

# The Role of $\beta$ -Arrestin2 in the Mechanism of Morphine Tolerance in the Mouse and Guinea Pig Gastrointestinal Tract<sup>§</sup>

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## ABSTRACT

$\beta$ -Arrestin2 has been reported to play an essential role in analgesic tolerance. Analgesic tolerance without concomitant tolerance to constipation is a limiting side effect of chronic morphine treatment. Because tolerance to morphine develops in the mouse ileum but not the colon, we therefore examined whether the role of  $\beta$ -arrestin2 in the mechanism of morphine tolerance differs in the ileum and colon. In both guinea pig and mouse, chronic *in vitro* exposure (2 h, 10  $\mu$ M) to morphine resulted in tolerance development in the isolated ileum but not the colon. The  $IC_{50}$  values for morphine-induced inhibition of electrical field stimulation contraction of guinea pig longitudinal muscle myenteric plexus shifted rightward in the ileum from  $5.7 \pm 0.08$  ( $n = 9$ ) to  $5.45 \pm 0.09$  ( $n = 6$ ) ( $p < 0.001$ ) after morphine exposure. A significant shift was not observed in the colon. Similar differential tolerance was seen between the

mouse ileum and the colon. However, tolerance developed in the colon from  $\beta$ -arrestin2 knockout mice.  $\beta$ -Arrestin2 and extracellular signal-regulated kinase 1/2 expression levels were determined further by Western blot analyses in guinea pig longitudinal muscle myenteric plexus. A time-dependent decrease in the expression of  $\beta$ -arrestin2 and extracellular signal-regulated kinase 1/2 occurred in the ileum but not the colon after 2 h of morphine (10  $\mu$ M) exposure. Naloxone prevented the decrease in  $\beta$ -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 a decrease in  $\beta$ -arrestin2 occurred. 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 limited however due to the presence and persistence of some of its 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, which collectively are referred to as “opioid-induced bowel dysfunction.” Although tolerance develops toward 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 tolerance mice (Ross et al., 2008). The *in vivo* 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) and *in vivo* (Goldstein and Schulz, 1973). Likewise, tolerance also develops to gastrointestinal transit as well 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 cellular mechanisms could account for morphine tolerance in the ileum but not the colon.

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**ABBREVIATIONS:** GRK, G protein-coupled receptor kinase; ANOVA, analysis of variance; EFS, electrical field stimulation; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LMMP, longitudinal muscle myenteric plexus; TTX, tetrodotoxin; Ct, cycle threshold.

The mechanisms underlying morphine tolerance are complex and not fully understood. Studies examining morphine tolerance have focused largely on analgesic tolerance and the neuronal circuitry associated with it in heterologously expressed cell lines. Much less is known with regard to the 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 after receptor phosphorylation by G protein-coupled receptor kinases (GRKs) and the recruitment of  $\beta$ -arrestins. The  $\beta$ -arrestins bind to phosphorylated receptors, uncouple them from G proteins, and facilitate internalization, thus resulting in desensitization. Antinociceptive tolerance is reduced in  $\beta$ -arrestin2 knockout mice (Bohn et al., 2000), which suggests that increased levels of  $\beta$ -arrestin2 may be involved importantly 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 the expression of  $\beta$ -arrestin2 in enteric neurons.

## Materials and Methods

**Animals.** Adult male albino guinea pigs (200–250 g) ( $n = 35$ ) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). The breeding pairs for the  $\beta$ -arrestin2 knockout mice on a C57BL/6 background were obtained from Dr. Robert J. Lefkowitz (Duke University, Durham, NC) and housed within the transgenic facility at Virginia Commonwealth University. All of the 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 daily using the following paradigm: on day 1, 10 mg/kg b.i.d.; days 2 and 3, 20 mg/kg b.i.d.; days 4, 5, and 6, 40 mg/kg b.i.d.; day 7, 80 mg/kg b.i.d. Morphine or vehicle was injected at intervals of 12 h, and the animals were euthanized on day 8.

**Preparation of Longitudinal and Circular Muscle from the Ileum and Colon.** Guinea pigs were sacrificed by  $\text{CO}_2$  asphyxiation, and mice were sacrificed by cervical dislocation. The distal colon (approximately 1 cm from the anus) and ileum (approximately 5 cm from the ileocecal junction) were dissected immediately and placed in a dissecting dish containing Krebs' solution (118 mM NaCl, 4.6 mM KCl, 1.3 mM  $\text{NaH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 25 mM  $\text{NaHCO}_3$ , 11 mM glucose, and 2.5 mM  $\text{CaCl}_2$ ). For the guinea pig experiments, the segments of the tissues were placed longitudinally on glass rods through the lumen, and the longitudinal muscle with the myenteric plexus (LMMP) was scraped gently with a cotton-tipped applicator. Approximately 2-cm-long LMMP preparations were suspended in the axis of the longitudinal muscle tied to a glass hook under 1 g of passive tension in 25-ml siliconized organ baths. For the experiments with mice, circular muscle preparations were used. The distal colon (approximately 1 cm proximal to the colon) and the ileum (approximately 2 cm proximal to the ileocecal junction) were dissected. In mice, unlike the guinea pig, the myenteric plexus strongly adheres to the circular muscle (Furukawa et al., 1986). We have shown previously that morphine induces robust contractions of the circular muscle due to the removal of a neurogenic inhibitory tone. This was confirmed in preliminary experiments whereby blocking

neuronal activation with tetrodotoxin (TTX) (1–10  $\mu\text{M}$ ) abolished morphine-induced contractions. Circular muscle rings were dissected and mounted in the organ bath using a metal triangle on one end and a glass hook on the other end (Ross et al., 2008). Tissues were bathed in Krebs' solution maintained at  $37^\circ\text{C}$  and bubbled with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  and equilibrated for 60 min before the start of the 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 3.82 software (BIOPAC Systems, Inc., Santa Barbara, CA). Acetylcholine (3  $\mu\text{M}$ ) 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 exposures of 10 min or an exposure of 2 h. For repeated exposures, tissues were incubated with morphine (10  $\mu\text{M}$ ) (Mallickrodt, Hazelwood, MO) for 10 min, followed by washout in Krebs' solution. After the recovery of EFS-induced contractions, morphine was applied again to the bath at the same concentration. This was repeated four to five 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 after longer exposures (2 h), a cumulative dose-response curve to morphine was measured after tissue equilibration. The percentage inhibition of EFS-induced contractions was measured at each successive concentration. Tissues then were incubated with morphine (10  $\mu\text{M}$ ) for 2 h and washed in Krebs' solution, and the cumulative dose-response curve measurement was repeated.

