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Role of the guanine nucleotide binding protein, $G\alpha_o$, in the development of morphine tolerance and dependence

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Abstract

Rationale—The use of morphine and other opioids for chronic pain is limited by the development of analgesic tolerance and physical dependence. Morphine produces its effects by activating the μ opioid receptor, which couples to $G\alpha_{i/o}$ -containing heterotrimeric G proteins. Evidence suggests that the antinociceptive effects of morphine are mediated by $G\alpha_o$. However, the role of $G\alpha_o$ in the development of morphine tolerance and dependence is unknown.

Objectives—Evaluate the contribution of $G\alpha_o$ to the development of morphine tolerance and dependence in mice.

Methods—129S6 mice lacking one copy of the $G\alpha_o$ gene ($G\alpha_o$ +/–) were administered morphine acutely or chronically. Mice were examined for tolerance to the antinociceptive action of morphine using the 52°C hot plate as the nociceptive stimulus and for dependence by evaluating the severity of naltrexone-precipitated withdrawal. Wild type littermates of the $G\alpha_o$ +/– mice were used as controls. Changes in μ receptor number and function were determined in midbrain and hindbrain homogenates using radioligand binding and μ agonist-stimulated [³⁵S]GTP γ S binding, respectively.

Results—Following either acute or chronic morphine treatment, all mice developed antinociceptive tolerance and physical dependence, regardless of genotype. With chronic morphine treatment, $G\alpha_o$ +/– mice developed tolerance faster and displayed more severe naltrexone-precipitated withdrawal than did wild type littermates. Morphine tolerance was not associated with changes in μ receptor number or function in brain homogenates from either wild type or $G\alpha_o$ +/– mice.

Conclusions—These data suggest that the guanine nucleotide binding protein $G\alpha_o$ offers some protection against the development of morphine tolerance and dependence.

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Keywords

antinociception; G protein; morphine; μ receptor; receptor binding; tolerance; withdrawal

Introduction

Morphine is a highly effective analgesic drug. Unfortunately, chronic use of morphine and other opioids results in the development of behavioral adaptations such as tolerance and dependence, which reduce the effectiveness of these drugs over the long-term. In humans, morphine analgesic tolerance is characterized by a decrease in analgesic potency such that higher doses of morphine (in some cases more than 10-fold) are required for the continued therapeutic management of pain (Buntin-Mushock et al. 2005). Further, repeated use of morphine results in physical dependence, which is characterized by an unpleasant, though non-life threatening withdrawal syndrome upon abrupt discontinuation of treatment (Farrell 1994; Kosten and George, 2002). Despite all we know regarding morphine tolerance and dependence, the inherent complexity of these adaptations requires that they be further studied before better analgesic treatments that are devoid of such adaptations can be proposed.

Morphine elicits the majority of its behavioral effects, including physical dependence, by activating the μ opioid peptide (MOP) receptor (μ receptor) (Matthes et al. 1996), a member of the GPCR superfamily of 7-transmembrane domain receptors (Huang et al. 2015). GPCRs are characterized by their coupling to heterotrimeric G proteins composed of a $G\alpha$ subunit, bound to a $G\beta\gamma$ heterodimer. At the cellular level, acute stimulation of μ receptors by morphine results in the activation of $G\alpha_{i/o}$ -containing G proteins that inhibit the activity of adenylate cyclase, activate K^+ channels, inhibit Ca^{2+} channels, and stimulate mitogen-activated kinase phosphorylation, among other pathways (Law et al. 2000). Coupling of the μ receptor to $G\alpha_{i/o}$ proteins, and in particular, $G\alpha_o$, is important for the production of μ agonist-mediated antinociception in mice (Lamberts et al. 2011).

Chronic/continuous morphine exposure has been associated with the uncoupling of several μ receptor-dependent, $G\alpha_{i/o}$ -mediated signaling pathways in both mice and rats (Bagley et al., 2005a; Eitan et al. 2003; Fyfe et al. 2010). This loss of μ receptor signaling following chronic morphine treatment is thought to result from either a reduction in the expression of $G\alpha_{i/o}$ subunits (Selley et al. 1997; Yoburn et al. 2003) and/or a decrease in the interaction between μ receptors and $G\alpha_{i/o}$ proteins (Elliott et al. 1997; Maher et al. 2001; Sim-Selley et al. 2007; Sim et al. 1996; Smith et al. 2007; Wang et al. 2005).

On the other hand, μ receptor uncoupling from $G\alpha_{i/o}$ proteins is not always observed following chronic morphine treatment (Contet et al. 2008; Madia et al. 2011), and in fact several studies have reported that chronic morphine induces the upregulation of $G\alpha_{i/o}$ protein expression in various brain regions (Fabian et al. 2002; Nestler et al. 1989; Terwilliger et al. 1991; Wang et al. 2004). There is also evidence of enhanced μ receptor-dependent signaling *via* $G\alpha_{i/o}$ following chronic morphine treatment (Ingram et al. 2008). Moreover, both knockdown and overexpression of $G\alpha_{i2}$ have been shown to ameliorate morphine antinociceptive tolerance in mice (Garzon and Sanchez-Blazquez 2001; Sanchez-

Blazquez and Garzon 1994; Yoburn et al. 2003). Thus, although $G\alpha_{i/o}$ activation represents a critical first step in the initiation of μ receptor signaling, the role of this early phase of μ receptor activity in the adaptations associated with chronic morphine use remains controversial.

