# THE ROLE OF $\beta$ -ARRESTIN2 IN THE MECHANISM OF MORPHINE TOLERANCE IN THE MOUSE AND GUINEA PIG GASTROINTESTINAL TRACT

Minho Kang, Hercules T. Maguma, Tricia H. Smith, Gracious R. Ross, William L. Dewey, and Hamid I. Akbarali

Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA 23298 (M.K., H.T.M, T.H.S., G.R.R.,W.L.D.H.I.A.).

JPET #186320 2

a) Running Title:  $\boldsymbol{\beta}$  -arrestin2 in morphine tolerance

b) Corresponding author:
Hamid I. Akbarali, Ph.D.
Department of Pharmacology and Toxicology,
Virginia Commonwealth University
1112 E. Clay St., McGuire Hall 317
Richmond, VA 23298
Tel (804) 828 9688

Email: hiakbarali@vcu.edu

c) TEXT PAGES: 28

FIGURES: 8

**REFERENCES: 32** 

ABSTRACT: 250 words

**INTRODUCTION: 467** 

DISCUSSION: 1509 words

d) ABBREVIATIONS: LMMP- Longitudinal muscle myenteric plexus, EFS- Electrical Field Stimulation,  $plC_{50}$  – Half maximal inhibition ( $lC_{50}$ ) (-Log M)

e) Section: Neuropharmacology

#### **ABSTRACT**

β-arrestin2 has been reported to play an essential role in analgesic tolerance. Analgesic tolerance without concomitant tolerance to constipation are limiting side-effects of chronic morphine treatment. Since tolerance to morphine develops in the mouse ileum but not the colon, we therefore examined whether the role of β-arrestin2 in the mechanism of morphine tolerance differs in the ileum and colon. In both guinea pig and mouse, chronic in-vitro exposure (2 hours, 10 µM) to morphine resulted in tolerance development in the isolated ileum but not the colon. The pIC<sub>50</sub> for morphine-induced inhibition of electrical field-stimulated (EFS) contraction of guinea pig longitudinal muscle myenteric plexus (LMMP) rightward shifted in the ileum from  $5.7 \pm 0.08$  (n=9) to  $5.45 \pm 0.09$  (n=6) (p<0.001) following morphine exposure. A significant shift was not observed in the colon. Similar differential tolerance was seen between the mouse ileum and colon. Tolerance, however, developed in the colon from β-arrestin2 knock-out mice. β -arrestin2 and ERK 1/2 expression levels were further determined by Western blots in guinea pig LMMP. A time-dependent decrease in expression of β -arrestin2 and ERK 1/2 occurred in the ileum but not the colon following 2 hour morphine (10 µM) exposure. Naloxone prevented the decrease in β –arrestin2. In the isolated ileum from guinea pigs chronically treated in-vivo with morphine for 7 days, neither additional tolerance to in-vitro exposure of morphine nor decrease in  $\beta$ -arrestin2 occured. We conclude that a decrease in  $\beta$ arrestin2 is associated with tolerance development to morphine in the gastrointestinal tract.

#### INTRODUCTION

Morphine remains one of the most frequently prescribed drugs for the treatment of moderate to severe pain. The clinical efficacy of morphine is however limited due to the presence and persistence of some of the adverse side-effects in chronically treated patients (Pappagallo, 2001; Holzer et al., 2009). Side effects to morphine treatment include nausea and vomiting, abdominal pain and constipation that are collectively referred to as "opioid-induced bowel dysfunction". While tolerance develops towards many of the effects of morphine, including analgesia, constipation remains resistant to tolerance such that in many cases patients choose to limit or discontinue opioid treatment (Moore and McQuay, 2005; Holzer et al., 2009; Tuteja et al., 2010). Consistent with the persistence of morphine-induced constipation in humans, tolerance to morphine-induced retardation of colonic transit does not develop in either moderate (5.5 fold) or high (52 fold) antinociceptive tolerant mice (Ross et al., 2008). The invivo findings correlate with the lack of tolerance development to repeated morphine administration in isolated colonic segments, an important site for the induction of constipation. Interestingly, unlike the colon, tolerance to morphine develops in the ileum. Tolerance to the effects of opioids in the guinea pig ileum has been well documented both in vitro (Paton, 1957) (Rezvani et al., 1983) as well as in-vivo (Goldstein and Schulz, 1973). Similarly tolerance also develops to gastrointestinal transit as well as in isolated segments of mouse ileum upon repeated or prolonged incubation with morphine (Ross et al., 2008). Thus, a major question that arises from these studies is whether differences in the cellular mechanisms could account for morphine tolerance in ileum but not in the colon.

The mechanisms underlying morphine tolerance are complex and not fully understood.

Studies examining morphine tolerance have largely focused on analgesic tolerance and the

neuronal circuitry associated with it and in heterologously expressed cell lines. Much less is known with regard to mechanisms of tolerance development or the lack of it in enteric neurons. One of the canonical pathways associated with opiate tolerance is the process of desensitization/internalization following receptor phosphorylation by G-protein receptor kinases (GRK) and recruitment of  $\beta$ -arrestins. The  $\beta$ -arrestins bind to phosphorylated receptors, uncouples them from G-proteins and facilitates internalization resulting in desensitization. Antinociceptive tolerance is reduced in  $\beta$ -arrestin2 knock-out mice (Bohn et al., 2000) which suggests that increased levels of  $\beta$ -arrestin2 may be importantly involved in the mechanism of tolerance. In this study we investigated whether the difference in morphine tolerance in the ileum and colon was due to differences in the role of  $\beta$ -arrestin2 in these two tissues. To address whether  $\beta$ -arrestin2 is involved in tolerance development in the gastrointestinal tract, we examined functional development of tolerance in the isolated ileum and colon from mice and guinea pigs upon repeated or prolonged administration of morphine and measured expression of  $\beta$ -arrestin2 in enteric neurons.

#### **METHODS**

Animals: Adult male albino guinea pigs (200-250g) (N= 35) were purchased from Charles River Laboratories (Wilmington, MA). The breeding pairs for the β-arrestin2 knock-out mice on C57/Bl6 background were obtained from Dr Lefkowitz (Duke University) and housed within the transgenic facility at Virginia Commonwealth University. All procedures with animals were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. Chronic parenteral exposure to morphine or saline was carried out over 7 days using the following schedule (Li et al., 2010; Patierno et al., 2011): Morphine or saline was injected subcutaneously twice/day using the following paradigm: On day 1, 10 mg/kg b.i.d.; day 2 and 3, 20 mg/kg b.i.d.; days 4, 5, and 6, 40 m/kg b.i.d.; day 7, 80 mg/kg b.i.d. Morphine or vehicle was injected at 12 hour intervals and the animals were euthanized on day 8.

## Preparation of longitudinal and circular muscle from ileum and colon.

Guinea- pigs were sacrificed by CO<sub>2</sub> asphyxiation and mice by cervical dislocation. The distal colon (approx. 1 cm from the anus) and ileum (approx. 5 cm from the ileo-cecal junction) were dissected out immediately and placed in a dissecting dish containing Krebs solution (118 mM NaCl, 4.6 mM KCl, 1.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 11 mM glucose, and 2.5 mM CaCl<sub>2</sub>). For the guinea pig experiments, the segments of tissues were placed longitudinally on glass rods through the lumen and the longitudinal muscle with the myenteric plexus (LMMP) were scrapped gently with a cotton- tipped applicator. About 2 cm long LMMP preparations were suspended in the axis of the longitudinal muscle tied to a glass hook under 1g passive tension in 25 ml of siliconized organ baths. For the experiments with mice, circular muscle preparations were used. The distal colon (approx. 1 cm proximal to the colon) and the ileum (approx.2 cm proximal to the ileo-cecal junction) were dissected. In mice, unlike the

guinea pig, the myenteric plexus strongly adheres to the circular muscle (Furukawa et al., 1986). We have previously shown that morphine induces robust contractions of the circular muscle due to removal of an neurogenic inhibitory tone. This was confirmed in preliminary experiments whereby blocking neuronal activation with tetrodotoxin (1 – 10  $\mu$ M) abolished morphine-induced contractions. Circular muscle rings were dissected and mounted in the organ bath using a metal triangle on one end and fixed to a glass hook on the other end (Ross et al., 2008). Tissues were bathed in Krebs solution maintained at 37°C and bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and equilibrated for 60 minutes before start of experiments. Tissues were washed in Krebs solution every 15 min.

**Neurogenic contractions:** Electrical field stimulation (EFS; 50 V, 7.5 Hz, unless stated otherwise) was applied through concentric electrodes over LMMP to produce neurogenic contractions. Isometric contractions were recorded by a force transducer (GR-FT03; Radnoti, Monrovia, CA) connected to a personal computer using Acqknowledge 382 software (BIOPAC Systems, Inc., Santa Barbara, CA). Acetylcholine (3 μM) contractions were measured at the beginning of each experiment as the reference control.