Morphine-induced contractions of the circular muscle preparations of the mouse ileum and colon were measured as described previously (Ross et al., 2008). In brief, morphine (3  $\mu\text{M}$ ) was applied four times at intervals of 30 min with repeated washings in Krebs' solution between these exposures. The amplitude of the initial response was taken as 100%, and percentage response was calculated against this for each successive response.

**Isolated Myenteric Ganglia.** The sheets of LMMP were incubated for 6 min at  $37^\circ\text{C}$  in Krebs' solution containing 0.1% protease, 0.2% collagenase type IV, and 0.1% bovine serum albumin for enzymatic digestion. The tissue was bubbled consistently with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ , accompanied by frequent trituration with a wide-bore Pasteur pipette to free the ganglia. The partly digested tissue then was collected by filtering through 500- $\mu\text{m}$  Nitex mesh and incubated for 1 h at  $37^\circ\text{C}$  in enzyme-free Krebs' solution for further isolation. The suspension then was filtered to remove the undigested tissue, and the filtrate was centrifuged for 1 min at 1000 rpm. The supernatant containing smooth muscle cells and debris was discarded, and the pellet containing the ganglia was 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- $\mu\text{l}$  capillary tube under a microscope.

**Western Blot Analyses.** Protein samples from guinea pig LMMP tissues were prepared in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Inc., 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 (Thermo Fisher Scientific, Waltham, MA). Standard Western blot procedures were used as described previously (Kang et al., 2004). In brief, samples were boiled for 3 min and subjected to 10% SDS polyacrylamide gel electrophoresis and then electroblotted using the iBlot system (Invitrogen, Carlsbad, CA). Immunoblotting was performed on a SNAP i.d. system (Millipore Corporation, Billerica, MA) using anti-mouse  $\beta$ -arrestin2, anti-rab-

bit glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-mouse GRK2 (Santa Cruz Biotechnology, Inc.), anti-rabbit  $\beta$ III-tubulin (Abcam, Inc., Cambridge, MA), anti-mouse extracellular signal-regulated kinase (ERK), and anti-rabbit phospho-ERK (Cell Signaling Technology, Danvers, 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 the density of each band was measured using the LiCor Odyssey infrared imaging system (LiCor Biosciences). The use of separate wavelengths for the secondary antibodies allowed for the determination of the ratios of proteins on the same blot. To confirm that protein loading and density of the control  $\beta$ III-tubulin was not saturated, various amounts of protein concentrations (40–120  $\mu$ g/lane) were loaded, and the expression of  $\beta$ -arrestin2,  $\beta$ III-tubulin, and GAPDH were measured. For each Western blot, the density of the loading control was confirmed to lie within the linear range.

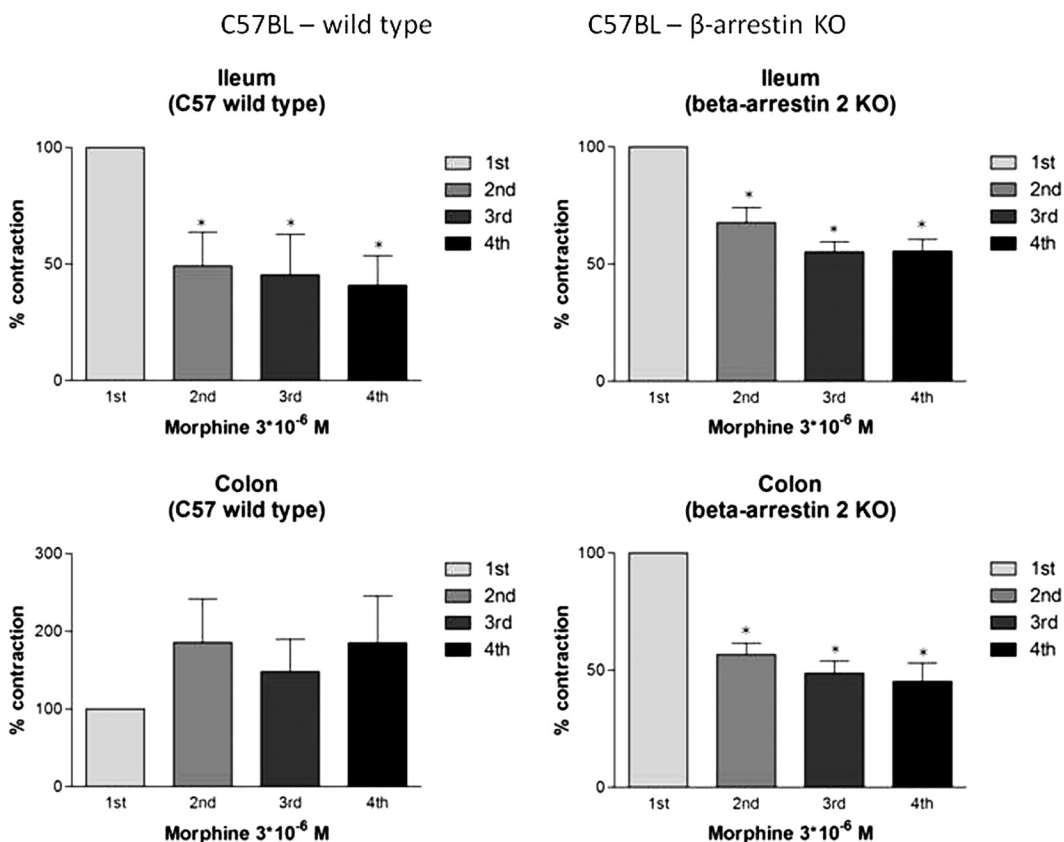
**Real-Time Polymerase Chain Reaction.** Total RNA was extracted with PureLink Micro-to-Midi Total RNA Purification System, as described by the manufacturer (Invitrogen). The first-strand cDNA was amplified using SensiMix One-Step Kit (Bioline, Taunton, MA) at 42°C for 30 min. Primers were designed using Vector NTI Suite software (Invitrogen). (See supplemental material for primer sequences and polymerase chain reaction methodology.)

