Gβγ That Interacts with Adenylyl Cyclase in Opioid Tolerance Originates from a Gs Protein

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ABSTRACT: We previously demonstrated that chronic morphine induces a change in G protein coupling by the mu opioid receptor (MOR) from Gβ/δ to Gs, concurrent with the instatement of an interaction between Gβγ and adenylyl cyclase types II and IV. These two signaling changes confer excitatory effects on the cell in place of the typical inhibition by opioids and are associated with morphine tolerance and dependence. Both signaling changes and these behavioral manifestations of chronic morphine are attenuated by cotreatment with ultra-low-dose naloxone. In the present work, using striatum from chronic morphine-treated rats, we isotyped the Gβ within Gs and Go heterotrimer that coupled to MOR and compared these to the Gβ isotype of the Gβγ that interacted with adenylyl cyclase II or IV after chronic morphine treatment. Isotyping results show that chronic morphine causes a Gs heterotrimer associated with MOR to release its Gβγ to interact with adenylyl cyclase. These data suggest that the switch to Gs coupling by MOR in response to chronic morphine, which is attenuated by ultra-low-dose opioid antagonist cotreatment, leads to a two-pronged stimulation of adenylyl cyclase utilizing both Gs and Gβγ subunits of the Gs protein novel to this receptor. © 2006 Wiley Periodicals, Inc. J Neurobiol 66: 1302–1310, 2006
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INTRODUCTION

Tolerance to the analgesic effects of opioids and the associated physical dependence that occurs after chronic administration are two major problems of current opioid therapy. The underlying mechanism of opioid tolerance has been postulated to be opioid receptor down-regulation and more recently receptor desensitization, or a decreased efficiency in G protein coupling. While receptor down-regulation is inconsistent and does not parallel opioid tolerance (Yoburn et al., 1993), the desensitization of opioid receptors has been established as a consequence of repeated or prolonged opioid treatment (Sim et al., 1996). Another theory hypothesizes that desensitization resulting in receptor internalization actually protects against tolerance (Finn and Whistler, 2001). An important but underemphasized cellular consequence of chronic opioid treatment is excitatory signaling by opioid receptors in place of the usual inhibitory signaling, possibly as a result of the decreased efficiency of coupling to the native G proteins that is the index of desensitization (Crain and Shen, 1992, 2000; Gintzler and Chakrabarti, 2001; Wang et al., 2005).

Excitatory signaling of opioid receptors was initially observed in an electrophysiological study using dorsal root ganglion cultures (Shen and Crain, 1989) that paralleled a facilitation rather than an inhibition of neuronal function in enteric ganglia preparations (Gintzler et al., 1987). In both cases, excitatory effects were caused by low doses of opioids and also by chronic exposure to higher doses, suggesting a
mechanism of opioid tolerance. The link between opioid tolerance and dependence and the excitatory effects of opioids observed in vitro was strengthened by a seminal report showing that cotreatment with ultra-low-dose opioid antagonists alleviated both the excitatory effects of opioids in vitro and opioid tolerance and dependence in vivo (Crain and Shen, 1995). Ultra-low-dose opioid antagonists were also shown to enhance and prolong the analgesic effects of opioids even acutely (Powell et al., 2002; Shen et al., 2002a,b) and to reverse the paradoxical hyperalgesic effect of low doses of opioids to provide strong analgesia (Crain and Shen, 2001). The effects of ultra-low-dose opioid antagonists have been extended to small clinical studies or clinical trials showing enhanced analgesia (Joshi et al., 1999; Hamman et al., 2004; Chindalore et al., 2005), opioid sparing effects (Gan et al., 1997), profound analgesia in a severely opioid tolerant patient (Cruciani et al., 2003), and, in a recent Phase III clinical trial, reduced physical dependence (Webster et al., 2006).

