

Mu-Opioid Receptor Coupling to $G\alpha_o$ Plays an Important Role in Opioid Antinociception

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Opioid analgesics elicit their effects *via* activation of the mu-opioid receptor (MOR), a G protein-coupled receptor known to interact with $G\alpha_{i/o}$ -type G proteins. Work *in vitro* has suggested that MOR couples preferentially to the abundant brain $G\alpha_{i/o}$ isoform, $G\alpha_o$. However, studies *in vivo* evaluating morphine-mediated antinociception have not supported these findings. The aim of the present work was to evaluate the contribution of $G\alpha_o$ to MOR-dependent signaling by measuring both antinociceptive and biochemical endpoints in a $G\alpha_o$ null transgenic mouse strain. Male wild-type and $G\alpha_o$ heterozygous null ($G\alpha_o +/−$) mice were tested for opioid antinociception in the hot plate test or the warm-water tail withdrawal test as measures of supraspinal or spinal antinociception, respectively. Reduction in $G\alpha_o$ levels attenuated the supraspinal antinociception produced by morphine, methadone, and nalbuphine, with the magnitude of suppression dependent on agonist efficacy. This was explained by a reduction in both high-affinity MOR expression and MOR agonist-stimulated G protein activation in whole brain homogenates from $G\alpha_o +/−$ and $G\alpha_o$ homozygous null ($G\alpha_o −/−$) mice, compared with wild-type littermates. On the other hand, morphine spinal antinociception was not different between $G\alpha_o +/−$ and wild-type mice and high-affinity MOR expression was unchanged in spinal cord tissue. However, the action of the partial agonist nalbuphine was compromised, showing that reduction in $G\alpha_o$ protein does decrease spinal antinociception, but suggesting a higher $G\alpha_o$ protein reserve. These results provide the first *in vivo* evidence that $G\alpha_o$ contributes to maximally efficient MOR signaling and antinociception.

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INTRODUCTION

Opioid analgesics are prescribed for the management of moderate to severe pain. Clinically used opioids elicit their effects by stimulation of the mu-opioid receptor (MOR), a member of the G protein-coupled receptor superfamily that interacts with heterotrimeric G proteins ($G\alpha\beta\gamma$), which are defined in terms of the $G\alpha$ subunit. Specifically, MOR couples to $G\alpha$ proteins of the pertussis toxin-sensitive $G\alpha_{i/o}$ family, which comprises $G\alpha_o$ (including splice variants $G\alpha_{o1}$ and $G\alpha_{o2}$), $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ (Laugwitz *et al*, 1993; Chakrabarti *et al*, 1995), as well as pertussis toxin-insensitive $G\alpha_z$ (Garzon *et al*, 1997). In the inactive state, $G\alpha\beta\gamma$ exists in complex with the receptor. On agonist stimulation, GDP bound to the $G\alpha$ subunit is exchanged for GTP, resulting in dissociation of active $G\alpha$ -GTP from the

$G\beta\gamma$ heterodimer (reviewed in Brown and Sihra, 2008); both $G\alpha$ -GTP and $G\beta\gamma$ modulate effectors downstream of MOR, including adenylyl cyclase (AC) (Yu and Sadee, 1988) and calcium channels (Hescheler *et al*, 1987; Moises *et al*, 1994). It has been shown that specific $G\alpha_{i/o}$ subunits differentially contribute to MOR-dependent behavioral responses, including morphine-mediated antinociception (Raffa *et al*, 1994; Sanchez-Blazquez *et al*, 2001). However, findings are inconsistent because of the variety of methods and models utilized in previous work, such that the contribution of each $G\alpha$ subunit to these responses is controversial.

$G\alpha_o$ is highly expressed in brain (Gierschik *et al*, 1986). Multiple lines of evidence suggest that opioid agonists can activate MOR-G protein complexes in a non-selective manner, especially in heterologous expression systems (Laugwitz *et al*, 1993; Clark *et al*, 2006a; Clark and Traynor, 2006b). On the other hand, the MOR-selective agonist [D -Ala², N -MePhe⁴, Gly-ol⁵]enkephalin (DAMGO) was found to activate $G\alpha_o$ to a greater extent than either $G\alpha_{i2}$ or $G\alpha_{i3}$ (Clark *et al*, 2008). Furthermore, in cultured neurons or neuronal-like cells, MOR has been shown to couple to AC (Carter and Medzihradsky, 1993) and N-type Ca^{2+} channels

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(Hescheler *et al*, 1987; Moises *et al*, 1994) primarily via activation of G α_o .

Despite the abundance of G α_o in the brain and evidence from *in vitro* studies that G α_o modulates signaling downstream of MOR, together with a recent report that G α_o may be involved in opioid dependence (Kest *et al*, 2009), findings *in vivo* have primarily implicated G α_{i2} and/or G α_z proteins as mediators of opioid agonist antinociception (Raffa *et al*, 1994; Sanchez-Blazquez *et al*, 1995, 2001; Standifer *et al*, 1996). These studies utilized mice administered *i.c.v.* antisense oligodeoxynucleotides (ODNs) against a specific G α subunit before antinociceptive testing of opioid agonists (*i.c.v.*) in the tail flick test (reviewed in Garzon *et al*, 2000). However, there are a number of inherent difficulties with this technique, including proper verification of the extent of protein knockdown. For most of these studies, knockdown of G α protein did not exceed ~50% in peri-ventricular regions (eg, periaqueductal gray) (Sanchez-Blazquez *et al*, 1995), while ODNs were less effective in brain regions more distal to the site of infusion (eg, thalamus), presumably due to poor diffusion (Sanchez-Blazquez *et al*, 1995; Standifer *et al*, 1996). However, in one study, in which greater (~60–80%) knockdown of G α subunits was achieved, ODNs directed against G α_o , in addition to other G α isoforms, suppressed morphine antinociception (Standifer *et al*, 1996). Clearly, inconsistencies in the efficacy and selectivity of G α protein knockdown complicate the interpretation of these studies. This previous work is further limited in that only a single measure of opioid antinociception was evaluated.

This study was designed to test the hypothesis that MOR coupling to G α_o is necessary for opioid antinociception using a constitutive G α_o knockout mouse strain (Duan *et al*, 2007). To probe the role of G α_o in MOR-mediated antinociception, opioid spinal and supraspinal antinociception were evaluated in response to noxious thermal stimuli; this is the first time that mice null for G α_o have been evaluated for alterations in MOR-dependent antinociception. Furthermore, to directly relate changes in opioid antinociception to alterations in MOR function, membrane homogenates from either whole brain or spinal cord of G α_o transgenic mice were evaluated for MOR expression and MOR agonist-stimulated G protein activity. These studies demonstrate that the abundant brain G protein, G α_o , is the primary G α subtype responsible for MOR-mediated signaling and antinociception.

MATERIALS AND METHODS

Transgenic Mice

Transgenic mice null for *Gnao1* (G α_o $-/-$), *Gnai2* (G α_{i2} $-/-$), or *Gnai3* (G α_{i3} $-/-$) were generated as previously described (Mortensen *et al*, 1992; Sowell *et al*, 1997; Duan *et al*, 2007) and were backcrossed onto the 129S6/SvEvTac strain for four generations. Transgenic mice and wild-type littermates were obtained by heterozygous breeding to control for genetic background. Adult, opioid-naïve male mice, matched for age, were utilized for all experiments. Mice were group-housed with food and water available *ad libitum*. Lights were maintained on a 12-h light-dark cycle (lights on at 0700 hours), and all testing was

performed during the light phase. Studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health and all experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals.

Antinociceptive Tests

The hot plate test was used to evaluate supraspinal antinociception. Mice were given two injections of saline (*i.p.*) to determine baseline latency, followed by three cumulative doses of agonist (*i.p.*) in 15-min intervals (nalbuphine) or 30-min intervals (morphine and methadone). Where four doses of drug were used, dose-effect curves were generated by pooling data from two overlapping, cumulative dose-effect measurements. Mice were placed on a 52 or 55 °C hot plate at the appropriate interval following each injection and the latency to lick forepaw(s) or jump was measured with a cutoff time of 60 or 45 s for the 52 or 55 °C hot plate temperatures, respectively, in order to prevent tissue damage.

The warm-water tail withdrawal test was used to evaluate spinal antinociception. Mice were given a single injection of saline (*i.p.*) to determine baseline latency, followed by four cumulative doses of agonist (*i.p.*) in 15-min intervals (nalbuphine) or 30-min intervals (morphine). The distal tip of the mouse's tail was placed in a 50 or 55 °C warm-water bath at the appropriate interval following each injection and the latency to tail flick was measured with a cutoff time of 20 or 15 s for 50 or 55 °C water, respectively, in order to prevent tissue damage.

For both antinociceptive tests, agonist-stimulated antinociception is expressed as a percentage of maximum possible effect (% MPE), where % MPE = (post-drug latency – baseline latency) \div (cutoff latency – baseline latency) \times 100.