**Data Analysis.** The data are expressed as mean  $\pm$  S.E. from three to five individual experiments for all of the Western blots. Dose-response curves to morphine inhibition of EFS-induced contractions were analyzed by repeated measures analysis of variance (ANOVA) followed by Bonferroni's post hoc test or by paired Student's *t* test where appropriate.  $IC_{50}$  values were calculated by nonlinear regression and reported as negative log values ( $-\log M$ ). The *p* values  $<0.05$  were considered significant. *N* refers to the number of animals, whereas *n* refers to the number of tissues.

## Results

**Tolerance in  $\beta$ -Arrestin2 Knockout 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  $\beta$ -arrestin2 knockout mice (Rae-hal 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 knockout mouse ileum and colon. We (Ross et al., 2008) have shown previously that morphine induces the contraction of circular muscle preparations and that repeated administration results in tolerance in the ileum but not the colon. Contractions of circular muscle preparations result from the disinhibition of constitutive inhibitory neurogenic tone (Grider and Makhlof, 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 (H. T. Maguma and H. I. Akbarali, unpublished observations).

After 1 h of equilibration of the isolated circular muscle from the ileum and colon, repeated administrations of morphine (3  $\mu$ M) were applied every 30 min, with in-between washes. Morphine produced individual contractions that waned with time (Supplemental Fig. 1) in both the ileum and the colon. The peak contractions after each administration of morphine were measured and normalized as a percentage of the first peak contraction in that tissue. As shown in Fig. 1 (and Supplemental Fig. 1, A and B), the peak contractile



**Fig. 1.** Morphine-induced contractions in wild-type C57BL/6 and  $\beta$ -arrestin2 knockout mice. Bar graphs depict percentile contractions to repeated morphine (3  $\mu$ M) exposures in circular muscle rings from the ileum and colon of wild-type C57BL/6 and  $\beta$ -arrestin2 knockout mice. The initial contraction to morphine was given a value of 100%, and each subsequent administration (second, third, and fourth) was normalized to the initial contraction (first). \*, *p*  $< 0.05$ , paired Student's *t* test; *n* = 4–6. See Supplemental Fig. 1 for typical raw tracings from the ileum and colon.



response to morphine was reduced markedly upon repeated administration in the ileum but not the colon in wild-type mice, which is indicative of the development of tolerance in the ileum but not the colon. Compared with the first response, morphine-induced contractions in the ileum were reduced significantly after the second exposure ( $50 \pm 10\%$ ,  $p < 0.05$ , paired Student's *t* test;  $n = 4$ ) and subsequently remained reduced up to the fourth application ( $42 \pm 9\%$ ). Likewise, in the  $\beta$ -arrestin2 knockout mice, significant tolerance developed after the second ( $67 \pm 6\%$ ,  $p < 0.05$ , paired Student's *t* test;  $n = 5$ ), third ( $65 \pm 4\%$ ), and fourth ( $66 \pm 5\%$ ) administrations. However, there was 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 knockout mice. In comparison to the initial response, the second exposure resulted in contractions that were reduced to  $54 \pm 5\%$  ( $p < 0.05$ ,  $n = 6$ ) and  $45 \pm 7\%$  at the fourth 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 knockout mice were not statistically different (ileum,  $42.6 \pm 7.1$  ( $n = 6$ ) versus  $65.4 \pm 12.8$  ( $n = 9$ ) mg/mg tissue weight;  $p = 0.20$ , *t* test) (colon,  $55.1 \pm 12.5$  ( $n = 10$ ) versus  $71.4 \pm 17.3$  ( $n = 10$ ) mg/mg tissue weight;  $p = 0.45$ , *t* test) (Supplemental Fig. 2).

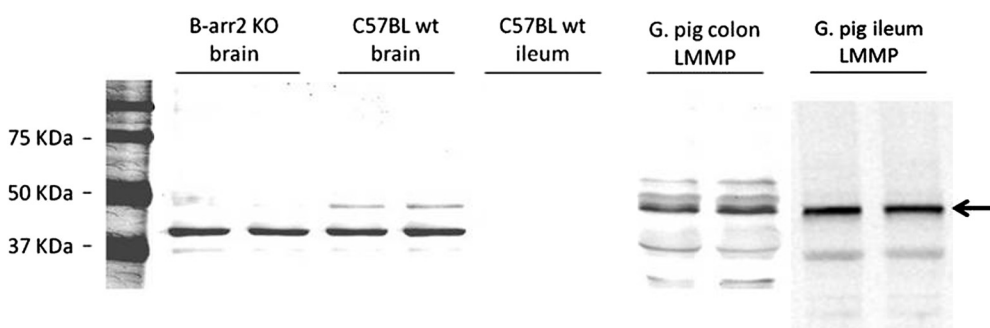
The above data show that repeated morphine treatment causes tolerance in the colon in the absence of  $\beta$ -arrestin2. To further investigate the role of  $\beta$ -arrestin2 in morphine tolerance, we determined the expression of  $\beta$ -arrestin2 in the mouse by Western blot analysis. The specificity of the  $\beta$ -arrestin2 antibody was tested by preabsorption with the antigen peptide (Supplemental Fig. 3). Initially, experiments were carried out to examine the feasibility of using the  $\beta$ -arrestin2 antibody in the mouse. An approximately 50-kDa protein corresponding to  $\beta$ -arrestin2 was detected in the mouse brain of wild-type but not  $\beta$ -arrestin2 knockout mice (Fig. 2). However,  $\beta$ -arrestin2 was barely detectable in the mouse ileum even when cell lysates from three 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, therefore making them difficult to separate from the muscle layers. To obtain a more enriched preparation of myenteric neurons, we used the guinea pig LMMP. Distinct bands corresponding to  $\beta$ -arrestin2 were detected in the guinea pig ileum and colon (Fig. 2). Therefore, to further explore the role of  $\beta$ -arrestin2 in morphine toler-

ance 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.