We have previously demonstrated that mice with a 50% reduction in $G\alpha_o$ protein ($G\alpha_o +/-$ mice) exhibit a significant decrease in morphine-mediated antinociception (Lamberts et al. 2011). Mice expressing $G\alpha_o$ proteins that are insensitive to negative modulation by regulator of G protein signaling proteins also show alterations in morphine-mediated antinociception (Lamberts et al., 2013). Furthermore, it has been hypothesized that genetic variation in the expression level of the $G\alpha_o$ gene, *Gnao1*, determines the severity of morphine dependence (Kest et al. 2009). Here, we determined whether $G\alpha_o$ plays a role in the adaptations that accompany chronic morphine treatment by evaluating the development of morphine tolerance and dependence in $G\alpha_o +/-$ mice alongside their wild type littermate controls. Additionally, biochemical endpoints were examined in brain homogenates from mice treated chronically with morphine to determine whether morphine tolerance was associated with changes in μ receptor number and G protein activation in brain regions that are thought to play a role in the development of antinociceptive tolerance to morphine (Morgan et al. 2005).

Materials and methods

Transgenic mice

$G\alpha_o +/-$ mice were generated on a pure 129S6 background as described (Duan et al., 2007; Lamberts et al., 2011). $G\alpha_o$ homozygous null ($G\alpha_o -/-$) mice do not frequently survive until weaning (Lamberts et al. 2011) and so were not examined in this study. $G\alpha_o +/-$ mice exhibit 50% of the $G\alpha_o$ protein levels of their wild-type littermates (Lamberts et al., 2011). Mice were group-housed in a facility where lights were maintained on a 12 h light/dark cycle (lights on at 07:00) and had unlimited access to food and water. Opioid-naïve mice between 10 and 20 weeks of age were utilized for all experiments and all testing was performed between 07:00 and 19:00. Male mice were used for all behavioral testing; due to a limited availability of transgenic mice, both male and female mice were used in the biochemical studies. All protocols were approved by the University of Michigan Committee on the Use and Care of Animals and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

Behavior

Hot plate test—For all tolerance studies, morphine antinociception was evaluated using the 52°C hot plate test as previously described (Lamberts et al. 2011). Hot plate responses were measured by placing mice on a hot plate analgesia meter maintained at $52.0 \pm 0.2^\circ\text{C}$ (Columbus Instruments, Columbus, OH), and the latency to lick forepaw(s) or jump was measured. The cutoff latency was set at 60 s to prevent tissue damage. Data are plotted as %MPE, where %MPE = (Drug latency – Baseline latency) / (Cutoff latency – Baseline latency).

Acute morphine treatment—Acute antinociceptive tolerance was induced in male wild type and $G\alpha_o$ +/- mice through administration of a single injection of morphine (128 mg/kg, i.p.). After 6 h, hot plate latencies were recorded immediately prior to a 10 mg/kg morphine (i.p.) challenge to determine baseline latency and again 30 min after mice received the challenge. Control mice were administered saline (i.p.) 6 h prior to the 10 mg/kg morphine challenge.

To measure acute physical dependence, wild type and $G\alpha_o$ +/- mice that received both the morphine treatment (128 mg/kg, i.p.) and the morphine challenge (10 mg/kg, i.p.) were injected 2 h later with the nonselective opioid receptor antagonist naltrexone (10 mg/kg, s.c.) to precipitate morphine withdrawal. Withdrawal behaviors were counted for 30 min as described below.

Chronic morphine treatment—Chronic antinociceptive tolerance was elicited in male wild type and $G\alpha_o$ +/- mice during an 8-day morphine treatment scheme. On day 1, morphine antinociception was evaluated using a cumulative dosing paradigm. The cumulative dosing procedure was chosen to minimize the total number of animals needed for this study, given the limited availability of transgenic mice. Briefly, mice were given two injections of saline (i.p.) to determine baseline latency, followed by three cumulative doses of morphine [10 mg/kg, 22 mg/kg (total 32 mg/kg), and 68 mg/kg (total 100 mg/kg), i.p.] in 30 min intervals, and hot plate latencies were recorded 30 min after each injection. The same dosing procedure was used for all mice. On day 2, mice received two injections of either 56 or 128 mg/kg morphine (i.p.) separated by 12 h. Control mice received two injections of saline (i.p.) according to the same schedule. Twice-daily injections continued for a total of 6 days, ending on day 7. Hot plate latencies were recorded immediately prior to each morning injection of saline or morphine to determine baseline latency and again 30 min after mice received the injection. On day 8, morphine antinociception was re-evaluated in the hot plate test using cumulative dosing, either 10–100 mg/kg morphine, as above, or 32–320 mg/kg morphine [32 mg/kg, 68 mg/kg (total 100 mg/kg), and 220 mg/kg (total 320 mg/kg), i.p.] Tolerance ratios were calculated similarly to Stevens and Yaksh (1989) for purposes of qualitative comparison only.

To measure physical dependence, wild type and $G\alpha_o$ +/- mice that received twice-daily injections of either 56 or 128 mg/kg morphine were injected with naltrexone (10 mg/kg, s.c.) 2 h after the final cumulative morphine dose on day 8 to precipitate morphine withdrawal. Withdrawal behaviors were then counted for 30 min as described below.

