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Morphine-Induced Physiological and Behavioral Responses in Mice Lacking G Protein-Coupled Receptor Kinase 6

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Abstract

G protein-coupled receptor kinases (GRKs) are a family of intracellular proteins that desensitize and regulate the responsiveness of G protein-coupled receptors (GPCRs). In the present study, we assessed the contribution of GRK6 to the regulation and responsiveness of the G protein-coupled mu-opioid receptor (μOR) in response to morphine in vitro and in vivo using mice lacking GRK6. In cell culture, overexpression of GRK6 facilitates morphine-induced beta-arrestin2 recruitment and receptor internalization, suggesting that this kinase may play a role in regulating the μOR. In vivo, we find that acute morphine treatment induces greater locomotor activation but less constipation in GRK6 knockout (GRK6-KO) mice compared to their wild-type (WT) littermates. The GRK6-KO mice also appear to be “presensitized” to the locomotor stimulating effects induced by chronic morphine treatment, yet these animals do not display more conditioned place preference than WT mice. Furthermore, several other morphine-mediated responses which were evaluated, including thermal antinociception, analgesic tolerance, and physical dependence, were not affected by ablation of the GRK6 gene. Collectively, these results suggest that GRK6 may play a role in regulating some, but not all morphine-mediated responses. In addition, these findings underscore that the contribution of a particular regulatory factor to receptor function can differ based upon the specific cell composition and physiology assessed, and illustrate the need for using caution when interpreting the importance of interactions observed in cell culture.

Keywords

mu-opioid receptor; G protein-coupled receptor kinase; morphine; constipation; locomotor activity; sensitization

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1. Introduction

Morphine is an effective analgesic for the treatment of pain, but its clinical utility is often limited by a number of undesirable side effects, including the development of analgesic tolerance, physical dependence, constipation, and addiction. Numerous pharmacological and knockout mouse studies have demonstrated that the physiological and behavioral effects of morphine are mediated primarily through activation of the mu-opioid receptor (μOR) (Matthes et al., 1996; Sora et al., 1997; Roy et al., 1998; Kieffer et al., 1999). As a G protein-coupled receptor (GPCR), the μOR is subject to regulation by G protein receptor kinases (GRKs). This family of regulatory proteins act in concert with beta-arrestins (βarrestins) to desensitize receptors and therefore, can impact the overall responsiveness of GPCRs following agonist activation (reviewed by Premont and Gainetdinov, 2007).

Seven distinct GRK subtypes (GRKs 1–7) have been identified which have different distribution patterns. GRK1 and GRK7 expression is primarily restricted to the visual system (Weiss et al., 1998; Chen et al., 1999) while GRKs 2, 3, 4, 5 and 6 have been shown to be more widely distributed throughout the body (Kunapuli and Benovic, 1993; Sallese et al., 2000; Hurlé et al., 2001; Erdtmann-Vourliotis et al., 2001; Fan et al., 2002; Sanada et al., 2006). Cellular studies reveal that μORs are weakly phosphorylated following morphine treatment and that the overexpression of GRK2 or GRK3 is sufficient to enhance receptor phosphorylation and facilitate subsequent trafficking events such as βarrestin recruitment, desensitization of receptor signaling, and internalization (Zhang et al., 1998; Whistler and von Zastrow, 1998; Kovoor et al., 1998; Bohn et al., 2004; Groer et al., 2007).

Mouse models in which individual GRK genes are ablated have provided a useful tool for elucidating the contribution of specific GRKs to opioid drug responses in vivo. Mice lacking GRK3 develop significantly less antinociceptive tolerance in response to chronic fentanyl administration (a μOR-directed analgesic) compared to their wildtype (WT) littermates, although no differences between the genotypes were observed following acute treatment (Terman et al., 2004). GRK2-KO mice are embryonically lethal and therefore, the biological significance of GRK2 regulation of opioid effects *in vivo* has not been conclusively studied. However, the different GRKs may not necessarily perform interchangeable functions and there may be some specificity in regard to their individual ability to regulate certain GPCRs under specific conditions (Premont and Gainetdinov, 2007). Moreover, while GRK2 and GRK3 are cytosolic proteins, GRKs 4, 5, & 6 are palmitoylated or contain polybasic domains, allowing them to reside at the cellular membrane (Premont and Gainetdinov, 2007); therefore, their contributions to μOR regulation may fundamentally differ from GRK2 and GRK3. The current study was undertaken to investigate the specific role of GRK6 in regulating the morphine-activated μOR across a wide range of biological responses.

Relative to several of the other GRKs, GRK6 is highly expressed in brain regions and gastrointestinal tissues associated with many of the physiological responses elicited by morphine (Erdtmann-Vourliotis et al., 2001; Gainetdinov et al., 2003). Here, we asked whether GRK6 could regulate the morphine-bound μOR in cells and evaluated whether morphine-mediated responses, including thermal antinociception, antinociceptive tolerance, locomotor activity, conditioned place preference, physical dependence and constipation were altered in the absence of GRK6 using GRK6 knockout (GRK6-KO) mice. We find that GRK6 can regulate the μOR in cell culture by facilitating morphine-induced βarrestin2 recruitment and receptor internalization. In vivo, however, only morphine-induced locomotor activity, sensitization, and constipation were altered in GRK6-KO mice. These findings suggest that while GRK6 can contribute to μOR regulation in vitro, its effects on morphine-induced responses in vivo are more complex and depend upon the immediate cellular environment and the physiology being assessed.

2. Methods

2.1. Animals

WT and GRK6-KO mice were generated from crossing heterozygous GRK6 animals as previously described (Gainetdinov et al., 2003). The parental mouse strains C57BL/6 and 129SvJ were used to generate this mouse line (Gainetdinov et al., 2003). Genotyping was performed on DNA extracted from tissue punches taken from the ear of each mouse to prevent damage to the tail. Mice used in these studies were age-matched (3–8 months old), males weighing between 25 and 35 grams housed in groups of five in Plexiglas chambers in a temperature-controlled room on a 12-hour light-dark cycle with lights on at 6:00 AM. Mice had free access to food and water before any experiments unless otherwise noted and were examined during between 8:00 AM and 5:00 PM and used only once. All studies were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and with approved animal protocols by The Ohio State University and Duke University Animal Care and Use Committees.

2.2. Drugs

Morphine sulfate pentahydrate (Sigma-Aldrich, St. Louis, MO), naloxone hydrochloride (Tocris Bioscience, Ellisville, MO), and cocaine methiodide (generously supplied by the National Institute on Drug Abuse Drug Program, Rockville, MD) were dissolved in 0.9% physiological saline and were given at a volume of 10 μL/g body weight for systemic injections. Morphine was injected subcutaneously (s.c.) or intraperitonially (i.p.) (the route of administration was determined experimentally not to impact upon the measures recorded here, data not shown), while naloxone and cocaine methiodide were administered i.p. For studies using osmotic pumps, morphine was dissolved in sterile distilled water. All drug solutions were freshly prepared prior to use.

2.3. In Vitro Procedures

2.3.1. βarrestin2-GFP translocation—HEK-293 cells were transiently transfected with N-terminally haemagglutinin-tagged μOR (HA-μOR) cDNA (5 μg) (GenBank accession # U26915.1) and βarrestin2-GFP (green fluorescent protein) cDNA (2 μg) (GenBank accession # 949986.1) as previously described (Groer et al., 2007). Another group of cells were also transfected with 5 μg of GRK6A cDNA (GenBank accession # AF040747.1) (Stoffel et al., 1994). Cells were incubated with anti-HA antibody conjugated to 594-Alexafluor (Molecular Probes, Carlsbad, CA) for 30 minutes in serum free media at 37°C prior to agonist treatment and live cell imaging. Images were collected by confocal microscopy (Olympus Fluoview 300) as previously described (Groer et al., 2007).