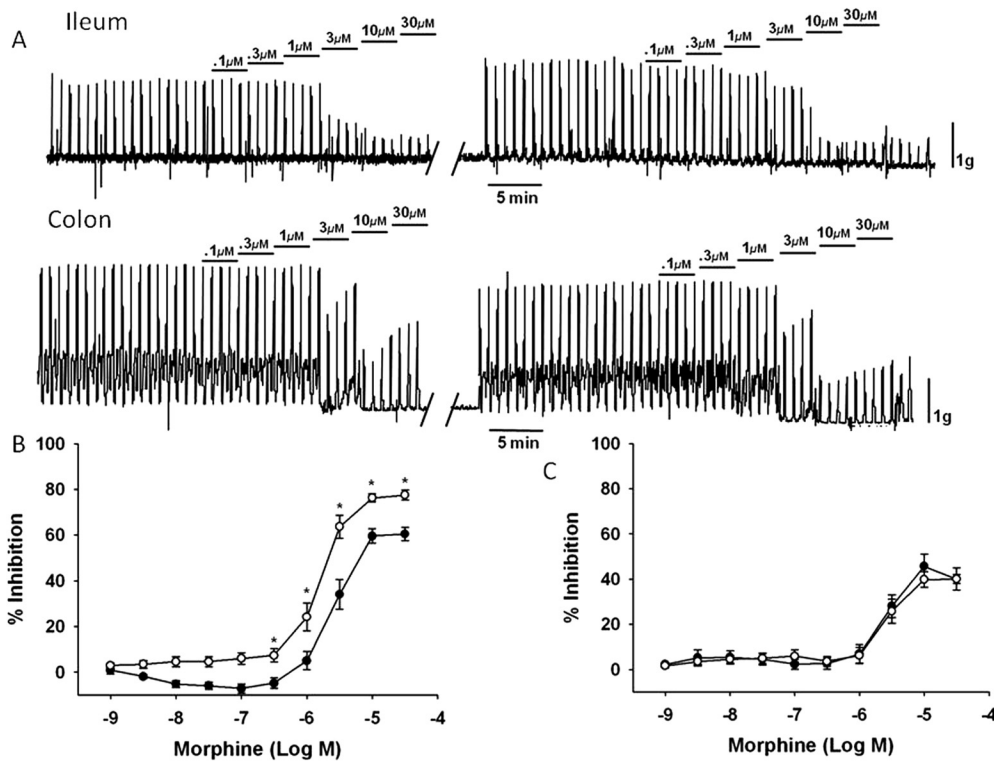
**Morphine Tolerance in the Guinea Pig LMMP.** It has been shown that morphine attenuates the EFS contractions of the guinea pig LMMP preparations due to presynaptic inhibition of acetylcholine release, and tolerance develops upon repeated morphine administration in the ileum (Collier et al., 1981). We investigated whether, 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 dose-response curve to morphine inhibition of EFS was determined before and after 2 h of incubation with  $10 \mu\text{M}$  morphine (Fig. 3). The exposure time to each concentration was  $<5$  min. We found that the cumulative dose-response curve to morphine was reproduced three times in both the ileum and the colon. The initial  $\text{IC}_{50}$  values ( $-\text{Log M}$ ) for morphine-induced inhibition of EFS were  $5.70 \pm 0.08$  ( $n = 9$ ) in the ileum and  $5.43 \pm 0.14$  ( $n = 7$ ) in the colon. After morphine incubation for 2 h, the  $\text{IC}_{50}$  value shifted to  $5.45 \pm 0.09$  ( $n = 6$ ) ( $p < 0.0001$ ) (repeated measures ANOVA) in the ileum, whereas it was  $5.48 \pm 0.17$  ( $n = 6$ ) ( $p = 0.829$ ) in the colon. Both the rightward shift in the dose-response curve and the decrease in maximal inhibition were significant in the ileum but not in the colon (Fig. 3). In the next series of experiments, individual concentrations of morphine were tested between washes. As we found in the tissues from mice, tolerance occurred in the ileum but not the colon of guinea pigs (Supplementary Fig. 4).

In the following experiments, LMMP strips from the guinea pig ileum and colon were incubated with a concentration of morphine ( $10 \mu\text{M}$ ) for 2 h 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 reduced markedly in the ileum but not the colon ( $n = 6$ ) (Fig. 4).

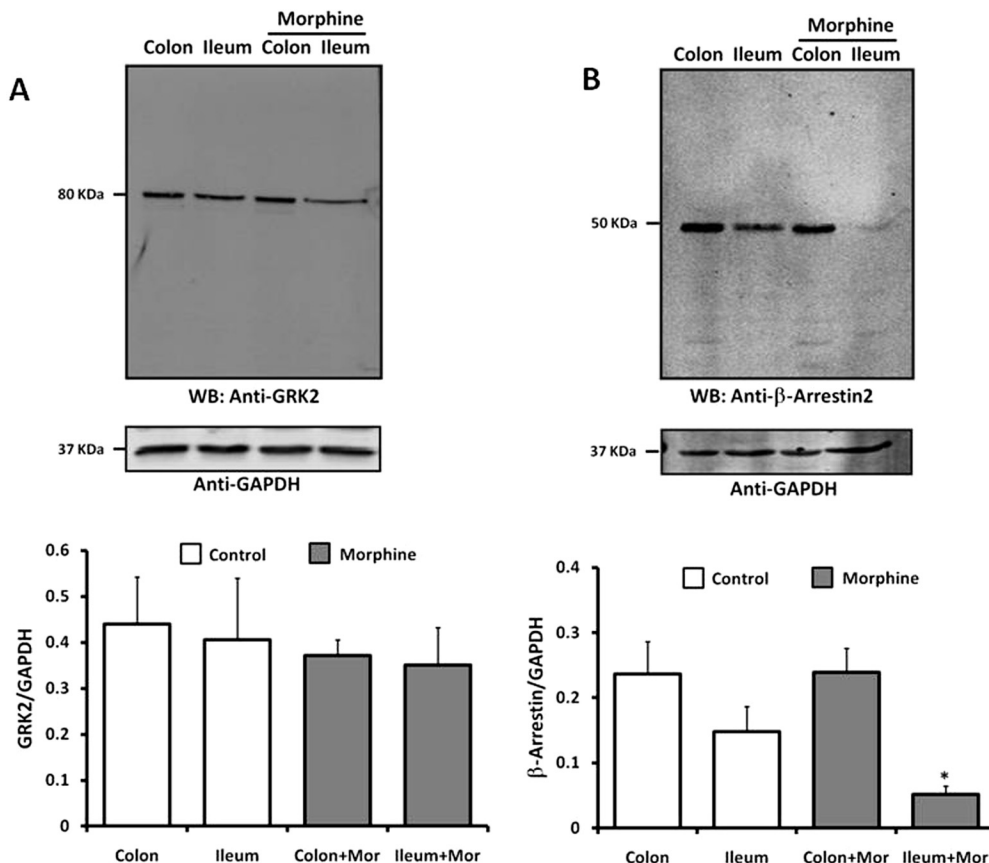
The expression of  $\beta$ -arrestin2 was examined in enzymatically isolated neurons from the ileum. The neuronal marker  $\beta$ III-tubulin (Tuj1) was used as the loading control. In whole-mount LMMP preparations,  $\beta$ -arrestin2 (Fig. 5A) and  $\beta$ III-tubulin staining was confined to myenteric neurons (Supplemental Fig. 5). In enzymatically isolated neuronal preparations,  $\beta$ -arrestin2 expression was reduced significantly in morphine-treated samples from the ileum by  $66 \pm 3\%$  ( $n = 3$ ) (Fig. 5B). To further determine whether the reduced levels of  $\beta$ -arrestin2 were dependent on  $\mu$ -opioid receptor-mediated activation by



