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Enhanced sensitivity of α3β4 nicotinic receptors in enteric neurons after chronic morphine: implication for opioid-induced constipation

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Running title: nAchR in opioid-induced constipation

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List of non-standard abbrevations:

AGID - Autoimmune gastrointestinal dysmotility
ENS - Enteric nervous system
LMMP - Longitudinal muscle myenteric plexus
nAChRs - Nicotinic acetylcholine receptors

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ABSTRACT

Opioid-induced constipation is a major side-effect that persists with chronic opioid use. Previous studies demonstrated that nicotine – induced contractions are enhanced following chronic morphine in guinea-pig ileum. In the present study we examined whether the increased sensitivity to nicotine could be observed in single enteric neurons following chronic morphine, determined the subunits in mouse enteric neurons and examined the effect of nicotine in reversing opioid-induced constipation. Nicotine (0.03 – 1 mM) dose-dependently induced inward currents from holding potential of -60 mV in isolated single enteric neurons from the mouse ileum. The amplitude of the currents, but not the potency to nicotine, was significantly increased in neurons exposed to long-term (16-24 hrs) but not short – term (10 mins) morphine. Quantitative mRNA analysis showed that nAchR subunit expression in the mouse ileum was $\alpha3 \geq \beta2 > \beta4 > \alpha5 > \alpha4 > \beta3 > \alpha6$. Nicotine induced currents were obtained in neurons from α7, β2, α5 and α6 knock-out mice. The currents were however inhibited by mecamylamine (10 μM) and the α3β4 blocker, α-conotoxin AUIB (3 μM) suggesting that nicotine-indcued currents were mediated by α3β4 subtype of nAChRs on enteric neurons. Conversely, NS3861, a partial agonist at α3β4 nAChR enhanced fecal pellet expulsion in a dose-dependent manner in chronic but not acute morphine treated mice. Overall, our findings suggest that the efficacy of nAChR agonists on enteric neurons is enhanced after chronic morphine exposure and activation of α3β4 subtype of nAChR reverses chronic but not acute morphine induced constipation.

INTRODUCTION

Opioids are commonly used for the treatment of pain. In spite of their highly efficacious analgesic properties, side effects such as tolerance, addiction and constipation significantly limit their long-term use. Many patients tend to discontinue the use of opioids due to the discomfort caused by these side effects, particularly constipation. While the analgesic effects of opioids are mediated through central actions, constipation is predominantly mediated through direct action on the peripheral μ-opioid receptors in the enteric nervous system. Morphine decreases GI transit largely as a result of diminishing the excitability of enteric neurons (North *et al.*, 1977), resulting in decreased excitatory neurotransmitter release (Paton, 1957). Tolerance to the analgesic effects develops upon repeated administration of morphine but does not occur in the colon. Thus, chronic morphine treatment continues to induce constipation. It is therefore of considerable interest to identify potential mechanisms that allow for the reversal or prevention of chronic opioid-induced constipation.

Inhibition of cholinergic transmission is a major mechanism of opioid action in the ENS. The μ-opioid receptors are localized at pre-synaptic nerve terminals as well as on cell bodies of both motor and interneurons in the myenteric plexus (Wood *et al.*, 2004). Activation of the μ-opioid receptors results in decreased excitability of neurons due to activation of K^+ channels, and/or inhibition of Ca^{2+} and Na^+ channels (Smith *et al.*, 2012; Wood *et al.*, 2004). Thus endogenous excitatory neurotransmitters such as acetylcholine have reduced activity during peristaltic reflex. Acetylcholine activates nicotinic receptors (nAChRs) within the myenteric ganglia stimulating both interneurons and motor neurons thereby facilitating peristalsis. Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels that are highly expressed in the enteric nervous system (Galligan, 2002; Galligan *et al.*, 2000) and allow for fast synaptic transmission. The five subunits comprising nAchR form as homomeric or heteromeric units. There are twelve separate subunits α2- α10 and β2- β4. Immunohistochemical, electrophysiological and pharmacological studies indicate that the predominant nAchR subunits in

myenteric neurons are the α3, α5, β2 and β4 subunits that generally form the heteromeric receptor (Zhou *et al.*, 2002). A significant implication for the role of the nAchR is that patients with anti-α3* -nAchR antibodies in the serum have autoimmune gastrointestinal dysmotility (AGID) (Meeusen *et al.*, 2013).

In the context of opioid-induced constipation, previous studies have reported enhanced sensitivity to nicotine and other agonists following chronic morphine in the guinea-pig ileum. These studies from the early 1970's demonstrated development of supersensitivity to several agonists in the morphine-tolerant guinea-pig ileum, including nicotine (Goldstein *et al.*, 1973; Johnson *et al.*, 1978). The mechanism for the supersensitivity is unclear, but has been suggested to involve increased depolarization of the enteric neurons. In view of the enhanced sensitivity to nicotine, we surmised that nAchRs provides a potential target for stimulating gastrointestinal motility in chronic opioid-induced constipation.

In this study, we have investigated the effects of nicotine on isolated enteric neurons from chronic morphine treated mice. We show that nicotine-induced currents in myenteric neurons from adult mouse small intestine are enhanced following chronic, but not acute, treatment with morphine. In addition, we demonstrate that the nAchR subunits α3 and β4 are predominant on the enteric neurons and that both nicotine and NS3681, a α3β4* partial agonist, reverse chronic opioid-induced constipation in vivo.

METHODS:

Sodium Chloride (NaCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), glucose, ATP disodium salt, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), EGTA(ethylene glycol tetraacetic acid), Nicotine hydrogen tartarate, mecamylamine and hexamethonium were purchased from Sigma-Aldrich (St. Louis, MO). Potassium chloride (KCl) was purchased from Fisher Scientific (Waltham, MA) and collagenase was purchased from Worthington (Lakewood, NJ). Laminin and Poly D-Lysine were purchased from BD bioscience (Franklin lanes, NJ), GDNF (glial cell-derived neurotrophic factor) was purchased from Neuromics (Edina, MN), FBS (fetal bovine serum) was purchased from Gemini Bio

products (West sacremento, CA), B-27, Trypsin and neurobasal A media were purchased from Thermofisher (Waltham, MA). NS3861 (3-(3-Bromo-2-thienyl)-8-methyl-8-azabicyclo[3.2.1]oct-2-ene fumarate) (Harpsoe *et al.,* 2013), α – conotoxin MII (Harvey *et al.,* 1997) and α – conotoxin AUIB (Luo *et al.,* 1998) were purchased from Tocris Bioscience (Bristol, UK). Morphine sulphate and morphine pellets (75 mg) were obtained from National Institutes of Drug Abuse (Bethesda, MD).

Animals: Adult male swiss-webster mice (25-30g) (Harlan Laboratories, Indianapoli, IN) housed in 12/12 light/dark cycle vivarium were used for the experiments. Mice null for the α5 and α7 (Jackson Laboratories, Sacramento, CA), α6 and β2 (Institut Pasteur, Paris, France) nAChR subunits and their wild-type (WT) littermates were bred in an animal care facility at Virginia Commonwealth University (Richmond, VA). These mice were backcrossed at least 12 to 15 generations to C57BL/6J mice (Jackson Laboratories). Mutant/transgenic and WT littermates were obtained from crossing heterozygous mice. The animal protocols were approved by Virginia Commonwealth University institutional animal care and use committee. Morphine or nicotine were administrated in mice through intraperitoneal (i.p.) route. Animals were exposed to morphine long term through surgical implantation of 75 mg morphine pellet for four days.

Surgical Implantation of Pellets: Pellets were implanted as previously described (Ross *et al.*, 2008). Mice were anesthetized with 2.5 % isoflurane and the hair on the back of their neck was shaved. Lack of response to a pinch on the toe and absence of righting reflex were used as signs to assess adequate anesthesia. Sterile surgical equipment was used for the process to minimize any potential contamination. Shaven skin was cleaned with povidone iodine (General Medical Corp., Prichard, WV) and rinsed with alcohol. A 1 cm horizontal incision was made at the base of the skull and the underlying subcutaneous space was moved towards the dorsal flanks using a glass rod. A placebo or morphine pellet was inserted in this space and closed using a 9 mm wound clips (BD bioscience, San Jose, CA). Iodine was applied again after closing the site and the animals were allowed to recover in their home cages.