Membrane Preparation

Mice were euthanized by cervical dislocation. Whole brain tissue, minus cerebellum, or thoracic and lumbar spinal cord was removed, immediately chilled in ice-cold 50 mM Tris base, pH 7.4, and membrane homogenates were prepared as previously described (Lester and Traynor, 2006). Final membrane pellets were resuspended in 50 mM Tris base, pH 7.4, aliquoted and stored at –80 °C. Protein content was determined using the method of Bradford (1976).

Western Blot Analysis

Membranes from whole brain (20 μ g protein) were mixed with sample buffer (63 mM Tris base, pH 6.8, 2% SDS, 10% glycerol, 0.008% bromophenol blue, and 50 mM dithiothreitol) and separated by SDS-PAGE on 10% (for detection of G α_o , G α_z , G α_{i1} , G α_{i2} , or G β_{1-4}) or 15% polyacrylamide gels (for detection of G γ_2). Proteins were then transferred to nitrocellulose membranes (Pierce, Rockford, IL) and probed with either rabbit polyclonal anti-G α_o (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-G α_z (1:200; Santa Cruz), rabbit polyclonal

anti-G α_{i1} (1:100; Santa Cruz), mouse monoclonal anti-G α_{i2} (1:1000; Millipore, Billerica, MA), rabbit polyclonal anti-G β_{1-4} (1:500; Santa Cruz), or rabbit polyclonal anti-G γ_2 (1:200; Santa Cruz). Membranes from spinal cord (20 μ g protein) were also evaluated for G α_o protein content, as above. All membranes were probed with mouse monoclonal anti- α -tubulin (1:1000; Sigma-Aldrich, St Louis, MO) as a loading control. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (1:10 000; Santa Cruz). Antibody immunoreactivity was detected by enhanced chemiluminescence using an EpiChem3 Benchtop Darkroom (UVP, Upland, CA) and band densities were quantified using Image J software (<http://rsbweb.nih.gov/ij/index.html>). Specifically, after background chemiluminescence was subtracted, G protein band densities were normalized to respective α -tubulin band densities and used to calculate expression relative to wild type for each G protein.

Radioligand-Binding Assays

For [3 H]diprenorphine ([3 H]DPN) binding, membranes from whole brain (100 μ g protein) or spinal cord (100–200 μ g protein) were incubated for 60 min at 25 °C with 4 nM [3 H]DPN in 50 mM Tris base, pH 7.4, with or without the MOR-selective antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH $_2$ (CTAP; 300 nM) to define MOR. For [3 H]DAMGO saturation binding, membranes from whole brain (100 μ g protein) were incubated for 60 min at 25 °C with increasing concentrations of [3 H]DAMGO (0.09–12 nM) in 50 mM Tris base, pH 7.4. Membranes from spinal cord (100–200 μ g protein) were incubated for 60 min at 25 °C with 12 nM [3 H]DAMGO in 50 mM Tris base, pH 7.4. For all radioligand-binding assays, nonspecific binding was evaluated in the presence of 10 μ M naloxone. Reactions were stopped by rapid filtration through a Brandel MLR-24 harvester (Brandel, Gaithersburg, MD), and bound radioligand was collected on GF/C filtermats (Whatman, Kent, UK) and rinsed three times with ice-cold 50 mM Tris base, pH 7.4. Filters were dried, saturated with EcoLume scintillation cocktail (MP Biomedicals, Solon, OH) and radioactivity was counted using a Wallac 1450 MicroBeta counter (PerkinElmer, Waltham, MA).

Agonist-Stimulated [35 S]GTP γ S-Binding Assays

To measure binding of the non-hydrolyzable GTP analog guanosine-5'-O-(3-[35 S]thio)triphosphate ([35 S]GTP γ S) to G α proteins, membranes from whole brain (10 μ g protein) or spinal cord (25–50 μ g protein) were pre-incubated for 10 min at 25 °C with or without various concentrations of the opioid agonists DAMGO, methadone, morphine, or nalbuphine in [35 S]GTP γ S-binding buffer (50 mM Tris base, pH 7.4, 5 mM MgCl $_2$, 100 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 100 μ M GDP, and 0.4 U/ml adenosine deaminase). After pre-incubation, 0.1 nM [35 S]GTP γ S was added and reactions were further incubated for 90 min at 25 °C. For saturation analysis of [35 S]GTP γ S binding, membranes from whole brain (10 μ g protein) were pre-incubated for 10 min at 25 °C with or without 10 μ M DAMGO in [35 S]GTP γ S-binding buffer, followed by incubation for 90 min at 25 °C with 0.1 nM [35 S]GTP γ S, with or without

various concentrations of unlabeled GTP γ S (0.8–50 nM). For all [35 S]GTP γ S-binding assays, nonspecific binding was evaluated in the presence of 10 μ M GTP γ S. Binding reactions were stopped by rapid filtration, rinsed three times with ice-cold wash buffer (50 mM Tris base, pH 7.4, 5 mM MgCl $_2$, and 100 mM NaCl), and bound radioactivity was measured by liquid scintillation counting, as above.

Drugs

Morphine sulfate was from RTI (Research Triangle Park, NC). Methadone and nalbuphine were obtained through the Narcotic Drug and Opioid Peptide Basic Research Center at the University of Michigan (Ann Arbor, MI). For behavioral experiments, all drugs were diluted in sterile water. [3 H]DPN, [3 H]DAMGO and [35 S]GTP γ S were purchased from PerkinElmer. Adenosine deaminase was obtained from Calbiochem (San Diego, CA). DAMGO, CTAP, GDP, GTP γ S, and all other chemicals were obtained from Sigma-Aldrich, unless otherwise noted.

Data Analysis

All data were analyzed using GraphPad Prism software, version 5.0. (GraphPad, San Diego, CA). Differences between genotypes were evaluated using Student's *t*-tests or one-way or two-way ANOVA with Bonferroni's post-tests, where appropriate. For all statistical tests, significance was set at $p < 0.05$. *In vivo* potency (ED $_{50}$) values were calculated by fitting the compiled data to an agonist *vs* normalized response curve (Hill slope = 1), and values are expressed as the mean (95% CI). Where antinociception was near or below 50% MPE, ED $_{50}$ values were extrapolated from the fitted data. Maximal radioligand-binding (B_{max}) and binding affinity (K_D) values were derived by fitting each experiment to a one-site saturation-binding curve fit (Hill slope = 1), while maximal [35 S]GTP γ S stimulation (E_{max}) and *in vitro* potency (EC $_{50}$) values were calculated by fitting individual experiments to an agonist *vs* response curve fit (Hill slope = 1); values are expressed as the mean \pm SEM.

RESULTS

Characterization of Transgenic Mice Lacking G α_o Protein

The full knockout, G α_o $-/-$ mice did not often survive until weaning (\sim 21 days), whereas wild-type and G α_o $+/-$ mice were obtained at frequencies predicted by Mendelian genetics (Table 1) ($\chi^2 = 11.07$, $df = 1$, $p < 0.001$). Peri-natal lethality was also noted in the initial reports of two independently generated G α_o null mouse strains (Valenzuela *et al*, 1997; Jiang *et al*, 1998). These previous studies also reported several neurological abnormalities in G α_o $-/-$ animals, including hyperactivity, tremor and turning behavior; however, no such gross behavioral abnormalities were noted for the G α_o $+/-$ or G α_o $-/-$ mice used in this study (Duan *et al*, 2007; unpublished observations). In adulthood ($>$ 8 weeks), body weight varied as a function of genotype (Table 1) ($F(2,23) = 14.54$, $p < 0.001$). *Post hoc* analysis revealed that those G α_o $-/-$ mice that did survive

weighed significantly less than their wild-type littermates, whereas $G\alpha_o$ $+/-$ mice did not differ from wild-type controls.

Supraspinal Antinociception in $G\alpha_o$ Transgenic Mice

To determine whether $G\alpha_o$ is involved in opioid antinociception, $G\alpha_o$ $+/-$ mice were evaluated for morphine antinociception in the hot plate test (Figure 1). In the 52 °C hot plate test, the baseline nociceptive threshold was not significantly different between wild-type (12.6 ± 0.6 s; $n=30$) and $G\alpha_o$ $+/-$ mice (12.2 ± 0.5 s; $n=32$; $t(60)=0.4885$, $p=0.627$). Morphine produced a dose-dependent increase in antinociception that was significantly reduced (~4-fold) in $G\alpha_o$ $+/-$ mice when compared with wild-type controls, with ED_{50} values of 47.7 mg/kg (31.2–72.9) and 11.4 mg/kg (5.9–22.1), respectively (Figure 1a). Although there was no significant interaction, there were significant main effects of dose and genotype (dose: $F(2,36)=19.88$; $p<0.001$; genotype: $F(1,36)=15.76$, $p<0.001$).