The precise signaling mechanism underlying the excitatory effects of opiates, as well as the mechanism of action of ultra-low-dose opioid antagonists, has been controversial. One proposal indicates that the excitatory signaling is mediated by a switch in mu opioid receptor (MOR) coupling from the Gi/o proteins that inhibit adenylyl cyclase to Gs, which would instead stimulate this enzyme (Crain and Shen, 1990, 2000; Shen and Crain, 1990). Another proposal suggests that the tolerance-associated excitatory signaling by MOR is mediated instead by Gβγ activation of adenylyl cyclase without a switch in G protein coupling (Wang and Gintzler, 1997; Gintzler and Chakrabarti, 2001). We recently confirmed that a switch in MOR-G protein coupling from Gi or Go to Gs occurs after chronic opioid treatment and that this switch and the associated tolerance and dependence are attenuated by ultra-low-dose naltrexone cotreatment (Wang et al., 2005). This study also noted the instatement of an interaction of Gβγ with adenylyl cyclase types II and IV after chronic morphine that was similarly attenuated by ultra-low-dose naltrexone cotreatment. The fact that Gs coupling to MOR and the Gβγ-adenyl cyclase interaction are both instated by chronic morphine and attenuated by ultra-low-dose naltrexone cotreatment suggests that the newly instated interaction of Gβγ was also a result of the switch to Gs coupling. Nevertheless, the origin of the Gβγ interacting with adenylyl cyclases after chronic morphine treatment was uncertain. The present work therefore aimed to define the origin of this Gβγ as either the Gs protein novel to this receptor or its native G protein by isotyping the Gβ contained in each.

**METHODS**

**Animals and Morphine Tolerance**

Pathogen-free, male 225–250 g Sprague-Dawley rats (Taconic, Germantown, NY) were maintained on a 12 h light/dark cycle with free access to food and water. All animal procedures were in compliance with the National Institutes of Health Guide for Care Use of Laboratory Animals and were approved by the City College of New York Animal Care and Use Committee. Four treatment groups of four rats each received subcutaneous injections twice daily for 7 days of saline (control), morphine (10 mg/kg), the combination of morphine (10 mg/kg) plus NLX (10 ng/kg), or NLX alone (10 ng/kg). Sixteen hours after the last injection, animals were sacrificed by decapitation, and dorsal striatum and thoracic spinal cord regions were harvested on ice immediately.

**DAMGO-Stimulated Coupling of Gβγ to Adenylyl Cyclase II or IV**

Types II and IV adenylyl cyclases are activated by Gβγ that results from the dissociation of G proteins following receptor stimulation. The coimmunoprecipitation procedure described below is an established procedure used to examine the association of GPCRs with G proteins (Friedman et al., 1993; Wang and Friedman, 1999; Jin et al., 2001; Zhang et al., 2001; Zhen et al., 2001; Cai et al., 2002; Wang et al., 2005). In addition, G proteins, adenylyl cyclase, and receptors interact via scaffolding proteins such as lipid rafts and caveolae, suggesting that such protein interactions survive a coimmunoprecipitation procedure (Insel et al., 2005). Further data show that GPCRs form stable signaling complexes with their effectors, including adenylyl cyclase, during receptor activation (Lavine et al., 2002). Because we used Gpp(NH)P with agonist to ensure persistent receptor activation, these signaling complexes should be stable under our experimental conditions.

To assess whether adenylyl cyclase type II or IV couples to Gβγ after stimulation of MOR, 200 μg of synaptic membranes prepared from striatum were incubated with 1 μM DAMGO in the presence of 1 nM Gpp(NH)p for 5 min at 37°C (Friedman and Wang, 1996). The addition of the non-hydrolyzable Gpp(NH)p prevents reassociation of the dissociated Gβγ with Gα-Gpp(NH)p. Membranes were solubilized in immunoprecipitation buffer containing 0.5% digitonin, 0.2% sodium cholate, and 0.5% NP-40 at 4°C with end-over-end rotation, and Gβγ was isolated by immunoprecipitation with an anti-panGβ antibody. The adenylyl cyclase-Gβγ complexes in the anti-Gβ immunoprecipitates were recovered by centrifugation at 4°C and the immunoprecipitates were washed three times with 1 mL phosphate-buffered saline (PBS), pH 7.2, and solubilized by boiling for 5 min in 200 μL of PAGE sample preparation buffer. The levels of adenylyl cyclase subtypes in the anti-Gβ immunoprecipitates were assessed by Western analyses using specific antibodies directed against adenylyl cyclase II or IV.