2.3.2. Receptor Internalization—HEK-293 cells were transiently transfected with μOR cDNA (2 μg) tagged on the C-terminus with yellow fluorescent protein (YFP). Another group of cells were also transfected with 5 μg of GRK6A cDNA (Stoffel et al., 1994). Cells were serum deprived for 30 minutes prior to live cell imaging as described above.

2.4. In Vivo Procedures

2.4.1. Antinociceptive Testing—Antinociception was evaluated by measuring response latencies to a thermal stimulus using a hot-plate test (56°C) or a warm-water tail-flick (54°C) assay as previously described (Bohn et al., 1999, 2000). Nociceptive latencies were assessed by measuring the time required for the mouse to either flick or lick its fore- or hindpaws (hot-plate) or to the first sign of a rapid flick of the tail (tail-flick). For both assays, basal latencies were measured prior to treatment and test latencies were measured at various times following morphine (5, 10, or 20 mg/kg, i.p.) injection. A cutoff latency of 30 seconds for the hot-plate

and 15 seconds for the tail-flick test were used to avoid tissue damage. Antinociception was reported as the percentage of maximum possible effect (% MPE) and calculated using the following formula: % MPE = 100% × [(drug response latency - basal latency)/ (cutoff time - basal latency)].

2.4.2. Antinociceptive Tolerance Studies—Antinociceptive tolerance was assessed by treating mice with morphine (5, 10, or 20 mg/kg, i.p.) once daily for 5 days. Basal latencies were determined in the hot-plate and warm water tail-flick assays prior to morphine treatment on day 1, and then latencies were assessed every other day 30 minutes following morphine treatment at the time of peak drug effect.

2.4.3. Locomotor Activity Studies—Locomotor activity measurements were made using the Versamax Animal Activity Monitoring System [20 × 20 cm^2] (Accuscan Instruments, Columbus, OH) as previously described (Bohn et al., 2003). Mice were individually placed into the activity monitoring boxes for 30 minutes to habituate the mice to the new environment, removed, injected with morphine (5, 10 or 20 mg/kg, i.p.), and immediately placed back into the boxes for 120 minutes of monitoring. For morphine locomotor sensitization experiments, mice were chronically treated with morphine (10 mg/kg, i.p.) once daily for 6 days. On days 1 and 7, locomotor activity was assessed as described above. Data were collected using Versadat software (Accuscan Instruments, Columbus, OH) and analyzed for the total number of beam breaks made by each animal in 5 minute increments.

2.4.4. Conditioned Place Preference—Conditioned place preference was measured using a three-compartment, commercially available place preference chamber for mice (Model ENV-3013, Med Associates, St. Albans, VT). Each place preference chamber was housed in a sound-attenuating cubicle, equipped with a fan for ventilation and white noise generation. Each chamber contained three distinct compartments separated by a manual guillotine door and was illuminated with a light source of adjustable intensity. The central compartment was 7.2 cm × 12.7 cm × 12.7 cm, with gray walls and plastic flooring. The two flanking choice compartments were 16.8 cm × 12.7 cm × 12.7 cm. One choice compartment was black with a stainless steel rod floor, and the other choice compartment was white with a stainless steel mesh floor. The chambers were controlled by an appropriate interface and the data were collected by a PC running a MED-PC IV software package (all from Med Associates, St. Albans, VT). The experiments were conducted in a designated mouse testing room in which no other activities took place during the test times. Mice of both genotypes were initially assessed for the time spent in either black or white compartment on the pre-conditioning day and assigned to receive morphine treatment in one of these compartments according to an “unbiased” experimental design as previously described (Bohn et al., 2003).

2.4.5. Induction of Physical Dependence and Withdrawal—Mice were made physically dependent on morphine (12 mg/kg/day or 24 mg/kg/day) using Alzet mini-osmotic pumps which continuously infused morphine at a rate of 1μL/hr for 7 days (Model 2001; Durect Corporation, Cupertino, CA). A single pump filled with morphine was implanted subcutaneously on the back of each mouse under light isoflurane anesthesia. To assess the extent of dependence, mice were administered naloxone (0.05 or 0.5 mg/kg, i.p), placed in Plexiglas cylinders (14.5 cm × 40.5 cm) lined with filter paper, and observed for the manifestation of different withdrawal signs over a 30 minute period (Bohn et al., 2000). The number of jumps, wet dog shakes, and paw tremors elicited by each mouse were counted in 5 minute intervals. The occurrence of diarrhea and mastication were also measured at 5 minute intervals and scored: a score of 0 was assigned if the behavior was absent, and a score of 1 was given if the behavior was present. The percent occurrence of these two signs was calculated by dividing the number of observed occurrences by 6 (the total number of occurrences possible)

and then multiplying by 100%. Weight loss was also determined by subtracting measured body weight after withdrawal from body weight prior to precipitating withdrawal. A global withdrawal score summarizing the results from the withdrawal signs collectively was calculated by multiplying withdrawal values for each mouse by a constant to make each sign of equal weight and then adding each average value. Signs were weighted as follows: jumps × 0.8; wet dog shakes × 1; paw tremors × 0.35; diarrhea score × 1.5; mastication score × 1.5 (weight loss was not included in the global score) (Berrendero et al., 2003).

2.4.6. Fecal Boli Accumulation—Mice were given an injection of saline (s.c.) or morphine (10 mg/kg, s.c.) and individually placed into small Plexiglas boxes (15.2 cm × 16.5 cm × 12.0 cm) lined with filter paper. Fecal boli were collected and weighed using an analytical balance every hour for 6 hours (Raehal et al., 2005).

2.4.7. Small Intestinal Transit—Small intestinal transit was measured using an orally administered charcoal meal as previously described (Raehal et al., 2005). At 48 hours prior to the experiment, mice were habituated to a modified cage containing a mesh wire insert in the presence of food and water and then fasted for 24 hours with free access to water. For the experiment, mice were given an injection of saline (s.c.) or morphine (1 or 5 mg/kg, s.c.) 20 minutes prior to receiving a charcoal meal consisting of 5% charcoal (2–12 μm powder, Sigma-Aldrich, St. Louis, MO), 10% gum arabic (Acros Organics, Morris Plains, NJ) in sterile water by oral gavage at a volume of 10 μL/g body weight. Thirty minutes later, mice were sacrificed by cervical dislocation and the small intestine from the pyloric sphincter to the ileal cecal junction was isolated and the mysentery removed. The distance traveled by the leading edge of the charcoal meal was measured relative to the total length of the small intestine and the percent of gastrointestinal transit for each treatment group was calculated as follows: % gastrointestinal transit= [(charcoal bolus distance)/ (small intestine length)] * 100%.

2.4.8. Large Intestinal Transit—Large intestine transit was measured using a bead expulsion assay as previously described (Raehal et al., 2005). Mice were first habituated in the same manner as the small intestinal transit assay. For the test, mice were given an injection of saline (s.c.) or morphine (1, 5, or 10 mg/kg, s.c.) and 5 minutes later, a round 3 mm glass bead (Thermo Fisher Scientific, Pittsburg, PA) was inserted 2 cm into the distal rectum using 2 mm round, flexible, plastic tubing. Mice were placed into small Plexiglas boxes (13.9 cm × 12.7 cm × 15.2 cm) lined with filter paper for observation and the time to bead expulsion was measured for each animal. Mice that did not expel their bead within a reasonable time period (4 hours) or produced feces before expelling the bead were eliminated from data analysis.