Morphine tolerance *in vitro* was measured in the guinea pig LMMP preparations using either repeated 10 min exposures, or a 2 hr exposure. For repeated exposures, tissues were incubated with morphine (10 µM) (Mallickrodt, Hazelwood, MO) for 10 min, followed by washout in Krebs solution. Following recovery of EFS-induced contractions, morphine was again applied to the bath at the same concentration. This was repeated 4-5 times. Tolerance was determined by measuring the percentage inhibition of EFS-induced contractions in the absence and presence of morphine at each successive exposure. For tolerance development following longer exposure (2 hour), a cumulative dose-response curve to morphine was carried out after tissue equilibration. The percent inhibition of EFS-induced contractions was measured at each

successive concentration. Tissues were then incubated with morphine (10  $\mu$ M) for 2 hours, washed in Krebs solution and the cumulative dose-response repeated.

Morphine induced contractions of the circular muscle preparations of the mouse ileum and colon were measured as previously described (Ross et al., 2008). Briefly, repeated exposure to morphine (3  $\mu$ M) was applied 4 times at intervals of 30 mins with repeated washings in Krebs between these exposures. The amplitude of the initial response was taken as 100% and percentage response calculated against this for each successive response.

Isolated myenteric ganglia. The sheets of LMMP were incubated for 6 minutes at 37°C in Krebs solution containing 0.1% protease, 0.2% collagenase type IV and 0.1% BSA for enzymatic digestion. The tissue was consistently bubbled with 95% O₂/5% CO₂, accompanied by frequently trituration with wide-bore pasteur pipette to free the ganglia. The partly digested tissue was then collected by filtering through 500 μm Nitex mesh and incubated for one hour at 37°C in enzyme-free Krebs solution for further isolation. The suspension was then filtered to remove the undigested tissue and filtrate was centrifuged for 1 minute at 1,000 rpm. The supernatant containing smooth muscle cells and debris was discarded, and the pellet containing the ganglia resuspended and washed twice in enzyme-free Krebs solution to remove cell debris. The pellet was resuspended and placed in a 10 cm culture dish. The ganglia were harvested by suction into a 20 μl capillary tube under microscope.

Western blots. Protein samples from guinea pig LMMP tissues were prepared in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA) containing protease inhibitors, 0.2 mM phenylmethylsulfonyl fluoride and 0.1 mM sodium orthovanadate using the Bullet Blender (Next Advance Inc, Averill Park, NY). Protein concentration was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL). Standard western blot procedures were used as described previously (Kang et al., 2004). Briefly, samples were boiled for 3 minutes and subjected to 10% SDS-

PAGE and then electroblotted using the iBlot system (Invitrogen, Carlsbad, CA). Immunoblotting was performed on a SNAPid system (Millipore, Billerica, MA) using anti-mouse-β-arrestin2, anti-rabbit-GAPDH, anti-mouse-GRK2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-rabbit-βIII-Tubulin (Abcam, Cambridge, MA), anti-mouse ERK and anti-rabbit phospho-ERK (Cell Signaling, Beverly, MA) as primary antibodies, and the goat anti-rabbit IRDye® 680 and goat anti-mouse IRDye® 800CW (LiCor Biosciences, Lincoln, NE) as secondary antibodies. The membranes were visualized and density of each band was measured using the LiCor Odyssey infrared imaging system (LiCor Biosciences, Lincoln, NE). The use of separate wavelengths of the secondary antibodies allowed for determining the ratios of proteins on the same blot. To confirm that protein loading and density of the control β-III tubulin was not saturated, various amounts of protein concentrations (40 to 120 μg/lane) were loaded and the expression of β-arrestin2, β III-tubulin and GAPDH were measured. For each western blot, the density of the loading control was confirmed to lie within the linear range.

## **Real-Time PCR**

Total RNA was extracted with PureLink Micro-to-Midi Total RNA Purification System, as described by the manufacturer (Invitrogen). The first strand cDNA was amplified using SensiMix One-Step Kit (Quantace) at 42°C for 30 min. Primers were designed using Vector NTI Suite software (Invitrogen). (see Supplementary Material for primer sequences and PCR methodology).

**Data analysis.** The data are expressed as means ± SE from 3-5 individual experiments for all Western blots. Dose-response to morphine inhibition of EFS contractions were analyzed by repeated measures ANOVA followed by Bonferroni's *post hoc* test or by paired Student's t-test where appropriate. Effective concentration of half-maximal inhibition (IC<sub>50</sub>) was calculated by

JPET Fast Forward. Published on November 30, 2011 as DOI: 10.1124/jpet.111.186320 This article has not been copyedited and formatted. The final version may differ from this version.

JPET #186320 10

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 10, 2024

non-linear regression and reported as negative log values (-Log M). *P* values <0.05 were considered significant. N refers to number of animals, n=number of tissues.

## **RESULTS**

#### Tolerance in β-arrestin2 knock-out mice.

Previous *in-vivo* studies have shown that an acute dose of morphine-induced a decrease in colonic transit and inhibition of fecal output in wild type but not in  $\beta$ -arrestin2 knock-out mice (Raehal et al., 2005). In addition, it has been shown that  $\beta$ -arrestin2 contributes to the development of tolerance to the antinociceptive effect of morphine in mice (Bohn et al., 1999). We now have investigated the role of  $\beta$ -arrestin2 in the development of tolerance to morphine in circular muscle preparations in wild-type and  $\beta$ -arrestin2 knock out mouse ileum and colon. We (Ross et al., 2008) have previously shown that morphine induces contraction of circular muscle preparations and repeated administration results in tolerance in the ileum but not the colon. Contractions of circular muscle preparations result from dis-inhibition of a constitutive inhibitory neurogenic tone (Grider and Makhlouf, 1987a) and both morphine and TTX produce contractions. In the presence of TTX (1 -10  $\mu$ M), morphine induced contractions were abolished in the mouse (data not shown).

After 1 hr of equilibration of the isolated circular muscle from ileum and colon, repeated administrations of morphine (3  $\mu$ M) were applied every 30 mins, with in-between washes. Morphine produced individual contractions that waned with time (supplementary figure 1) in both ileum and colon. The peak contractions following each administration of morphine were measured and normalized as a percent of the first peak contraction in that tissue. As shown in Figure 1 (and supplementary figure 1A and B), the peak contractile response to morphine was markedly reduced upon repeated administration in the ileum but not the colon in wild-type mice indicative of the development of tolerance in the ileum but not the colon. Compared to the 1<sup>st</sup> response, morphine-induced contractions in the ileum were significantly reduced following the  $2^{nd}$  exposure (50 ± 10%, p<0.05, paired Student's t-test; n=4) and subsequently remained reduced upto the  $4^{th}$  application (42 ± 9%). Similarly, in the  $\beta$  -arrestin2 knock-out mice,

significant tolerance developed following the  $2^{nd}$  (67 ± 6%, p<0.05, paired Student's t-test; n=5),  $3^{rd}$  (65 ± 4%) and  $4^{th}$  (66 ± 5%) administrations. There was however no significant difference in the peak contractions to morphine in the colon of wild-type mice upon repeated administration of morphine. However significant tolerance developed in the colon from  $\beta$ -arrestin2 knock- out mice. In comparison to the initial response, the  $2^{nd}$  exposure resulted in contractions that were reduced to  $54 \pm 5\%$  (p<0.05, n=6), and  $45 \pm 7\%$  at the  $4^{th}$  administration. Thus tolerance developed to repeated morphine exposure in the colon in the absence of  $\beta$  arrestin2 but not in tissues taken from wild type mice. The initial contractile force produced by morphine in the ileum and colon of wild-type and  $\beta$ -arrestin2 knock-out mice were not statistically different (ileum:  $42.6 \pm 7.1$  (n=6) vs  $65.4 \pm 12.8$  (n=9) mg/mg tissue weight; p = 0.20 ; t-test) (colon:  $55.1 \pm 12.5$  (n=10) vs  $71.4 \pm 17.3$  (n=10) mg/mg tissue weight; p = 0.45, t-test) (supplementary figure 2).