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In order to determine the source of Gβγ, the subtype of Gβ associated with adenylyl cyclase II and IV was determined by coimmunoprecipitation of Gβγ and adenylyl cyclase II or IV using antibodies directed against adenylyl cyclase II or IV. Two hundred micrograms of synaptosomal membranes prepared from striatum were incubated with 1 μM DAMGO in the presence of 1 nM Gpp(NH)p for 5 min at 37°C (Friedman and Wang, 1996). Membranes were solubilized in immunoprecipitation buffer containing 0.5% digitonin, 0.2% sodium cholate, and 0.5% NP-40 at 4°C with end-over-end rotation, and Gβγ that couples to the specific adenylyl cyclase was isolated by immunoprecipitation with immobilized anti-adenyl cyclase II or IV antibodies. The specific adenylyl cyclase-Gβγ complexes in the immunoprecipitates were recovered by centrifugation at 4°C, washed three times each with 1 mL PBS, and solubilized by boiling for 5 min in 200 μL of PAGE sample preparation buffer. Following centrifugation to remove agarose-protein G-antibody complexes, the levels of Gβ subtypes in the anti-adenyl cyclase immunoprecipitates were assessed by Western analyses using specific antibodies directed against Gβ subclasses.

**Determination of Gβ Subtype in MOR-Coupled Gs versus Go Proteins**

Using striatal tissue from the four different treatment groups, the Gβ subtypes in MOR-coupled Gs versus Go proteins were determined by a double coimmunoprecipitation method. Two hundred micrograms of striatal synaptic membranes were solubilized in immunoprecipitation buffer containing 0.5% digitonin, 0.2% sodium cholate, and 0.5% NP-40 at 4°C with end-over-end rotation. The MOR-G protein complexes in the lysates were first isolated by immunoprecipitation with anti-MOR antibodies. Next, the MOR-Gs and MOR-Go complexes were isolated by passing through immunoaffinity columns coated either with immobilized anti-Go or anti-Go. The MOR-Gs and MOR-Go complexes were then eluted with 190 μL antigen elution buffer (pH 2.8) and neutralized immediately by adding 10 μL of 1.5 M Tris solution (pH 8.8). The neutralized MOR-Gs and MOR-Go complexes were solubilized and denatured by boiling for 5 min after addition of 200 μL 2X PAGE sample preparation buffer. The levels of Gβ subtypes in MOR-associated Gs or Go were determined by Western blotting using specific antibodies directed against Gβ subclasses. The blot was stripped and reprobed with anti-MOR, -Go, or -Go to assess the levels of MOR, Go, and Go immuno precipitated by the respective antibodies.

**Western Blot Analysis**

Solubilized immunoprecipitates (100 μL) derived from coimmunoprecipitation assays were separated by 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were washed with PBS and blocked overnight at 4°C with 10% milk followed by washing with PBS with 0.1% Tween-20 (PBST) and incubation at room temperature for 2 h with antibodies against specific Gα proteins (separately, at 1:1000 dilutions) and anti-MOR antibodies (1:500 dilution). After washing, membranes were incubated for 1 h with antirabbit IgG-HRP (1:5000 dilution) and washed with 0.3% PBST followed by washing with 0.1% PBST. Immunoreactivity was visualized by reacting with ECL reagent (Pierce-ENDOGEN) for exactly 5 min and immediate exposure to x-ray film. Specific bands were quantitated by densitometric scanning (GS-800 calibrated densitometer; Bio-Rad Laboratories).

**Specificity of Antibodies Used in Coimmunoprecipitation**

The specificities of the antibodies for adenylyl cyclase II or IV were demonstrated by Western blots and rigorous peptide blocking procedures using 100 μL of eluates derived from covalently immobilized anti-adenyl cyclase I, II, IV, or V columns (Wang et al., 2005). Specificities for antibodies against Go1, Goα, Goβ, and Goα/II were similarly demonstrated in that publication. Likewise, the specificities of anti-Gβ2 and anti-Gβ3 antibodies were assessed here by Western blots of column eluates derived from immunoaffinity columns with covalently immobilized anti-Gα1, 2, 3, or 4, with and without Gβ2 and Gβ3 peptide blockade. Anti-Gβ2 is weakly cross-reactive with Gβ3 and anti-Gβ3 mildly detects Gβ2 (Fig. 1).

**Statistical Analysis**

All data are presented as mean ± standard error of the mean. Treatment effects were evaluated by two-way ANOVA followed by Newman-Keul’s test for multiple comparisons. Two-tailed Student’s t test was used for post hoc pairwise comparisons. The threshold for significance was p < 0.05.