**Fig. 2.**  $\beta$ -Arrestin2 expression in mouse and guinea pig. Western blot analyses of  $\beta$ -arrestin2 (arrow) from protein samples of mouse brain and ileum and guinea pig ileum and colon LMMP preparations are shown.  $\beta$ -Arrestin2 was detected from the brain samples of C57BL/6 mice but only faintly seen in the mouse ileum and absent in the brain samples from the  $\beta$ -arrestin2 knockout mice. A robust band was present in the guinea pig colon and ileum LMMP. The specificity of the antibody was confirmed further by preabsorption with antigen peptide (Supplemental Fig. 2).



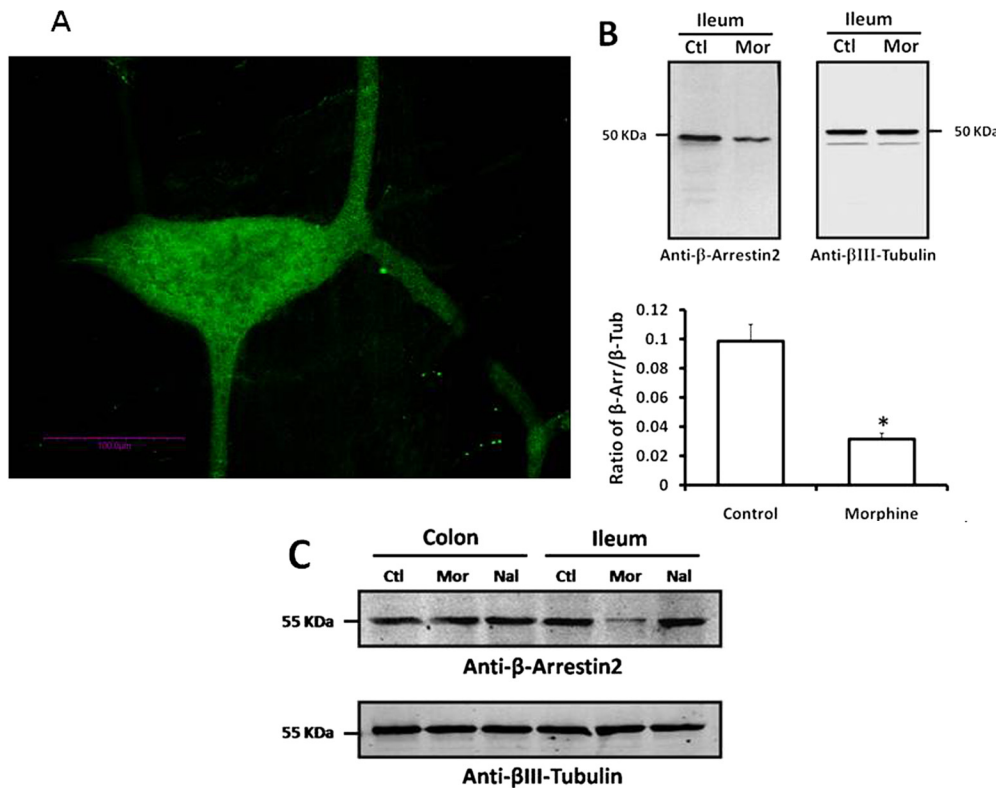
**Fig. 3.** A, raw tracing of dose-response curves to morphine on EFS-induced contractions in the ileum (top) and colon (bottom) from guinea pig LMMP. An initial dose-response curve to morphine was followed by 2 h of incubation with 10  $\mu$ M morphine (indicated by the gap in the trace). A dose-response curve to morphine then was repeated. B and C, dose-response curves for morphine-induced inhibition of EFS-induced contractions in the guinea pig ileum (B) and colon (C) LMMP. Open circles represent control and closed circles after 2 h of incubation with morphine (10  $\mu$ M). Cumulative dose-response curves were carried out on each strip ( $n = 6-9$  strips from at least five guinea pigs). A two-way ANOVA followed by Bonferroni's post hoc test was used to measure significance. \*,  $p < 0.001$ .



**Fig. 4.** Down-regulation of  $\beta$ -arrestin2 in the guinea pig ileum by chronic morphine exposure. A and B, 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 h) and were subject to immunoblot with anti-GRK2 (A) or anti- $\beta$ -arrestin2 (B) antibodies. Morphine treatment (10  $\mu$ M for 2 h) did not alter the expression levels of GRK2 in either tissue but markedly reduced  $\beta$ -arrestin2 expression in the ileum. Equal amounts of protein loading were confirmed by the anti-GAPDH antibody. Bar graphs represent the ratio of GRK2 to GAPDH (A) or  $\beta$ -arrestin2 to GAPDH (B). Data represent mean  $\pm$  S.E. ( $n = 6$ ). \*,  $p < 0.05$  versus control sample, unpaired  $t$  test.

morphine, the ileum and colon LMMP preparations were pretreated with naloxone (10  $\mu$ M) for 30 min before morphine exposure for 2 h. Naloxone prevented the decrease in  $\beta$ -arrestin2 expression in the ileum (Fig. 5C) indicative of a  $\mu$ -opioid receptor-mediated effect ( $N = 2$ ).