Enteric Neuron Cell Isolation: Enteric neurons were isolated as previously described (Smith *et al.*, 2013) (Smith *et al.*, 2012). Ileum tissue was obtained immediately from a euthanized mice and placed in ice cold Krebs solution (in mM: 118 NaCl, 4.6 KCl, 1.3 NaH2PO4, 1.2 MgSO4, 25 NaHCO3, 11 glucose and 2.5 CaCl2) and bubbled with carbogen (95% O2/5% CO2)). The luminal contents were flushed with ice-cold Krebs and the tissues threaded on a plastic rod through the lumen. The longitudinal muscle layer with the adherent myenteric plexus was gently stripped using a cotton-tip applicator. The Isolated longitudinal-myenteric plexus (LMMP) was then minced and subjected to digestion with collagense for one hour and with trypsin for 7 mins at 37^{o}C in the water bath. Tissue was triturated and collected using centrifugation after each digestion step. The isolated cells were then washed and plated on laminin and poly d-lysine coated cover slips in neurobasal A media containing 1% FBS, 1X B-27 and 10 ng/ml glial cell derived neurotropic factor (GDNF) and penicillin/streptomycin.

Electrical Recordings: Standard whole cell configuration was used for all recordings. EPC 10 amplifier (HEKA, Bellmore, NY) was used for recordings. All patch clamp recordings were carried out in enteric neurons within two days following isolation. Coverslips with attached cells were placed in a recording chamber under an inverted microscope and continuously perfused with external solution containing (in mM) 135 NaCl, 5.4 KCl, 0.3 NaH2PO4, 1 MgCl2 , 5 glucose and 2 CaCl2 (PH adjusted to 7.4 using 1M NaOH). The patch pipettes were prepared using Flaming-Brown horizontal puller (P-87; Sutter Instruments, Novato, CA) and fire polished. Resistance of the pipettes used was 1.5-2.5 MΩ when filled with internal solution containing (in mM): 100 K-aspartic acid, 30 KCl, 4.5 ATP, 1 $MgCl_2$, 10 HEPES, and 0.1 EGTA. Series resistance was less than 10 MΩ and not compensated. The voltage clamp recordings were performed at a holding potential of -60 mv. The currents were measured by either giving a series of voltage pulses or recorded using a gap free protocol at -60 mV. Action potentials were induced in the neurons by a series of current injections in current clamp mode at resting membrane potential. Nicotine and ATP were applied to the cell via bath perfusion. The cells were washed after each nicotine exposure for 3 -5 mins before exposing to the next concentration.

PCR: Quantitative real time PCR was performed on RNA extracted from the ileum LMMP. LMMP tissue was first collected in trizol and RNA was extracted using the manufacturer's protocol (Life technologies, Cat # 15596, Carlsbad, CA). PCR experiments were performed following the BIO-RAD (Hercules, CA) iTaq Universal SYBR Green One-Step Kit. In order to quantify the mRNA of nAChRs from LMMP, a standard curve was first plotted using different concentrations of genomic DNA with each primer. The standard curve was plotted based on the Ct value obtained at each concentration of DNA. The quantity of mRNA in the LMMP sample was determined based on the position of Ct value of the sample in the standard curve. Copy number was calculated using the online tool available at scienceprimer.com. The primers used were as follows β2-Forward: TGCTCCAACTCTATGGCGCT β2-Reverse: CACCAGCTCAGAGCCATTAG, β3-Forward: CAGGCTTCCTACGGGTCTTC β3-Reverse: GGGCGGACACATTTCTGATA, β4-Forward: CCCTGCTCCTCGTCTCTCTGTT β4-Reverse: TGGAGATGAGCTGGGAGGAG, α3-Forward: CCGCTGTCCATGCTGATGCT α3-Reverse: GCCACAGGTTGGTTTCCATG, α4-Forward: TGCCGCTCCTGCTGCTCTTA α4-Reverse: GCGGACAAGGACCACATCTG, α5-Forward: GTTGCCTGAGCTATCCTCTG α5-Reverse: CCACGTCCACTAACTGAGAT, α6-Forward: GACCAGGGAAACCTGCACTC α6-Reverse: GATCGGAGACATTCTCCACC. Experiments were performed in triplicates from three separate biological samples. 18s rRNA was used as an internal control to quantify the normalized fold change.

Total fecal pellet output : Fecal pellet output was measured as an indication of the total gastrointestinal motility. These studies were conducted in a blinded fashion with pellet counting done by an observer oblivious to the group being tested. Mice were divided into the following groups. 1) Control (Placebo-pelleted (4-days)) 2) Acute morphine –treated (Placebo-pelleted + single 10 mg kg^{-1} morphine injection) and 3) chronic morphine (75 mg morphine-pelleted (4-days)). Mice in each group were injected with a single acute saline or 0.175, 0.35, 0.525 and 1.575 mg kg^{-1} nicotine (i.p.) and placed in an empty cage. The number of fecal pellets expelled between thirty and sixty mins after the injection were counted. Acute morphine injections were given twenty mins prior to the saline or nicotine injections. A similar separate

group of mice were tested for the effects of NS3681 (0.01, 0.05, 0.1 and 0.5 mg kg^{-1}, i.p.). All the nicotine doses are calculated by multiplying the dose of salt with 0.35 (Base/Anhydrous salt) as described earlier (Matta *et al.*, 2007).

Data Analysis: Sigma Plot 11.0 and GraphPad Prism 6 were used for data analysis. Data are presented as mean ± EM and scattered plots used to show the distribution. P values less than 0.05 were considered significant. Two tailed t-test was used to compare the differences between two different groups, One-Way ANOVA, Two-Way ANOVA with tukey Kramer post hoc test or fisher's LSD were used for comparison between multiple groups. Specific tests are provided under figure legends. The EC50 values were calculated using lease square linear regression analysis followed by calculation of confidence limits (Bliss, 1967).

RESULTS:

Long term exposure to morphine enhanced the nicotine induced excitability: Nicotine-induced currents were examined in isolated neurons from the adult mouse myenteric plexus. In this primary culture preparation, both neurons and glia are isolated from the myenteric plexus (Smith *et al.*, 2012; Smith *et al.*, 2013). In order to record nicotinic currents from neurons, each cell was examined for its ability to elicit an action potential (Fig 1A). Cells exhibiting action potentials also displayed inward currents when depolarized in voltage clamp mode (Fig 1B). The average resting membrane potential of enteric neurons from the mouse ileum was -47.6 ± 0.6 mV (n=73) and long-term exposure to morphine (16-20 hrs) (3 uM) did not significantly affect the resting potential -48.3 ± 1 mV; (n=42) (Fig 2A). We have previously shown that 16-20 hr treatment of morphine results in neurons developing tolerance and dependence , similar to those isolated from morphine-pelleted mice (Smith *et al.*, 2014). In these cells, overnight treatment with morphine (3 μM) enhances excitability upon precipitated withdrawal similar to neurons obtained from morphine-pelleted mice (Smith *et al.*, 2014). Previous reports have also suggested that the enhanced nicotine induced contractions in long term morphine treated guinea pigs may be mediated due

to depolarization of enteric neurons by chronic morphine (Kong *et al.*, 1997; Leedham *et al.*, 1992). While long-term morphine did not alter the resting potential, the enteric neurons were significantly more sensitive to nicotine following morphine treatment. As shown in figure 2B, nicotine at 1 μM slightly depolarized control neurons by 2.2 ± 0.3 mV, however after long-term morphine treatment, the depolarization (3.8 ± 1.2 mV) resulted in spontaneous action potentials in 3/8 cells (Fig 2B). At 3 μM nicotine, the depolarization in control cells was 9.7 ± 1.2 mV and 16.1 ± 2.2 mV in long term morphine treated cells (Fig 2C). These data suggested that neurons were more sensitive to nicotine following long-term morphine treatment.