**RESULTS**

**Ultra-Low-Dose NLX Attenuates Gβγ-Adenylyl Cyclase Interaction**

To test whether Gβγ contributes to chronic morphine-associated adenylyl cyclase super-activation, we examined the direct coupling of Gβγ to adenylyl cyclase types II and IV, subtypes known to be regulated by Gβγ (Tang and Gilman, 1991). Striatal membranes from rats of the four treatment groups (n = 4) were stimulated in vitro for 10 min with DAMGO in the presence of the nonhydrolyzable GTP analogue, Gpp(NH)p, which was added to prevent G protein cycling. The Gβγ coupled to adenylyl cyclase II or IV was communoprecipitated with a pan-Gβ antibody. Western blots of the immunoprecipitates were first probed with an antibody against
adenyl cyclase II and subsequently reprobed with an antibody to adenyl cyclase IV. While coupling between Gβγ and the cyclases was not detectable in vehicle-treated tissues, chronic morphine treatment elicited coupling of Gβγ to adenyl cyclase II and IV in striatum in response to *in vitro* stimulation with DAMGO (Fig. 2). This morphine-induced effect was markedly attenuated by NLX cotreatment, demonstrated by 55.8% and 62.4% reductions in levels of adenyl cyclase II and IV protein, respectively, in Gβ immunoprecipitates (Fig. 2). In tissues obtained from treatment with NLX alone, no detectable association between Gβ and the adenyl cyclases was detected either at baseline or after MOR stimulation. These changes in coupling were not due to treatment-induced changes in expression levels of Gβ or adenyl cyclases (Wang et al., 2005).

**Gβγ Interacting with Adenylyl Cyclase Originates from the Gs Heterotrimer**

We hypothesized that Gβ3 in MOR-coupled Gs and Go are of different subclasses and designed a double-immunoprecipitation strategy to isotype the Gβ in order to identify the source of Gβγ that interacts with adenyl cyclase II and IV. Previously, we have shown that heterotrimeric G proteins can be isolated by an anti-Gα immunopurification method (Friedman and Wang, 1996). Using a two-tiered coimmunopurification method, MOR-coupled heterotrimeric Gs and Go in solubilized striatal tissue from vehicle, morphine, morphine + NLX, and NLX groups were separately isolated with MORs as evidenced by the presence of Gβ subunits together with respective Gα and MOR proteins (Fig. 3). While Gβ2 was found in MOR-Gs complexes, Gβ3 was found in MOR-Go

![Figure 1](image1.png) Gβ2 and Gβ3 antibodies are relatively specific to the targeted Gβ subclass. The specificities of anti-Gβ2 and -Gβ3 antibodies were evaluated using the column eluates derived from immunoaffinity columns of the covalently immobilized anti-Gβ1, -Gβ2, -Gβ3, and -Gβ4 by Western blotting with specific anti-Gβ2 and -Gβ3 antibodies (bottom blots). The blots were stripped and reprobed with the same two antibodies that were preabsorbed for 30 min at 25°C with 25 μg of the alternate antigen peptide (i.e., anti-Gβ2 and -Gβ3 antibodies were preabsorbed respectively with Gβ3 and Gβ2) (middle blots). The blots were stripped once again and reprobed with these two antibodies after they were preabsorbed for 30 min at 25°C with 25 μg of their respective antigen peptides (top blots). Although anti-Gβ2 and -Gβ3 cross-reacted mildly with each other, they did not recognize Gβ1 or Gβ4. The specificities of the anti-Gβ2 and -Gβ3 antibodies were also supported by the demonstration that preadsorption with the respective antigen peptides eliminated the detection of the intended Gβ proteins.