Morphine withdrawal scores—Morphine withdrawal behaviors were scored essentially as described (Divin et al. 2008), with slight modifications. Immediately following injection of naltrexone (10 mg/kg, s.c.), mice were placed individually in empty Plexiglas boxes and withdrawal behaviors were observed for 30 min. The number of occurrences of jumping, wet dog shakes, and paw tremors was recorded in 5 min intervals and totaled for the full 30 min observation period. The presence of ptosis, chewing, and diarrhea was scored during each 5 min interval, as follows: intervals during which the behavior was absent were given a score of 0, whereas intervals in which the behavior was present were given a score of 1, for a maximum possible score of 6 during the 30 min observation period. Mice were also weighed

immediately preceding and immediately following the 30 min observation period, and weight loss was calculated as % decrease in body weight.

Biochemical assays

Tissue collection and preparation—For biochemistry studies, male and female wild type and $G\alpha_o$ +/- mice were treated chronically with morphine and brain tissue was removed and stored for future analysis. On Day 1, mice were treated with increasing cumulative doses of morphine (10–100 mg/kg, i.p.), as above. Mice were then treated twice-daily for 6 days (Days 2–7) with 128 mg/kg morphine (i.p.). Control mice received an equivalent number of saline injections (i.p.) on Day 1, followed by twice-daily injections of saline (i.p.) on Days 2–7. On the morning of Day 8, mice were euthanized and a midbrain section containing the periaqueductal gray (PAG) and a hindbrain section containing the rostral ventromedial medulla (RVM) were dissected out and rapidly frozen in isopentane. Brain tissue was then stored at -80°C until use.

Frozen brain tissue was brought to 4°C in ice-cold 50 mM Tris, pH 7.4 (Tris buffer), and membrane homogenates were prepared as previously described (Lester and Traynor 2006). Final membrane pellets were resuspended in Tris buffer and assayed immediately. Total protein in the samples was determined using the BCA protein assay (ThermoFisher Scientific, Waltham, MA).

Agonist-stimulated [^{35}S]GTP γ S binding assay to assay μ -receptor activity—Incorporation of the slowly-hydrolyzed GTP analog [^{35}S]GTP γ S into activated $G\alpha$ subunits was monitored *in vitro* (Traynor and Nahorski 1995). Brain homogenates prepared as above (10 μg protein) were incubated for 2 h at 25°C in [^{35}S]GTP γ S binding buffer (50 mM Tris, 5 mM MgCl_2 , 100 mM NaCl and 1 mM EDTA, pH 7.4, with 2 mM dithiothreitol, 100 μM GDP and 0.4 U/mL adenosine deaminase) with 0.1 nM [^{35}S]GTP γ S and a maximal concentration (10 μM) of either DAMGO or morphine. Non-specific binding was evaluated in the presence of 10 μM unlabeled GTP γ S. Reactions were stopped by rapid filtration through GF/C filtermats (Whatman, Kent, UK) using a Brandel MLR-24 harvester (Brandel, Gaithersburg, MD), and bound radioactivity was determined by liquid scintillation counting using a Wallac 1450 MicroBeta counter (PerkinElmer, Waltham, MA).

Radioligand binding assays—Total opioid receptor expression was evaluated by incubating brain homogenates (50 μg protein) in Tris (4 ± 0.4 nM) of the radiolabeled, nonselective opioid receptor antagonist [^3H]-diprenorphine (DPN). Expression of the μ receptor was defined using the μ receptor-selective antagonist CTAP (300 nM). To measure high-affinity μ receptor expression, brain membrane homogenates (50 μg protein) were incubated in Tris buffer with a saturating concentration (12 ± 1.2 nM) of the radiolabeled μ receptor-selective agonist [^3H]DAMGO. All binding reactions were incubated for 60 min at 25°C . Non-specific binding was evaluated in the presence of the nonselective opioid receptor antagonist naloxone (10 μM). Binding reactions were stopped by rapid filtration and bound radioactivity was measured by liquid scintillation counting, as above.

Data analysis

All data were analyzed using GraphPad Prism 6 software (San Diego, CA). Differences between groups were evaluated using unpaired *t*-test or two-way ANOVA with Bonferroni's post-test, where appropriate. For all statistical tests, significance was set at $p < 0.05$. ED₅₀ (potency) was calculated by fitting the compiled antinociception data to an agonist *versus* response curve (Hill slope=1). All data are reported as the mean $\pm$ SEM.

Drugs and chemicals

Morphine sulfate was from RTI (Research Triangle Park, NC) and naltrexone hydrochloride was from Endo Pharmaceuticals (Newark, DE). For behavioral experiments drugs were diluted in sterile water. Radiochemicals including [³H]DPN, [³H]DAMGO, and [³⁵S]GTP γ S, were purchased from PerkinElmer. Adenosine deaminase was obtained from Calbiochem (San Diego, CA). DAMGO, CTAP, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

Results

Baseline characteristics of the G α_o +/- mice

As previously reported, G α_o +/- mice did not display any gross behavioral abnormalities, and their body weight at weaning was equivalent to that of wild type littermates (Lamberts et al., 2011). Although G α_o +/- mice exhibit a significant decrease in morphine-mediated antinociception, they do not differ from wild type mice in terms of baseline nociceptive threshold in the 52°C hot plate test (wild type 14.0 ± 0.3 s; G α_o +/- 14.0 ± 0.5 s) in agreement with our previous findings (Lamberts et al., 2011).

Acute morphine tolerance and dependence

Mice were first subjected to a short (acute) tolerance paradigm, in which they were given a single dose of 128 mg/kg morphine or saline and challenged with morphine (10mg/kg, i.p.) 6 h later. The 128 mg/kg dose of morphine corresponds to the ED₁₀₀ and ED₈₀ dose for antinociception in the 52°C hot plate test in wild type and G α_o +/- mice, respectively. Although this high dose produced some observable locomotor suppression, it did not result in overdose-induced death in these mice. This is consistent with a higher morphine requirement in several 129 mouse strains (Kest et al, 2002).