Long term but not short term exposure to morphine enhances nicotine induced currents: To study the short term and long term effects of morphine on nAChRs, enteric neurons were treated with 3 μM morphine for a period of 10 min in the bath or over-night for a period of 16-20 hours in culture. nAChR activity was then assessed on these cells using nicotine as an agonist in a whole-cell voltage clamp mode (fig 3A). Cells were held at -60 mV and the peak amplitudes of inward currents were plotted against the nicotine concentration. The peak amplitude of nicotine-induced currents were significantly greater in long-term morphine treated cells. The dose-response curve showed significantly greater currents at 300 μM and 1mM nicotine in the prolonged morphine exposed cells but not in the short exposure group when compared to the control drug naïve group (Fig 3A&B). While the peak amplitudes were enhanced, there was no difference in the potency of nicotine. The EC50 values were 44 μM (95 % C.I 27 - 61), 39 μM (95 % C.I 25 ± 53) and 42 μM (95 % C.I 28 – 56) in control, short-term and after prolonged exposure, respectively (Fig 3C). We further tested the effect of nicotine upon a single application to ensure that desensitization is not a confounding factor. The amplitude of maximal currents induced by a single individual exposure to 1 mM nicotine was 139.4 ± 34.3 pA/pF in control, 106.51 ± 11.1 pA/pF in short-term treated neurons, and 228 ± 14.7 pA/pF after long-term exposure to morphine (Fig 3D). Pretreatment with the opioid-receptor antagonist, naloxone (1 μM) blocked the long-term morphine induced nicotinic currents (Fig 3D).

Long term exposure to morphine did not alter ATP induced current: We next tested if long term morphine effects are specific for nicotine induced currents by examining another ligand gated channel, the P2X receptor. 1mM ATP, an agonist at P2X receptor induced inward current at a holding potential of -60 mV. ATP induced currents were examined in cells exposed to long term morphine. There was no significant differences seen among the currents induced by ATP in control vs long term morphine treated cells. The amplitude of currents induced by 1 mM ATP are 73.6 ± 17 pA/pF in control and 56.1 ± 12.4 pA/pF long term morphine treated cells. (Fig 4)

α3 and β4 nAChR subunits are highly expressed in the mouse enteric neurons: Previous studies in the neonatal guinea pig ileum have suggested the functional expression of α3, β4, β2 and α5 subunits on the enteric neurons (Zhou *et al.*, 2002). Data from qPCR quantifying the mRNA levels of nAChRs in the LMMP of ileum demonstrated a higher expression in the order of α3 > β2 > β4 > α5 > α4 > β3 > α6 subunits (Supl figure 1). However the contribution of these subunits in nicotine induced responses is not clear. In order to further examine the functional role of these subunits in the enteric neurons, we used a genetic and a pharmacological approach. Enteric neurons were isolated from C57BL/6J mice as these formed the background strain for all nicotinic knock-outs. Figure 5A shows nicotine-induced inward currents from α7, α5, α6 and β2 knock out mice. Exposure to nicotine (3 uM – 300 uM) induced concentration-dependent inward currents in all knock-out mice and were not significantly different in maximal amplitude to the C57BL/6J background (Fig 5B and Supplemental Fig 2). This indicates that these receptor subunits were not specifically involved in nicotine-induced currents in the enteric neurons. However the nicotine induced currents from α5 knock out enteric neurons showed a delayed desensitization compared to the other subtype knock out mice suggesting that they may be involved in regulating the desensitization of the nAChR expressed in enteric neurons (Fig 5 A & B).

To further define the composition of nAChRs mediating nicotine's effects, we used α3β4 and α3β2/α6β2 nicotinic antagonists with different selectivity toward these nAChR subtypes. Mecmylamine (10 μM)

and hexamethonium (10 μM) significantly blocked the nicotine induced currents at concentrations previously reported to be more preferential for α3β4 receptors (Papke *et al.*, 2010). This was further confirmed by inhibition of nicotine-induced currents by the α-conotoxin, AUIB which has been shown to be highly specific for α3β4 expressed receptor (Harvey *et al.*, 1997; Luo *et al.*, 1998). A submaximal dose of AUIB (3 μM) that is specific for α3β4 significantly blocked the nicotine induced currents in enteric neurons. α-conotoxin M-II (100 nM), a nAChR antagonist specific for α3β2/α6β2 subtypes did not significantly block the nicotine induced currents in the enteric neurons (Fig 6 A & B). These findings suggested that the nicotine induced currents in the enteric neurons are primarily mediated through the α3β4* nAChR receptors with possible co-expression of α5 subunit modulating receptor desensitization.

Effects of Nicotine and NS3861 on GI motility: The above studies indicate that chronic morphine treatment leads to enhanced nicotine-induced effects on the myenteric neurons The cellular studies in enteric neurons suggest that the nAchR mediating nicotinic responses are likely to be α3β4. To determine the role of α3β4 nAChRs on the opioid induced decreased gastrointestinal motility, we used nicotine and NS3861, a compound with high affinity and partial agonist properties at α3β4 expressed nAChRs (Harpsoe *et al.*, 2013). As shown in the figure 7, the number of pellets (measured over a 30 min interval) following saline injection in placebo-pelleted mice was 5 ± 1.1 pellets. Nicotine significantly decreased the number of fecal pellets expelled with increasing doses (0.175, 0.35, 0.525 and 1.575mg kg^{-1}) (Fig 7A). In mice treated with acute morphine (10 mg kg^{-1}), there was complete inhibition of pellet expulsion during the first hour and nicotine did not stimulate pellet expulsion (Fig 7B). However, in morphine pelleted animals, nicotine 0.175 mg kg^{-1} significantly enhanced the number of fecal pellets expelled to 3.3 ± 1. There was however, no significant differences seen with other nicotine doses tested (0.35, 0.525 and 1.575 mg kg^{-1}) (Fig 7C).

Similarly, NS3861 did not significantly alter the number of fecal pellets expelled at 0.01, 0.05, 0.1 mg kg^{-1} and decreased the number of pellets with a dose of 0.5 mg kg^{-1} when given i.p. In mice treated with

acute morphine (10 mg kg^{-1}), there was complete inhibition of pellet expulsion and NS3861 did not stimulate pellet expulsion. However, in the morphine pelleted mice, the number of fecal pellets expelled was 1.2 ± 0.58 and the number of fecal pellet expelled increased in a dose-related manner with a significant increase seen at 0.1 mg kg^{-1} NS3861 (Fig 8).

Enhanced responses to nicotine are not associated with dependence in enteric neurons: Long term exposure to morphine was previously shown to induce tolerance and dependence in single enteric neurons from ileum but not in the colon (Ross *et al.*, 2008; Smith *et al.*, 2014). In order to identify if the enhanced responses to nicotine are associated with morphine dependence we studied the effects of long term morphine on response to nicotine in enteric neurons from the colon. Nicotine induced currents were significantly enhanced in colonic cells treated with long term morphine. The amplitudes of currents induced by 1 mM nicotine were 148 ± 28.5 pA/pF in control and 222 ± 24.4 pA/pF in long term morphine treated cells respectively. The EC50 values of nicotine were 18.4 µM (95 % C.I 11.6 – 29.1) in control and 7.1 µM (95 % C.I 3.2 – 15.7) long term morphine treated cells respectively. (Fig 9)

α3β4 mRNA expression is not altered after prolonged exposure to morphine: In order to test if the enhanced responses to nicotine seen after prolonged exposure to morphine are mediated through an increase in transcription of the α3β4 nAChR receptor, we examined the α3β4 mRNA expression in LMMP from mice pelleted with either placebo or 75 mg morphine for four days. The normalized fold change of mRNA were not significantly different among the two tested groups. (Fig 10).