2.5. Data Analysis

Results for each experiment are expressed as mean ± S.E.M. Studies evaluating time course or dose-response effects between genotypes were analyzed using a standard two-way analysis of variance (ANOVA) test, followed by Bonferroni post-hoc analysis where appropriate. When two groups were compared for a single response, a Student's *t* test was used. The criterion for significance was set at $p < 0.05$. All statistics were calculated using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

3. Results

To determine if GRK6 is capable of regulating the μOR in vitro, we used confocal microscopy to assess both the translocation of βarrestin2-GFP to the plasma membrane of HEK-293 cells as well as internalization of μOR-YFP, with or without overexpression of GRK6A which is the variant predominantly expressed in brain (Firsov and Elalouf, 1997). In the absence of GRK6 overexpression, morphine treatment induced weak βarrestin2-GFP translocation

(Figure 1A) and μOR-YFP internalization (Figure 1B) in cells expressing the μOR. Overexpression of GRK6, however, was capable of augmenting morphine-induced βarrestin2-GFP translocation (Figure 1A) and μOR-YFP internalization (Figure 1B). Importantly, cells not expressing the μOR did not display βarrestin2 recruitment with or without morphine treatment (data not shown).

The contribution of GRK6 to morphine-mediated thermal antinociceptive responses was assessed in GRK6-KO mice and their WT littermates using hot-plate (56°C) and warm water tail-flick (54°C) assays. Basal response latencies did not differ between WT and GRK6-KO mice in either the hot-plate ($p>0.05$, Student's *t* test) or tail-flick ($p>0.05$, Student's *t* test) test (Figures 2A and 2B, *insets*). Following acute treatment with a moderate dose of morphine (10 mg/kg, i.p.), both genotypes displayed similar time-dependent antinociceptive responses in the hot-plate (two-way ANOVA for genotype: $F(1,77)=1.52$, $p>0.05$; for time: $F(6,77)=13.75$, $p<0.0001$; for genotype × time: $F(6,77)=0.13$, $p>0.05$) and the tail-flick test (two-way ANOVA for genotype: $F(1,77)=3.20$, $p>0.05$; for time: $F(6,77)=34.75$, $p<0.0001$; for genotype × time: $F(6,77)=0.85$, $p>0.05$) (Figures 2A and 2B). Furthermore, no differences were observed between WT and GRK6-KO mice in response to several doses of morphine (5, 10, or 20 mg/kg, i.p.) measured at 30 minutes, the time of peak drug effect, in either the hot-plate (two-way ANOVA for genotype: $F(1,28)=0.06$, $p>0.05$; for dose: $F(2,28)=52.10$, $p<0.0001$; for genotype × dose: $F(2,28)=0.01$, $p>0.05$) or tail-flick test (two-way ANOVA for genotype: $F(1,28)=0.01$, $p>0.05$; for dose: $F(2,28)=165.53$, $p<0.0001$; for genotype × dose: $F(2,28)=2.58$, $p>0.05$) (Figures 2C and 2D). Moreover, morphine-induced tolerance developed to an equal extent and at a similar rate in WT and GRK6-KO mice in response several doses of morphine in both the hot-plate (for 5 mg/kg two-way ANOVA for genotype: $F(1,24)=0.08$, $p>0.05$; for time: $F(2,24)=13.05$, $p>0.05$; for genotype × time: $F(2,24)=0.78$, $p>0.05$; for 10 mg/kg two-way ANOVA for genotype: $F(1,33)=0.97$, $p>0.05$; for time: $F(2,33)=7.63$, $p>0.05$; for genotype × time: $F(2,33)=0.859$, $p>0.05$; for 20 mg/kg two-way ANOVA for genotype: $F(1,27)=0.57$, $p>0.05$; for time: $F(2,27)=9.99$, $p>0.05$; for genotype × time: $F(2,27)=0.63$, $p>0.05$) and the tail-flick test (for 5 mg/kg two-way ANOVA for genotype: $F(1,24)=1.90$, $p>0.05$; for time: $F(2,24)=19.85$, $p>0.05$; for genotype × time: $F(2,24)=0.00$, $p>0.05$; for 10 mg/kg two-way ANOVA for genotype: $F(1,33)=0.23$ $p>0.05$; for time: $F(2,33)=38.91$, $p>0.05$; for genotype × time: $F(2,33)=0.67$, $p>0.05$; for 20 mg/kg, two-way ANOVA for genotype: $F(1,27)=0.00$, $p>0.05$; for time: $F(2,27)=102.06$, $p>0.05$; for genotype × time: $F(2,27)=1.38$, $p>0.05$) (Figures 2E and 2F).

Morphine exerts a stimulatory effect on locomotor activity in mice. Therefore, GRK6-KO mice and their WT littermates were monitored for morphine-induced locomotor activity. During a 30 minute habituation period, both WT and GRK6-KO mice became less active to a similar extent (two-way ANOVA for genotype: $F(1,120)=1.49$, $p>0.05$; for time: $F(5,120)=6.21$, $p<0.0001$; for genotype × time: $F(5,120)=0.57$, $p>0.05$). Following acute treatment with morphine (10 mg/kg, i.p.), both genotypes showed a marked increase in locomotor activation; however, the GRK6-KO mice showed a significantly greater increase in locomotor activity as measured by the number of beam breaks compared to WT controls (two-way ANOVA for genotype: $F(1,480)=171.04$, $p<0.0001$; for time: $F(23,480)=7.77$, $p<0.0001$; for genotype × time: $F(23,480)=1.59$, $p<0.05$) (Figure 3A). Moreover, the GRK6-KO mice displayed enhanced locomotor activity over the total 120 minute test period at several doses of morphine compared to WT mice (two-way ANOVA for genotype: $F(1,62)=6.41$, $p<0.05$; for dose: $F(3,62)=28.43$, $p<0.0001$; for genotype × dose: $F(3,62)=4.16$, $p=0.01$; WT vs KO, $*p<0.05$, $**p<0.01$, Bonferroni post-hoc analysis) (Figure 3B).

Chronic morphine treatment in mice can also lead to the development of sensitization to the locomotor response in which repeated drug treatment leads to enhanced locomotor behaviors over that observed following the first treatment with drug (Robinson and Berridge, 2001).

Following single daily morphine injections over 6 days, there was a significant difference in the locomotor stimulating effects of a challenge dose of morphine given on day 7 between WT and GRK6-KO mice (two-way ANOVA for genotype: $F(1,480)=5.76$, $p<0.05$; for time: $F(23,480)=6.21$, $p<0.0001$; for genotype × time: $F(23,480)=0.33$, $p>0.05$) (Figure 3C). Furthermore, there were significant differences in the total number of beam breaks made over the 120 test period locomotor activity between the genotypes on day 1 compared to day 7, especially between WT and GRK6-KO mice on day 1 (two-way ANOVA for genotype: $F(1,40)=9.00$, $p<0.01$; for time: $F(1,40)=1.79$, $p>0.05$; for genotype × time: $F(1,40)=3.83$, $p>0.05$, WT vs KO, $^{**}p<0.01$, Bonferroni post-hoc analysis) (Figure 3D). Moreover, the WT mice showed a significantly enhanced locomotor response on day 7 as compared to day 1 ($p<0.05$, Student's *t* test), while GRK6-KO mice displayed a similar locomotor activity profile on day 7 as on the first day of treatment ($p>0.05$, Student's *t* test) (Figure 3D).