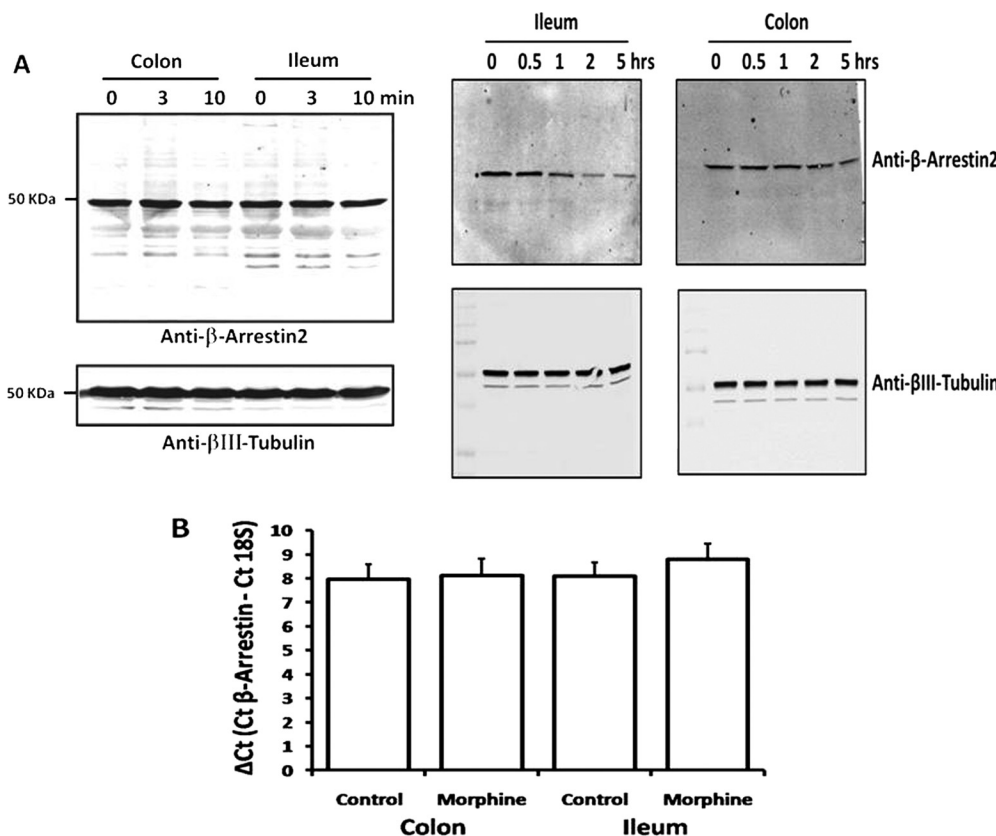
Agonist-stimulated degradation of  $\beta$ -arrestin2 may be transient (Shenoy et al., 2001) and therefore raises the possibility that the kinetics of  $\beta$ -arrestin2 down-regulation between the ileum and the colon may differ due to differences in this process. We therefore examined the expression of  $\beta$ -ar-



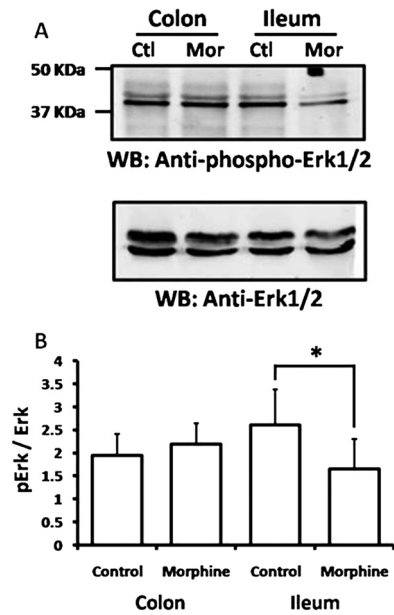
**Fig. 5.** Expression of  $\beta$ -arrestin2 in guinea pig ileum neurons. A, immunohistochemical localization of  $\beta$ -arrestin2 immunoreactivity in a whole-mount preparation showing localization within enteric ganglia. B, myenteric neurons were isolated enzymatically from control and morphine-treated ileum LMMP.  $\beta$ -Arrestin2 expression was decreased significantly in morphine-treated neurons. Equal loading of neuronal samples was confirmed by anti- $\beta$ III-tubulin antibody as a neuronal marker. Results are expressed as the ratio of  $\beta$ -arrestin2 to  $\beta$  III-tubulin (bar graph). All of the experiments were performed in triplicate. Data represent mean  $\pm$  S.E.M. \*,  $p < 0.01$  versus control sample, unpaired  $t$  test;  $n = 3$ . Ctl refers to the control, and Mor refers to the samples from morphine-treated tissues. C,  $\beta$ -arrestin2 expression from the ileum and colon LMMP treated with morphine (Mor) or morphine plus naloxone (Nal). In the presence of naloxone (10  $\mu$ M), morphine does not down-regulate  $\beta$ -arrestin2 expression. A similar result was obtained from two separate guinea pigs.

restin2 in both the ileum and the colon at multiple time points. As shown in Fig. 6A, loss of  $\beta$ -arrestin2 expression was not evident at 3 or 10 min in the ileum or colon. In the ileum, the decrease in  $\beta$ -arrestin2 expression was significant at 2 h of morphine exposure and persisted up to 5 h.

In the colon, a decrease in  $\beta$ -arrestin2 expression was not observed at any time point. The mRNA expression of  $\beta$ -arrestin2 was not altered by chronic morphine exposure (Fig. 6B), indicating that the down-regulation was not due to decreased synthesis.



