}, nM	I_{MAX} %
20.6 ± 5	49 ± 5	143 ± 66	28 ± 2

Figure 7. Novel Antagonist competition in KOR-KO/MOR-KO

Evaluation of candidate selective KOR antagonists in striatal membranes prepared from MOR- and KOR-KO mouse striata. A. Compound 1 structure and chemical name (Frankowski et al., 2014). B. Binding affinities and K_i ratios for Compound 1 in CHO-MOR, CHO-KOR and CHO-DOR expressing CHO cell lines. Competition binding assays were performed against 2–4 nM [^{3}H]U69,593 (KOR), 2–3 nM [^{3}H]DAMGO (MOR) or 0.1–0.3 nM [^{3}H]Diprenorphine (DOR). Nonspecific binding was determined by 10 μM NorBNI (KOR), Naloxone (MOR) or Naltrindole (DOR) in the cell lines from this laboratory as previously described (Zhou et al., 2013). C. Compound 1 and NorBNI displace [^{3}H]U69,593 (2–6 nM) binding in WT C57Bl/6 mouse striatal membranes (10 μM NorBNI determining nonspecific binding). Potency values are given in the legend with mean ± SEM (n=5). D. Compound 1 partially blocks DynB(1-9) stimulated [^{35}S]GTPγS in MOR-KO striatal membrane and to a lesser extent in KOR-KO striatal membranes while NorBNI abolishes all DynB(1-9) signaling regardless of genotype (NorBNI data are from Figure 6, shown in this graph for comparison to compound 1). E. Pharmacological parameters are derived from GraphPad Prism, nonlinear regression analysis, *n* = 9–12; data are presented as Mean ± SEM.

Table 1

Pharmacological characterization of agonist-stimulated [^{35}S]GTPγS binding in WT, KOR-KO, and MOR-KO striatal membranes.

	WT		KOR-KO		MOR-KO	
Agonist	**EC_{50} (μM)**	**Fold Stimulation**	**EC_{50} (μM)**	**Fold Stimulation**	**EC_{50} (μM)**	**Fold Stimulation**
DynA(1-17)	2.1 ± 0.2	3.1 ± 0.1	1.5 ± 0.2	2.8 ± 0.1	3.6 ± 0.3	2.6 ± 0.1
DynA(1-13)	1.4 ± 0.2	2.9 ± 0.1	1.4 ± 0.3	2.6 ± 0.2	2.0 ± 0.3	2.4 ± 0.1
DynB(1-13)	1.1 ± 0.1	2.5 ± 0.1	1.9 ± 0.7	2.2 ± 0.2	1.4 ± 0.3	2.1 ± 0.1
DynB(1-9)	0.64 ± 0.04	2.3 ± 0.1	0.89 ± 0.2	2.0 ± 0.2	0.87 ± 0.1	1.9 ± 0.03
U69,593	0.47 ± 0.14	1.32 ± 0.02	NC		0.32 ± 0.09	1.31 ± 0.08
DAMGO	0.34 ± 0.02	1.68 ± 0.02	0.53 ± 0.11	1.71 ± 0.14	NC	

Pharmacological parameters were derived from nonlinear regression of concentration response studies using GraphPad Prism software, 6.0. Since the dynorphin-stiumlated response curves did not always plateau, a maximal effect was confined at 20 μM to impose a maximum response for calculating potencies in GraphPad Prism, 6.0. Maximal responses (Fold Stimulation) at 20 μM agonist concentrations are reported as efficacy values for the dynorphin peptides. For U69,593 and DAMGO, standard nonlinear regression analysis was used to derive E_{MAX} values, reported also as Fold Stimulation in this table. NC indicates not converged. N = 4–9 individual assays; means are presented ± S.E.M. Curves for these data are presented in Figures 1–4.

Table 2

Antagonism of 1 μM DynA(1-17)- or 1 μM DynB(1-9)-stimulated [^{35}S]GTPγS binding in WT, KOR-KO, and MOR-KO striatal membranes.

1 μM DynA(1-17)						
Antagonist	**WT**		**KOR-KO**		**MOR-KO**	
	IC_{50} (nM)	**I_{MAX} %**	**IC_{50} (nM)**	**I_{MAX} %**	**IC_{50} (nM)**	**I_{MAX} %**
NorBNI	46.0 ± 5.2	88 ± 4	66.7 ± 20.4	86 ± 8	19.0 ± 3.0	85 ± 9
5′-GNTI	17.3 ± 2.1	100 ± 9	11.7 ± 4.5	100 ± 13	11.5 ± 0.8	87 ±10
CTOP	115.9 ± 26.5	53 ± 5	86.9 ± 26.4	59 ± 5	NC	
Naloxone	10.7 ± 2.0	75 ± 9	8.1 ± 2.1	93 ± 4	39.5 ± 10.2	65 ± 6

1 μM DynB(1-9)						
Antagonist	**WT**		**KOR-KO**		**MOR-KO**	
	IC_{50} (nM)	**I_{MAX} %**	**IC_{50} (nM)**	**I_{MAX} %**	**IC_{50} (nM)**	**I_{MAX} %**
NorBNI	28.1 ± 2.9	84 ± 4	43.1 ± 10.1	93 ± 11	9.1 ± 2.3	99 ± 5
5′-GNTI	11.4 ± 2.0	96 ± 5	10.9 ± 1.5	120 ± 3	4.7 ± 0.7	93 ± 10
CTOP	263.6 ± 106.9	47 ± 6	106.6 ± 81.4	75 ± 13	NC	
Naloxone	21.9 ± 2.8	84 ± 4	21.3 ± 2.9	91 ± 8	27.1 ± 3.5	85 ± 6

Pharmacological parameters were derived from nonlinear regression of concentration response studies using GraphPad Prism software, 6.0. I_{MAX} percentages reflect % inhibition of 1 μM dynorphin-stimulated G protein signaling within each assay. NC indicates not converged; N ≥ 3 individual assays; means are presented ± S.E.M.