DISCUSSION:

In the present study we show that 1) Chronic morphine treatment increases the efficacy for nicotine in isolated single neurons, 2) α3β4 subtype of nAChRs mediates the nicotine induced currents in the adult mouse enteric neurons, 3) NS3861, a partial agonist at the α3β4 nAChR reversed morphine induced constipation in chronic but not acute morphine treated mice, 4) Morphine effects were specific for

nAChRs and not seen on P2X receptors expressed on the enteric neurons, and 5) the enhanced efficacy at nAChR is not due to development of opioid dependence as similar findings are also seen from the neurons isolated from colon.

Morphine induced constipation is a major problem limiting its clinical utility. Peripheral mu-opioid antagonists have recently become available as an option for this treatment, however there are limitations with their use, including potential cardiovascular side-effects. In this study we have found that the nAChR α3β4 sensitivity is significantly enhanced after prolonged opioid treatment. nAChR subtypes are highly expressed on the enteric neurons and an α3β4 nicotinic agonist provides a prokinetic effect following chronic but not acute opioid exposure.

Previous reports have suggested that the increase in the potency of nicotine after prolonged exposure to morphine (Johnson *et al.*, 1978) (Goldstein *et al.*, 1973) may derive from depolarization of enteric neurons by chronic morphine due to reduced sodium potassium pump activity (Kong *et al.*, 1997; Leedham *et al.*, 1992). However, in isolated neurons that were treated with morphine overnight and demonstrated enhanced response to nicotine, showed no significant difference in the resting membrane potential. Furthermore, the responses to ATP remained unchanged, although ATP and acetylcholine are generally co-transmitters in the myenteric plexus. This suggests that chronic morphine induced enhancement of nAchR is specific and may involve either a membrane delimited pathway or alterations in intracellular signaling. It is noteworthy that α3β4 nicotinic antagonists and partial agonists also reverse morphine withdrawal symptoms, including diarrhea in the mouse. (Muldoon *et al.*, 2014).

We further examined the specific receptor subtype affected by chronic opioids. In the present study we approached to answer this question by examining the mRNA expression levels and by using transgenic models along with a pharmacological approach in mouse enteric neurons. nAChRs from the LMMP of ileum displayed mRNA expression in the order α3 > β2 > β4 > α5 > α4 > β3 > α6 subunits (Supplemental Fig 1) in agreement with previous reports from neonatal guinea pig ileum (Zhou *et al.*, 2002). However

the contribution and function of each of these subunits in the enteric neurons is not clear. The amplitude of nicotine induced currents measured from enteric neurons of α7, α6, α5, β2 subunit knock out mice were not significantly different from control mice suggesting that they are not the subunits mediating nicotine induced currents in the enteric neurons. However, nicotine induced currents from α5 knock out mice displayed a delayed desensitization pattern suggesting that α5 may be also a potential subunit modifying the inactivation of the nicotine induced currents. The current profile of α5 knock-out enteric neurons is similar to that seen by Gerzanich et al in α3 expressing oocytes (Gerzanich *et al.*, 1998). While further studies are needed to examine in the α3 and β4 knock out mice, the main limitation is that α3 null mutant mice do not survive to adult hood (Xu *et al.*, 1999). Mecamylamine, a nicotinic antagonist with a preferential selectivity for α3β4, completely abolished the nicotine induced currents at a concentration of 10 μM. Similarly, hexamethoium (10 μM), a non-selective antagonist significantly decreased the nicotine induced currents. α-Conotoxin AUIB (3 μM) at a concentration highly specific for α3β4 also significantly reduced nicotine induced currents (Harvey *et al.*, 1997; Luo *et al.*, 1998; Zhou *et al.*, 2002). Collectively, these data suggest that α3 and β4 subunits mediate the nicotine induced currents in enteric neurons. α5 subunit may be co-expressed with this nicotinic subtype and modulate its desensitization properties.

Fecal pellet output assay performed using nicotine showed an increase in fecal pellet output with a dose of 0.175 mg kg^{-1}. However, an inverted U shaped response was seen with increasing doses of nicotine. The inverted U shaped dose-response to nicotine is typicaly also seen in many behavioral assays (Picciotto, 2003) that may involve activation of other subunits. NS3861, a partial agonist and with highest affinity at α3β4 compared to other subtypes of nAChR displayed a dose-dependent enhancement in the number of fecal pellets expelled in chronic but not acute morphine treated mice. However, at higher doses of NS3861 there was a decrease in the transit. This may be due to the desensitization of the receptor or off target effects at α3β2 receptors. This suggests that the α3β4 subtype of nAChR provides a potential target to reverse chronic morphine induced constipation.

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Long term exposure to morphine leads to tolerance and dependence (North *et al.*, 1978; Smith *et al.*, 2014). In order to examine if the enhanced response to nicotine is associated with morphine dependence in a single isolated cell, we tested the nicotine induced currents post prolonged exposure to morphine in enteric neurons from colon. We have previously shown that overnight exposure to morphine induces dependence in enteric neurons in the ileum but not colon (Smith *et al.*, 2014). Enhanced responses to nicotine were seen in cells isolated from the colon after long term morphine treatment (Fig 9). This suggested that the enhanced response to nicotine may not be associated with dependence.

An increase in the nicotine induced currents may be due to an increase in the receptor number or a post translational modification leading to changes in biophysical properties of the receptor. Increase in the receptor number can be mediated through an increase in the transcription of the receptor gene or alteration in membrane trafficking of the receptor. mRNA levels of α3β4 were not significantly changed in the LMMP of ileum from morphine pelleted mice (4-days) suggesting that the change in the transcription of α3β4 is not the cause behind the enhanced response to nicotine. However, it is possible that protein expression is altered. Further studies will be required to determine the receptor protein expression of these subunits following chronic morphine. Previous reports have shown that nAChR activity may be enhanced by phosphorylating or altering membrane trafficking (Govind *et al.*, 2009; Govind *et al.*, 2012; Walsh *et al.*, 2008; Wecker *et al.*, 2010). Govind *et al.*, 2012 demonstrated that nicotine-indcued upregulation of α4β2 nAchR occurs by multiple processes that include a fast transient component and a slower component that involves inhibition of proteosomal degradation of the β2 subunit resulting in increased nAchR assembly. It remains to be established whether similar processes occurs due to chronic morphine exposure in enteric neurons. Increased nAChR numbers have been reported in brains of morphine dependent mice (Neugebauer *et al.*, 2013).

Since constipation persists with chronic morphine due to the lack of tolerance development in the colon (Ross et al., 2008), our present findings suggest that identifying α3β4 nAchR agonists with peripheral

selectivity may be useful strategy in the treatment of opioid-induced constipation in patients with long-term opioid treatment.

AUTHORSHIP CONTRIBUTIONS:

Participated in research design: Gade, Kang, Damaj, Dewey, Akbarali .

Conducted experiments: Gade, Khan, Kang.

Contributed Transgenic mice: Damaj.

Performed data analysis: Gade, Khan, Kang, Akbarali

Wrote or contributed to the writing of the manuscript: Gade, Grider, Damaj, Dewey, Akbarali.

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LEGENDS FOR FIGURES

Figure 1:. A) Current clamp recording from the same cell showing action potentials upon giving a positive currents injection of 60 pA. B) Whole-Cell voltage clamp recordings from neurons showing inward sodium current induced upon giving a series of voltage steps from -120 mV to 0 mV.