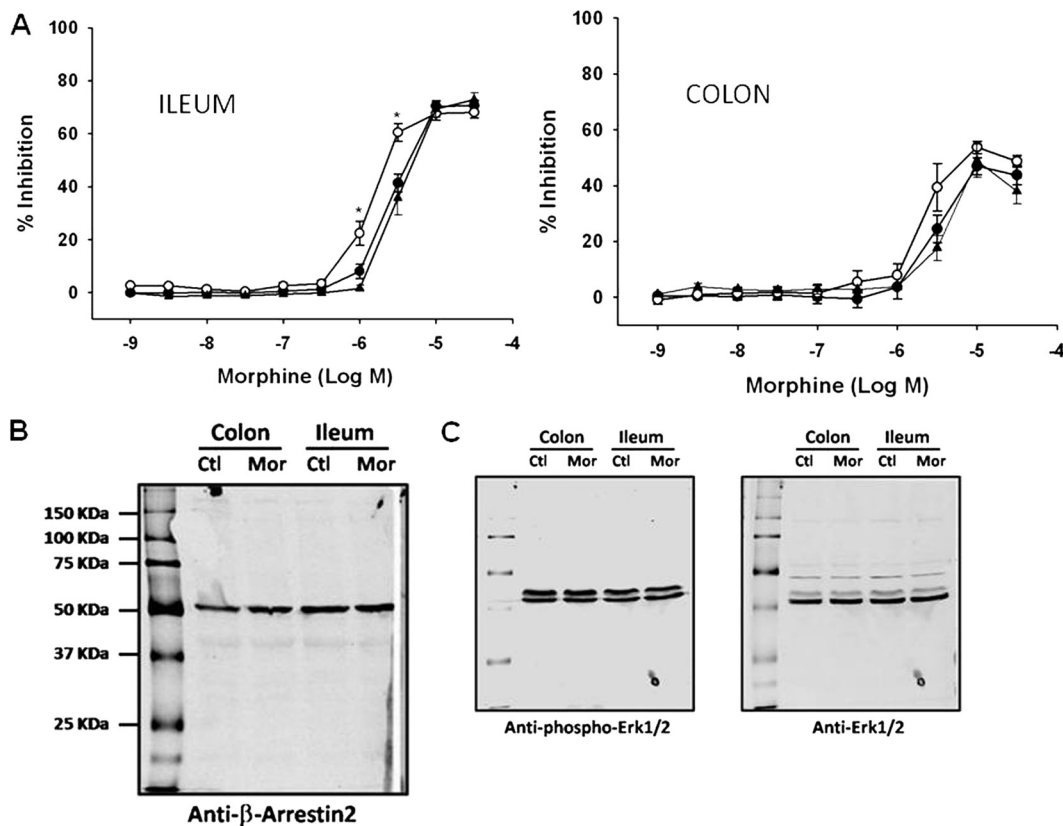
**Fig. 6.** Time course of the down-regulation of  $\beta$ -arrestin2 protein (A) and mRNA (B) expression by morphine in the guinea pig. A, the LMMP from the ileum and colon were treated with 10  $\mu$ M morphine from 0 to 5 h, and the expression of  $\beta$ -arrestin2 was detected by anti- $\beta$ -arrestin2 antibody at various time points. Left, expression of  $\beta$ -arrestin2 from the ileum and colon after 3 and 10 min of exposure to 10  $\mu$ M morphine. Right, expression of  $\beta$ -arrestin2 after 0.5, 1, 2, and 5 h of exposure to morphine in the ileum. Significant decreases were observed at 2 h. Similar results were obtained in two separate runs. B, mRNA expression of  $\beta$ -arrestin2 in controls and after morphine treatment (10  $\mu$ M; 2 h) in the ileum and colon LMMP preparations. Data are presented as  $\Delta$ Ct values calculated as the Ct value of  $\beta$ -arrestin2 minus the Ct value of 18S ribosomal RNA for each sample. No differences were observed in mRNA expression between the ileum and the colon after morphine treatment.



**Fig. 7.** Down-regulation of phospho-ERK by chronic morphine in the guinea pig LMMP. A, Western blot analysis of phospho-ERK1/2 in the ileum and colon in the absence and presence of morphine (10  $\mu$ M) treatment. Ctl, control; Mor, morphine-treated samples. Total ERK1/2 was measured in the same samples. B, bar graph representing ratios of phospho-ERK to total ERK in the ileum and colon (\*,  $p < 0.05$ , paired  $t$  test;  $n = 3$ ).

$\beta$ -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  $\beta$ -arrestin2 in the ileum but not the colon after morphine exposure (Fig. 7).

**Chronic In Vivo Morphine Treatment.** We tested whether tolerance to morphine inhibition of EFS was induced in the guinea pig LMMP from animals treated with morphine in vivo for 7 days (twice daily). A cumulative dose-response curve to morphine was carried out in LMMP from guinea pigs chronically treated with morphine or saline. The  $IC_{50}$  value ( $-\log M$ ) for the inhibition of EFS-induced contractions in the ileum from saline treated animals was  $5.81 \pm 0.07$  ( $n = 8$ ;  $N = 3$ ), which was not statistically significant from the ileum of noninjected naïve animals. In the chronic morphine-treated animals, the dose-response curve was shifted significantly ( $p < 0.0001$ ) to the right with an  $IC_{50}$  value of  $5.4 \pm 0.05$  ( $n = 19$ ;  $N = 6$ ), which is indicative of tolerance development (Fig. 8A). The  $IC_{50}$  value for morphine in the colon from saline-treated guinea pigs was  $5.62 \pm 0.12$  ( $n = 5$ ;  $N = 3$ ) and was not significantly different from that of the colon of chronic morphine-treated guinea pigs ( $5.41 \pm 0.12$  ( $n = 12$ ;  $N = 6$ )). 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



**Fig. 8.** Tolerance and  $\beta$ -arrestin2 expression in LMMP from in vivo chronic morphine-treated guinea pigs. A, dose-response curves to morphine-induced inhibition of EFS-induced 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 dose-response curve was measured after 2 h of incubation with morphine (10  $\mu$ M) 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. \*,  $p < 0.05$  two-way ANOVA followed by Bonferroni's post hoc. B, Western blot analyses of  $\beta$ -arrestin2 expression in the colon and ileum from morphine tolerant guinea pigs. Expression was measured in the absence of morphine exposure (Ctl) and after 2 h of morphine exposure (Mor). C, expression of phospho-ERK and total ERK in the colon and ileum from morphine tolerant guinea pigs in the absence of morphine exposure (Ctl) and after 2 h of morphine exposure (Mor).