![Figure 2](image2.png) Chronic morphine treatment caused direct association of Gβγ proteins with adenyl cyclase type II and IV, shown by coimmunoprecipitation of Gβ proteins with adenyl cyclase II and IV proteins. This morphine-induced Gβγ-adenyl cyclase II and IV interaction was attenuated by the 10 ng/kg NLX cotreatment. Adenylyl cyclase type II and IV is detected in Western blots of Gβ protein immunoprecipitates derived from striatal membranes that were stimulated with DAMGO for 10 min in the presence of Gpp(NH)p. The blots were stripped and reprobed with an anti-Gβ antibody to demonstrate equivalent Gβ protein levels were precipitated in each lane. The change in adenylyl cyclase levels was realized by densitometric quantitation of bands (previously published in Wang et al., 2005). The blots shown here are the representatives of four separate experiments, each using a single rat from each treatment group.
Despite low-level cross-reactivity between antibodies to Gβ2 and Gβ3 (Fig. 1), these associations appeared to be mutually exclusive. Further, in contrast to strong detection of Gβ2 and Gβ3, negligible amounts of Gβ1 or Gβ4 were noted in MOR-Gs and Go complexes (data not shown).

After confirming that Gβγ2 is found in Gs and Gβγ3 in Go associated with MOR, we next examined which Gβγ associates with adenyl cyclase II and IV after in vitro MOR stimulation in order to reveal the origin of the Gβγ interacting with adenyl cyclase II and IV. The levels of Gβ subtypes in adenyl cyclase immunoprecipitates from striatal membranes from the four treatment groups were assessed. Robust Gβ2 but not Gβ3 was detected in precipitates of either adenyl cyclase II or IV obtained from rats treated with chronic morphine or morphine + NLX but not from vehicle- or NLX-treated rats (Fig. 4). These data support the notion that the Gβ2-containing Gβγ dimer (Gβ2γ) that interacts with adenyl cyclase II and IV in vitro is produced from Goα and MOR complexes. Despite low-level cross-reactivity between antibodies to Gβ2 and Gβ3 (Fig. 1), these associations appeared to be mutually exclusive. Further, in contrast to strong detection of Gβ2 and Gβ3, negligible amounts of Gβ1 or Gβ4 were noted in MOR-Gs and Go complexes (data not shown).

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cyclase in chronic morphine-treated rats originates from Gs but not Go after the coupling switch. In addition, the reduced Gβ2 in adenyl cyclase precipitates in striatal membranes from rats treated with morphine + NLX versus morphine alone is consistent with the suppression of the adenyl cyclase - Gβγ interaction observed after treatment with morphine combined with ultra-low-dose NLX.

**DISCUSSION**

We previously demonstrated that chronic morphine induces a switch in G protein coupling from the native Gi/o proteins to Gs, and that this switch was concurrent with the instatement of an interaction of Gβγ with adenyl cyclase II and IV (Wang et al., 2005). Both chronic morphine-induced changes were attenuated by chronic cotreatment with ultra-low-dose NLX. The similar treatment effects on the G protein switch and on the Gβγ interaction with adenyl cyclase II or IV suggested that this Gβγ was derived from the Gs heterotrimer. The present experiments were designed to clarify the origin of the Gβγ interacting with adenyl cyclase II or IV after chronic morphine treatment.

By isotyping the adenyl cyclase-associated Gβ as well as the Gβ of the MOR-associated Go and Gs proteins, we demonstrated that the Gβ2γ dimer signaling to adenyl cyclase after chronic morphine originates from the Gs heterotrimer that is the result of the chronic morphine-induced G protein coupling.

**Figure 4** Chronic morphine treatment enabled the interaction of the Gβ2γ dimer with adenyl cyclase II and IV (AC II and AC IV, respectively), shown by representative Western blots (A) and the average (±SEM) densitometric quantitations of protein bands from four separate experiments (B). To identify the Gβ subtype associated with AC II and AC IV, striatal lysates (200 μg) from each treatment group were immunoprecipitated with immobilized anti-AC II or anti-AC IV antibodies. The Gβ subtype in the adenyl cyclase immunoprecipitates was determined by Western blotting with specific antibodies to Gβ2 and Gβ3 subclasses. Although the levels of immunoprecipitated AC II and AC IV did not differ between treatment groups (top two panels), the exclusive presence of Gβ2 indicates that the Gβ2γ dimer interacting with AC II and AC IV is derived from the MOR-coupled Gs that emerges with chronic morphine treatment. These morphine-induced Gβ2-adenyl cyclase associations were also attenuated by NLX cotreatment (p < 0.05). Asterisk indicates p < 0.05 in morphine versus vehicle comparisons; plus sign indicates p < 0.05 in morphine + NLX versus morphine comparisons.
switch. We chose striatal tissue for these studies because our previous data showed MORs in striatum of opioid naive animals are coupled exclusively to Go proteins, in contrast to MORs in other areas that utilize both Go and Gi proteins (Wang et al., 2005). Because the isotypes of the Gaβδs contained in the MOR-associated Gs and Go proteins happened to be different, this isotyping approach was successful in revealing the origin of the Gaβγ interacting with adenylyl cyclase II or IV as one G protein and not the other. Hence, the chronic opioid-induced MOR coupling switch to Go also instigates the interaction of Gaβγ with adenylyl cyclase II and IV. The excitatory effect on the cell that occurs during opioid tolerance and dependence therefore appears to be an additive or synergistic effect of a loss of adenylyl cyclase inhibition by Goα/o and a stimulation of adenylyl cyclases by both Go s and its associated Gaβγ.