We hypothesized that increasing the efficacy requirements of the nociceptive system might further exaggerate this observed genotype difference; thus, the hot plate temperature was raised to 55 °C and $G\alpha_o$ transgenic mice were again evaluated for morphine supraspinal antinociception (Figure 1b). As expected, a decreased baseline nociceptive threshold was observed at the elevated hot plate temperature, and there were no significant differences between wild-type (7.5 ± 0.8 s; $n=9$) and $G\alpha_o$ $+/-$ mice at baseline (6.6 ± 0.6 s; $n=10$; $t(17)=0.8940$, $p=0.384$). Morphine dose-dependently produced antinociception in both wild-type and $G\alpha_o$ $+/-$ mice, but the ED_{50} was shifted ~6-fold for $G\alpha_o$ $+/-$ mice, with a value of 62.7 mg/kg (42.9–91.5) compared with 9.9 mg/kg (5.8–17.1) for wild-

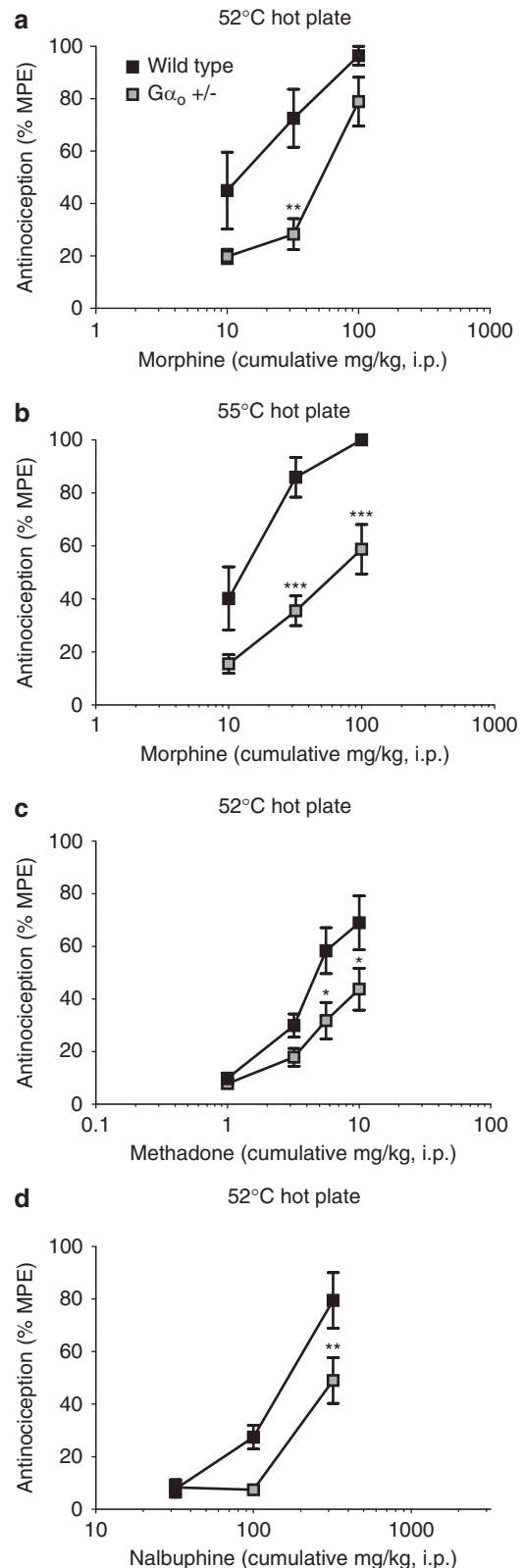
type littermates (Figure 1b). There were significant main effects of both dose ($F(2,51)=25.37$; $p<0.001$) and genotype ($F(1,51)=41.98$, $p<0.001$), although there was no significant interaction.

Table 1 Physical Characteristics of Wild-Type and $G\alpha_o$ Transgenic Mice

Genotype	Body weight (g)	Genotype frequency at weaning (%)	
		Expected	Observed ($n=347$)
Wild type	28.6 ± 1.3 ($n=10$)	25.0	35.7 ($n=124$)
$G\alpha_o$ $+/-$	27.6 ± 0.8 ($n=12$)	50.0	59.4 ($n=206$)
$G\alpha_o$ $-/-$	18.2 ± 1.4 ($n=4$)*	25.0	4.9 ($n=17$)

Asterisk indicates a statistical difference vs wild type by Bonferroni's post-test ($p<0.001$).

Figure 1 Supraspinal antinociception produced by morphine, methadone, and nalbuphine in the hot plate test in $G\alpha_o$ transgenic mice. Antinociception was measured in wild-type and $G\alpha_o$ $+/-$ mice 30 min following morphine in the (a) 52 °C or (b) 55 °C hot plate test, (c) 30 min following methadone in the 52 °C hot plate test, and (d) 15 min following nalbuphine in the 52 °C hot plate test. Data represent the mean \pm SEM for morphine at 52 °C ($n=7$) and 55 °C ($n=9-10$), for methadone ($n=7-15$), and for nalbuphine ($n=8-10$). Legend in panel (a) also describes panels (b) through (d). Asterisks indicate a statistical difference vs wild type by Bonferroni's post-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).



To determine whether G α_o has a role in the antinociception produced by opioid agonists other than morphine, G α_o transgenic mice were evaluated for either methadone or nalbuphine antinociception in the 52 °C hot plate test (Figures 1c and d). Like morphine, methadone produced a dose-dependent increase in antinociception (Figure 1c). The ED₅₀ value for wild-type mice was 13.0 mg/kg (10.1–16.8), which was ~2-fold higher than the extrapolated ED₅₀ value for G α_o +/– mice of 5.8 mg/kg (4.4–7.6). There was no significant interaction; however, there were significant main effects of both dose ($F(3,82) = 33.12$, $p < 0.001$) and genotype ($F(1,82) = 19.87$, $p < 0.001$). The partial agonist nalbuphine also produced a dose-dependent stimulation of antinociception that was significantly reduced for G α_o +/– mice, compared with wild-type littermates (Figure 1d), with an ED₅₀ value of 170.2 mg/kg (108.3–267.6) for wild-type mice. Extrapolation of the dose-response curve for G α_o +/– mice gave an ED₅₀ value of 432.0 mg/kg (289.0–645.9), representing a ~3-fold shift. There were significant main effects of both dose ($F(2,48) = 48.62$, $p < 0.001$) and genotype ($F(1,48) = 11.09$; $p = 0.002$), as well as a significant dose \times genotype interaction ($F(2,48) = 3.377$, $p = 0.043$).

Spinal Antinociception in G α_o Transgenic Mice

G α_o transgenic mice were also evaluated in the warm-water tail withdrawal test (Figure 2), the same antinociceptive measure that was utilized in the majority of antisense ODN studies (Raffa *et al*, 1994; Sanchez-Blazquez *et al*, 1995, 2001). In the 50 °C tail withdrawal test, the baseline tail flick latency was not significantly different between wild-type (3.2 ± 0.4 s; $n = 13$) and G α_o +/– mice (4.2 ± 0.6 s; $n = 15$; $t(26) = 1.399$, $p = 0.174$). Morphine produced a dose-dependent increase in antinociception in both wild-type and G α_o +/– mice, with ED₅₀ values of 5.2 mg/kg (2.7–9.8) and 4.1 mg/kg (2.3–7.2), respectively (Figure 2a). There was a significant main effect of dose ($F(3,44) = 14.79$, $p < 0.001$), although the main effect of genotype ($F(1,44) = 0.1024$, $p = 0.751$) and the dose \times genotype interaction were not significant.

Given that morphine behaves as a full agonist in this test, which may preclude the identification of small differences between genotypes, it was hypothesized that increasing the efficacy requirement of the system by raising the water bath temperature to 55 °C (Figure 2b) should allow for the identification of such differences. Again, as predicted, a decreased baseline nociceptive threshold was observed at the elevated water temperature, and there were also no significant differences between wild-type (1.9 ± 0.1 s; $n = 8$) and G α_o +/– mice in this test (1.8 ± 0.2 s; $n = 8$; $t(14) = 0.6932$, $p = 0.500$). Against the 55 °C stimulus, morphine produced a dose-dependent increase in antinociception that was equivalent between wild-type and G α_o +/– mice, with ED₅₀ values of 8.2 mg/kg (5.5–12.5) and 7.4 mg/kg (5.5–10.0), respectively (Figure 2b). There was no significant interaction or significant effect of genotype (genotype: $F(1,56) = 0.2371$, $p = 0.628$), but there was a significant main effect of dose (dose: $F(3,56) = 124.0$, $p < 0.001$).