The above data show that repeated morphine treatment causes tolerance in the colon in the absence of  $\beta$ -arrestin2. In order to further investigate the role of  $\beta$ -arrestin2 in morphine tolerance, we determined the expression of  $\beta$ -arrestin2 in the mouse by western blots. The specificity of the  $\beta$ -arrestin2 antibody was tested by pre-absorption with the antigen peptide (supplementary figure 3). Initially, experiments were carried out to examine the feasibility of using the  $\beta$ -arrestin2 antibody in the mouse. A ~50kDa protein corresponding to  $\beta$ -arrestin2 was detected in the mouse brain of wild-type but not  $\beta$ -arrestin2 knock- out mice (Figure 2). However,  $\beta$ -arrestin2 was barely detectable in the mouse ileum even when cell lysates from 3 mice were pooled. This reflects the low protein yields of enteric neurons obtained from the tissues of the mouse gastrointestinal wall. This could be due to the myenteric neurons strongly adhering to the circular muscle and therefore difficult to separate out from the muscle layers. To obtain a more enriched preparation of myenteric neurons, we used the guinea pig longitudinal muscle-myenteric plexus preparation (LMMP). Distinct bands corresponding to  $\beta$ -

arrestin2 was detected in the guinea pig colon and ileum (Figure 2). Therefore, in order to further explore the role of  $\beta$ -arrestin2 in morphine tolerance in the ileum and colon, we studied the relationship between morphine tolerance and  $\beta$  arrestin2 levels in the guinea pig LMMP ileum and colon preparations.

## Morphine tolerance in the guinea pig LMMP

It has been shown that morphine attenuates the EFS contractions of the guinea pig LMMP preparations due to presynaptic inhibition of acetylcholine release and tolerance develops upon repeated morphine administration in the ileum (Collier et al., 1981). We investigated if similar to mice, the guinea pig colon is resistant to the development of tolerance to morphine. In the present experiments, tolerance development to morphine in the ileum and colon was examined in two different ways. In the first series of experiments, a cumulative doseresponse to morphine inhibition of EFS was determined prior to and following a 2 hour incubation with 10 µM morphine (Figure 3). The exposure time to each concentration was less than 5 min. We found that the cumulative dose-response curve to morphine was reproduced three times in both ileum and colon, The initial IC<sub>50</sub> value (-Log M) for morphine-induced inhibition of EFS in the ileum was  $5.70 \pm 0.08$  (n=9) and  $5.43 \pm 0.14$  (n=7) for the colon. After morphine incubation for 2 hours the IC<sub>50</sub> shifted to  $5.45 \pm 0.09$  (n=6) (p<0.0001) (repeated measures ANOVA) in the ileum whereas it was 5.48 ± 0.17 (n=6) (p=0.829) in the colon. Both the rightward shift in dose-response and the decrease in maximal inhibition were significant in the ileum but not in the colon (Figure 3). In the next series of experiments, individual concentrations of morphine were tested between washes. As we found in tissues from mice, tolerance occurred in the ileum but not the colon of guinea pigs (supplementary figure 4).

In the following experiments, LMMP strips from guinea pig ileum and colon were incubated with a concentration of morphine (10 µM) for 2 hours that has been shown to induce

tolerance in the ileum, and levels of  $\beta$ -arrestin2 and GRK2 expression were determined by immunoblots. Morphine treatment did not alter GRK2 expression in either tissue, however, the expression of  $\beta$ -arrestin2 was markedly reduced in the ileum but not the colon (n=6) (Figure 4).

The expression of  $\beta$ -arrestin2 was examined in enzymatically isolated neurons from the ileum. The neuronal marker,  $\beta$ -III tubulin (Tuj1) was used as the loading control. In whole mount LMMP preparations,  $\beta$ -arrestin2 (figure 5A) and  $\beta$ -III tubulin staining was confined to myenteric neurons (supplementary figure 5). In enzymatically isolated neuronal preparations,  $\beta$ -arrestin2 expression was significantly reduced in the morphine treated samples from the ileum by  $66 \pm 3$ % (n=3) (figure 5B). To further determine if the reduced levels of  $\beta$ -arrestin2 were dependent on  $\mu$ -receptor mediated activation by morphine, the colon and ileum LMMP preparations were pretreated with naloxone (10  $\mu$ M) for 30 min prior to morphine exposure for 2 hours. Naloxone prevented the decrease in  $\beta$ -arrestin2 expression in the ileum (figure 5C) indicative of a  $\mu$  receptor mediated effect (N=2).

Agonist-stimulated degradation of  $\beta$ -arrestin2 may be transient (Shenoy et al., 2001) and therefore raises the possibility that the kinetics of  $\beta$ -arrestin2 down-regulation between the colon and ileum may differ due to differences in this process. We therefore examined expression of  $\beta$ -arrestin2 in both ileum and colon at multiple time points. As shown in figure 6A, loss of  $\beta$ -arrestin2 expression was not evident at 3 or 10 min in the colon or ileum. In the ileum, the decrease in  $\beta$ -arrestin2 was significant at 2 hours of morphine exposure and persisted up to 5 hours. In the colon, a decrease in  $\beta$ -arrestin2 was not observed at any time point. The mRNA expression of  $\beta$ -arrestin2 was not altered by chronic morphine exposure (figure 6B) indicating that the down-regulation was not due to decreased synthesis.

β-arrestins act as scaffolding proteins and have been shown to activate mitogen activated protein kinases such as ERK1/2 (DeWire et al., 2007). There was a significant

reduction in phospho-ERK1/2 concomitant with a decrease in β-arrestin2 in the ileum but not the colon following morphine exposure (figure 7A and B).

## Chronic in vivo morphine treatment

We tested whether tolerance to morphine inhibition of EFS was induced in guinea pig LMMP from animals treated with morphine in-vivo for 7-days (twice daily). A cumulative doseresponse to morphine was carried out in LMMP from guinea pigs chronically treated with morphine or saline. The IC<sub>50</sub> (-LogM) for inhibition of EFS-induced contractions in the ileum from saline treated animals was 5.81 ± 0.07 (n=8;N=3) that was not statistically significant from the ileum of non-injected naïve animals. In the chronically morphine treated animals the doseresponse was significantly (p<0.0001) shifted to the right with an IC<sub>50</sub> of  $5.4 \pm 0.05$  (n=19:N=6) indicative of tolerance development (Figure 8A). The IC<sub>50</sub> for morphine in the colon from saline treated guinea pigs was 5.62 ± 0.12 (n=5:N=3) and was not significantly different from the colon of chronic morphine treated guinea pigs (5.41 ± 0.12 (n=12:N=6). These results in isolated organs from guinea pigs treated chronically in-vivo with morphine were similar to those seen in isolated organs from drug naïve guinea pigs i.e. tolerance was observed in the ileum but not the colon. To further establish that tolerance had developed following chronic parental administration of morphine, LMMP strips were incubated for 2 hours with 10 µM morphine. As shown in Figure 8A, in both the ileum (A; filled triangles) and the colon (B; filled triangles), no further shifts were observed after 2 hour morphine exposure. Thus tolerance develops in the ileum following chronic parenteral administration as the dose-response is shifted compared to saline treated, and that further 2 hour incubation does not induce a shift. Tolerance did not develop to morphine in LMMP from colon as the dose-response is similar to saline treated animals after chronic in-vivo treatment or after additional in-vitro incubation with morphine.

We tested whether the *in vitro* incubation of morphine altered the level of  $\beta$ -arrestin2 and p-ERK1/2 in guinea pigs treated for 7 days with morphine. LMMP preparations from the colon and ileum of guinea pigs treated for 7 days *in vivo* were further exposed for 2 hours incubation with morphine (10 µM) and subjected to Western blot analysis. Consistent with the lack of additional functional tolerance in the ileum, the levels of  $\beta$ -arrestin2 expression were not altered by further in vitro 2 hour treatment with morphine (figure 8B and supplementary figure 6). Similarly, no changes were observed in p-ERK1/2 expression in the ileum following morphine treatment (figure 8C).

## **DISCUSSION**

Our findings show that a decrease in the  $\beta$ -arrestin2 levels accompanies the development of tolerance to morphine in the gastrointestinal tract. Here we demonstrate that tolerance to morphine occurs in guinea pig and mouse ileum but not in the colon of either species. The expression of  $\beta$ -arrestin2 is significantly decreased in isolated tissues of the guinea pig ileum following two hour morphine exposure. On the other hand, neither tolerance nor a decrease in  $\beta$ -arrestin2 levels are observed in the colon. Since genetic deletion of  $\beta$ -arrestin2 in the knock-out mice results in tolerance development in the colon, this implies that levels of  $\beta$ -arrestin2 play an important role in morphine-induced tolerance in the gastrointestinal tract.