Figure 2: Long term exposure to morphine enhanced the nicotine induced excitability: A) Resting membrane potentials of neurons from control (n=73) and long term morphine group (n=42) in a current clamp mode. B) Raw traces showing the depolarization induced by the 1 µM nicotine in control and after long term morphine treatment.C) Amplitude of depolarization induced by 1 µM and 3 µM concentrations of nicotine in control and long term morphine treated cells. Student's t-test. *P < 0.05

Figure 3: Short term but not long term exposure to morphine enhances nicotine induced currents: A) Rawtraces of recording from neurons with different concentrations of nicotine. B) Concentration-response curves of peak currents at different concentrations of nicotine. C) Concentration-response curve plotted as a function of maximum response in each group D) Scattered plot showing the distribution of data of currents induced by 1mM nicotine from control (n=15), long term morphine (n=17), in the presence of 1 µM naloxone (n=5) and Long term morphine in the presence of 1 µM naloxone (n=5). Two-Way ANOVA. Tukey kramer Post Hoc test. *P < 0.05

Figure 4: Long term exposure to morphine did not alter ATP induced current: A) Raw trace displaying the inward current induced by 1 mM concentration of ATP. B) Amplitude of current induced by 1 mM ATP in control (n=7) and long term morphine treated cells (n=5). Student's t-test. *P < 0.05

Figure 5: A) Raw traces of currents induced by 0.3 mM nicotine from neurons isolated from LMMP of α7, c-57, α5, α6 and β2 K.O mice. B) Concentration response curves of nicotine induced currents in cells from neurons isolated from LMMP of α7, c-57, α5, α6 and β2 K.O mice. Two-Way ANOVA. Tukey kramer Post Hoc test. *P < 0.05

Figure 6: Hexamethonium, mecamylamine and α-Conotoxin AUIB but not M-II block the nicotine induced currents in neurons isolated from mouse LMMP: A) Raw traces of nicotine induced currents in control cells and in the presence of 10 μM hexamethonium (n=5), 10 μM mecamylamine (n=5), 100 nM M-II (n=9) and 3 μM AUIB (n=8) B) Scatter plot showing the distribution of the amplitude of current in control and in the presence of 10 μM hexamethonium,10 μM mecamylamine, 100 nM M-II and 3 μM AUIB. *P < 0.05

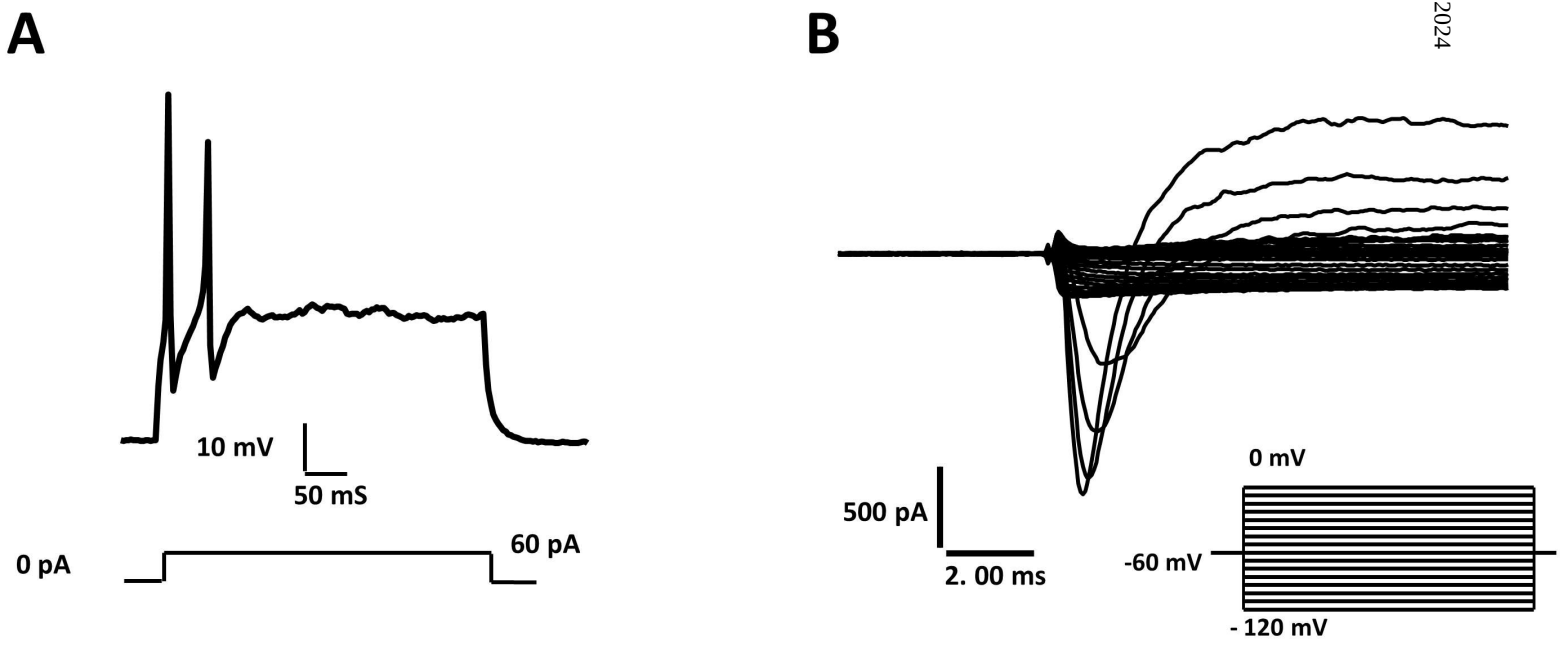
Figure 7: Effects of nicotine on fecal output: Scatter plot displaying the number of fecal pellets expelled after a single I.P injection of saline or different concentrations of nicotine in A) Placebo pelleted mice, B) Placebo pelleted mice receiving 10 mg kg^{-1} morphine and C) Morphine Pelleted mice (N = 5-10 in each group). Scatter plot displaying the % distance moved by charcoal after a single I.P injection of saline or different concentrations of nicotine in D) Placebo pelleted mice, E) Placebo pelleted mice receiving 10 mg kg-1 morphine and F) Morphine Pelleted mice (N = 5 in each group) One-Way ANOVA. Fisher's LSD. *P < 0.05

Figure 8: Effects of NS3861 on fecal output: Scatter plot displaying the number of fecal pellets expelled after a single I.P injection of saline or different concentrations of NS3861 in A) Placebo pelleted mice, B) Placebo pelleted mice receiving 10 mg kg^{-1} morphine and C) Morphine Pelleted mice (N = 5 in each group) One-Way ANOVA. Tukey Kramer post-hoc test. *P < 0.05

Figure 9: Nicotine induced currents in neurons isolated from mouse colon: A) Concentration response curves of nicotine induced currents in neurons isolated from mouse colon. B) Scattered plot showing the distribution of data peak inward currents induced by 1 mM Nicotine in control (n=6) and long term morphine treated cells (n=5). Two-Way ANOVA. Tukey kramer Post Hoc test. *P < 0.05

Figure 10: α3β4 mRNA expression is not altered after prolonged exposure to morphine: Normalized fold change of the mRNA of A) β4 and B) α3 subunits in placebo pelleted (PP) and morphine pelleted (MP) animals. (N=5) Student's t-test *P < 0.05