Locomotor activation and behavioral sensitization have been generally correlated with the rewarding properties of drugs as well as addiction (Robinson and Berridge, 2001; De Vries and Shippenberg, 2002). To determine if the rewarding or reinforcing properties of morphine were also affected by the loss of GRK6, a conditioned place preference paradigm was used. Although differences in locomotor activity and sensitization were observed between WT and GRK6-KO mice, there were no significant differences between the genotypes in response to saline or with increasing doses of morphine as both the WT and GRK6-KO mice showed an equivalent and dose-dependent increase in preference for the morphine-paired chamber (two-way ANOVA for genotype: $F(1,76)=0.08$, $p>0.05$; for dose: $F(4,76)=1.79$, $p<0.001$; for genotype × dose: $F(4,76)=1.50$, $p>0.05$) (Figure 4).

Prolonged exposure to morphine leads to the development of physical dependence, which can be assessed by measuring antagonist-precipitated withdrawal behaviors. Both WT and GRK6-KO mice displayed prominent withdrawal signs following naloxone (0.05 and 0.5 mg/kg, i.p.) administration, indicating that they had become dependent on morphine at the doses tested. However, with the exception of the number of paw tremors, there were no significant differences in the number of jumps (2-way ANOVA for genotype: $F(1,39)=1.12$, $p>0.05$; for dose: $F(1,39)=0.04$, $p>0.05$; for genotype × dose: $F(1,39)=0.25$, $p>0.05$), wet dog shakes (2-way ANOVA for genotype: $F(1,39)=0.17$, $p>0.05$, for dose: $F(1,39)=0.19$, $p>0.05$; for genotype × dose: $F(1,39)=0.30$, $p>0.05$), paw tremors (2-way ANOVA for genotype: $F(1,39)=5.52$, $p>0.05$; for dose: $F(1,39)=0.17$, $p>0.05$; for genotype × dose: $F(1,39)=0.08$, $p>0.05$), diarrhea (2-way ANOVA for genotype: $F(1,39)=3.36$, $p>0.05$; for dose: $F(1,39)=0.28$, $p>0.05$; for genotype × dose: $F(1,39)=2.47$, $p>0.05$), mastication (2-way ANOVA for genotype: $F(1,39)=3.32$, $p>0.05$; for dose: $F(1,39)=0.07$, $p>0.05$; for genotype × dose: $F(1,39)=0.40$, $p>0.05$) and weight loss (2-way ANOVA for genotype: $F(1,39)=2.17$, $p>0.05$; for dose: $F(1,39)=3.35$, $p>0.05$; for genotype × dose: $F(1,39)=0.06$, $p>0.05$) between the two genotypes in mice treated with a 24 mg/kg/day dose of morphine and two different doses of naloxone (0.05 and 0.5 mg/kg, i.p.) (Figure 5A). As shown in figure 5B, GRK6-KO mice treated with 12 and 24 mg/kg/day morphine and 0.5 mg/kg naloxone showed significant differences in the occurrence of mastication (two-way ANOVA for genotype: $F(1,33)=5.91$, $p<0.05$; for dose: $F(1,33)=0.14$, $p>0.05$; for genotype × dose: $F(1,33)=1.70$, $p>0.05$), while there were no differences with any of the other withdrawal signs measured including jumps (2-way ANOVA for genotype: $F(1,33)=0.66$, $p>0.05$; for dose: $F(1,33)=5.05$, $p>0.05$; for genotype × dose: $F(1,33)=0.11$, $p>0.05$), wet dog shakes (2-way ANOVA for genotype: $F(1,33)=0.75$, $p>0.05$; for dose: $F(1,33)=2.36$, $p>0.05$; for genotype × dose: $F(1,33)=0.02$, $p>0.05$), paw tremors (2-way ANOVA for genotype: $F(1,33)=3.55$, $p>0.05$; for dose: $F(1,33)=0.41$, $p>0.05$; for genotype × dose: $F(1,33)=0.00$, $p>0.05$), diarrhea (2-way ANOVA for genotype: $F(1,33)=0.12$, $p>0.05$; for dose: $F(1,33)=0.01$, $p>0.05$; for genotype × dose: $F(1,33)=0.01$, $p>0.05$), and weight loss (2-way ANOVA for genotype: $F(1,33)=1.57$, $p>0.05$; for dose: $F(1,33)=0.44$, $p>0.05$; for genotype × dose: $F(1,33)=0.03$, $p>0.05$). Furthermore, there were no differences in overall global scores

which simultaneously account for all withdrawal parameters measured between the two genotypes at the doses of naloxone tested (two-way ANOVA for genotype: $F(1,35)=2.43$, $p>0.05$; for dose: $F(1,35)=1.99$, $p>0.05$; for genotype × dose: $F(1,35)=0.10$, $p>0.05$) (Figure 5C). Similarly, there were no differences in the overall global scores between the two genotypes at the doses of morphine assessed (two-way ANOVA for genotype: $F(1,39)=3.06$, $p>0.05$; for dose: $F(1,39)=0.00$, $p>0.05$; for genotype × dose: $F(1,39)=0.23$, $p>0.05$) (Figure 5D).

Morphine treatment also affects gastrointestinal function in a manner that produces constipation. Therefore, GRK6-KO mice were monitored for differences in their gastrointestinal responses to morphine. The constipating effect of morphine was initially assessed by measuring fecal boli production over 6 hours in response to saline (s.c.) or morphine (10 mg/kg, s.c.) treatment. Both WT and GRK6-KO mice produced equivalent amounts of feces over the 6 hour period in response to saline (two-way ANOVA for genotype: $F(1,168)=0.01$, $p>0.05$; for treatment: $F(5,168)=7.33$, $p<0.0001$; for genotype × treatment: $F(5,168)=0.07$, $p>0.05$), indicating that normal gastrointestinal function in not altered in the absence of GRK6 (Figure 6A). However, GRK6-KO mice produced significantly more fecal boli over the test period compared to their WT littermates following morphine treatment (two-way ANOVA for genotype: $F(1,144)=5.95$, $p<0.05$; for treatment: $F(5,144)=13.19$, $p<0.0001$; for genotype × treatment: $F(5,144)=1.16$, $p>0.05$) (Figure 6A). To determine if differences in eating behaviors was responsible for these differences, food consumption was determined for each cage of animals in a 24 hour period among 3 cages containing the same genotype. There were no significant differences in food intake between the WT and GRK6-KO mice (WT 4.57 ± 0.32 g/ 3 days vs KO 4.12 ± 0.36 g/ 3 days; $p= 0.4460$, Student's *t* test; n= 8/genotype; data not shown).

To further assess how the loss of GRK6 is affecting gastrointestinal responses, the effect of morphine on inhibition of small intestinal transit in WT and GRK6-KO mice was evaluated using a charcoal meal assay. There were no significant differences in the % gastrointestinal transit measured between the two genotypes in response to saline ($p=0.929$, Student's *t* test) (Figure 6B). While morphine dose-dependently reduced small intestinal transit, there were no significant differences between GRK6-KO mice and WT controls at any of the doses tested (two-way ANOVA for genotype: $F(1,12)=0.23$, $p>0.05$; for dose: $F(1,12)=5.10$, $p<0.05$; for genotype × dose: $F(1,12)=0.42$, $p>0.05$) (Figure 6B). Since differences in the overall amount of fecal boli were apparent, potential differences in large intestinal transit were assessed using a colonic bead expulsion assay. Again, there were no differences in bead expulsion times in response to saline between the two genotypes ($p>0.05$, Student's *t* test). However, while morphine dose-dependently delayed bead expulsion in the WT and GRK6-KO mice, the GRK6-KO mice expelled their beads more rapidly than WT controls (two-way ANOVA for genotype: $F(1,45)=12.63$, $p<0.001$; for dose: $F(2,45)=192.10$, $p<0.0001$; for genotype × dose: $F(2,45)=1.37$, $p>0.05$; WT vs KO $*p<0.05$, Bonferroni post-hoc analysis) (Figure 6C), which is consistent to the results observed in the whole gut transit study.