Notwithstanding the prevalence, suffering and economic burden of chronic pain, opiate-based therapy remains under-utilized largely as a result of adverse side-effects. Clinical reports suggest that of the several adverse effects of long term opiate use, constipation is one of the most debilitating (Grond et al., 1994; Kurz and Sessler, 2003; Droney et al., 2008; Tuteja et al., 2010). Studies in several species including the mouse and the guinea pig have demonstrated that morphine alters gastrointestinal motility and produces constipation via its effect on  $\mu$  opioid receptors. (Roy et al., 1998; Sternini et al., 2004). In this study we found that like the mouse (Ross et al., 2008), tolerance develops in the guinea pig ileum but not the colon. Due to the reported role of  $\beta$ -arrestin2 in antinociceptive tolerance, we investigated the role of  $\beta$ -arrestin2 in morphine tolerance in the gastrointestinal tract.

Morphine and other opioids inhibit both excitatory and inhibitory neurotransmitter release in the gastrointestinal tract resulting in inhibition of a) peristaltic contractions, and b) elevation of tone and enhanced non-propulsive contractions leading to constipation. In the isolated organs, the inhibition by morphine of excitatory cholinergic –mediated contractions induced by EFS partly reflects the inhibition of neurogenic control of peristaltic contractions, and has been

classically studied in the guinea pig ileum LMMP preparations. Morphine and other opioids also contract circular muscle preparations via inhibition of a constitutive inhibitory neurogenic tone. Studies by Grider and Makhlouf (Grider and Makhlouf, 1987b) showed that addition of tetrodotoxin (TTX) induces contractions of rat colon circular muscle preparations and the effects of endogenous opioids can be partially abolished in the presence of TTX. In the mouse circular muscle preparations, addition of TTX also induces tone and blocks morphine-induced contractions (data not shown). Unlike the guinea pig where the myenteric plexus is largely attached to the longitudinal muscle, in the mouse the myenteric plexus strongly adheres to the circular muscle (Furukawa et al., 1986). We therefore chose to use the circular muscle preparations to examine morphine-induced tolerance in the mouse. Using the circular muscle preparations, we found as in our previous study (Ross et al., 2008) that tolerance develops to repeated administration of morphine in the ileum but not the colon, however tolerance develops in the colon from the  $\beta$ -arrestin2 knock-out mice. Interestingly, the absence of  $\beta$ -arrestin2 did not prevent the acute effects of morphine. The amplitude of the initial contractile response to morphine was not significantly different in the knock-out mice compared to those produced in the wild-type. This suggests that the acute effects of morphine do not require β-arrestin2 signaling. However, a decrease in β-arrestin2 appears to be necessary to produce tolerance i.e. decrease in agonist responsiveness following chronic treatment. Similar acute effects of morphine have been observed in-vivo towards inhibition of fecal output in the β-arrestin2 knockout mice. Raehal et al. (Raehal et al., 2005) showed that morphine initially suppressed fecal output in the β-arrestin2 knock-out mice but it recovered after 2 hours. The recovery may be due to tolerance to morphine –induced inhibition of defecation developing over time in the knock-out mice in the absence of β-arrestin2. Similarly, acute morphine inhibits gastrointestinal transit to the same extent in wild type and β-arrestin2 knock-out mice supporting the hypothesis that acute effects of morphine do not require signaling via  $\beta$ -arrestin2 in the ileum or colon. Although recovery from morphine-induced inhibition of gastrointestinal transit was not observed

in the  $\beta$ -arrestin2 knock-out mice (Raehal et al., 2005), it is possible that tolerance may not have developed 50 mins post-morphine. Further studies may be required to determine the extent of tolerance to morphine-induced inhibition of gastrointestinal transit in the  $\beta$ -arrestin2 knock-out mice. We have previously shown that tolerance develops to inhibition of gastrointestinal transit in morphine-pelleted mice (Ross et al., 2008).

There were two major advantages of using the guinea pig to measure changes in the expression levels of  $\beta$ -arrestin2 within the ileum and colon for this study: 1) The relative increased ease of obtaining LMMP with an intact ganglionic plexus, 2) historically, morphine tolerance and dependence have been well studied in this model (Collier et al., 1981; Rezvani et al., 1983). In addition, the  $\beta$ -arrestin2 antibody showed specificity in the guinea pig allowing for biochemical measurements. The latter is of particularly relevance since our experiments with mouse ileum showed low expression, although  $\beta$ -arrestin2 could be detected from the brain of wild-type and absent in the knock-out mice. Moreover, the antibody recognized a distinct band in the guinea pig LMMP and isolated neuronal preparations.

While chronic morphine resulted in a decrease in  $\beta$ -arrestin2 levels in the ileum, a similar decrease was not observed in the colon. Thus mechanisms associated with recruitment/downregulation of  $\beta$ -arrestin2 likely differ between these two tissues. Recruitment of  $\beta$ -arrestin2 to the  $\mu$  receptor can result in internalization, degradation and/or recycling of the receptor upon agonist-mediated activation. A number of studies support the notion that the cellular environment affects  $\mu$  receptor regulation (Connor et al., 2004; Raehal et al., 2011). Much less is known regarding  $\beta$ -arrestin2 regulation. Recent studies suggest that  $\beta$  -arrestin may be ubiquitinated following agonist stimulation and is a necessary step in the trafficking and degradation of GPCR (Shenoy et al., 2001). Ubiquitination would potentially direct  $\beta$ -arrestin2 towards degradation by the proteosome. The  $\mu$ -opioid receptors belong to "Class A" type receptors which have been shown to induce transient ubiquitination of  $\beta$ -arrestin2 and also

promote its de-ubiquitination that leads to dissociation with the receptor complex and decreased ERK activation (Shenoy et al., 2009). The transient nature of  $\beta$ -arrestin2 ubiquitination is due to the recruitment of deubiquitnating enzymes following receptor stimulation. It is possible that differences between the ileum and colon may relate to mechanisms associated with  $\beta$ -arrestin2 ubiquitination/deubiquitination thus altering the time-course of  $\beta$ -arrestin2 levels in these tissues following morphine. The mechanisms associated with receptor trafficking and degradation of  $\beta$ -arrestin2 merits further investigation.

In the isolated ileum from chronically treated guinea pigs, the two hour in-vitro exposure to morphine did not alter  $\beta$ -arrestin2 levels. This would appear at first to be in contrast to our in-vitro findings from drug naïve animals. However, the absence of further tolerance development during the 2 hour in-vitro incubation is consistent with the observation that  $\beta$ -arrestin2 did not decrease. Although,  $\beta$ -arrestin2 levels were detectable in the ileum after 7 days of treatment, the effects of in-vivo chronic morphine may either result in uncoupling of the receptor from  $\beta$ -arrestin2 or that mechanisms associated with its down-regulation are altered such that with respect to tolerance development it resembles the colon. Presently, it is not clear as to whether the cellular basis for tolerance induced in-vitro is the same as that seen in-vivo. It is well known that more than one mechanism of morphine tolerance may exist. Nevertheless, our studies demonstrate that downregulation and/or uncoupling of receptor with  $\beta$ -arrestin2 accompanies tolerance development in the gastrointestinal tract. The temporal relationship of  $\beta$ -arrestin2 and chronic morphine exposure *in-vivo* needs further examination to clarify this issue.

We also observed a parallel decrease in phospho-ERK with β-arrestin2 in the ileum but not the colon. The significance of this finding is not entirely clear, however it has been suggested that ERK mediated signaling plays an important role in preventing tolerance and it is likely that chronic morphine disrupts the β-arrestin2/p-ERK complex in the ileum but not the

colon. Previous studies have demonstrated that opioids may either increase or decrease the MAP kinase signaling pathway depending on the time course or system under investigation (Muller and Unterwald, 2004).  $\beta$ -arrestin2 also serves as a scaffolding protein, and it is likely that the decrease in phospho-ERK follows from the decreased levels of  $\beta$ -arrestin2.

Many pathways have been associated with opioid tolerance including the role of phospholipase C, protein kinases A and C, protein phosphatases, G-receptor kinases and CD-38 pathway (Taylor and Fleming, 2001; Bailey et al., 2006; Smith et al., 2007). Differences in the rate and degree of tolerance have been reported for high and low efficacy agonists and in different organs. It is noteworthy that most of these studies utilized cell based assays, or antinociception in-vivo or in-vitro studies of neurons involved in reward pathways. The present study further highlights that extrapolation of the mechanisms from one paradigm i.e. analgesia to another i.e. gastrointestinal function may not be justified. Tolerance in the gastrointestinal tract is associated with decreased levels of β-arrestin2 in contrast to antinociceptive tolerance where β-arrestin2 knock-out results in prevention of tolerance (Bohn et al., 1999).

## **AUTHORSHIP CONTRIBUTION:**

Contributed equally to this work: Kang, Maguma

Participated in research design: Kang, Maguma, Ross, Akbarali, Dewey

Conducted experiments: Kang, Maguma, Ross, Smith Performed data analysis: Kang, Maguma, Ross, Smith

Wrote the manuscript. Akbarali, Dewey

## **REFERENCES**

Bailey CP, Smith FL, Kelly E, Dewey WL and Henderson G (2006) How important is protein kinase C in mu-opioid receptor desensitization and morphine tolerance? *Trends Pharmacol Sci* 27:558-565.

Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ and Caron MG (2000) Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* 408:720-723.

Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG and Lin FT (1999) Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* 286:2495-2498.

Collier HO, Cuthbert NJ and Francis DL (1981) Model of opiate dependence in the guineapig isolated ileum. *Br J Pharmacol* 73:921-932.

Connor M, Osborne PB and Christie MJ (2004) Mu-opioid receptor desensitization: is morphine different? *Br J Pharmacol* 143:685-696.

DeWire SM, Ahn S, Lefkowitz RJ and Shenoy SK (2007) Beta-arrestins and cell signaling. *Annu Rev Physiol* 69:483-510.

Droney J, Ross J, Gretton S, Welsh K, Sato H and Riley J (2008) Constipation in cancer patients on morphine. *Support Care Cancer* 16:453-459.

Furukawa K, Taylor GS and Bywater RA (1986) An intracellular study of myenteric neurons in the mouse colon. *J Neurophysiol* 55:1395-1406.

Goldstein A and Schulz R (1973) Morphine-tolerant longitudinal muscle strip from guinea-pig ileum. *Br J Pharmacol* 48:655-666.

Grider JR and Makhlouf GM (1987a) Role of opioid neurons in the regulation of intestinal peristalsis. *Am J Physiol* 253:G226-231.

Grider JR and Makhlouf GM (1987b) Suppression of inhibitory neural input to colonic circular muscle by opioid peptides. *J Pharmacol Exp Ther* 243:205-210.

Grond S, Zech D, Diefenbach C and Bischoff A (1994) Prevalence and pattern of symptoms in patients with cancer pain: a prospective evaluation of 1635 cancer patients referred to a pain clinic. *J Pain Symptom Manage* 9:372-382.

Holzer P, Ahmedzai SH, Niederle N, Leyendecker P, Hopp M, Bosse B, Spohr I and Reimer K (2009) Opioid-induced bowel dysfunction in cancer-related pain: causes, consequences, and a novel approach for its management. *J Opioid Manag* 5:145-151.

Kang M, Morsy N, Jin X, Lupu F and Akbarali HI (2004) Protein and gene expression of Ca2+ channel isoforms in murine colon: effect of inflammation. *Pflugers Arch* 449:288-297.

Kurz A and Sessler DI (2003) Opioid-induced bowel dysfunction: pathophysiology and potential new therapies. *Drugs* 63:649-671.

Li P, Maguma HT, Thayne K, Davis B and Taylor DA (2010) Correlation of the time course of development and decay of tolerance to morphine with alterations in sodium pump protein isoform abundance. *Biochem Pharmacol* 79:1015-1024.

Moore RA and McQuay HJ (2005) Prevalence of opioid adverse events in chronic non-malignant pain: systematic review of randomised trials of oral opioids. *Arthritis Res Ther* 7:R1046-1051.

Muller DL and Unterwald EM (2004) In vivo regulation of extracellular signal-regulated protein kinase (ERK) and protein kinase B (Akt) phosphorylation by acute and chronic morphine. *J Pharmacol Exp Ther* 310:774-782.

Pappagallo M (2001) Incidence, prevalence, and management of opioid bowel dysfunction. *Am J Surg* 182:11S-18S.

Patierno S, Anselmi L, Jaramillo I, Scott D, Garcia R and Sternini C (2011) Morphine Induces mu Opioid Receptor Endocytosis in Guinea Pig Enteric Neurons Following Prolonged Receptor Activation. *Gastroenterology* 140:618-626.

Paton WD (1957) The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br J Pharmacol Chemother* 12:119-127.

Raehal KM, Schmid CL, Groer CE and Bohn LM (2011) Functional Selectivity at the {micro}-Opioid Receptor: Implications for Understanding Opioid Analgesia and Tolerance. *Pharmacol Rev* 63:1001-1019.

Raehal KM, Walker JK and Bohn LM (2005) Morphine side effects in beta-arrestin 2 knockout mice. *J Pharmacol Exp Ther* 314:1195-1201.

Rezvani A, Huidobro-Toro JP, Hu J and Way EL (1983) A rapid and simple method for the quantitative determination of tolerance development to opiates in the guinea-pig ileum in vitro. *J Pharmacol Exp Ther* 225:251-255.

Ross GR, Gabra BH, Dewey WL and Akbarali HI (2008) Morphine tolerance in the mouse ileum and colon. *J Pharmacol Exp Ther* 327:561-572.

Roy S, Liu HC and Loh HH (1998) mu-Opioid receptor-knockout mice: the role of mu-opioid receptor in gastrointestinal transit. *Brain Res Mol Brain Res* 56:281-283.

Shenoy SK, McDonald PH, Kohout TA and Lefkowitz RJ (2001) Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science* 294:1307-1313.

Shenoy SK, Modi AS, Shukla AK, Xiao K, Berthouze M, Ahn S, Wilkinson KD, Miller WE and Lefkowitz RJ (2009) Beta-arrestin-dependent signaling and trafficking of 7-transmembrane receptors is reciprocally regulated by the deubiquitinase USP33 and the E3 ligase Mdm2. *Proc Natl Acad Sci U S A* 106:6650-6655.

Smith FL, Gabra BH, Smith PA, Redwood MC and Dewey WL (2007) Determination of the role of conventional, novel and atypical PKC isoforms in the expression of morphine tolerance in mice. *Pain* 127:129-139.

Sternini C, Patierno S, Selmer IS and Kirchgessner A (2004) The opioid system in the gastrointestinal tract. *Neurogastroenterol Motil* 16 Suppl 2:3-16.

Taylor DA and Fleming WW (2001) Unifying perspectives of the mechanisms underlying the development of tolerance and physical dependence to opioids. *J Pharmacol Exp Ther* 297:11-18.

Tuteja AK, Biskupiak J, Stoddard GJ and Lipman AG (2010) Opioid-induced bowel disorders and narcotic bowel syndrome in patients with chronic non-cancer pain. *Neurogastroenterol Motil* 22:424-430, e496.

JPET Fast Forward. Published on November 30, 2011 as DOI: 10.1124/jpet.111.186320 This article has not been copyedited and formatted. The final version may differ from this version.

JPET #186320 25

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 10, 2024

**Footnotes:** This work was supported by National Institute of Health grants [R01DA024009] [T32DA007027] and [P50DA005274].

# Figure Legends

**Figure 1**: Morphine induced contractions in WT C57/Bl6 and β-arrestin2 knock-out mice. Bar graphs depict percentile contractions to repeated morphine (3 μM) exposure in circular muscle rings from ileum and colon of WT C57/Bl6 and β-arrestin2 knock-out mice. The initial contraction to morphine was given a value of 100 % and each subsequent administration ( $2^{nd}$ ,  $3^{rd}$ ,  $4^{th}$ ) were normalized to the initial contraction ( $1^{st}$ ). (\* p< 0.05: n=4-6; paired Student's test). See supplementary figure 1 for typical raw tracings from ileum and colon.

**Figure 2**: β-arrestin2 expression in mouse and guinea pig. Western blot of β-arrestin2 (arrow) from protein samples of mouse brain and ileum and guinea pig colon and ileum LMMP preparation. β-arrestin2 was detected from the brain samples of C57/Bl6 mice but only faintly seen in the mouse ileum and absent in the brain samples from the β-arrestin2 knock-out mice. A robust band was present in the guinea pig colon and ileum LMMP. Specificity of the antibody was further confirmed by pre-absorption with antigen peptide (supplementary figure 2).

**Figure 3:.** A) Raw tracing of dose-response to morphine on EFS contractions in the ileum (top) and colon (bottom) from guinea-pig LMMP. An initial dose-response to morphine was followed by a 2 hour incubation with 10μM morphine (indicated by gap in tracing). A dose-response to morphine was then repeated. B) Dose response curves for morphine-induced inhibition of EFS contractions in the guinea pig ileum (left panel) and colon (right panel) LMMP. Open circles represent control and closed circles after 2 hour incubation with morphine (10 μM). Cumulative dose-response were carried out on each strip (n=6-9 strips from at least 5 guinea pigs). A two-way ANOVA followed by Bonferroni's post-hoc test was used to measure significance, \* p<0.001).