(i.e., tolerance was observed in the ileum but not the colon). To further establish that tolerance had developed after chronic parental administration of morphine, LMMP strips were incubated for 2 h with 10  $\mu$ M morphine. As shown in Fig. 8A, in both the ileum (filled triangles) and the colon (open triangles), no further shifts were observed after 2 h of morphine exposure. Thus, tolerance develops in the ileum after chronic parenteral administration, because the dose-response curve is shifted compared with that of saline treatment and a further 2 h of incubation does not induce a shift. Tolerance did not develop to morphine in LMMP from the colon, because the dose-response curve is similar to that of 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 phospho-ERK1/2 in guinea pigs treated for 7 days with morphine. LMMP preparations from the ileum and colon of guinea pigs treated for 7 days *in vivo* were exposed further for 2 h of incubation with morphine (10  $\mu$ M) 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 h treatment with morphine (Fig. 8B; Supplemental Fig. 6). Likewise, no changes were observed in phospho-ERK1/2 expression in the ileum after morphine treatment (Fig. 8C).

## Discussion

Our findings show that a decrease in the  $\beta$ -arrestin2 levels accompanies the development of tolerance to morphine in the gastrointestinal tract. In this study, we demonstrate that tolerance to morphine occurs in the guinea pig and mouse ileum but not in the colon of either species. The expression of  $\beta$ -arrestin2 is decreased significantly in isolated tissues of the guinea pig ileum after 2 h of morphine exposure. However, neither tolerance nor a decrease in  $\beta$ -arrestin2 levels is observed in the colon. Because genetic deletion of  $\beta$ -arrestin2 in the knockout 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 underused 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 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 1) the inhibition of peristaltic contractions and 2) the elevation of tone and enhanced nonpropulsive contractions leading to constipation. In 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 studied classically in the guinea pig ileum LMMP preparations. Morphine and other opioids also contract circular muscle preparations via the inhibition of a constitutive inhibitory neurogenic tone. Studies by Grider and Makhlof (1987b) showed that the addition of 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 mouse circular muscle preparations, the addition of TTX also induces tone and blocks morphine-induced contractions (H. T. Maguma and H. I. Akbarali, unpublished observations). Unlike the guinea pig, where the myenteric plexus is attached largely 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 circular muscle preparations to examine morphine-induced tolerance in the mouse. Using 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  $\beta$ -arrestin2 knockout 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 knockout mice compared with that produced in wild-type mice. This suggests that the acute effects of morphine do not require  $\beta$ -arrestin2 signaling. However, a decrease in  $\beta$ -arrestin2 expression appears to be necessary to produce tolerance (i.e., a decrease in agonist responsiveness after chronic treatment). Similar acute effects of morphine have been observed *in vivo* toward the inhibition of fecal output in  $\beta$ -arrestin2 knockout mice. Raehal et al. (2005) showed that morphine initially suppressed fecal output in  $\beta$ -arrestin2 knockout mice, but it recovered after 2 h. The recovery may be due to tolerance to morphine-induced inhibition of defecation developing over time in knockout mice in the absence of  $\beta$ -arrestin2. Likewise, acute morphine inhibits gastrointestinal transit to the same extent in wild-type and  $\beta$ -arrestin2 knockout 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  $\beta$ -arrestin2 knockout mice (Raehal et al., 2005), it is possible that tolerance may not have developed 50 min after morphine administration. Further studies may be required to determine the extent of tolerance to morphine-induced inhibition of gastrointestinal transit in the  $\beta$ -arrestin2 knockout mice. We have shown previously that tolerance develops to the 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) LMMP with an intact ganglionic plexus can be obtained with relative increased ease; 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, thus allowing for biochemical measurements. The latter is of particular relevance, because our experiments with the mouse ileum



showed low expression, although  $\beta$ -arrestin2 could be detected from the brain of wild-type mice and was absent in the knockout mice. Moreover, the antibody recognized a distinct band in the guinea pig LMMP and isolated neuronal preparations.

Although 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 the recruitment/down-regulation of  $\beta$ -arrestin2 likely differ between these two tissues. Recruitment of  $\beta$ -arrestin2 to the  $\mu$ -opioid 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  $\mu$ -opioid 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 after agonist stimulation and that ubiquitination is a necessary step in the trafficking and degradation of G protein-coupled receptors (Shenoy et al., 2001). Ubiquitination would potentially direct  $\beta$ -arrestin2 toward degradation by the proteasome. The  $\mu$ -opioid receptors belong to the "class A" type receptors, which have been shown to induce transient ubiquitination of  $\beta$ -arrestin2 and also promote its deubiquitination 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 deubiquitinating enzymes after receptor stimulation. It is possible that differences between the ileum and the colon may relate to the mechanisms associated with  $\beta$ -arrestin2 ubiquitination/deubiquitination, thus altering the time course of  $\beta$ -arrestin2 levels in these tissues after morphine administration. The mechanisms associated with receptor trafficking and degradation of  $\beta$ -arrestin2 merit further investigation.

In the isolated ileum from chronically treated guinea pigs, 2 h of 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 in vitro incubation for 2 h 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 alter mechanisms associated with its down-regulation such that with respect to tolerance development it resembles the colon. Presently, it is not clear 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 down-regulation and/or uncoupling of the 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  $\beta$ -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  $\beta$ -arrestin2/p-ERK complex in the ileum but not the colon. Previous studies have demonstrated that

opioids may either increase or decrease the mitogen-activated protein 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 levels follows from the decreased levels of  $\beta$ -arrestin2.

Many pathways have been associated with opioid tolerance, including phospholipase C, protein kinases A and C, protein phosphatases, GRK, and CD38 (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 used cell-based assays or antinociception in vivo or in vitro studies of neurons involved in reward pathways. The present study highlights further 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  $\beta$ -arrestin2 compared with antinociceptive tolerance where  $\beta$ -arrestin2 knockout results in the prevention of tolerance (Bohn et al., 1999).

#### Authorship Contributions

*Participated in research design:* Kang, Maguma, Ross, Dewey, and Akbarali.

*Conducted experiments:* Kang, Maguma, Smith, and Ross.

*Performed data analysis:* Kang, Maguma, Smith, and Ross.

*Wrote or contributed to the writing of the manuscript:* Dewey and Akbarali.

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