Wild-type mice challenged with morphine (i.p.) showed a $57 \pm 26\%$ decrease in antinociception in the 52°C hot plate test compared with mice that had received saline (Figure 1a). Similarly, morphine-treated G α_o +/- mice exhibited an $86 \pm 37\%$ reduction in morphine antinociception, as compared to saline-treated G α_o +/- mice (Figure 1a). These values are not different ($p = 0.55$). Thus, a single morphine injection was capable of producing acute tolerance to a challenge dose of morphine given 6 h later in both wild type and G α_o +/- mice [main effect of treatment: $F(1,29)=19.8$, $p < 0.001$]. The degree of morphine antinociception was lower in the G α_o +/- mice compared to their wild-type littermates, independent of pretreatment condition [main effect of genotype: $F(1,29)=6.74$, $p=0.015$], as expected from previous work (Lamberts et al. 2011). The level of antinociception after morphine treatment was not significantly different across the genotypes

[treatment $\times$ genotype interaction: $F(1,29)=0.00$, $p=0.987$], indicating that under these conditions, a similar level of antinociception was obtained between wild type and $G\alpha_o$ +/- mice.

Following the morphine challenge, mice were given the non-selective opioid receptor antagonist naltrexone (10 mg/kg, s.c.) to precipitate a withdrawal syndrome, and withdrawal signs were scored for 30 min. The number of naltrexone-elicited behaviors was significantly greater following acute morphine treatment than following acute saline injection for both wild type and $G\alpha_o$ +/- mice (data not shown). Neither wild type nor $G\alpha_o$ +/- mice exhibited any jumping behavior when withdrawal was precipitated following acute morphine treatment, but wet dog shakes, paw tremors, ptosis, chewing behavior, and diarrhea were observed (Figure 1b). Only diarrhea was different between genotypes, with $G\alpha_o$ +/- mice exhibiting significantly more diarrhea during withdrawal from acute morphine compared with wild type littermates ($t(11)=3.47$, $p=0.005$; Figure 1b).

Chronic morphine tolerance

Mice were randomly assigned to receive repeated injections of saline, 56 mg/kg/injection morphine or 128 mg/kg/injection morphine (i.p.). In preliminary studies, 56 and 128 mg/kg morphine corresponded to the ED_{80} dose for antinociception in the 52°C hot plate test in wild type and $G\alpha_o$ +/- mice, respectively.

Morphine-dose response curves were established for all mice on day 1 and again on day 8 following twice-daily treatment with saline, 56 mg/kg morphine or 128 mg/kg morphine (Figure 2a-b). Morphine potency was determined by calculating the ED_{50} , and potencies measured on day 8 were compared with the initial potency measured on day 1 (Table 1). On Day 1, the potency of morphine was significantly lower in the $G\alpha_o$ +/- mice than in their wild-type littermates ($t(49)=9.00$, $p<0.001$; Table 1), as predicted from a previous study (Lamberts et al. 2011). Morphine antinociception was decreased in $G\alpha_o$ +/- mice compared with wild type littermates, independent of treatment [main effect of genotype: $F(1,45)=456$, $p<0.01$]. In both wild type and $G\alpha_o$ +/- mice, chronic morphine treatment produced a dose-dependent decrease in morphine potency, indicating the presence of tolerance [main effect of treatment: $F(2,45)=1667$, $p<0.01$]. However, the degree of tolerance was significantly lower in $G\alpha_o$ +/- mice [treatment $\times$ genotype interaction: $F(2,45)=498$, $p<0.01$]. Bonferroni's post-hoc analysis revealed a significant decrease in morphine potency in wild type mice treated with either 56 mg/kg ($p<0.001$) or 128 mg/kg morphine ($p<0.001$), whereas in $G\alpha_o$ +/- mice, morphine potency was only reduced following 128 mg/kg morphine treatment ($p<0.001$; Table 1).

Comparison of the time course of tolerance development to these same doses of morphine (56 mg/kg and 128 mg/kg) in $G\alpha_o$ +/- mice and wild-type littermates was determined by measuring hot plate latency over 6 days, 30 min after each first daily injection of morphine (Figure 2c-d). Morphine antinociception significantly declined over time in wild type mice treated with either 56 mg/kg or 128 mg/kg in a similar manner [main effect of treatment: $F(2,138)=83.5$, $p<0.001$; main effect of day: $F(5,138)=4.87$, $p<0.001$; treatment $\times$ day interaction: $F(10,138)=0.655$, $p=0.764$]. Bonferroni's post-test demonstrated a significant decrease in morphine antinociception on days 5–7 in wild type mice treated with either 56

mg/kg ($p<0.05$) or 128 mg/kg morphine ($p<0.05$; Figure 2c). Morphine antinociception also decreased over time in $G\alpha_o$ +/- mice; this effect was exaggerated in those mice treated with 128 mg/kg morphine compared with the 56 mg/kg treatment group [main effect of treatment: $F(2,132)=64.4$, $p<0.001$; main effect of day: $F(5,132)=5.49$, $p<0.001$; treatment $\times$ day interaction: $F(10,132)=2.01$, $p=0.037$]. Post-hoc analysis revealed a significant decrease in morphine antinociception on days 3–7 in $G\alpha_o$ +/- mice treated with 128 mg/kg morphine ($p<0.01$; Figure 2d). In contrast, $G\alpha_o$ +/- mice exhibited more variable effects of 56 mg/kg morphine treatment over time, with a significant decreased in morphine antinociception observed on days 4 ($p<0.05$) and 6 only ($p<0.001$; Figure 2d).