As an alternative method of evaluating whether the efficacious antinociception produced by morphine was masking a mediatory role for G α_o , spinal antinociception

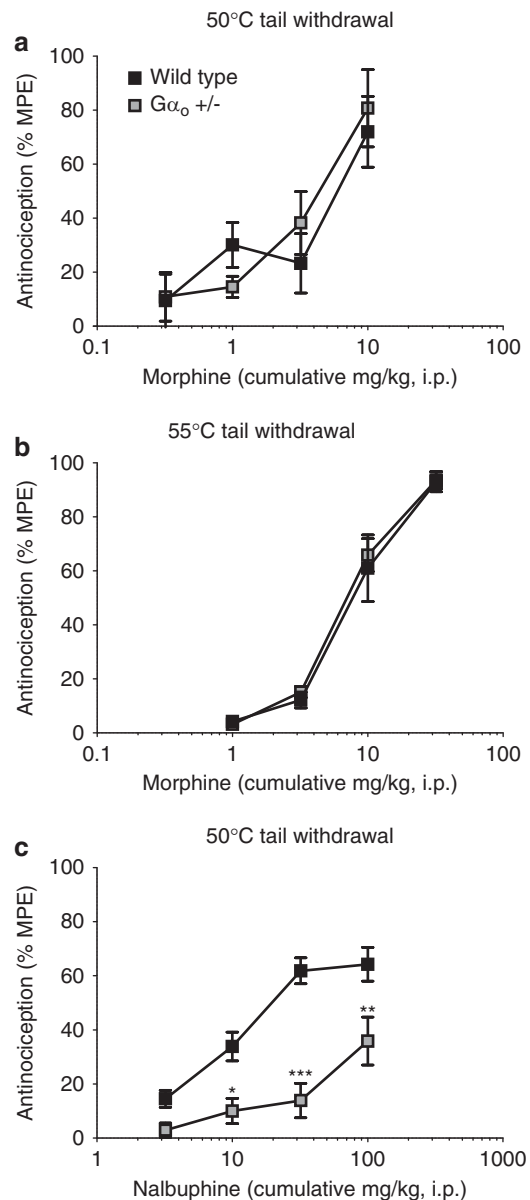


Figure 2 Ability of morphine and nalbuphine to induce spinal antinociception in the warm-water tail withdrawal test in G α_o transgenic mice. Antinociception was measured in wild-type and G α_o +/– mice 30 min following morphine in the (a) 50 °C or (b) 55 °C warm-water tail withdrawal test and (c) 15 min following nalbuphine in the 50 °C warm-water tail withdrawal test. Data represent the mean \pm SEM for morphine at 50 °C ($n = 6-7$) and 55 °C ($n = 8$) and for nalbuphine ($n = 7-8$). Legend for panels (b) and (c) is the same as for panel (a). Asterisks indicate a statistical difference vs wild type by Bonferroni's post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

was measured in the 50 °C warm-water tail withdrawal test in response to the low-efficacy agonist, nalbuphine (Figure 2c). Nalbuphine produced a dose-dependent stimulation of spinal antinociception that was significantly reduced (~7-fold) in G α_o +/– mice when compared with wild-type littermates, with wild-type mice exhibiting an ED₅₀ value of 24.2 mg/kg (17.5–33.4) (Figure 2a). Extrapolation of the nalbuphine dose-response for G α_o +/– mice revealed an ED₅₀ value of 176.1 mg/kg (113.3–273.8).

There were significant main effects of dose and genotype (dose: $F(3,52) = 20.35$, $p < 0.001$; genotype: $F(1,52) = 48.62$, $p < 0.001$), as well as a significant dose \times genotype interaction ($F(3,52) = 3.552$, $p = 0.021$).

Antinociception in $G\alpha_{i2}$ and $G\alpha_{i3}$ Transgenic Mice

To confirm the importance of $G\alpha_o$ for opioid antinociception, transgenic mice lacking either $G\alpha_{i2}$ ($G\alpha_{i2}$ heterozygous null, $G\alpha_{i2} +/ -$; $G\alpha_{i2}$ homozygous null, $G\alpha_{i2} -/ -$) or $G\alpha_{i3}$ ($G\alpha_{i3}$ heterozygous null, $G\alpha_{i3} +/ -$; $G\alpha_{i3}$ homozygous null, $G\alpha_{i3} -/ -$), together with their respective wild-type littermates, were evaluated in the 52 °C hot plate and 50 °C warm-water tail withdrawal tests (Figure 3). Both the $G\alpha_{i2}$ and the $G\alpha_{i3}$ transgenic mouse strains were generated in parallel with $G\alpha_o$ transgenic mice, and inactivation of the appropriate $G\alpha$ subunit has been previously confirmed by western blot analysis (Sowell *et al*, 1997; Duan *et al*, 2007). In the 52 °C hotplate test (Figures 3a and b), the baseline response latency was equivalent among all $G\alpha_{i2}$ transgenic mouse genotypes (wild-type: 12.0 ± 0.7 s, $n = 10$; $G\alpha_{i2} +/ -$: 11.6 ± 0.7 s, $n = 9$; $G\alpha_{i2} -/ -$: 12.9 ± 0.7 s, $n = 8$; $F(2,24) = 0.8112$, $p = 0.456$). Morphine produced a dose-dependent increase in antinociception that was not different between wild-type, $G\alpha_{i2} +/ -$ and $G\alpha_{i2} -/ -$ mice, with ED_{50} values of 9.4 mg/kg (5.8–15.3), 12.0 mg/kg (6.9–20.8), and 10.6 mg/kg

(6.0–18.8), respectively (Figure 3a). There was a significant main effect of dose ($F(2,72) = 59.03$, $p < 0.001$), but the main effect of genotype ($F(2,72) = 0.3274$, $p = 0.722$) and the dose \times genotype interaction were not significant. Similarly, in $G\alpha_{i3}$ transgenic mice, morphine produced a dose-dependent increase in antinociception that was equivalent between wild-type, $G\alpha_{i3} +/ -$ and $G\alpha_{i3} -/ -$ mice, with ED_{50} values of 13.2 mg/kg (8.5–20.4), 10.6 mg/kg (6.0–18.9) and 8.1 mg/kg (4.4–15.0), respectively (Figure 3b). There was a significant main effect of dose ($F(2,69) = 37.38$, $p < 0.001$), but not genotype ($F(2,69) = 0.7686$, $p = 0.468$), and no significant interaction. There were also no genotype-dependent differences observed in the baseline nociceptive threshold for these mice (wild-type: 13.3 ± 1.4 s, $n = 9$; $G\alpha_{i3} +/ -$: 16.8 ± 1.2 s, $n = 9$; $G\alpha_{i3} -/ -$: 16.2 ± 1.4 s, $n = 8$; $F(2,23) = 2.139$, $p = 0.141$).

$G\alpha_{i2}$ and $G\alpha_{i3}$ transgenic mice were also evaluated for spinal antinociception in the 50 °C tail withdrawal test (Figures 3c and d). Baseline response latencies in this test were equivalent among all $G\alpha_{i2}$ (wild-type: 5.0 ± 0.4 s, $n = 9$; $G\alpha_{i2} +/ -$: 4.4 ± 0.6 s, $n = 8$; $G\alpha_{i2} -/ -$: 3.9 ± 0.5 s, $n = 6$; $F(2,20) = 1.050$, $p = 0.368$) and $G\alpha_{i3}$ transgenic mouse genotypes (wild-type: 5.6 ± 0.4 s, $n = 8$; $G\alpha_{i3} +/ -$: 4.2 ± 0.5 s, $n = 9$; $G\alpha_{i3} -/ -$: 4.4 ± 0.8 s, $n = 6$; $F(2,20) = 1.990$, $p = 0.163$). Morphine produced a dose-dependent increase in antinociception that was not different between