Figure 1



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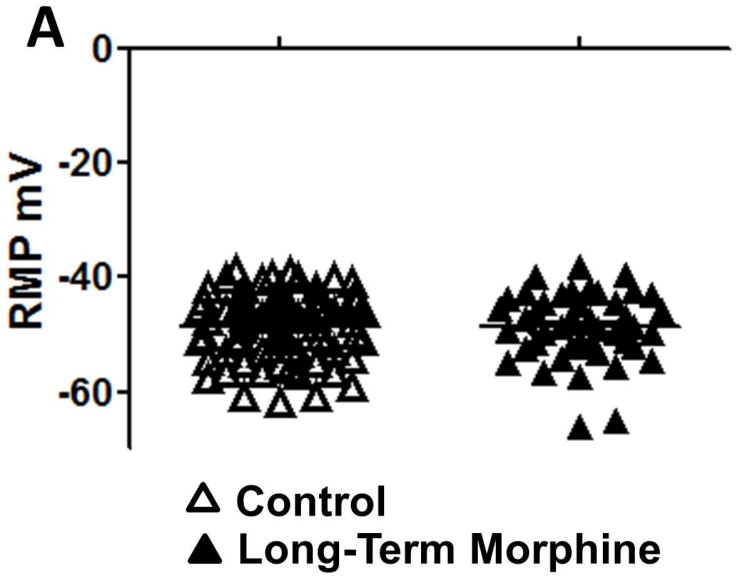
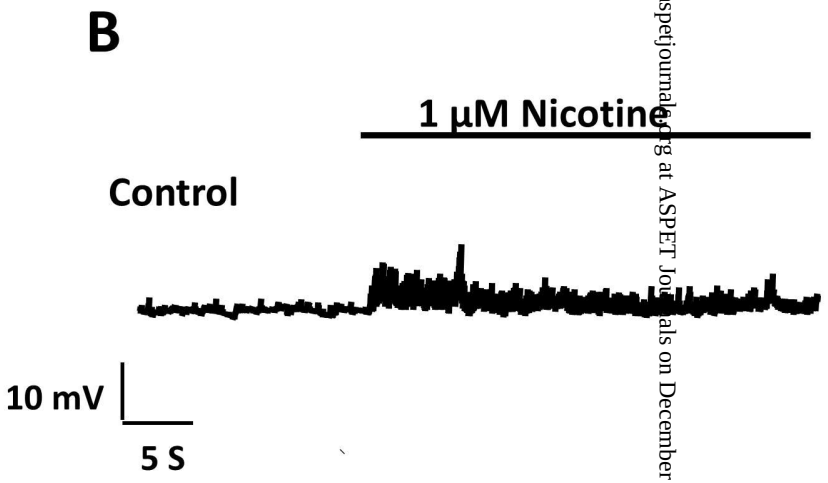
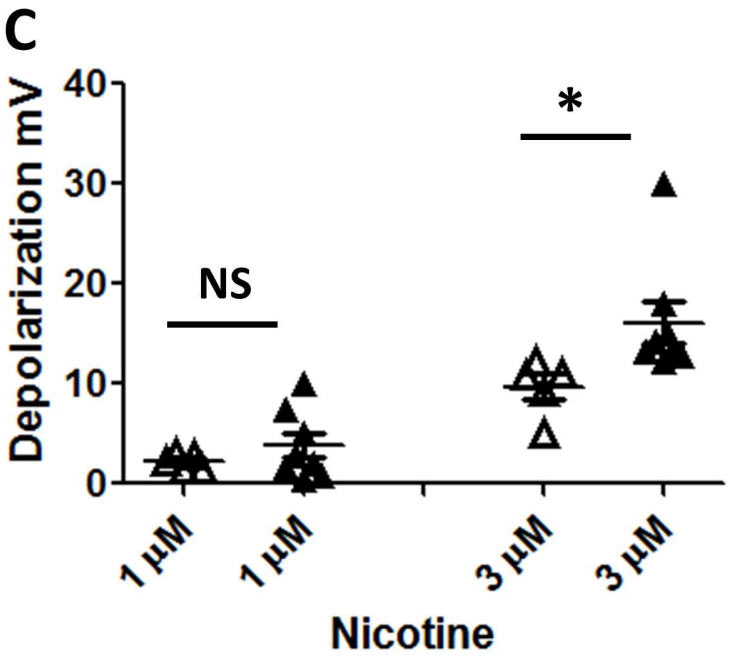






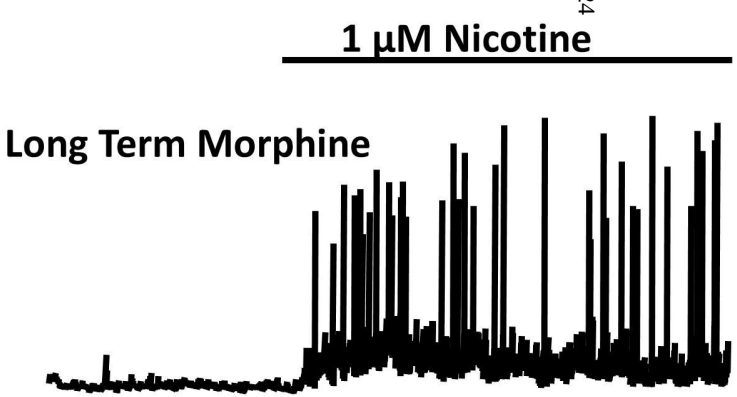
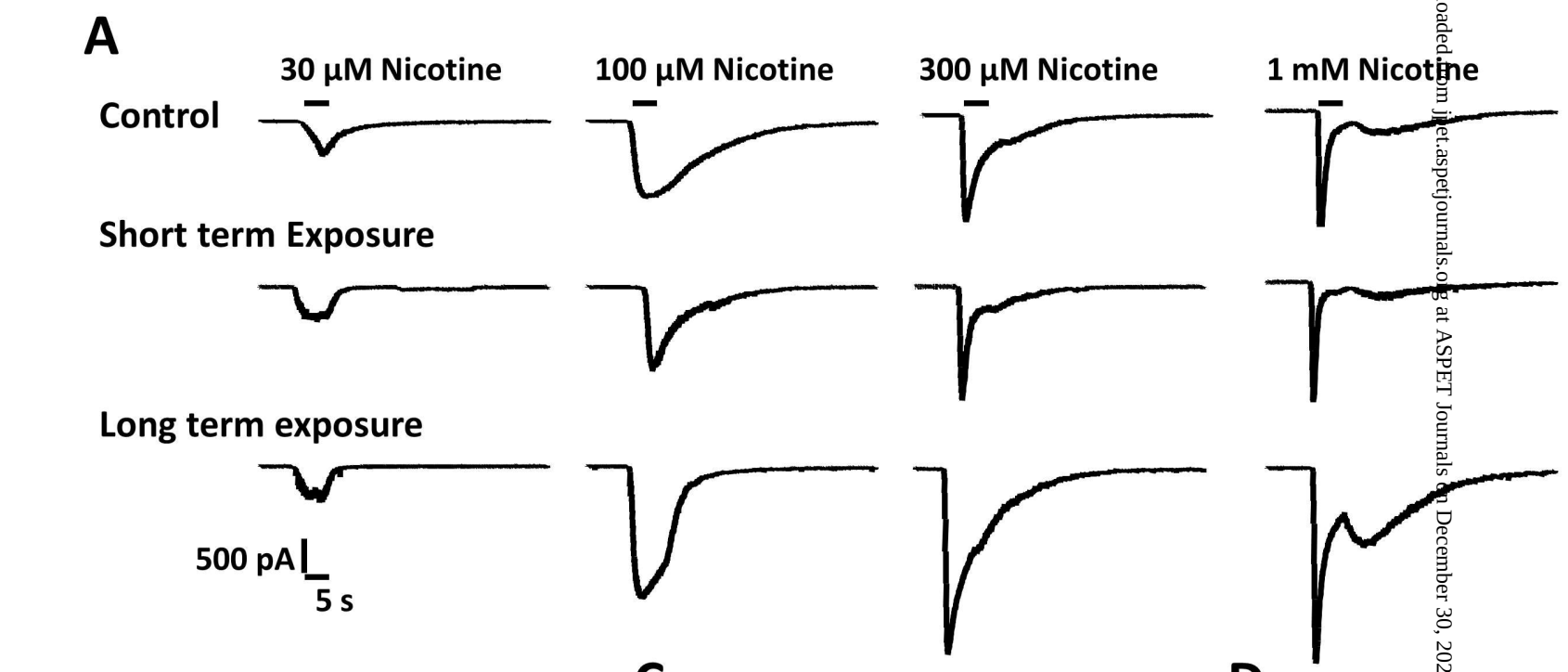
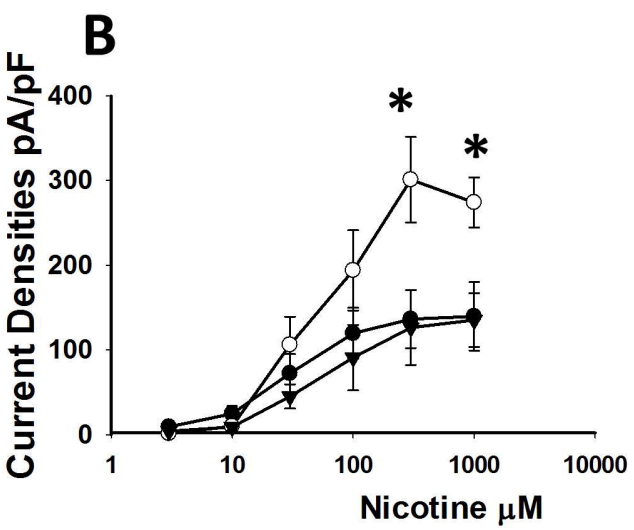
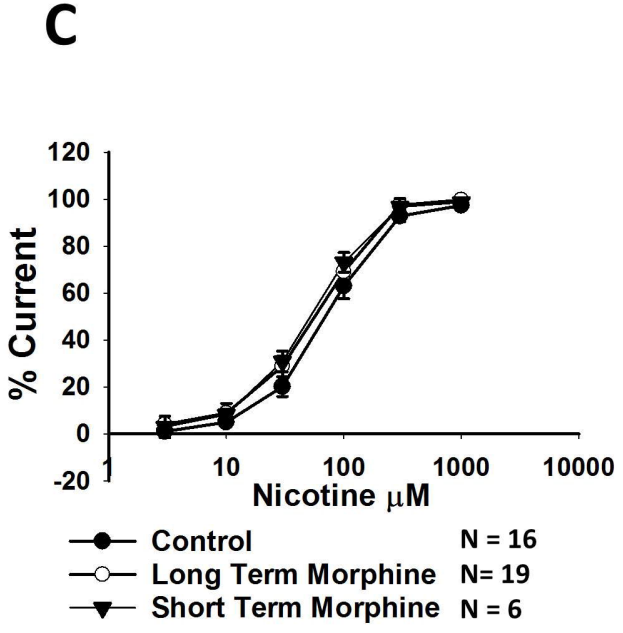
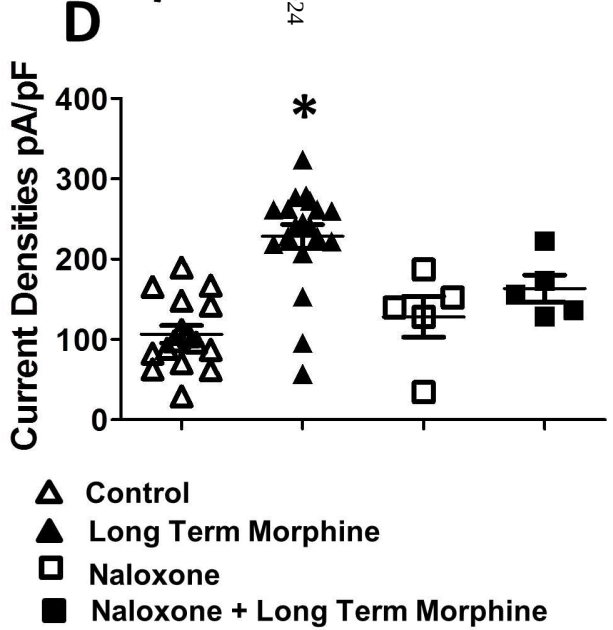