In addition to opioid receptors, activation of dopamine receptors has been shown to inhibit gastrointestinal motility (Walker et al., 2000; Al-Jahmany et al., 2004; Li et al., 2006). We have previously shown that GRK6 has been shown to play a role in regulating D2 dopamine receptors in vivo (Gainetdinov et al., 2003). Therefore, we assessed possible dopamine contributions to gastrointestinal transit in the GRK6-KO mice using the colonic bead expulsion assay. Cocaine methiodide, a quaternary salt that does not cross the blood brain barrier at low concentrations (Abraham et al., 1992), was used to increase dopamine level in the enteric nervous system. As shown in figure 6D, acute treatment with a single dose of cocaine methiodide (5 mg/kg, i.p.) significantly delayed bead transit in WT but not GRK6-KO mice ($p<0.01$, Student's *t* test).

4. Discussion

In this study we find that GRK6 can regulate the morphine-activated μOR in cell culture by facilitating morphine-induced βarrestin2-GFP recruitment and receptor internalization (Figure 1), which is analogous to studies in which GRK2 overexpression was shown to also facilitate morphine-induced βarrestin2-GFP recruitment and μOR internalization (Zhang et al., 1998;Whistler and von Zastrow, 1998;Bohn et al., 2004;Groer et al., 2007). However, the role of GRKs in regulating the responsiveness of the μOR to morphine in vivo appears to be diverse. In vivo, morphine-induced locomotor activity (Figure 3), sensitization (Figure 3), and constipation (Figure 6) were altered in GRK6-KO mice. However, there were no significant differences in thermal antinociceptive responses between WT and GRK6-KO mice following acute morphine treatment in both the hot-plate and warm water tail-flick assays (Figure 2). Furthermore, there were no differences in the onset of antinociceptive tolerance (Figure 2) or the development of physical dependence (Figure 5) following chronic morphine treatment, suggesting that GRK6 does not play a predominant role in regulating these biological responses to morphine. Taken together with previous studies, our findings suggest that μOR may be regulated by multiple GRKs rather than predominantly by a single GRK, and the extent of regulation by each GRK may vary among cell types.

While GRK6 does not appear to play a role in thermal antinociception, analgesic tolerance and physical dependence, the loss of GRK6 does affect the locomotor stimulating effects of morphine. Acute morphine treatment resulted in enhanced locomotor activity at several doses in GRK6-KO mice compared to their WT littermates (Figure 3). While WT mice become sensitized to the locomotor-stimulating effects of morphine following chronic treatment, GRK6-KO mice display the same locomotor activation profile following chronic treatment as they did in response to acute treatment, indicating that the loss of GRK6 “presenstizes” these mice to the locomotor activating effects induced by morphine. The locomotor activity profiles observed in the GRK6-KO mice in response to morphine resemble those seen following treatment with the psychostimulants cocaine and *d*-amphetamine (Gainetdinov et al., 2003); wherein GRK6-KO mice displayed enhanced locomotor activation and “presensitization” to the locomotor activating effects of these drugs compared to WT mice (Gainetdinov et al., 2003). In this study, the locomotor stimulating effects of these drugs are correlated to an increase in extracellular dopamine levels in the striatum (Gainetdinov et al., 2003). Furthermore, the enhanced locomotor activity in the GRK6-KO mice compared to WT animals associated with an increase in G protein-coupling to dopamine D2 receptors in striatal membranes in the absence of GRK6 (Gainetdinov et al., 2003). Interestingly, it is well established that opioid treatment indirectly causes increased dopamine signaling within the striatum and nucleus accumbens, brain regions involved in mediating morphine-induced locomotion (Di Chiara and North, 1992;Hnasko et al., 2005). Therefore, the increase in locomotor activation and “presensitization” observed in the GRK6-KO mice in response to morphine may be due to enhanced D2 dopamine receptor activity in the striatum of GRK6-KO mice, rather than a direct result of altered regulation of the μOR.

Although the GRK6-KO mice were supersensitive to morphine-induced locomotor activation, there was no significant difference in their development of conditioned place preference to morphine compared to WT mice (Figure 4). This may be considered paradoxical since increases in mesolimbic dopamenergic activity and locomotor sensitization have traditionally been correlated with the rewarding properties of drugs of abuse (reviewed by Robinson & Berridge, 2001). However, there are a number of studies which demonstrate that the mechanisms involved in drug reward are independent of those involved in locomotor activity (Spielewoy et al., 2000;Rouge-Pont et al., 2002;Hnasko et al., 2005;De Vries and Shippenberg, 2002). A similar dichotomy was observed in βarrestin2-KO mice, which displayed decreased locomotor activity compared to their WT counterparts, but showed enhanced conditioned place preference

in response to morphine (Bohn et al., 2003). Furthermore, dopamine-deficient mice develop conditioned place preference to morphine without displaying enhanced locomotor activity, demonstrating that dopamine release is not required for morphine-mediated conditioned place preference (Hnasko et al., 2005). Therefore, while GRK6 regulation of dopamine receptors may be responsible for the increase in locomotor activity observed in the GRK6-KO mice, it is possible that other mechanisms are responsible for the conditioned place preference that develops following morphine treatment.

Given the role that GRKs play in desensitizing G protein-coupled receptors, it is interesting that morphine-induced constipation was reduced in the GRK6-KO mice, an affect that appears to be mediated at the level of the colon (Figure 6). This physiological response suggests that GRK6 may be acting to mediate μOR signaling in the gastrointestinal tract rather than as a desensitizing agent. A similar reduction in morphine-induced constipation and colonic motility was also observed in μarrestin2-KO mice (Raehal et al., 2005). Following GRK-mediated phosphorylation of an activated GPCR, βarrestins interact with the receptor and, for some GPCRs, can serve as scaffolding molecules for components of signaling cascades such as the mitogen activated (MAP) kinases (Lefkowitz and Shenoy, 2005). Several in vivo studies have demonstrated that βarrestin2 can function as a positive mediator of receptor signaling and that these signaling pathways have physiological effects (Schmid and Bohn, 2008). Importantly, GRK6 has been shown to facilitate βarrestin2-mediated signaling pathways. For example, in the heart, GRK6 can phosphorylate angiotensin II receptors to facilitate a G protein-independent, βarrestin2-mediated pathway leading to inotropy (Rajagopal et al., 2006). Therefore, GRK6 may also be involved in positively regulating morphine-mediated constipation by facilitating activation of a similar pathway in colon.

We have also considered that the differences observed in morphine-induced constipation between the GRK6-KO mice and their WT littermates may not be due to direct regulation of the μOR, but rather could also be due to GRK6 regulation of other neurotransmitter systems that affect gastrointestinal motility. Since it is known that the dopamine transporter is expressed in the gastrointestinal tract (Walker et al., 2000) and that the activation of peripheral dopamine receptors can decrease gastrointestinal motility (Walker et al., 2000; Al-Jahmany et al., 2004; Li et al., 2006), we evaluated the possible contribution of the dopamine system by using cocaine methiodide, a peripherally restricted dopamine transporter blocker. As shown in Figure 6, this treatment delayed colonic motility in WT but not GRK6-KO mice. While these results indicate an altered regulation of dopamine signaling in the gastrointestinal tract of mutant mice, the exact mechanism of such alterations is not immediately clear. One possible explanation could be that in the absence of GRK6, dopamine D2 receptor signaling in the gut is disrupted. Previously, Beaulieu et al. (2005) described decreased D2 dopamine receptor function in the absence of βarrestin2 in the striatum following treatment with psychostimulants. The deletion of the GRK6 gene could potentially decrease D2 dopamine receptor-βarrestin2 interactions in gut, which could impact the physiological effects observed here by interrupting a downstream βarrestin2-mediated signaling pathway. Further studies examining the contribution of dopamine and other neurotransmitter systems on morphine-induced constipation and signal transduction in the gut are underway.