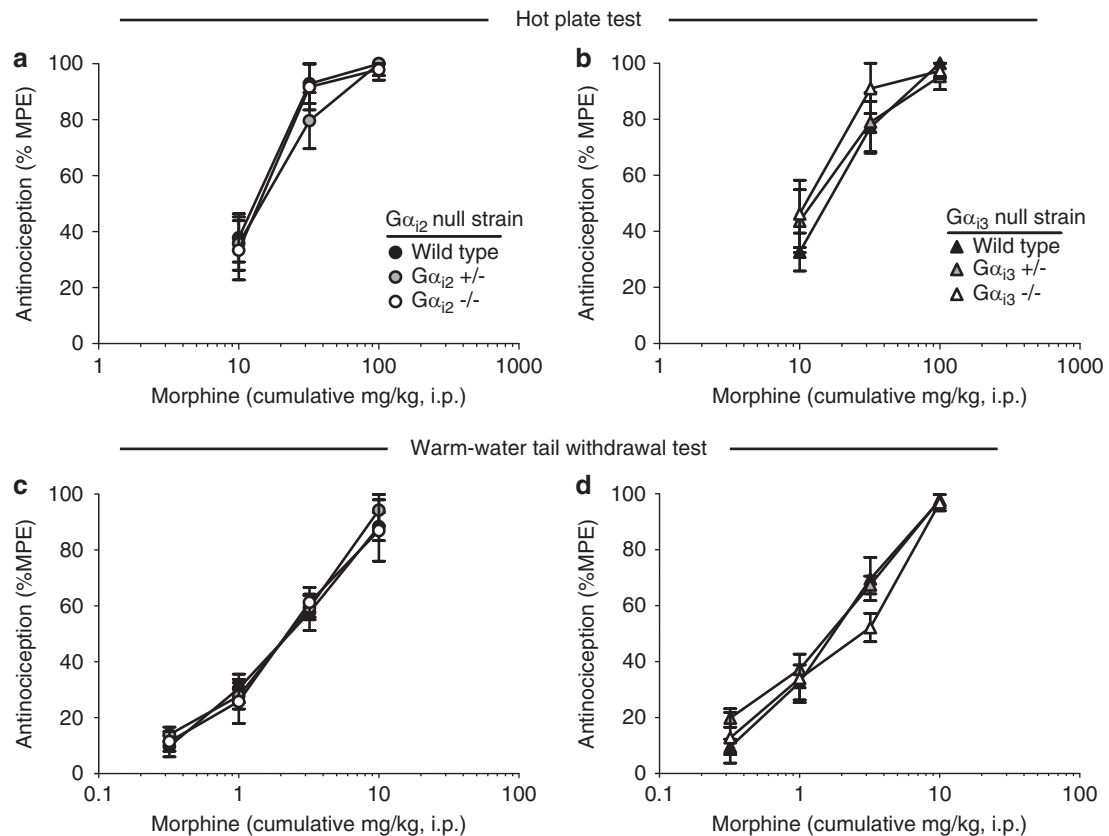


Figure 3 Morphine supraspinal and spinal antinociception in $G\alpha_{i2}$ and $G\alpha_{i3}$ transgenic mice. Antinociception produced 30 min following morphine was evaluated in the 52 °C hot plate test in (a) $G\alpha_{i2} +/ -$ and $G\alpha_{i2} -/ -$ mice and (b) $G\alpha_{i3} +/ -$ and $G\alpha_{i3} -/ -$ mice and in the 50 °C warm-water tail withdrawal test in (c) $G\alpha_{i2} +/ -$ and $G\alpha_{i2} -/ -$ mice and (d) $G\alpha_{i3} +/ -$ and $G\alpha_{i3} -/ -$ mice, together with their respective wild-type littermates. Data represent the mean \pm SEM for $G\alpha_{i2}$ mice in the hot plate ($n = 8$ –10) and tail withdrawal tests ($n = 6$ –9) and for $G\alpha_{i3}$ mice in the hot plate ($n = 8$ –9) and tail withdrawal tests ($n = 6$ –9). Legends for panels (c) and (d) are the same as for panels (a) and (b), respectively.

wild-type, G α_{i2} +/– and G α_{i2} –/– mice, with ED₅₀ values of 2.2 mg/kg (1.6–2.9), 2.0 mg/kg (1.6–2.6), and 2.2 mg/kg (1.5–3.3), respectively (Figure 3c). There was no significant interaction or main effect of genotype (F(2,80) = 0.2412, p = 0.786), but there was a significant main effect of dose (F(3,80) = 113.5, p < 0.001). Similarly, in G α_{i3} transgenic mice, morphine produced a dose-dependent increase in antinociception that was equivalent in wild-type, G α_{i3} +/– and G α_{i3} –/– mice, with ED₅₀ values of 1.6 mg/kg (1.2–2.2), 1.4 mg/kg (1.2–1.7), and 2.0 mg/kg (1.4–3.1), respectively (Figure 3d). Although there was no significant interaction or main effect of genotype (F(2,80) = 1.936, p = 0.151), there

was a significant main effect of dose (F(3,80) = 171.1, p < 0.001).

G Protein Expression in G α_o Transgenic Mouse Brain

Western blot analysis of G protein expression in whole brain membrane samples confirmed the loss of G α_o protein in G α_o –/– mice (Figure 4a). Quantification of western blot images for G α_o revealed that, in comparison with wild-type controls, G α_o +/– mice express ~60% less G α_o protein, which is close to the expected 50% reduction (Figure 4a). Across a panel of G protein subunits, including G $\alpha_{i/o}$, G β ,

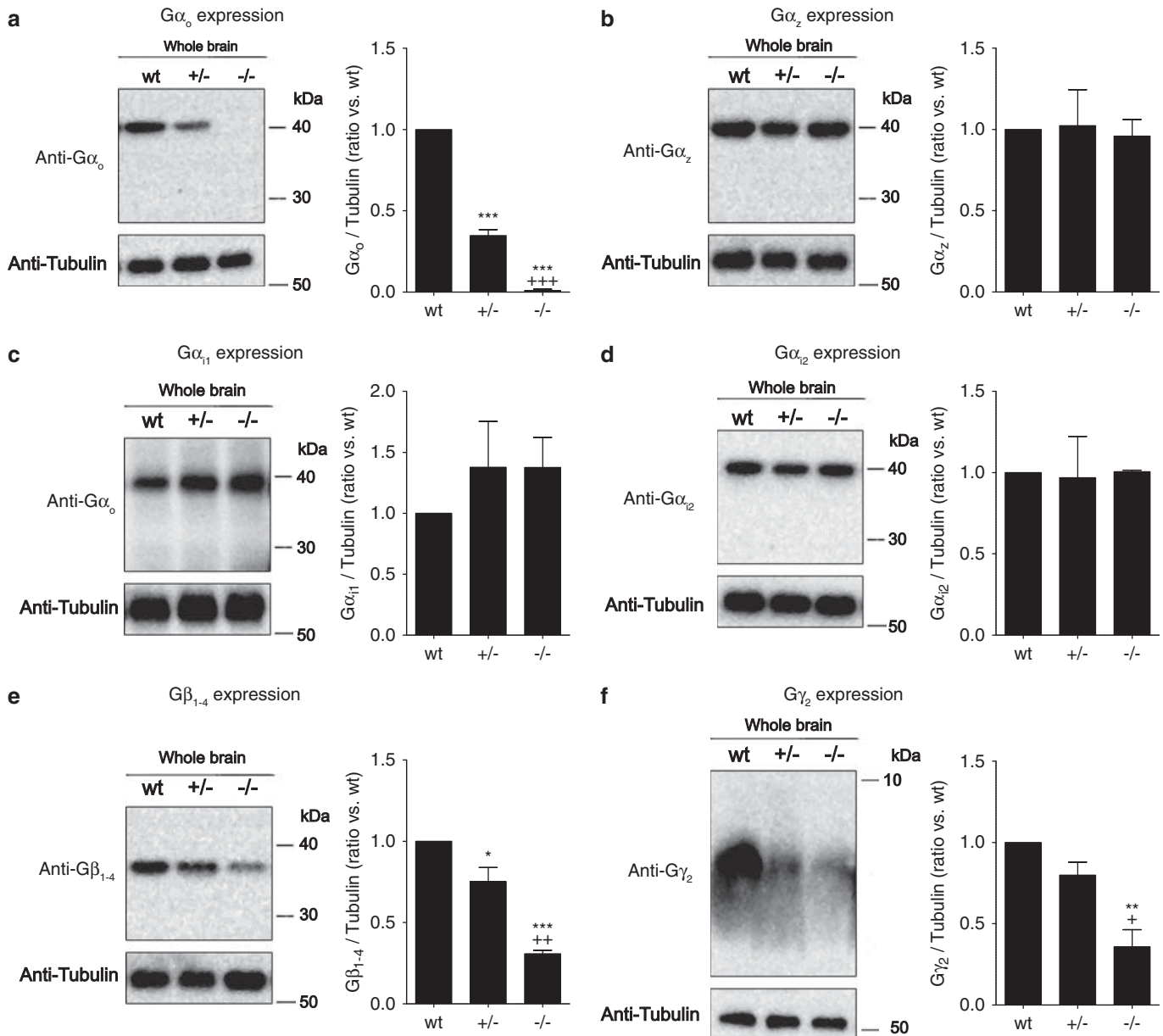


Figure 4 G protein expression in whole brain homogenates from G α_o transgenic mice. Membranes from whole brain of wild-type (wt), G α_o +/– (+/–), and G α_o –/– (–/–) mice were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed for the expression of (a) G α_o , (b) G α_z , (c) G α_{i1} , (d) G α_{i2} , (e) G β_{1-4} , or (f) G γ_2 using selective antibodies (see Materials and Methods section); membranes were also probed for tubulin as a loading control. G protein expression was quantified in Image J by normalizing G protein band intensity to tubulin band intensity, and data are plotted as a ratio of wt expression. Data represent the mean \pm SEM (n = 3). Symbols indicate a statistical difference vs wt (* p < 0.05, ** p < 0.01, *** p < 0.001) or +/- (+ p < 0.05, ++ p < 0.01, +++ p < 0.001) by Bonferroni's post-test.

and G γ proteins (Figure 4), the expression of G α_o (Figure 4a) ($F(2,6) = 527.9$, $p < 0.001$), G β_{1-4} (Figure 4e) ($F(2,6) = 46.53$, $p < 0.001$), and G γ_2 (Figure 4f) ($F(2,6) = 18.45$, $p = 0.003$) were significantly decreased as a function of genotype. In contrast, there were no compensatory changes noted for the expression of either G α_z (Figure 4b) ($F(2,6) = 0.0548$, $p = 0.947$), G α_{i1} (Figure 4c) ($F(2,6) = 0.6938$, $p = 0.536$), or G α_{i2} (Figure 4d) ($F(2,6) = 0.0189$, $p = 0.981$).