Withdrawal from chronic morphine is more severe in $G\alpha_o$ +/- mice

Immediately following the re-evaluation of morphine potency on Day 8 in the above experiment, mice were injected with naltrexone (10 mg/kg, s.c.), and withdrawal signs were counted during the next 30 min. In initial testing, significantly more naltrexone-elicited behaviors were observed following chronic morphine treatment than following an equivalent number of saline injections in both wild type and $G\alpha_o$ +/- mice (data not shown). Compared with wild type littermates, $G\alpha_o$ +/- mice exhibited a significantly greater number of jumps, independent of treatment [main effect of genotype: $F(1,33)=7.42$, $p=0.010$]. Post-hoc testing showed that $G\alpha_o$ +/- mice treated with 56 mg/kg morphine jumped over 4 times more often than did their wild type littermates treated with the same dose of morphine ($p<0.05$; Figure 3). Given that a similar number of jumps was observed in wild type mice treated with either 56 mg/kg or 128 mg/kg morphine [main effect of treatment: $F(1,33)=0.033$, $p=0.858$], the 56 mg/kg dose appears to be maximal in producing withdrawal effects in these mice. No significant interaction was observed [genotype $\times$ treatment interaction: $F(1,33)=1.30$, $p=0.262$].

There were also more wet dog shakes observed in $G\alpha_o$ +/- mice compared with wild type littermates, independent of treatment dose [main effect of genotype: $F(1,33)=4.69$, $p=0.038$]. Similarly, there was no difference in number of wet dog shakes between treatment groups [main effect of treatment: $F(1,33)=1.39$, $p=0.246$], nor was there a significant interaction [genotype $\times$ treatment interaction: $F(1,33)=1.39$, $p=0.246$]. There were no differences between genotypes in the occurrence of ptosis, chewing, diarrhea, or weight loss. In summary, some, but not all, withdrawal behaviors were increased in $G\alpha_o$ +/- mice compared with wild type littermates. Further, treatment twice-daily for 6 days with 56 mg/kg morphine appears to produce a maximal number of withdrawal-elicited behaviors in wild type 129S6 mice, as treatment with 128 mg/kg does not greatly increase the occurrence of these behaviors.

Chronic morphine treatment is not associated with changes at the level of the μ receptor

To evaluate if morphine tolerance and dependence were associated with changes at the receptor level in wild type and $G\alpha_o$ +/- mice, μ receptor expression and function was measured in membrane homogenates prepared from the brains of mice treated chronically with either morphine or saline (Table 2; Figure 4). After chronic treatment with saline (i.p.) or 128 mg/kg morphine (i.p.), a midbrain section containing the periaqueductal gray (PAG) and a hindbrain section containing the rostral ventromedial medulla (RVM) were removed.

Membrane homogenates prepared from these sections were subjected to radioligand binding and agonist-stimulated [35 S]GTP γ S binding analyses to measure μ receptor expression (Table 2) and μ receptor activity (Figure 4), respectively.

To measure total opioid receptor expression, brain membranes from wild type and $G\alpha_o$ +/- mice were incubated with a saturating concentration (4nM) of [3 H]DPN, which binds the μ receptor as well as δ (DOP) and κ (KOP) opioid receptors. Total μ receptor number was measured by displacing [3 H]DPN with the μ receptor-selective antagonist CTAP (300 nM). There was no effect of morphine treatment on total opioid receptor expression in either midbrain or hindbrain homogenates from wild type and $G\alpha_o$ +/- mice (Table 2). Similarly, μ receptor expression was not affected by morphine treatment in either wild type or $G\alpha_o$ +/- mice (Table 2). High-affinity μ receptor expression can be measured with the agonist [3 H]DAMGO (Lamberts et al. 2011). No effect of chronic morphine treatment was seen on the level of high-affinity μ receptors in either midbrain or hindbrain from wild type and $G\alpha_o$ +/- mice, as measured by the binding of 12 nM [3 H]DAMGO (Table 2).

As expected from previous work, (Lamberts et al., 2011) $G\alpha_o$ +/- mice (Figure 4b) exhibited a ~50% decrease in maximal [35 S]GTP γ S stimulation by either DAMGO or morphine compared to their wild-type counterparts (Figure 4a). Chronic morphine treatment did not alter maximal DAMGO- or morphine-stimulated G protein activation in either midbrain or hindbrain homogenates from wild type and $G\alpha_o$ +/- mice (Figure 4).

Discussion

In this study, we demonstrate that both wild type and $G\alpha_o$ +/- mice on a 129S6 background develop antinociceptive tolerance. There were no appreciable differences between genotypes in these adaptations following acute morphine treatment, but $G\alpha_o$ +/- mice developed tolerance to chronic morphine at a faster rate than their wild type littermates, as measured using the 52°C hot plate test. After an acute bolus dose of morphine followed by naltrexone, qualitatively and quantitatively similar withdrawal signs were observed across the genotypes, although diarrhea was more pronounced in the $G\alpha_o$ +/- mice. Naltrexone-precipitated withdrawal following long-term morphine administration was more severe in $G\alpha_o$ +/- mice, mostly due to an increase in jumping behavior. Overall, these studies add to previous literature (Kest et al. 2009) suggesting that $G\alpha_o$ protein may be protective against morphine tolerance and dependence.