These data further resolve the controversy over the mechanism of excitatory signaling of opioid receptors after exposure to chronic morphine. Clearly, both Gs coupling and Gaβγ signaling to adenylyl cyclase II or IV contribute to the excitatory effects observed after chronic opioid treatment. The present data confirm the origin of the Gaβγ interacting with adenylyl cyclase II or IV as the Gs heterotrimer that emerges to couple to the MOR after chronic opioid treatment as opposed to the Go protein that normally couples to this receptor in striatum. Additionally, our findings of chronic morphine-induced MOR-Gs coupling were recently replicated using the same communoprecipitation procedure by the group initially proposing a Gaβγ stimulation of adenylyl cyclase without an alteration in the heterotrimeric G protein (Chakrabarti et al., 2005).

What remains unclear, however, is which signaling moiety may be responsible for analgesia or analgesic tolerance versus physical dependence. The analgesic effects of MOR activation have been predominantly attributed to the Gaβγ dimer released from Go/o, which activates G protein-activated inwardly rectifying potassium (GIRK) channels (Ikeda et al., 2000) and inhibits voltage-dependent calcium channels (VDCCs) (Saegusa et al., 2000), thereby suppressing cellular activities by hyperpolarization. Therefore, analgesic tolerance could result simply from the loss of this hyperpolarization when MOR couples to Gs instead of Go/o. However, adenylyl cyclase inhibition may also contribute to opioid analgesia, or its activation may contribute to analgesic tolerance, because over-expression of adenylyl cyclase type 7 in the CNS of mice led to more rapid tolerance to morphine (Yoshimura et al., 2000). Wang and Gintzler (1997) also suggest that adenylyl cyclase activation mediates analgesic tolerance and tolerance-associated hyperalgesia. Nevertheless, the superactivation of adenylyl cyclase after chronic opioid administration is more often viewed as a hallmark of opioid dependence than as a mediator of tolerance (Nestler, 2001).

Whether such chronic opioid-induced alterations at the MOR may also contribute to the addictive potential of opioids is another point to consider. Ultra-low-dose naltrexone cotreatment blocked the acute rewarding effects of analgesic doses of morphine or oxycodone in the conditioned place preference paradigm (Olmstead and Burns, 2005) and reduced the rewarding potency of the opiate and the subsequent vulnerability to relapse in the self-administration and reinstatement model of addiction (Leri and Burns, 2005). These findings suggest that excitatory signaling of opioid receptors may contribute to or augment the acute rewarding effects of opioids as well as their longer-term addictive potential.

In summary, we have successfully utilized an isotyping approach to identify the origin of the MOR-associated Gaβγ that signals to adenylyl cyclase II or IV after chronic opioid administration as a novel Gs protein but not the Go protein that normally couples to MOR in striatum. These results suggest that the stimulation of adenylyl cyclase by Gaβγ previously hypothesized to mediate opioid tolerance (Gintzler and Chakrabarti, 2001) is a direct result of the switch to Gs coupling by MOR, and that both Go and Gaβγ proteins of this Gs heterotrimer contribute to the excitatory effects of opioids after chronic administration. Moreover, there are multiple signaling consequences of the switch to Gs coupling by MORs chronically exposed to opioids, and each may contribute differently to the various behavioral effects of long-term opioid administration such as analgesic tolerance, physical dependence, and the possibility of addiction. This notion may explain the multiple beneficial effects of ultra-low-dose opioid antagonist cotreatment that have been shown to restore the normal G protein coupling profile of MOR (Wang et al., 2005).

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