In summary, we have shown that morphine-induced responses like locomotor activity, sensitization, and constipation are affected by the loss of GRK6, while other responses such as thermal antinociception, analgesic tolerance, physical dependence and conditioned place preference are not. It is not clear whether the differences in responses are due to direct GRK6-mediated regulation of the μOR, or to altered regulation of other neurotransmitter systems such as dopamine. Therefore, additional studies will need to be conducted to further elucidate the potential contribution of other systems. Importantly, the results of these studies lend further support to the idea that the cellular environment and exact GRK repertoire play a key role in

determining GPCR responsiveness and highlight that studying receptors in their endogenous setting is critical to understanding the mechanisms involved in receptor signaling and drug effects.

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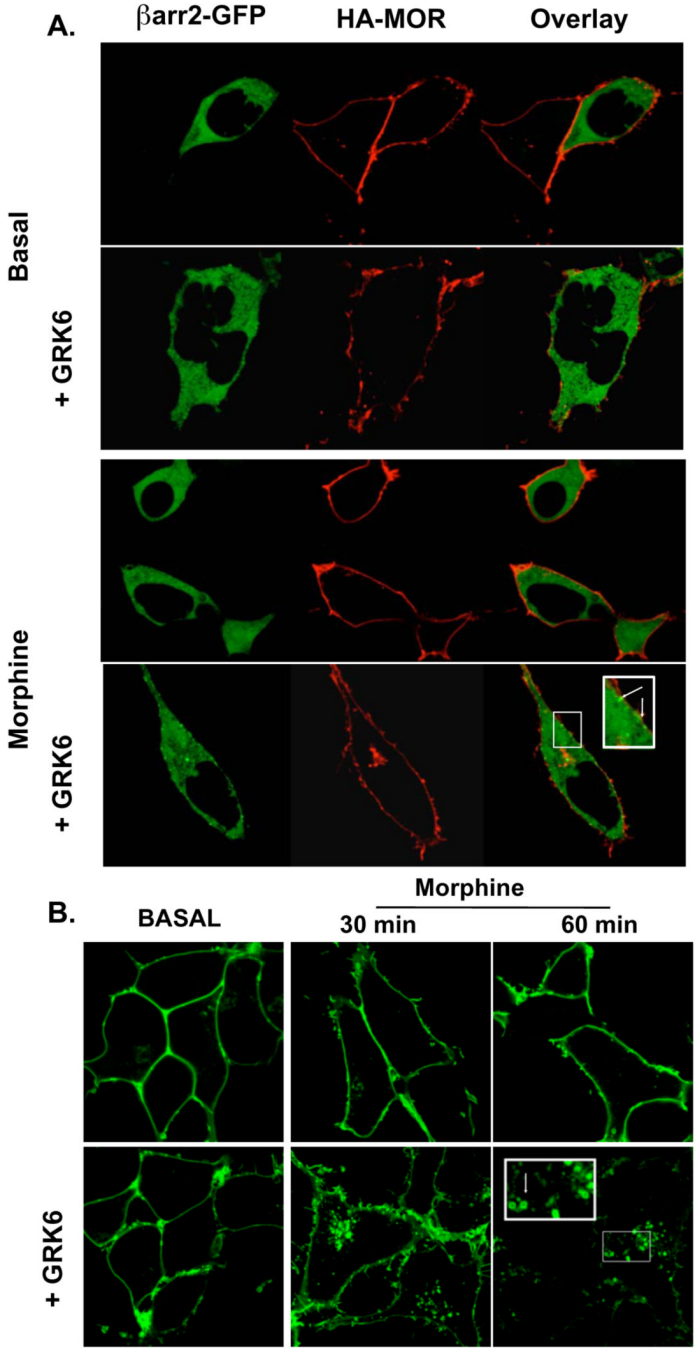

Figure 1.

GRK6 facilitates morphine-induced βarrestin2 recruitment and μOR trafficking in vitro. (A) HEK-293 cells expressing HA-tagged μOR and βarrestin2-GFP (green) were labeled with an HA antibody conjugated to Alexafluor 594 (red) and monitored via live cell confocal microscopy (100X objective). Morphine treatment (10 μM, 10 minutes) alone did not cause detectable recruitment of βarrestin2-GFP to the μOR. In contrast, morphine was able to induce recruitment of βarrestin2-GFP to the μOR in cells overexpressing GRK6, as indicated by arrows (inset: 2X magnification). (B) HEK-293 cells expressing μOR-YFP display robust receptor internalization following morphine treatment (10 μM, times indicated) only in cells

overexpressing GRK6 as indicated by arrows (inset: 2X magnification). Images are representative of several images collected from 3 independent transfections.