**Figure 4**: Downregulation of β-arrestin2 in the guinea pig ileum by chronic morphine. Protein samples were obtained from isolated LMMP from guinea pig colon and ileum that were exposed to either Krebs solution (control) or morphine (10  $\mu$ M for 2 hour) and were subject to immunoblot with A) anti-GRK2 (left panel) or B) anti-β-Arrestin2 (right panel) antibodies. Morphine treatment (10  $\mu$ M for 2 hours) did not alter the expression levels of GRK2 in either tissues, but markedly reduced β-arrestin2 expression in the ileum. Equal amount of protein loading was confirmed by anti-GAPDH antibody. Bar graphs represent the ratio of GRK2 to GAPDH (left) or β-arrestin2 to GAPDH (right). Data represent mean  $\pm$  SE (n=6). \*P < 0.05 versus control sample, unpaired t-test.

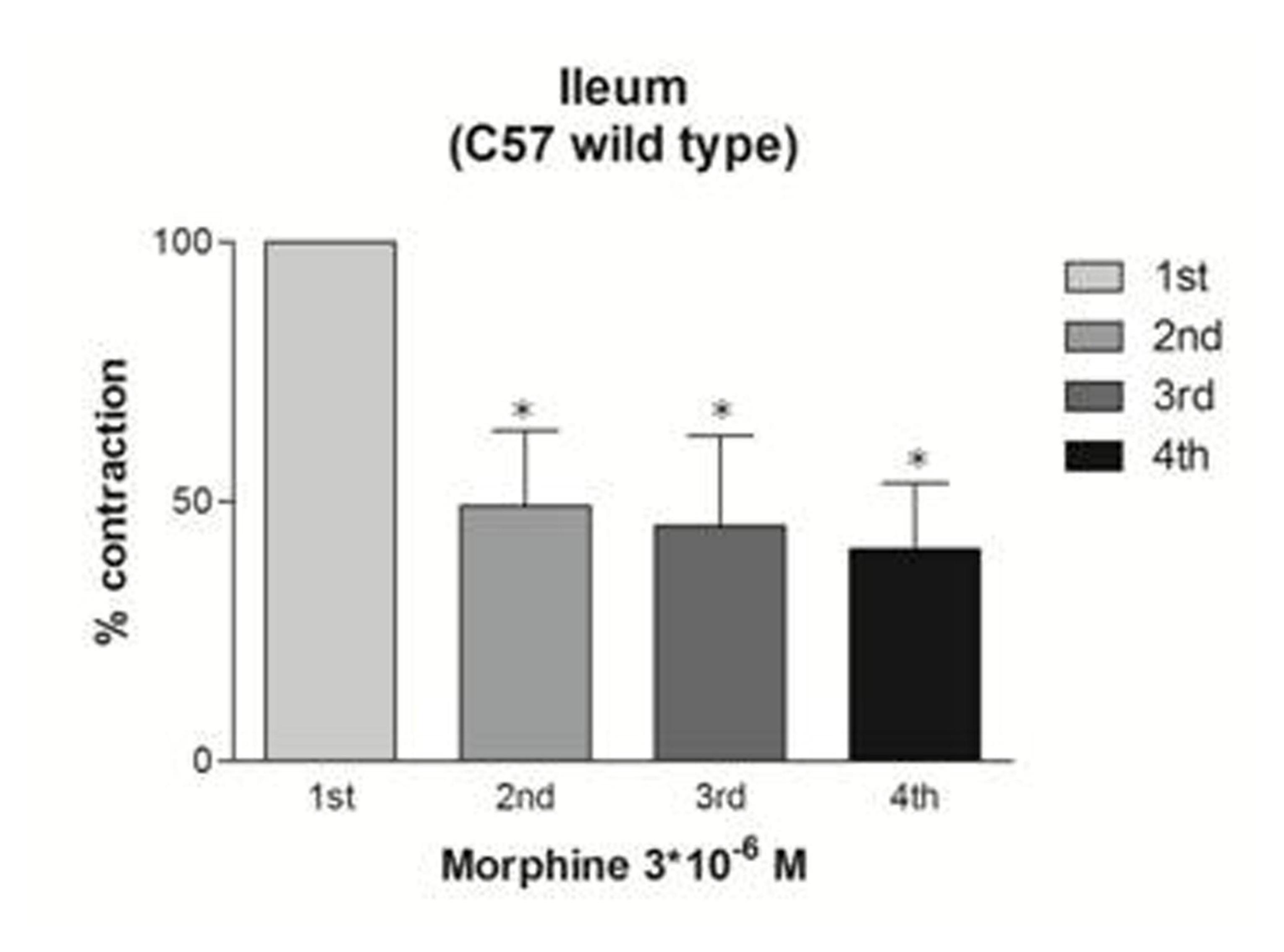
**Figure 5**: Expression of β-arrestin2 in guinea pig ileum neurons. A) Immunohistochemical localization of β-arrestin2 immunoreactivity in whole mount preparation showing localization within enteric ganglia. B) Myenteric neurons were enzymatically isolated from control and morphine-treated ileum LMMP. β-arrestin2 expression was significantly decreased in morphine-treated neurons. Equal loading of neuronal samples was confirmed by anti-β III-tubulin antibody as a neuronal marker. Results are expressed as the ratio of β-arrestin2 to β III-tubulin (bar graph). All experiments were performed in triplicates. Data represent mean SEM. \* $^*P$  < 0.01 versus control sample, unpaired  $^*P$ -test;  $^*P$  = 3. Ctl refers to the control and Mor refers to samples from morphine treated tissues. C) β-arrestin2 expression from ileum and colon LMMP treated with morphine (Mor) or morphine plus naloxone (Nal). In the presence of naloxone (10 μM), morphine does not down-regulate β-arrestin2 expression. Similar result was obtained from two separate guinea pigs.

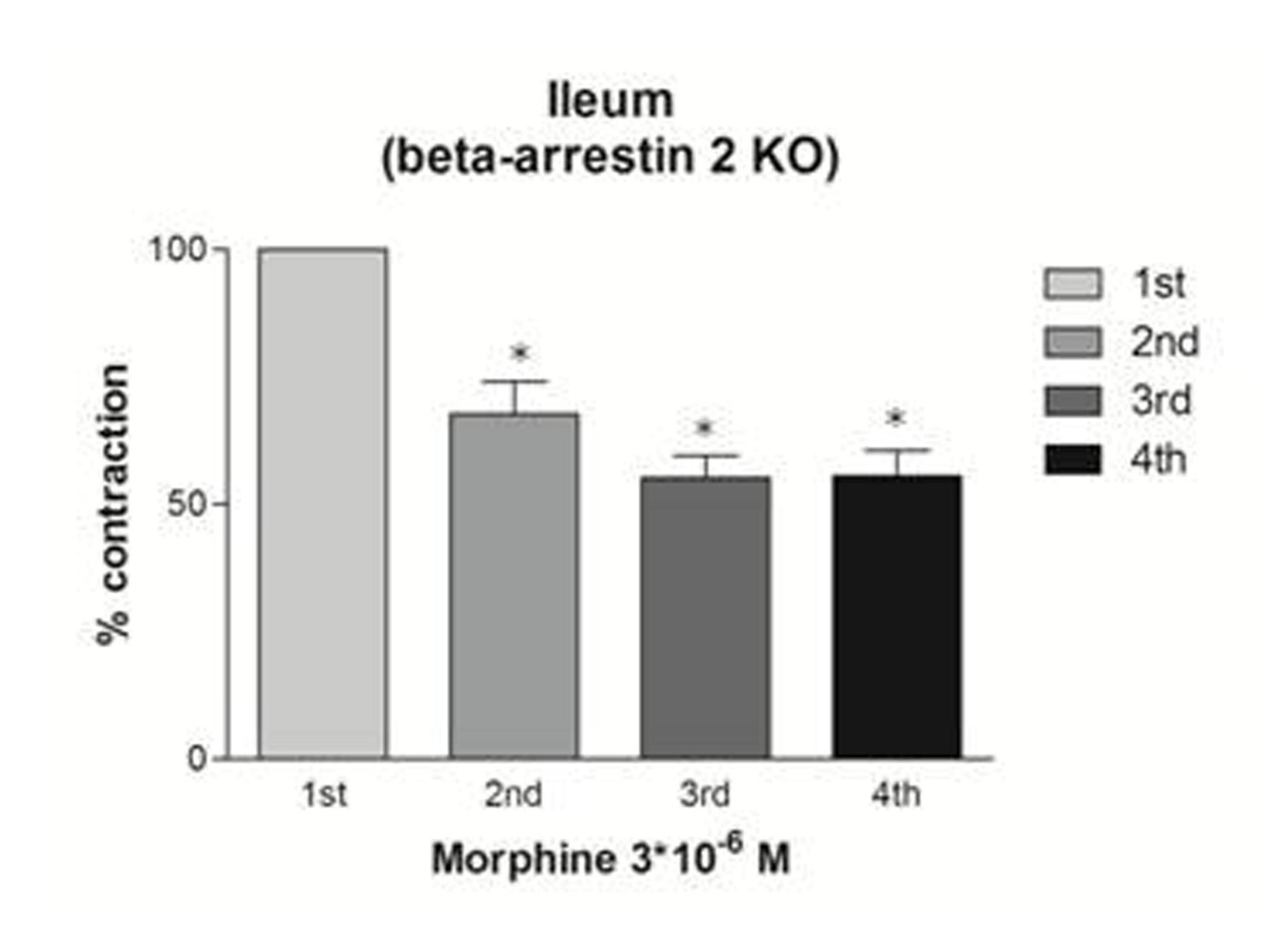
**Figure 6**: Time course of the down-regulation of  $\beta$ -arrestin2 protein (A) and mRNA (B) expression by morphine in the guinea pig. A) the LMMP from the ileum and colon were treated with 10 μM morphine from 0 to 5 hours and the expression of  $\beta$ -arrestin2 was detected by anti-β-Arrestin2 antibody at various time points. Left panel: expression of  $\beta$ -arrestin2 from ileum and