Wild type 129S6 mice receiving twice daily injections of 56 mg/kg morphine developed significant antinociceptive tolerance in the 52°C hot plate test. In contrast, $G\alpha_o$ +/- mice treated similarly did not develop tolerance in the same test, although results were variable, suggesting that 56 mg/kg/injection morphine may be a sub-threshold dose for producing tolerance in mice with reduced $G\alpha_o$ protein expression. In contrast, a similar level of tolerance (~6-fold) was observed for both wild type and $G\alpha_o$ +/- mice treated with 128 mg/kg/injection morphine. Our previous work has demonstrated that the G protein: μ receptor ratio is 34:1 in wild type mice and 24:1 in $G\alpha_o$ +/- mice (Lamberts et al., 2011). The simplest hypothesis for these findings is that the ratio of G proteins to μ receptors directly determines the degree of both morphine antinociception and morphine tolerance

such that reduced antinociception at a given morphine dose translates to reduced morphine tolerance at that same dose.

Whereas morphine responsiveness in wild type mice treated with 128 mg/kg morphine was significantly attenuated beginning on Day 5, $G\alpha_o$ +/- mice treated with the same dose of morphine exhibited a decrease in antinociception as early as the second day of injections that was maintained throughout the treatment period. It is unclear why morphine tolerance would develop more quickly in $G\alpha_o$ +/- mice since antinociception is reduced in these animals (Lamberts et al. 2011). A possible explanation for this observation is that $G\alpha_o$ +/- mice exhibit enhanced μ receptor desensitization following morphine treatment. Desensitization, or reduced μ receptor coupling to $G\alpha_{i/o}$, is known to occur in the continued presence of μ receptor agonists due to processes including receptor phosphorylation and recruitment of arrestins (Gainetdinov et al., 2004; Williams et al., 2013). Thus, with the 50% reduction in $G\alpha_o$ protein in $G\alpha_o$ +/- mice, signaling systems may be less efficient and therefore have a decreased receptor reserve, which could make $G\alpha_o$ +/- mice more susceptible to the development of desensitization, and subsequently, tolerance (Morgan and Christie, 2011). On the other hand, the fact that acute antinociceptive tolerance, which is thought to more closely mimic initial μ receptor desensitization (Christie, 2008; Williams et al., 2001), was not different between wild type and $G\alpha_o$ +/- mice suggests that the mechanism by which chronic morphine tolerance develops more quickly in $G\alpha_o$ +/- mice may not involve differential desensitization.

Although the 129S6 mice in this study developed significant tolerance to chronic morphine treatment, these behavioral changes were not associated with alterations in μ receptor expression or activity in whole midbrain or hindbrain homogenates in either the $G\alpha_o$ +/- mice or their wild-type littermates. Consistent with the importance of the midbrain PAG and the hindbrain RVM for both morphine antinociception (Yaksh et al., 1988) and the development of morphine tolerance (Morgan et al., 2005), previous reports have shown that μ receptor coupling to $G\alpha_{i/o}$ proteins is reduced in these regions when rats (Sim et al., 1996; Wang et al., 2005; Smith et al., 2007) or mice (Sim-Selley et al., 2007) are treated chronically with morphine. In contrast, other studies have not found any changes in μ receptor-mediated activation of G proteins in mouse brain or spinal cord following chronic morphine treatment (Contet et al., 2008; Madia et al., 2011). Sim-Selley and colleagues (2007) have shown that the loss of agonist-stimulated [35 S]GTP γ S binding following chronic morphine treatment is highly dependent on the severity of the treatment regime and that this loss contributes to enhanced tolerance to morphine (rather than reduced tolerance). This is likely to be particularly true in 129S6 mice, which express higher levels of μ receptors compared to C57/BL6 mice (Lamberts et al., 2011), and so could possess a higher μ receptor reserve. A further caveat is that gross dissection of brain regions coupled with homogenization in the present study may mask alterations in μ receptor signaling that might have occurred within specific neuronal populations (Morgan et al., 2003; Sim-Selley et al., 2007) and/or subcellular locations (Fabian et al., 2002; Madia et al., 2011). Future studies could profitably utilize electrophysiological methods to measure μ receptor activity in specific cell types. Our results also do not rule out the possibility that there are changes downstream of G protein activation (Bagley et al. 2005a; Eitan et al. 2003; Fyfe et al. 2010). Nevertheless, it is unlikely that changes in μ receptor coupling to G protein alone can fully

account for behavioral tolerance to morphine in our model (Christie, 2008; Gintzler and Chakrabarti, 2006).

Compared with wild type littermates, $G\alpha_o$ +/- mice demonstrated enhanced diarrhea following acute morphine and increased jumping following chronic morphine, although other withdrawal measures were not different between the genotypes. Since dependence is a homeostatic response to increased signaling, this is opposite to the finding that might have been expected. Moreover, previous studies have shown that knockdown of $G\alpha_o$ expression with antisense oligonucleotides reduces the severity of naltrexone-precipitated withdrawal from chronic morphine (Kest et al., 2009; Sanchez-Blazquez and Garzon, 1994). However, there are several “anti-withdrawal” systems in the brain that, when activated, serve to dampen the severity of morphine withdrawal. Several of these systems, including the neuropeptides nociceptin/OFQ (Kest et al., 2001), galanin (Zachariou et al., 2000; 2003), activate $G\alpha_{i/o}$ -coupled receptors to oppose morphine withdrawal. In the $G\alpha_o$ +/- mice, these systems may have reduced activity such that “pro-withdrawal” systems dominate, thereby leading to a worsening of physical dependence to morphine.