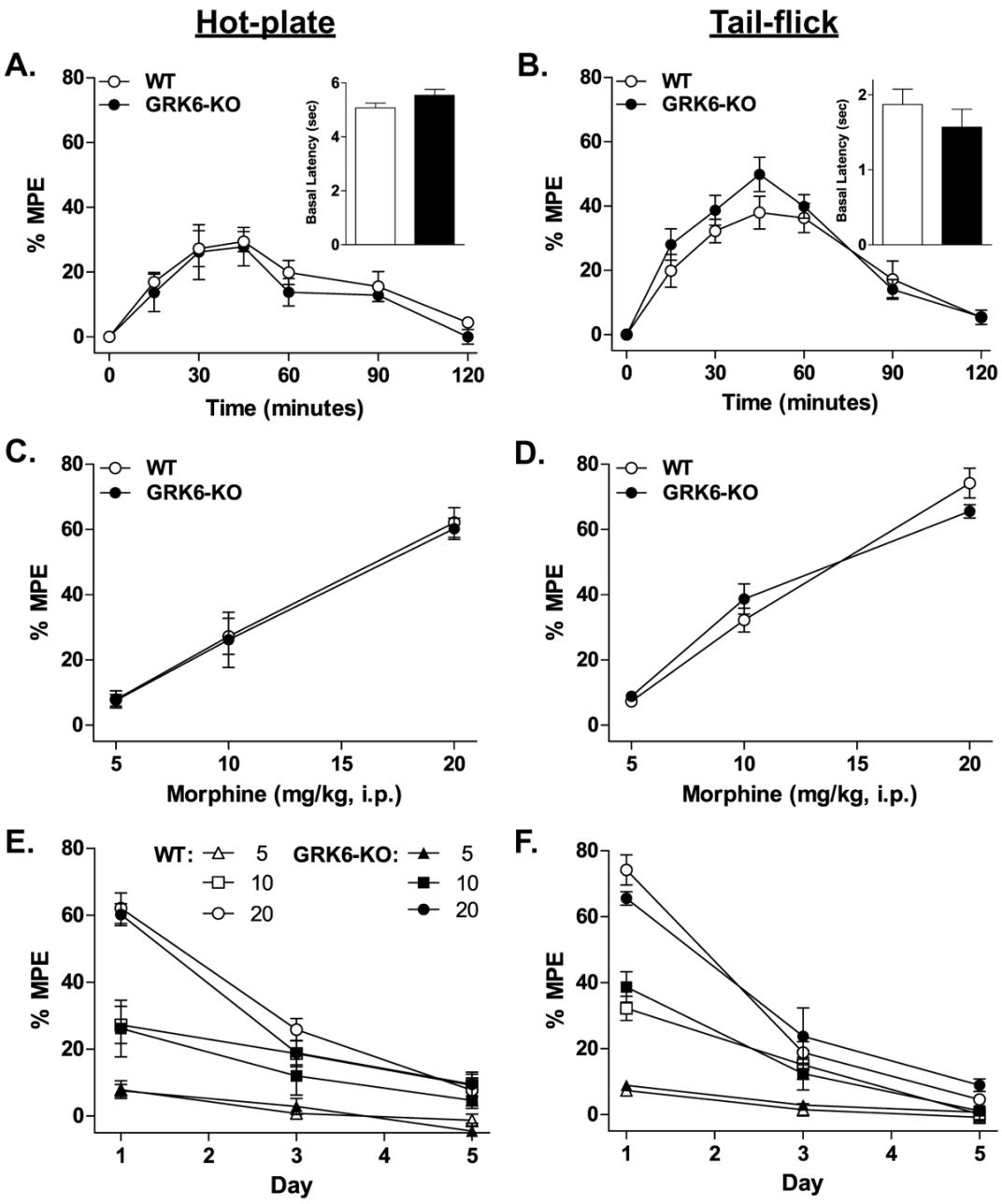

Figure 2.

Morphine produces similar thermal antinociceptive and analgesic tolerance profiles in WT and GRK6-KO mice. Data are presented as the mean ± S.E.M. (A & B) Mice were treated with morphine (10 mg/kg, i.p.) and antinociception was determined at various time points after treatment using a hot-plate (56°C) and warm-water tail-flick (54°) test (n=6–7/group). Basal response latencies are shown in the insets (n=17/group). (C & D) Mice were treated with morphine (5, 10, 20 mg/kg, i.p.) and antinociceptive profiles were assessed at 30 minutes post drug injection in the hot-plate and tail-flick test (n=5–7/group). (E & F) Morphine-induced tolerance was determined by measuring the latency to paw withdrawal 30 minutes after a 10 mg/kg injection (i.p.) on the days indicated (n=5–7/group).

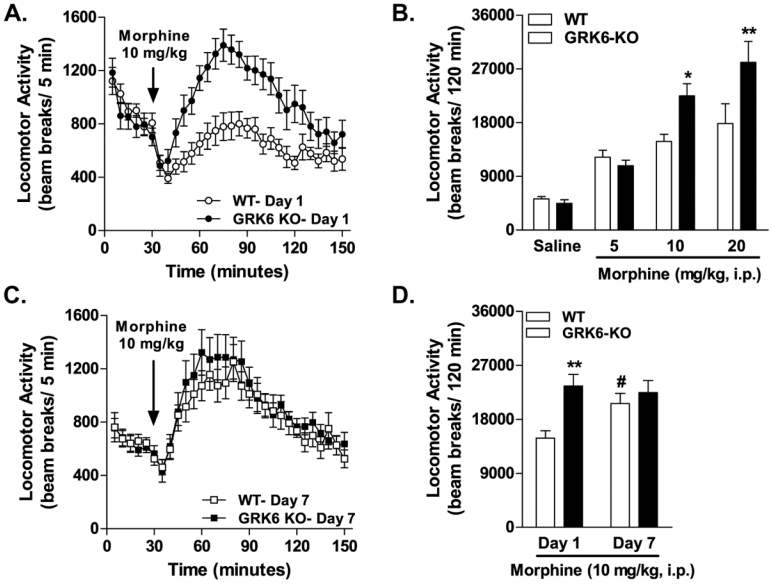

Figure 3.

GRK6-KO mice display increased locomotor activity and "presensitization" to the stimulating effects of morphine. Mice were habituated to the locomotor boxes for 30 minutes, given an i.p. injection of morphine, and then immediately placed back into the boxes for an additional 120 minutes of monitoring. Data are presented as the mean ± S.E.M. (A) Time course for locomotor activity in WT and GRK6-KO mice following acute morphine (10 mg/kg, i.p.) treatment (n=11/group). (B) The total number of beam breaks made by WT and GRK6-KO mice over the 120 min test period following treatment with several doses of morphine (5, 10, 20 mg/kg, i.p.) (WT vs KO, *p<0.05, **p<0.01; n=7–12/group). (C) Mice were chronically treated with morphine (10 mg/kg, i.p.) once daily for 6 days and locomotor activity was assessed following a challenge dose of morphine (10 mg/kg, i.p.) on day 7 in the same manner as above (n=11/group). (D) The total number of beam breaks made in 120 minutes by WT and GRK6-KO mice following chronic morphine (10 mg/kg, i.p.) treatment (WT day 1 vs KO day 1, **p<0.01; WT day 1 vs WT day 7, $^{\#}p$<0.05; n=11/group).