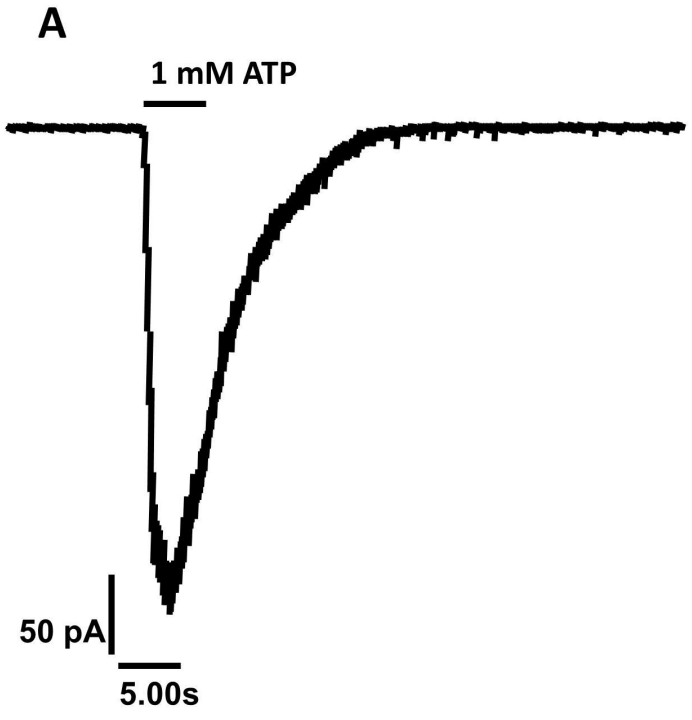
Figure 3










A
1 mM ATP
50 pA
5.00s

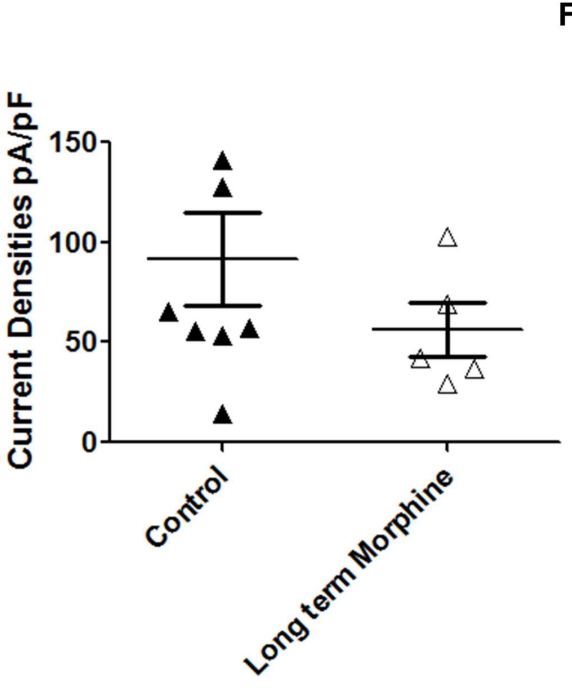
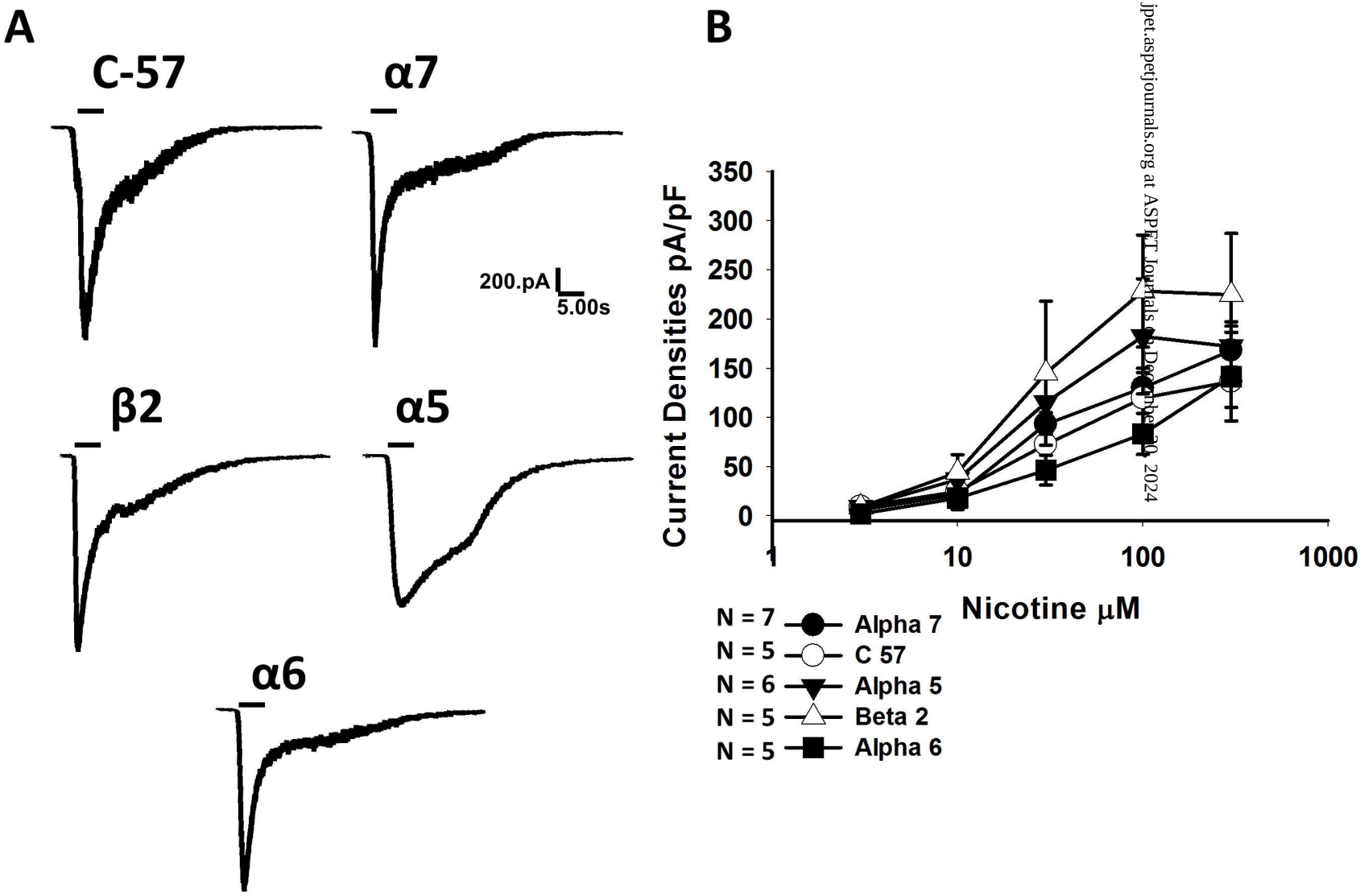