As with all studies employing constitutive transgenic mice, there is the complication that $G\alpha_o$ +/- mice express reduced $G\alpha_o$ protein organism-wide and throughout development. Given that morphine tolerance and dependence are characterized by alterations at the cellular, synaptic, and neuronal network levels (Christie, 2008; Williams et al., 2001), $G\alpha_o$ +/- mice may possess unknown developmental compensations that affect the development of these behavioral adaptations, consequently an appropriate approach would be to generate a $G\alpha_o$ conditional knockout mouse. Additional future studies could address the mechanism of the enhancement in morphine dependence in $G\alpha_o$ +/- mice by evaluating changes within systems that modulate the severity of morphine withdrawal, such as GABA, orexin, δ -opioid, nociceptin and α_2 -adrenergic receptors among others (Bagley et al., 2005b; Georgescu et al., 2003; Hao et al., 2011; Kest et al., 2001; Kest et al., 1996; Levitt et al., 2011; McNally and Akil, 2002; Valeri et al., 1989; Yu et al., 2008; Zachariou et al., 2003).

Finally, a general comment on morphine tolerance and dependence in 129 mouse strains is warranted. We demonstrate that 129S6 mice develop antinociceptive tolerance to morphine in the 52°C hot plate test following either single or repeated drug administration. These findings contrast with prior evidence in the literature that 129S6 mice do not develop tolerance to morphine in either the tail flick or the hot plate test. This lack of morphine tolerance in 129S6 mice has been ascribed to be due to a defect in the NMDA receptor (Kolesnikov et al., 1998; Nitsche et al., 2002) or neuronal levels of GM1 ganglioside (Crain and Shen, 2000). However, a major difference between the current study and previous reports in which chronic morphine tolerance was not observed, is the dose of morphine used. In particular, the 129S6 mice in our study received approximately ED_{80} doses, either 56 or 128 mg/kg morphine (i.p.), twice-daily for 6 days. In contrast, mice in previous studies received 75 mg morphine (s.c. pellet) over 3 or more days (Kolesnikov et al., 1998; Nitsche et al., 2002), 2.5 mg/kg morphine (i.p.) once-daily for 5 days (Kolesnikov et al., 1998), or 3 mg/kg morphine (s.c.) once-daily for 5 days (Crain and Shen, 2000). It should be noted that Bryant et al. (2006) were also able to demonstrate chronic morphine tolerance in 129S6 mice in both the hot plate and tail flick tests by giving once-daily injections of 10 – 40

mg/kg morphine (s.c.) for 6 days followed by a single challenge injection of 7.5 mg/kg morphine (s.c.). Thus, the ability to observe morphine tolerance in 129S6 mice depends upon both the nociceptive test and the dosing regimen employed. Nonetheless, significantly higher doses of morphine are required to elicit antinociceptive tolerance in 129S6 mice as compared with more “tolerance-sensitive” strains, such as CD-1 or C57BL/6 (Kest et al., 2002). Moreover, the severity of withdrawal exhibited by 129S6 mice in our hands was similar to that reported for 129P3/J mice and is much lower than the extent of withdrawal observed in other strains (Kest et al., 2002b). Thus, 129 mouse strains may possess genetic modifiers that have a dampening effect on the development of morphine tolerance and dependence, making them more resistant to these chronic adaptations. Future work could uncover these genetic modifiers as a step toward characterizing genes that protect against the development of morphine tolerance and dependence.

In conclusion, $G\alpha_o$ +/- mice that express 50% less $G\alpha_o$ protein than their wild-type littermates showed increased sensitivity to tolerance and more severe naltrexone-precipitated withdrawal behaviors following chronic morphine treatment, thus agreeing with the findings from genetic studies suggesting that $G\alpha_o$ signaling offers some protection against morphine antinociceptive tolerance and physical dependence (Kest et al. 2009). The differences observed were quantitatively minor, which is likely because $G\alpha_o$ +/- mice only express one null allele of *Gnao1*. Nevertheless, the finding that 129S6 mice developed morphine tolerance and dependence when administered high doses of morphine indicates that the 129S6 strain of mice and knockdown of $G\alpha_o$ may be useful models for evaluating characteristics that diminish or enhance morphine tolerance and dependence, respectively. The identification of factors that counteract morphine tolerance and dependence could help uncover novel targets for the development of opioid analgesics that are devoid of these adaptations.

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Highlights

- Morphine tolerance and dependence occur 129S6 mice
- $G\alpha_o$ heterozygous null mice develop morphine tolerance more quickly than wild type
- Withdrawal from chronic morphine is more severe in $G\alpha_o$ heterozygous null mice
- Chronic morphine does not alter μ -opioid agonist activation of G proteins in brain membranes

Figure 1.

Acute morphine tolerance and dependence. (a) Antinociception was measured in the 52°C hot plate test 30 min following a challenge injection of morphine (10 mg/kg) in wild type and $G\alpha_o$ +/- mice that were treated with either saline ($n=6-7$) or 128 mg/kg morphine ($n=10$) 6 h previously. ** $p<0.01$ compared with saline-treated mice of the same genotype by Bonferroni's post-test. (b) naltrexone (10 mg/kg)-precipitated withdrawal in wild type ($n=6$) and $G\alpha_o$ +/- mice ($n=7$) that were treated with 128 mg/kg morphine and challenged with 10 mg/kg morphine 6 h later. Withdrawal behaviors were scored for a period of 30 min (see

Materials and methods). ** $p < 0.01$ compared with wild type by unpaired t -test. All data are plotted as the mean $\pm$ SEM.