MOR Expression in G α_o Transgenic Mouse Brain and Spinal Cord

To evaluate whether the reduction in opioid antinociception observed in G α_o +/– mice could be explained by alterations at the receptor level, MOR expression was measured in membranes from whole brain or from spinal cord of G α_o transgenic mice (Table 2). Binding of a maximal concentration (4 nM) of the radiolabeled opioid antagonist [3 H]DPN, representing the entire pool of MOR, delta- and kappa-opioid receptors, was unaffected by genotype in either whole brain ($F(2,5) = 0.3542$, $p = 0.718$) or spinal cord ($t(4) = 0.0097$, $p = 0.993$). To measure total MOR expression, maximal [3 H]DPN binding was displaced using the MOR-selective antagonist CTAP (300 nM). Total MOR expression was also not different between genotypes (Table 2) in either whole brain ($F(2,5) = 0.6832$, $p = 0.547$) or spinal cord ($t(4) = 0.7611$, $p = 0.489$).

In whole brain, maximal binding (B_{max}) of [3 H]DAMGO (Table 2), which, as an agonist, recognizes only high-affinity MOR, was significantly decreased in G α_o +/– and G α_o –/– mice when compared with wild-type controls ($F(2,5) = 10.44$; $p = 0.016$). There was no change across genotypes in the affinity (K_D) of [3 H]DAMGO for high-affinity MOR sites ($F(2,5) = 1.398$; $p = 0.330$). In contrast, maximal (12 nM) [3 H]DAMGO binding was unchanged in the spinal cord of G α_o +/– mice when compared with wild-type littermate controls ($t(4) = 1.186$, $p = 0.301$).

G protein Activation in G α_o Transgenic Mouse Brain and Spinal Cord

To examine the importance of G α_o for MOR function, the [35 S]GTP γ S-binding assay was utilized to evaluate the first component of MOR signaling, namely, G protein activation, in membranes from either whole brain or spinal cord of G α_o transgenic mice (Figure 5; Table 3). In whole brain, basal levels of [35 S]GTP γ S incorporation (Table 3) were significantly reduced in G α_o +/– and G α_o –/– mice, compared with wild-type littermates ($F(2,5) = 20.06$, $p = 0.004$), suggesting that G α_o is responsible for some, but not all, basal G protein activity. The MOR-selective agonist DAMGO produced a dose-dependent stimulation of [35 S]GTP γ S binding that was reduced in G α_o +/– and G α_o –/– mice when compared with wild-type controls (Figure 5a). There was a significant concentration \times genotype interaction for this response ($F(14,39) = 6.700$, $p < 0.001$), including main effects of both concentration ($F(7,39) = 43.38$, $p < 0.001$) and genotype ($F(2,39) = 72.02$, $p < 0.001$). Maximal DAMGO-stimulated binding (E_{max}) (Table 3) was decreased in G α_o transgenic mice in a genotype-dependent manner ($F(2,5) = 64.69$, $p < 0.001$); this reduction in maximal stimulation was without a change in potency (EC_{50}) between wild-type and G α_o +/– mice ($t(2) = 1.307$, $p = 0.321$). Morphine also produced a dose-dependent stimulation of [35 S]GTP γ S binding that was significantly decreased in G α_o +/– and G α_o –/– mice when compared with wild-type littermates (Figure 5b). There were significant main effects of both concentration and genotype, as well as a significant concentration \times genotype interaction (concentration: $F(7,39) = 15.79$, $p < 0.001$; genotype: $F(2,39) = 51.45$, $p < 0.001$; concentration \times genotype: $F(14,39) = 3.468$, $p = 0.001$). The E_{max} for morphine (Table 3) was reduced as a function of genotype ($F(2,5) = 16.24$, $p = 0.007$), and was accompanied by a nonsignificant trend toward a reduction in the EC_{50} value for G α_o +/– mice, compared with wild-type littermates ($t(4) = 2.407$, $p = 0.074$).

Given that DAMGO-stimulated [35 S]GTP γ S incorporation was significantly attenuated, saturation analysis of

Table 2 Properties of Agonist and Antagonist Radioligand Binding in Membranes from Whole Brain or Spinal Cord of Wild-Type and G α_o Transgenic Mice

Tissue	Genotype	[3 H]DPN binding		[3 H]DAMGO binding	
		Total (fmol/mg protein)	MOR (fmol/mg protein) ^a	B _{max} (fmol/mg protein)	K _D (nM)
Whole brain	Wild type	366 \pm 24	218 \pm 13 ^b	246 \pm 29	2.5 \pm 0.4
	G α_o +/–	391 \pm 38	216 \pm 3	181 \pm 16	1.9 \pm 0.5
	G α_o –/–	407 \pm 38	233 \pm 12	121 \pm 17*	3.5 \pm 1.3
Spinal cord	Wild type	161 \pm 20	95 \pm 5	84 \pm 5 ^c	ND
	G α_o +/–	161 \pm 14	101 \pm 6	93 \pm 6 ^c	ND

Abbreviation: ND, not determined.

^aMOR expression was evaluated as the amount of bound [3 H]DPN at a maximal concentration that was displaced by the MOR-selective antagonist CTAP (300 nM).

^bIn wild-type whole brain, there is a trend for total MOR to be less than high-affinity MOR because binding was measured indirectly (see Materials and Methods).

^cIn spinal cord, B_{max} values were estimated using a single maximal concentration of [3 H]DAMGO.

Data represent the mean \pm SEM ($n = 2-3$ performed in at least duplicate). Asterisk indicates a statistical difference vs wild-type whole brain by Bonferroni's post-test ($p < 0.05$).

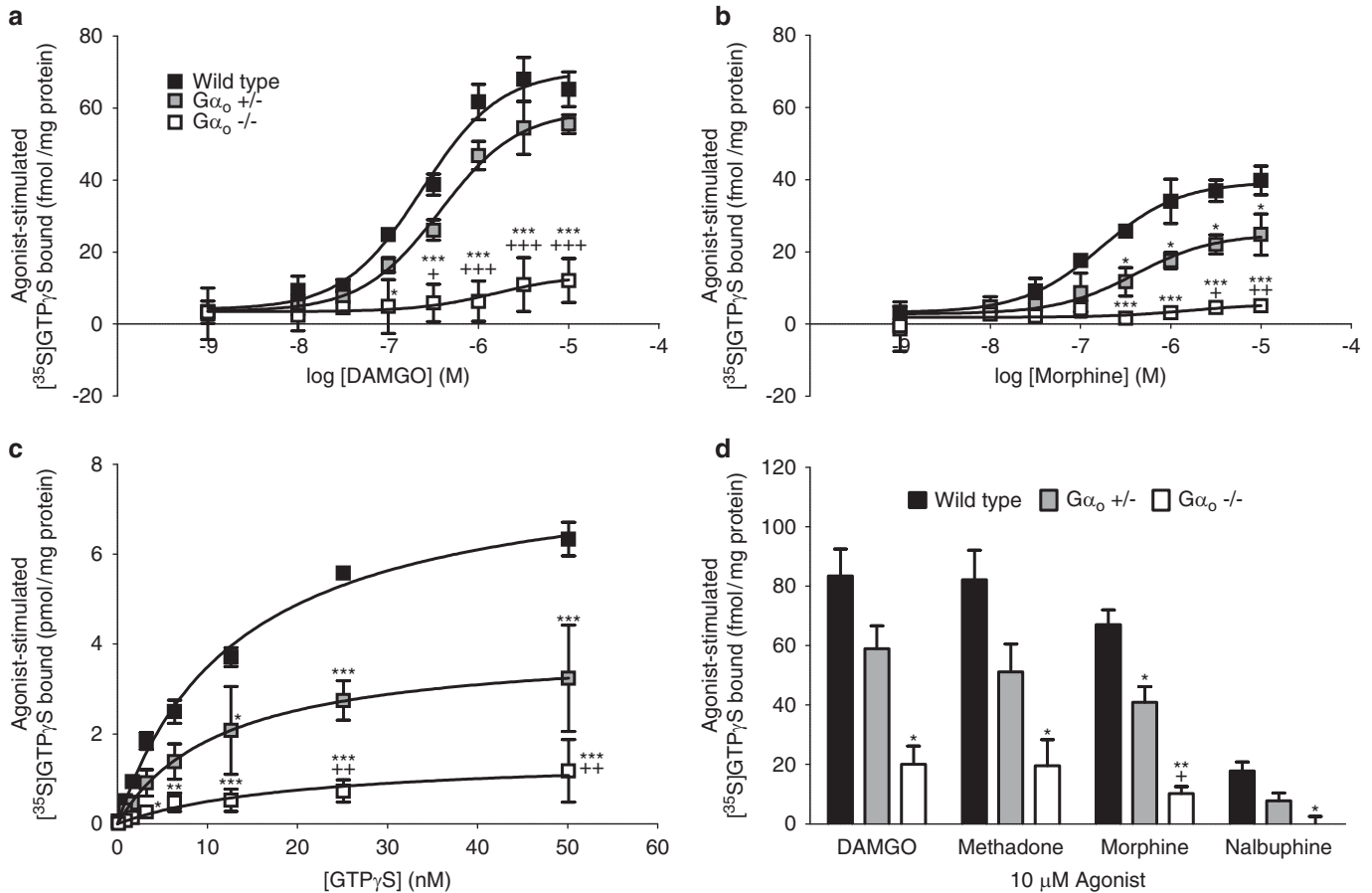


Figure 5 Ability of opioid agonists to stimulate G protein activity in whole brain homogenates from $G\alpha_o$ transgenic mice. Agonist-stimulated [³⁵S]GTPγS (0.1 nM) binding was measured in the presence of various concentrations of the opioid agonists (a) DAMGO or (b) morphine, (c) in the presence of 10 μM DAMGO plus increasing concentrations of unlabeled GTPγS, and (d) in the presence of 10 μM DAMGO, methadone, morphine, or nalbuphine in membrane homogenates from whole brain of wild-type, $G\alpha_o$ +/- and $G\alpha_o$ -/- mice. Nonspecific binding was evaluated in the presence of unlabeled GTPγS (10 μM). Data are plotted as agonist-stimulated [³⁵S]GTPγS binding, defined as the increase in [³⁵S]GTPγS incorporation in the presence of agonist over that of basal (measured in the absence of agonist), and represent the mean ± SEM ($n=2-3$ performed in at least duplicate). Legend in (a) also applies to panels (b) and (c). Symbols indicate a statistical difference vs wild type (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) or $G\alpha_o$ +/- (+ $p<0.05$, ++ $p<0.01$, +++ $p<0.001$) by Bonferroni's post-test.