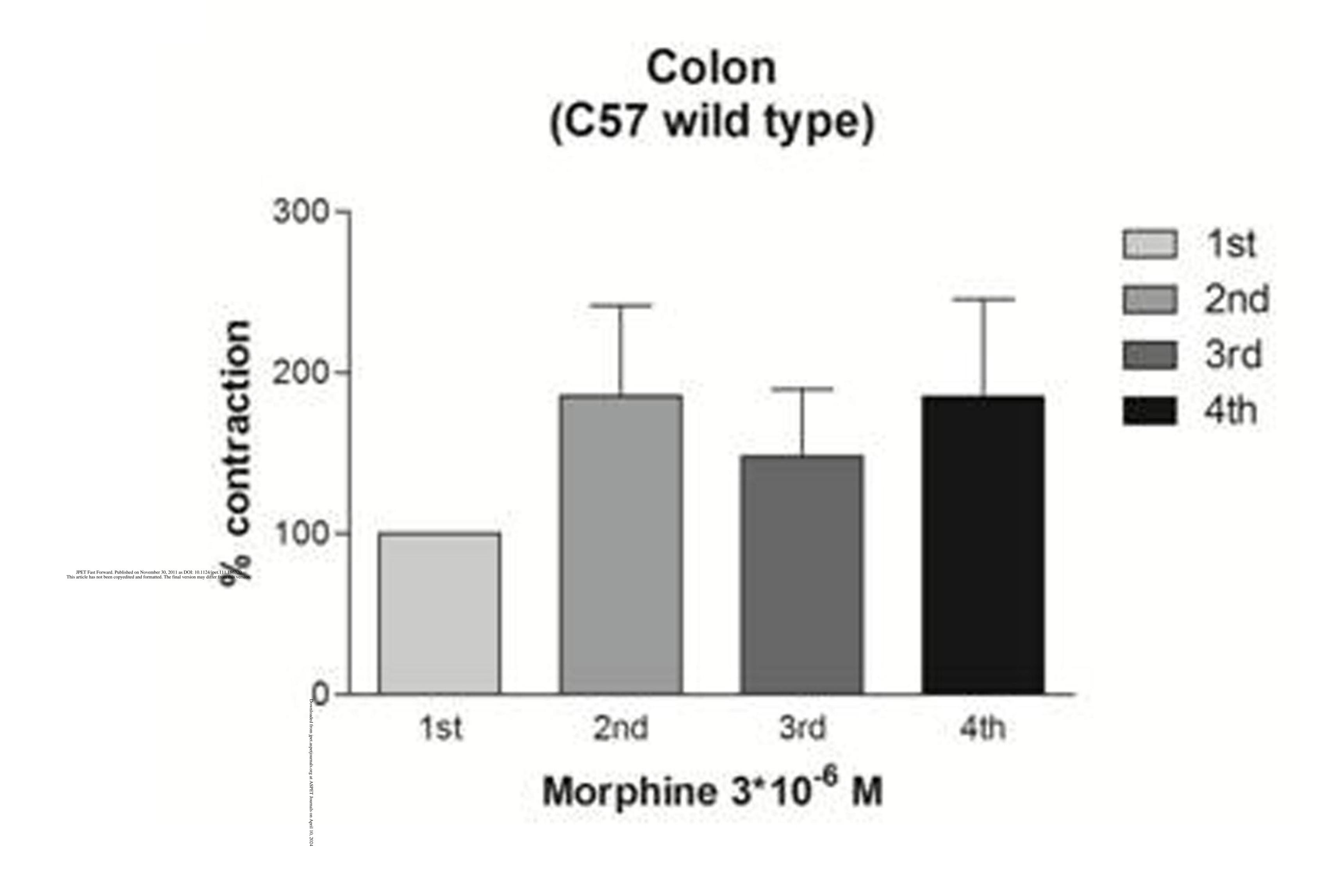
colon following 3 and 10 min exposure to 10  $\mu$ M morphine. Right Panel: expression of  $\beta$ -arrestin2 following 0.5, 1, 2 and 5 hour exposure to morphine in the ileum. Significant decreases were observed at 2 hours. Similar results were obtained in two separate runs. B) mRNA expression of  $\beta$ -arrestin2 in controls and after morphine treatment (10  $\mu$ M; 2hours) in the ileum and colonic LMMP preparations. Data are presented as  $\Delta$ Ct values calculated as Ct value of  $\beta$ -arrestin2 – Ct value of r18S for each sample. No differences were observed in mRNA expression between the ileum and colon following morphine treatment.

**Figure 7**: Down regulation of p-Erk by chronic morphine in the guinea pig LMMP. A) Western blot of phospho-Erk1/2 (top panel) in the ileum and colon in the absence and presence of morphine (10 μM) treatment. Ctl: control, Mor: morphine treated samples. Total Erk1/2 was measured in the same samples. B) Bar graph represents ratios of p-Erk to total Erk in ileum and colon (n= 3, paired t-test \*p<0.05).

**Figure 8:** Tolerance and β-arrestin2 expression in LMMP from in-vivo chronic morphine treated guinea pigs. A) Dose response curves to morphine – induced inhibition of EFS contractions in the ileum (left) and colon (right) from 7 day treated guinea pigs (n= 6-11). Morphine induced inhibition of EFS contractions in the LMMP preparations were examined from saline treated (open circles) and morphine-treated guinea pigs (closed circles). A second cumulative doseresponse was measured following 2 hour incubation with morphine (10 μM) in chronic in-vivo morphine treated guinea pigs (closed triangles). No further shifts were observed in either ileum or colon in morphine tolerant guinea pigs. B) Western blot of β-arrestin2 expression in the colon and ileum from morphine tolerant guinea pigs. Expression was measured in the absence (Ctl) and following 2 hour morphine exposure (Mor). C) Expression of phospho-Erk and total Erk in the colon and ileum from morphine tolerant guinea pigs in the absence (Ctl) and following 2 hour morphine exposure (Mor).