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Figure 2.

Chronic morphine tolerance. Morphine-mediated antinociception in (a) wild type and (b) $G\alpha_o$ +/- mice treated twice-daily with saline, 56 mg/kg morphine or 128 mg/kg morphine. Morphine antinociception was evaluated in the 52°C hot plate test using a cumulative dosing procedure in all mice on Day 1 and again in the same mice on Day 8 following chronic administration of saline, 56 mg/kg morphine or 128 mg/kg morphine. See Table 1 for numbers of subjects per group and statistical analysis. Time course of morphine-mediated antinociception in (c) wild type and (d) $G\alpha_o$ +/- mice treated twice-daily with saline, 56 mg/kg morphine or 128 mg/kg morphine. Antinociception was measured as above, 30 min following each morning injection of morphine. * p <0.05, ** p <0.01, *** p <0.001 compared with antinociception on Day 2 within the same treatment group by Bonferroni's post-test. All data are plotted as the mean $\pm$ SEM.

Figure 3.

Chronic morphine dependence. Naltrexone (10 mg/kg)-precipitated withdrawal in wild type ($n=9-10$) and $G\alpha_o +/ -$ mice ($n=9$) that were treated twice-daily with either 56 mg/kg morphine or 128 mg/kg morphine. Withdrawal behaviors were scored for a period of 30 min (See Materials and methods). * $p<0.05$ compared with wild type mice that received the same chronic treatment by Bonferroni's post-test. All data are plotted as the mean $\pm$ SEM.

Figure 4.

μ -agonist-stimulated G protein activity. Whole midbrain and hindbrain homogenates were prepared from (a) wild type ($n=3-4$) or (b) $G\alpha_o$ +/- mice ($n=3-4$) following twice-daily treatment with saline or 128 mg/kg morphine, and membranes prepared from these sections were evaluated for 10 μ M DAMGO- or 10 μ M morphine-stimulated [35 S]GTP γ S incorporation. Agonist-stimulated [35 S]GTP γ S binding was calculated by subtracting basal binding from binding that occurred in the presence of agonist. Basal [35 S]GTP γ S binding was as follows (fmol/mg protein): wild type midbrain (saline, 56 ± 6 ; morphine, 60 ± 3); wild type hindbrain (saline, 42 ± 2 ; morphine, 45 ± 2); $G\alpha_o$ +/- midbrain (saline, 38 ± 6 ;

morphine, 38 ± 1); $G\alpha_o$ +/-hindbrain (saline, 35 ± 4 ; morphine, 34 ± 1). All data are plotted as the mean $\pm$ SEM.

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Table 1

Morphine ED₅₀ in the 52°C hot plate test in wild type and Gα_o +/- mice treated chronically with morphine.

Day / Chronic Treatment ^a	Wild type			Gα _o +/-		
	Morphine ED ₅₀ (mg/kg)	n	Tolerance Ratio ^b	Morphine ED ₅₀ (mg/kg)	n	Tolerance Ratio ^b
Day 1	11.1 ± 1.1	26		25.1 ± 1.1 *	25	
Day 8 / Saline	20.8 ± 1.3	7	1.9 ± 0.2	35.9 ± 1.3	7	1.4 ± 0.1
Day 8 / 56 mg/kg Morphine	54.3 ± 1.3 #	9	4.9 ± 0.5	40.3 ± 1.3	8	1.6 ± 0.1
Day 8 / 128 mg/kg Morphine	66.9 ± 1.2 #	10	6.0 ± 0.6	133 ± 1.2 #	10	5.3 ± 0.2

^aChronic treatment refers to the stated agent being administered twice-daily (i.p.) for 6 days.

^bTolerance Ratio = Day 8 ED₅₀ / Day 1 ED₅₀. Data are presented as the mean ± SEM.

* *p*<0.001 compared with wild type mice on Day 1 by unpaired *t*-test.

p<0.001 compared with saline-treated mice of the same genotype by Bonferroni's post-test.

Table 2

Opioid receptor expression in brain homogenates from wild type and $G\alpha_o$ +/- mice treated chronically with morphine.

Brain Region	Chronic Treatment ^a	4 nM [³ H]DPN binding (fmol/mg protein)				12 nM [³ H]DAMGO binding (fmol/mg protein)			
		Wild type		$G\alpha_o$ +/-		Wild type		$G\alpha_o$ +/-	
		Total	MOR	Total	MOR	Total	MOR	Total	MOR
Midbrain	Saline	487 ± 36	355 ± 22	432 ± 103	306 ± 77	336 ± 36		267 ± 62	
	128 mg/kg Morphine	456 ± 45	298 ± 42	436 ± 7	306 ± 9	326 ± 50		362 ± 27	
Hindbrain	Saline	336 ± 16	199 ± 7	381 ± 56	274 ± 66	209 ± 12		238 ± 37	
	128 mg/kg Morphine	393 ± 35	262 ± 27	349 ± 21	245 ± 25	222 ± 24		223 ± 15	

^aChronic treatment refers to the stated agent being given twice-daily (i.p.) for 6 days. Data are presented as the mean ± SEM ($n=3-4$, each performed in triplicate).