DAMGO-stimulated binding was performed (Figure 5c; Table 3) to measure the maximal number of G proteins (B_{max}) activated by agonist-occupied MOR and the ability of agonist to induce formation of GTP-bound $G\alpha$ (K_D) (Traynor and Nahorski, 1995; Selley *et al.*, 1997). In membranes from whole brain, DAMGO-stimulated [³⁵S]GTPγS incorporation was increased as a function of increasing concentration of GTPγS, but was significantly reduced in $G\alpha_o$ +/- and $G\alpha_o$ -/- mice when compared with wild-type controls (Figure 5c). There were significant main effects of both concentration and genotype, as well as a significant concentration × genotype interaction (concentration: $F(7,44) = 37.50$, $p < 0.001$; genotype: $F(2,44) = 79.22$, $p < 0.001$; concentration × genotype: $F(14,44) = 7.482$, $p < 0.001$). This reduction was manifested as a decrease in B_{max} for GTPγS binding ($F(2,5) = 9.359$, $p = 0.020$), without an accompanying change in the K_D for GTPγS (Table 3) ($F(2,5) = 0.6608$, $p = 0.556$).

[³⁵S]GTPγS binding stimulated by a maximal concentration of DAMGO, morphine, methadone, or nalbuphine was evaluated in whole brain homogenates from $G\alpha_o$ transgenic mice (Figure 5d). In wild-type mice, the opioid agonists

tested elicited maximal [³⁵S]GTPγS stimulation according to the rank order of efficacy DAMGO = methadone > morphine >> nalbuphine. When compared with wild-type controls, $G\alpha_o$ +/- and $G\alpha_o$ -/- mice exhibited a reduction in G protein stimulation across all opioid agonists tested, including: DAMGO (wild-type: 83.4 ± 9.1 fmol/mg; $G\alpha_o$ +/-: 59.0 ± 7.7 fmol/mg; $G\alpha_o$ -/-: 20.1 ± 6.1 fmol/mg; $F(2,5) = 12.98$, $p = 0.011$), methadone (wild-type: 82.2 ± 9.9 fmol/mg; $G\alpha_o$ +/-: 51.2 ± 9.3 fmol/mg; $G\alpha_o$ -/-: 19.6 ± 8.7 fmol/mg; $F(2,5) = 9.407$, $p = 0.020$), morphine (wild-type: 67.1 ± 5.0 fmol/mg; $G\alpha_o$ +/-: 40.9 ± 5.3 fmol/mg; $G\alpha_o$ -/-: 10.3 ± 2.3 fmol/mg; $F(2,5) = 29.93$, $p = 0.002$), and nalbuphine (wild-type: 17.8 ± 3.0 fmol/mg; $G\alpha_o$ +/-: 7.7 ± 2.6 fmol/mg; $G\alpha_o$ -/-: 0.01 ± 2.49 fmol/mg; $F(2,5) = 8.770$, $p = 0.035$).

In spinal cord homogenates, basal levels of [³⁵S]GTPγS incorporation (Table 3) were not different between $G\alpha_o$ +/- mice and their wild-type littermates ($t(4) = 0.6431$, $p = 0.555$), suggesting that $G\alpha_o$ is not as important for basal G protein activity in the spinal cord. DAMGO-stimulated binding (Figure 6; Table 3) was significantly reduced in $G\alpha_o$ +/- mice when compared with wild-type

Table 3 Properties of Agonist-Stimulated [³⁵S]GTP γ S Binding in Membranes from Whole Brain of Wild-Type and G α_o Transgenic Mice

Tissue	Genotype	Basal [³⁵ S]GTP γ S binding (fmol/mg protein)		DAMGO		Morphine		DAMGO-stimulated [³⁵ S]GTP γ S saturation binding	
		E_{max} (fmol/mg protein)	EC_{50} (nM)	E_{max} (fmol/mg protein)	EC_{50} (nM)	E_{max} (fmol/mg protein)	EC_{50} (nM)	B_{max} (pmol/mg protein)	K D (nM)
Whole brain	Wild type	62.2 ± 2.5	287 ± 91	71.3 ± 3.2	287 ± 91	39.4 ± 4.5	165 ± 14	8.31 ± 1.01	14.2 ± 4.0
	G α_o +/-	49.1 ± 5.8	382 ± 23	59.4 ± 4.0	382 ± 23	25.6 ± 3.9	350 ± 76	4.26 ± 1.31	16.5 ± 6.7
	G α_o -/-	22.2 ± 0.3***+	NC	13.1 ± 2.3****+++	NC	5.2 ± 1.0**	NC	1.18 ± 0.53*	7.1 ± 4.4
Spinal cord	Wild type	59.0 ± 10.9	ND	46.4 ± 5.4	ND	36.3 ± 4.0	ND	ND	ND
	G α_o +/-	49.0 ± 11.1	ND	35.0 ± 6.7#	ND	19.5 ± 3.1##	ND	ND	ND

Abbreviations: NC, not calculated; ND, not determined.

Agonist-stimulated [³⁵S]GTP γ S binding is defined as the increase in [³⁵S]GTP γ S incorporation in the presence of agonist, over that of basal (measured in the absence of agonist). Data represent the mean ± SEM ($n = 2-3$ performed in duplicate). Symbols indicate a statistical difference vs wild type whole brain (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) or G α_o +/- whole brain (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) by Bonferroni's post-test or vs wild-type spinal cord by Student's paired t -test (# $p < 0.05$, ## $p < 0.01$).

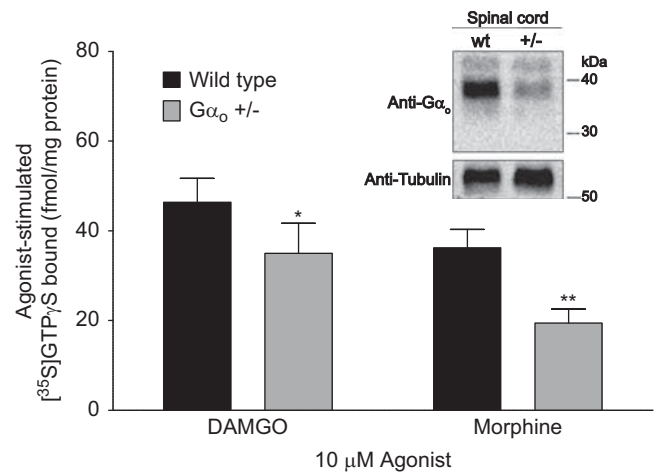


Figure 6 DAMGO- and morphine-stimulated G protein activity in spinal cord homogenates from G α_o transgenic mice. [³⁵S]GTP γ S (0.1 nM) incorporation stimulated by 10 μ M DAMGO or morphine was evaluated in membrane homogenates from spinal cord of wild-type and G α_o +/- mice. Nonspecific binding was evaluated in the presence of unlabeled GTP γ S (10 μ M). Data are plotted as agonist-stimulated [³⁵S]GTP γ S binding, defined as the increase in [³⁵S]GTP γ S binding in the presence of agonist over that of basal (measured in the absence of agonist), and represent the mean ± SEM ($n = 3$ performed in quadruplicate). Asterisks indicate a statistical difference vs wild type by Student's paired t -test (* $p < 0.05$, ** $p < 0.01$). Inset, representative western blot in spinal cord membranes showing reduced G α_o protein expression in G α_o +/- mice (+/-) when compared with wild-type (wt) controls; membranes were probed for tubulin as a loading control.

controls ($t(2) = 7.072$, $p = 0.019$). Similarly, morphine-stimulated [³⁵S]GTP γ S binding in spinal cord (Figure 6; Table 3) showed a decrease in G α_o +/- mice, as compared with wild-type littermates ($t(2) = 17.54$, $p = 0.003$). Western blot analysis of G α_o expression in spinal cord membranes confirmed that there was a significant reduction in G α_o protein levels in these samples, as compared with the loading control tubulin (Figure 6, inset).