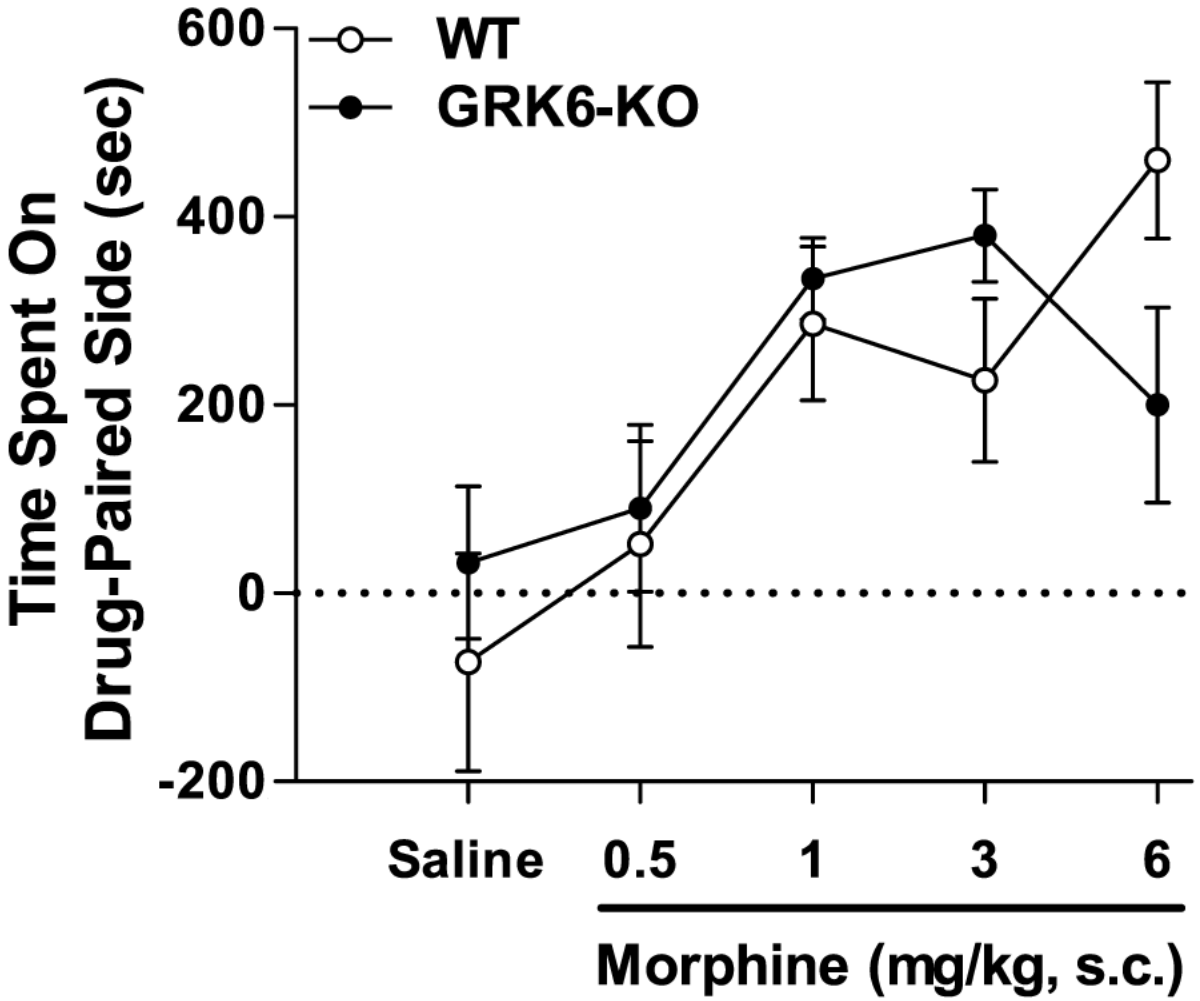

Figure 4.

Morphine produces similar conditioned place preference responses in WT and GRK6-KO mice. Following a 6 day conditioning regimen (drug on days 1, 3, and 5; saline on days 2, 4, and 6), the amount of time spent in each compartment in the absence of drug was assessed in a 30 minute test (post-conditioning). Data are shown as the difference in the time spent in the morphine-paired compartment between the post-conditioning day and the pre-conditioning day (n=5–12/group). Data are presented as the mean ± S.E.M.

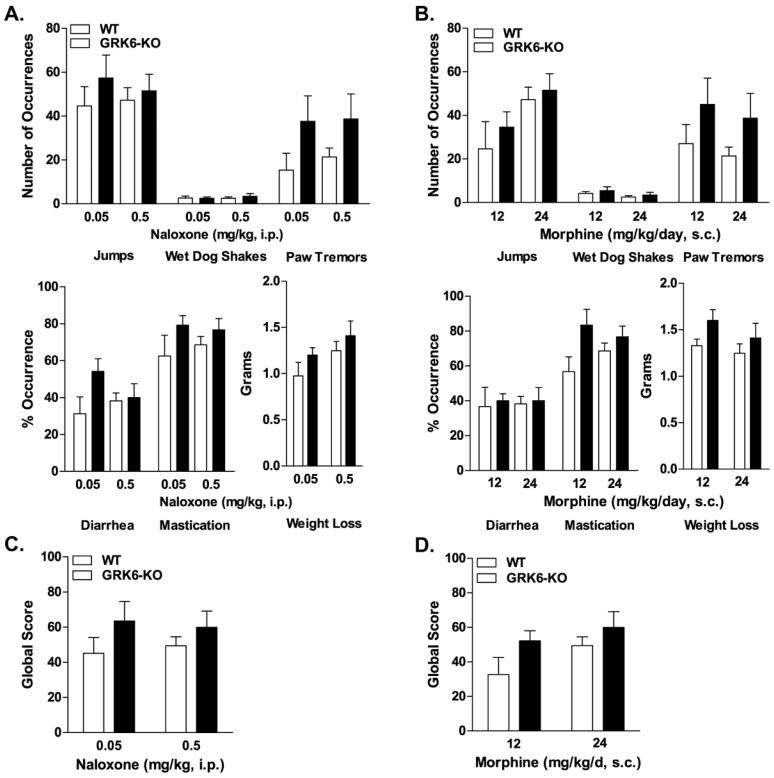

Figure 5.

Morphine-induced physical dependence develops to the same extent in both WT and GRK6-KO mice. Data are presented as the mean ± S.E.M. (A & B) Antagonist-precipitated withdrawal responses measured over a 30 minute period in WT and GRK6-KO mice using naloxone (0.05 or 0.5 mg/kg, i.p.) following 7 days of chronic infusion of morphine (12 or 24 mg/kg/day, s.c.) (n=6–17/group). (C & D) The calculated global withdrawal score which collectively accounts for all of the naloxone-precipitated withdrawal responses (except for weight loss) in WT and GRK6-KO mice made dependent on morphine (see text for details) (n=5–17/group).

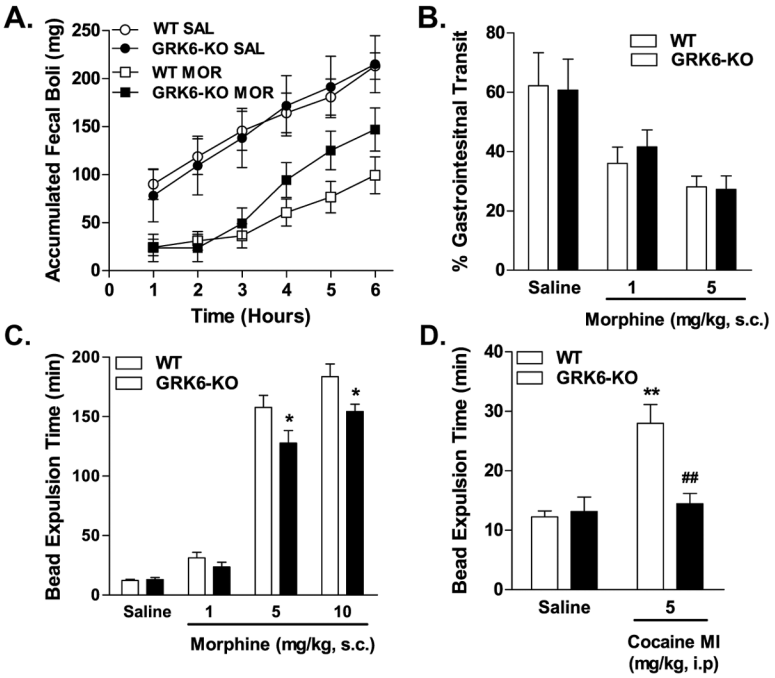

Figure 6.

Gastrointestinal responses following acute treatment with morphine and cocainemethiodide in WT and GRK6-KO mice. Data represent the mean ± S.E.M. (A) Mice were treated with saline (s.c.) or morphine (10 mg/kg, s.c.) and fecal boli were weighed at 1 hour intervals over a 6 hour period (n=12–15/group). (B) Small intestinal transit was determined by giving mice an injection of saline (s.c.) or morphine (1, 5 mg/kg, s.c.) 20 minutes prior to receiving an orally administered charcoal meal. Thirty minutes later, the small intestine from the pyloric sphincter to the ileal cecal junction was isolated and the distance traveled by the leading edge of the charcoal meal was measured relative to the total length of the small intestine and the percent of gastrointestinal transit for each treatment group was calculated (n=3–4/group). (C) Large intestinal transit was determined using a colonic bead expulsion assay in which WT and GRK6-KO mice were treated with saline (s.c.) or morphine (1, 5, 10 mg/kg, s.c.) and five minutes later a 3-mm glass bead was inserted 2 cm into the distal rectum and the time to bead expulsion was measured (WT vs KO, $*p<0.05$; n=4–8). (D) Mice were treated with cocaine methiodide (cocaine MI, 5 mg/kg, i.p.) and colonic motility was measured using the colonic bead expulsion assay as described above (WT vs KO cocaine MI, $^{\#\#}p<0.01$; WT saline vs cocaine MI, $**p<0.01$, n=5/group).