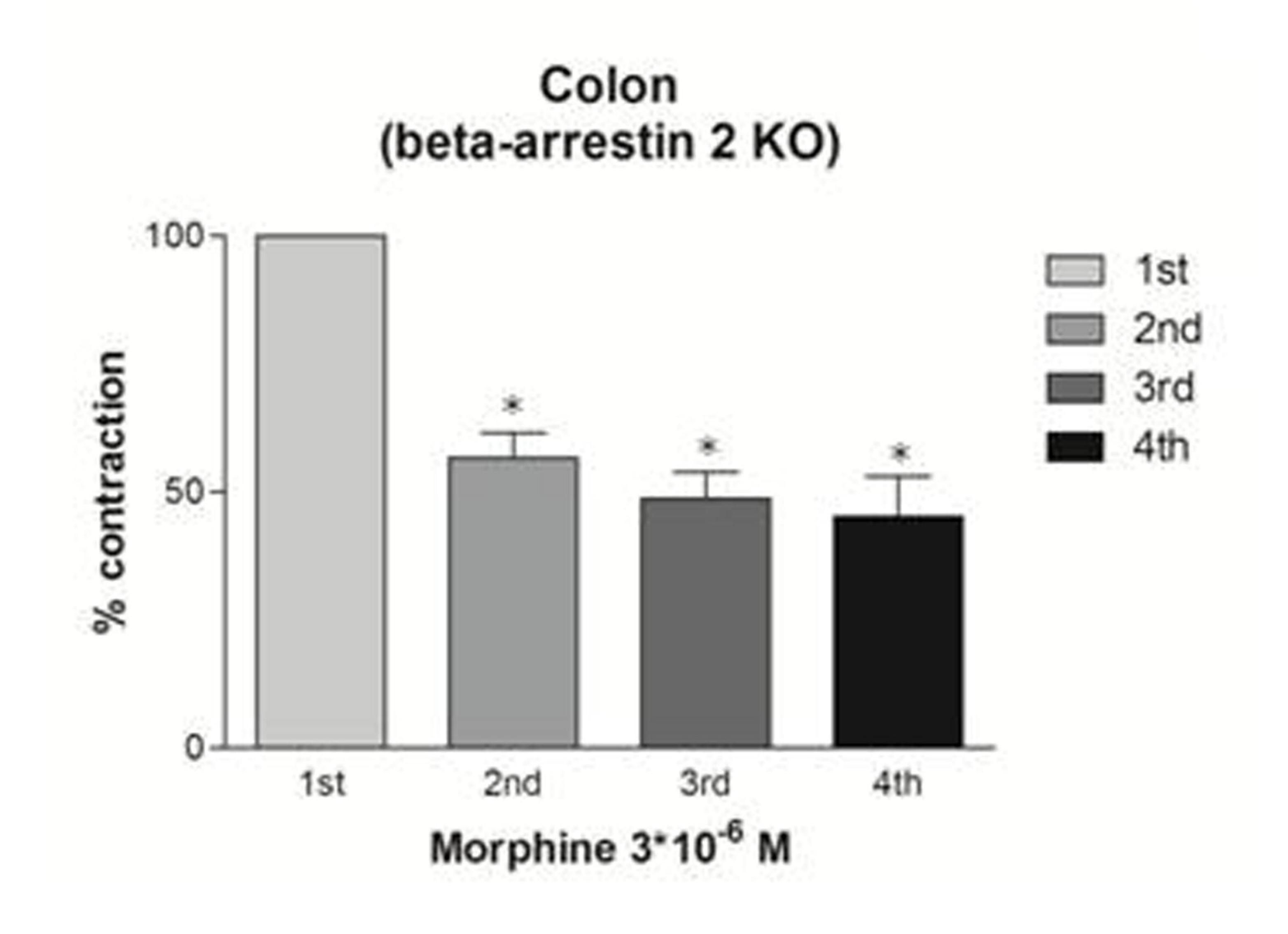


Figure 2

JPET Fast Forward. Published on November 30, 2011 as DOI: 10.1124/jpet.111.186320 This article has not been copyedited and formatted. The final version may differ from this version.

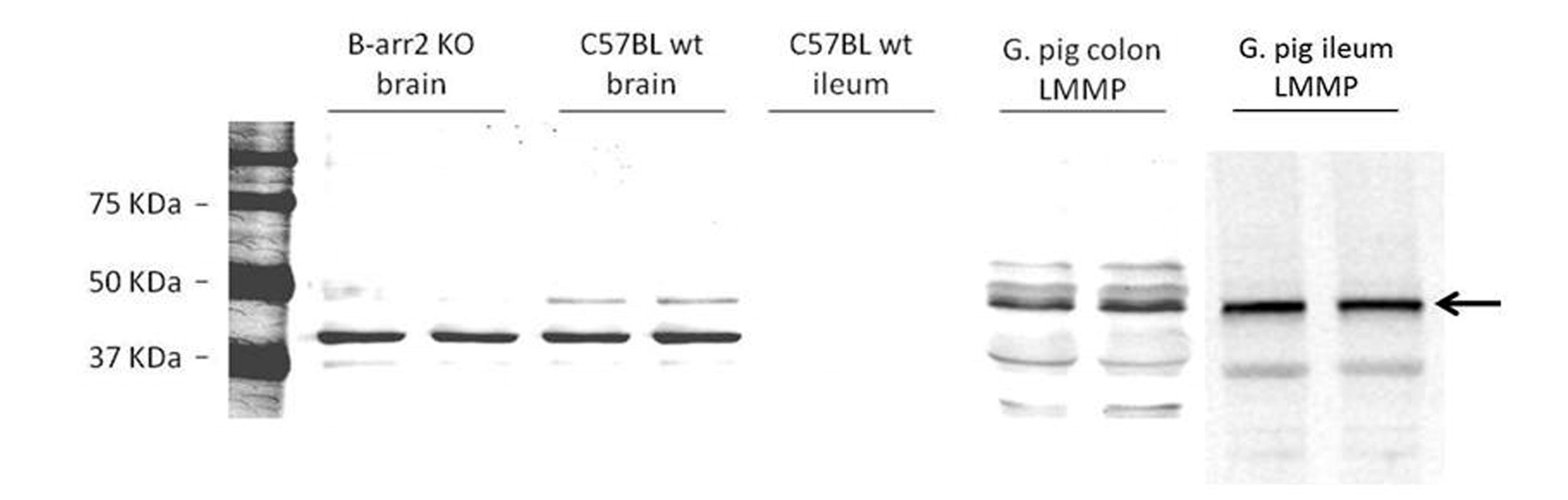


Figure 3


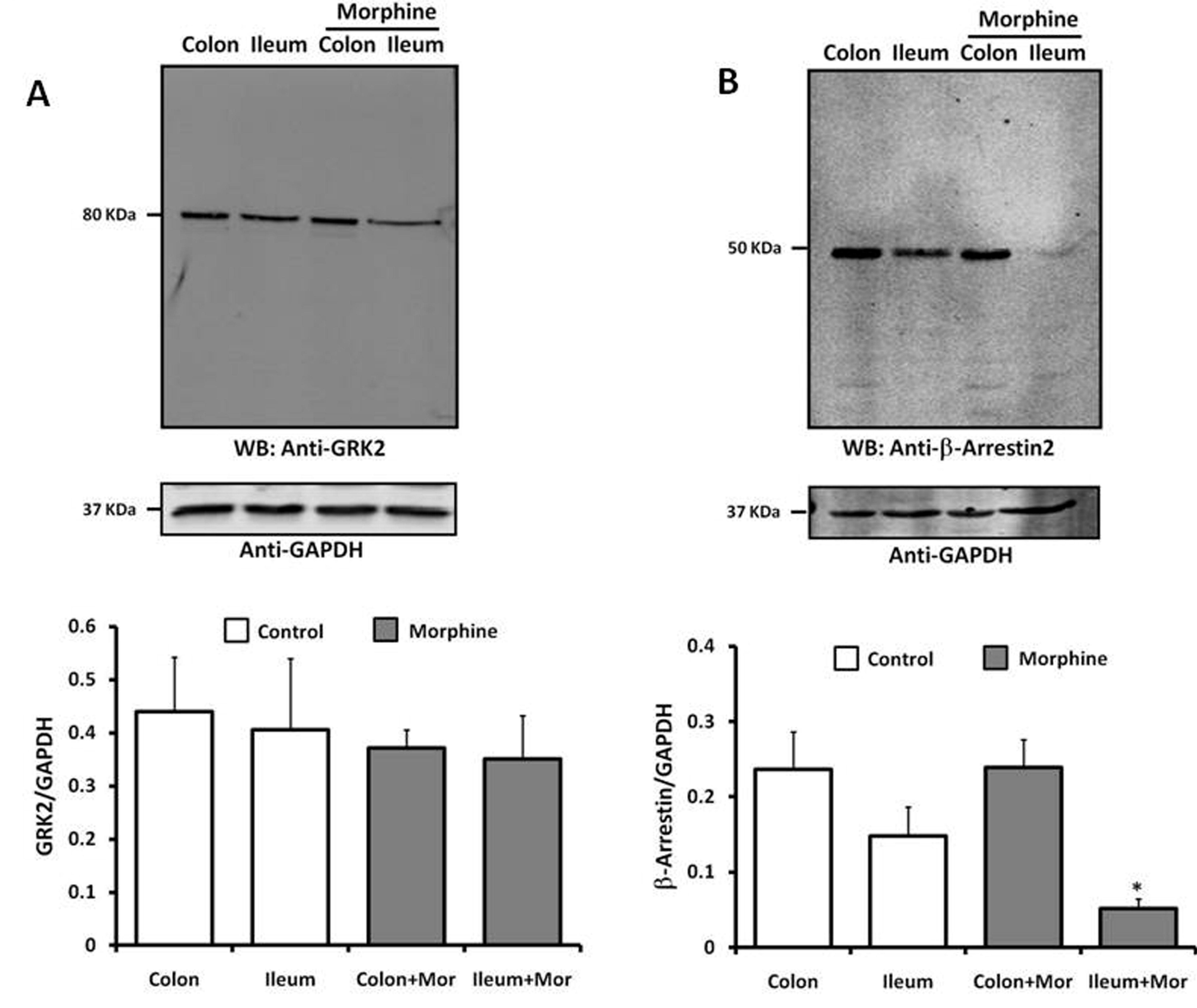



Figure 4








Figure 7



WB: Anti-phospho-Erk1/2



WB: Anti-Erk1/2