DISCUSSION

This study shows that reduction in the expression of the inhibitory G α isoform, G α_o , attenuates MOR agonist-mediated antinociception in mice at both the supraspinal and the spinal level. However, whether a genotype-dependent difference was seen depended on the efficacy of the agonist and the strength of the noxious stimulus; a greater effect of the G α_o +/- genotype was manifested in the presence of the partial agonist nalbuphine or against a higher temperature stimulus. In contrast, there were no differences observed in the antinociceptive response to morphine in mice that were null for either G α_{i2} or G α_{i3} , compared with their respective wild-type littermates, at either the supraspinal or the spinal level. Furthermore, the loss of G α_o protein in G α_o -/- mice resulted in a decrease in G β and G γ expression, a reduction in the number of high-affinity MOR-binding sites, and consequently, attenuation of MOR agonist-stimulated [³⁵S]GTP γ S binding. Together, these results provide strong evidence that MOR coupling to G α_o is important for opioid antinociception.

MOR Agonist-Mediated Antinociception

In wild-type mice, there was no difference in the potency of morphine observed at the higher hot plate temperature of 55 °C when compared with 52 °C, and morphine remained fully effective at both temperatures. However, this effect of temperature was exaggerated in G α_o +/- mice such that a larger shift in the potency of morphine was realized at the higher hot plate temperature, and even at 100 mg/kg, full antinociception was not attained. This suggests a reduced efficiency of antinociceptive processing in the G α_o +/- mice, leading to a higher agonist efficacy requirement. In confirmation of this, methadone, which has higher efficacy than morphine (Adams *et al*, 1990; Peckham and Traynor, 2006; McPherson *et al*, 2010), showed a smaller genotype difference. These findings confirm a role for G α_o in opioid agonist-mediated supraspinal antinociception against a thermal stimulus, but also indicate that in the G α_o +/- mice, sufficient G α_o protein remains to give a robust response and/or that other G $\alpha_{i/o}$ proteins are involved in the response. However, this latter suggestion is less likely given the absence of a difference between G α_{i2} or G α_{i3} null mice and their wild-type littermates and the lack of compensatory changes in the expression of other G $\alpha_{i/o}$ proteins in G α_o null mice.

Surprisingly, in light of our findings using the hot plate test, but in agreement with previous ODN studies (Raffa *et al*, 1994; Sanchez-Blazquez *et al*, 1995, 2001; Standifer *et al*, 1996), we did not observe a genotype-dependent difference in the ability of systemic morphine to produce antinociception between wild-type and G α_o +/- mice using the tail withdrawal test. However, we did see a profound shift in the potency of the partial agonist nalbuphine, which has lower efficacy than morphine (Dykstra *et al*, 1997; Selley *et al*, 1998). This suggests, as with the hot plate test, that the relationship between the strength of the noxious stimulus and the efficacy of the ligand determines if a genotype difference is observed. These findings imply that blockade of spinal nociception, as measured in the tail withdrawal test, requires less agonist efficacy. As a result, even with a large reduction in G α_o protein, the system is still able to function efficiently.

Previous studies have shown that ODN knockdown of G α subunits inhibits antinociception in an agonist-specific manner, suggesting that different agonists may cause MOR to signal through different G α proteins. For example, antinociception induced by the partial agonist buprenorphine in the warm-water tail withdrawal test was significantly reduced after administration of antisense ODNs targeting G α_{i2} , G α_{i3} , G α_{o2} , G α_z , or G α_q , whereas morphine antinociception was only attenuated in the presence of ODNs targeting G α_{i2} or G α_z (Sanchez-Blazquez *et al*, 2001). However, in our study, morphine antinociception in the tail withdrawal test was not altered on loss of G α_o , G α_{i2} , or G α_{i3} . Our findings indicate this may be due to differences in relative agonist efficacy, which suggests that there is a G α_o protein reserve for full agonists such that even a significant knockdown of G α_o does not necessarily alter the ability of morphine to elicit antinociception, whereas a partial agonist, such as nalbuphine, is more susceptible. Indeed, Standifer *et al* (1996) reported a reduction in morphine antinociception in the radiant-heat tail flick assay in mice

exhibiting >60% knockdown of G α_o . On the other hand, the reason(s) why knockdown of G α_{i2} and other G α subunits affected antinociception in a ligand-dependent manner in previous studies is not clear, but may be due to differences in the route of administration (central *vs* peripheral) or the approach used (ODN *vs* constitutive knockdown). For example, in our constitutive knockdown, although no compensatory changes in G $\alpha_{i/o}$ protein expression were observed, other developmental changes may have occurred to substitute for the loss of G α_o specifically.

MOR-Dependent G Protein Activation

Loss of G α_o , as determined by western blot, was accompanied by a reduction in both G β and G γ subunits. Valenzuela *et al* (1997) observed a similar decrease in G β protein in ventricular membranes from a separately generated G α_o -/- mouse. This reduction in G $\beta\gamma$ is likely due to the instability of these subunits in the absence of sufficient concentrations of G α protein (Hwang *et al*, 2005). A mechanism of regulated G α and G $\beta\gamma$ expression would prevent the accumulation of free G $\beta\gamma$ dimers that are functionally competent in the absence of receptor agonist (Jiang *et al*, 1998). Reductions in free G β and G γ levels were not observed in brains from mice lacking either G α_{i2} or G α_{i3} (data not shown), presumably due to the lower expression levels of these G α proteins.

This decrease in G α_o and accompanying G β and G γ subunits, in addition to reducing the antinociceptive response, also reduced the ability of MOR agonists to stimulate [³⁵S]GTP γ S incorporation in whole brain or spinal cord homogenates. Indeed, DAMGO- and morphine-stimulated binding of 0.1 nM [³⁵S]GTP γ S was abolished in whole brain homogenates from G α_o -/- mice, confirming the importance of G α_o for MOR signaling (Jiang *et al*, 1998, 2001). The reduction in G α_o and cognate G β and G γ subunits also resulted in a decrease in high-affinity MOR-binding sites, but not total MOR sites, suggesting a reduction in heterotrimeric G protein coupling. However, high-affinity MOR binding was still present in the complete absence of G α_o , which could indicate that other G $\alpha_{i/o}$ subunits are taking the place of G α_o and providing a functional compensation, although there were no obvious increases in the levels of these isoforms. Indeed, analysis of DAMGO-stimulated [³⁵S]GTP γ S saturation binding revealed a G α protein to high-affinity MOR ratio (G α :MOR) of approximately 34:1 in wild-type mice, compared with 24:1 in G α_o +/- mice and 10:1 in G α_o -/- mice. These results suggest that, in the brain, G α proteins other than G α_o are able to form complexes with MOR. Such complexes might also help to translocate MOR to the cell surface, as with delta-opioid receptor (DOR)/G α_{i2} complexes that are preassembled in secretory vesicles before delivery to the plasma membrane (Zhao *et al*, 2011). However, G protein was not required for DOR translocation; if this was also true for MOR, it would explain the high level of low-affinity MOR present in the G α_o -/- mice.

In spinal cord homogenates, both total and high-affinity MOR numbers are considerably less than in whole brain of wild-type mice. Furthermore, there was no change in MOR expression observed in spinal cord tissue from G α_o +/-

mice. This could be because of an overabundance of G α_o compared with MOR in the spinal cord. It is unlikely that other G α subunits are making a bigger contribution in the spinal cord given that there is no difference in morphine antinociception in the tail withdrawal test between G α_{i2} or G α_{i3} null mice and their wild-type littermates. Similarly, differences between supraspinal and spinal antinociceptive circuitry have been demonstrated in a G α_z -deficient mouse (Hendry *et al*, 2000), although the mechanisms underlying these supraspinal *vs* spinal differences were not further characterized. Together, these findings suggest that MOR signaling in the spinal cord may be more efficient, such that full behavioral responses can be achieved at much lower MOR expression and/or on activation of a smaller fraction of the total pool of G proteins.

Concluding Remarks

The present results using G α_o +/– mice demonstrate that G α_o has an important role in opioid antinociception. Moreover, changes observed in opioid antinociception in G α_o +/– mice were paralleled by similar alterations in opioid-dependent signaling at the cellular level. This conclusion is further supported by the recent work of Kest *et al* (2009), who showed that G α_o expression modulates opioid dependence in mice by targeted knockdown of G α_o mRNA, which reduced the expression of withdrawal after chronic heroin or morphine. However, despite the strong evidence linking G α_o to opioid antinociception, these findings cannot be taken as absolute proof that MOR coupling to G α_o is required for morphine analgesia. G α_o is important for the signaling and activity of many neurotransmitter receptors in the central nervous system (reviewed in Jiang and Bajpayee, 2009). Thus, it is possible that non-opioid pathways are compromised in the G α_o +/– mice and contribute to the altered antinociceptive responses (Connor and Christie, 1999). These and other questions related to the consequences of regional knockdown of G α_o will be addressed in future studies. Nevertheless, the finding that in addition to antinociception, both high-affinity MOR expression and MOR agonist-stimulated G protein activity are reduced strongly supports the notion that the G α_o -MOR complex has a key role in opioid antinociception.

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DISCLOSURE

The authors declare no conflict of interest.

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