B
Current Densities pA/pF
150
100
50
0
Control
Long term Morphine

Figure 5



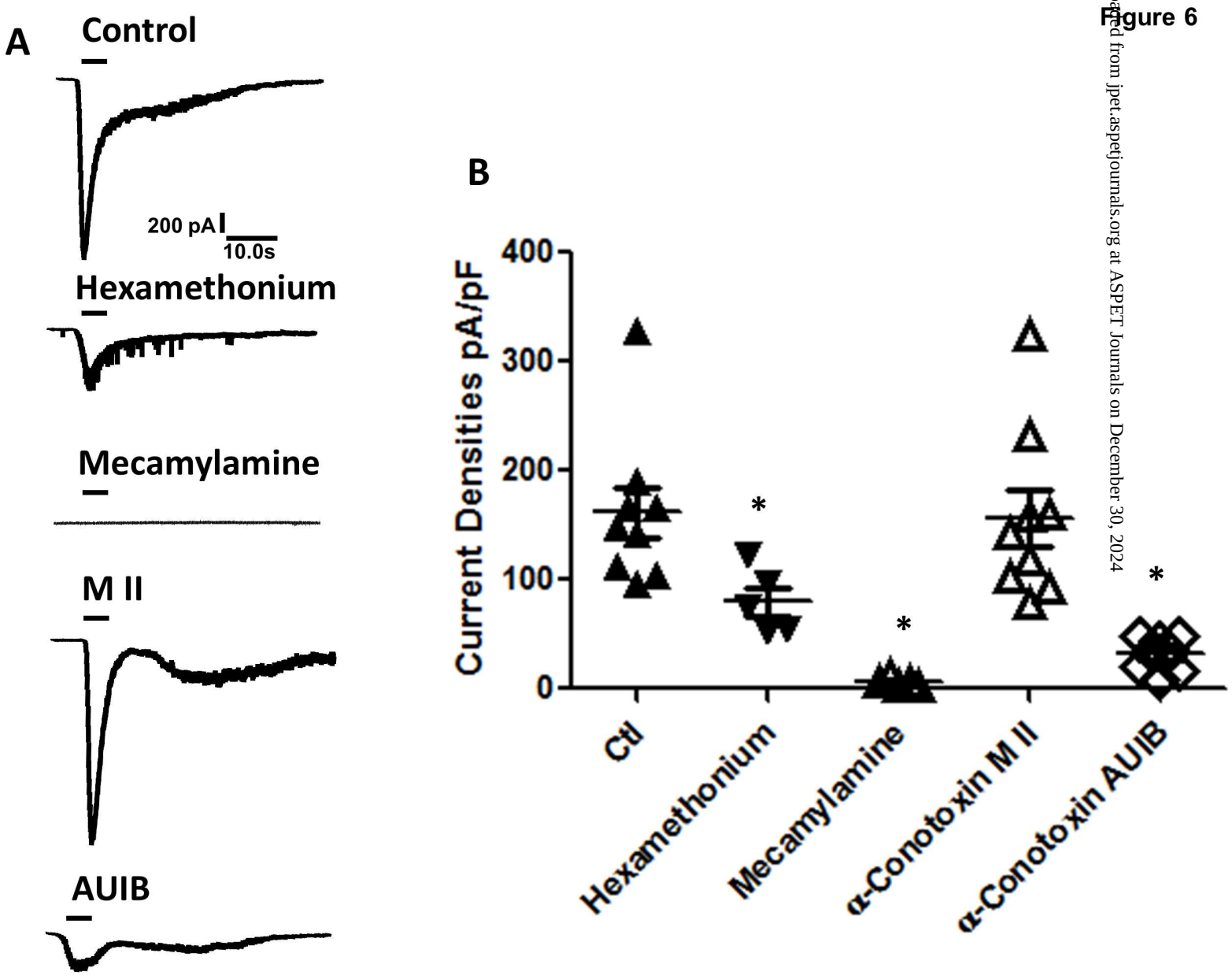


Figure 7

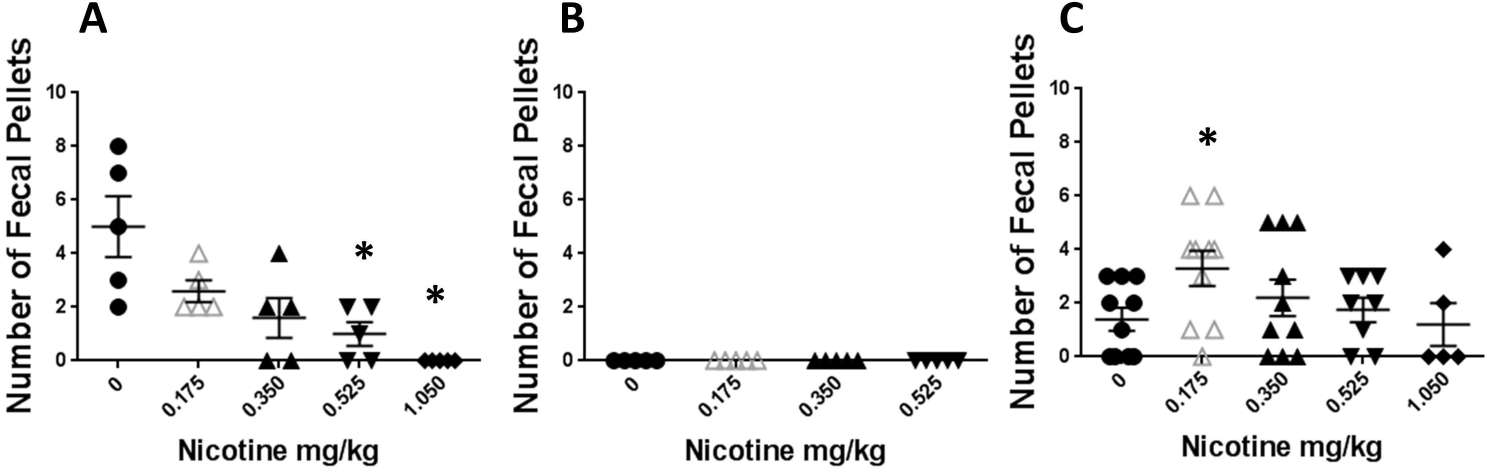
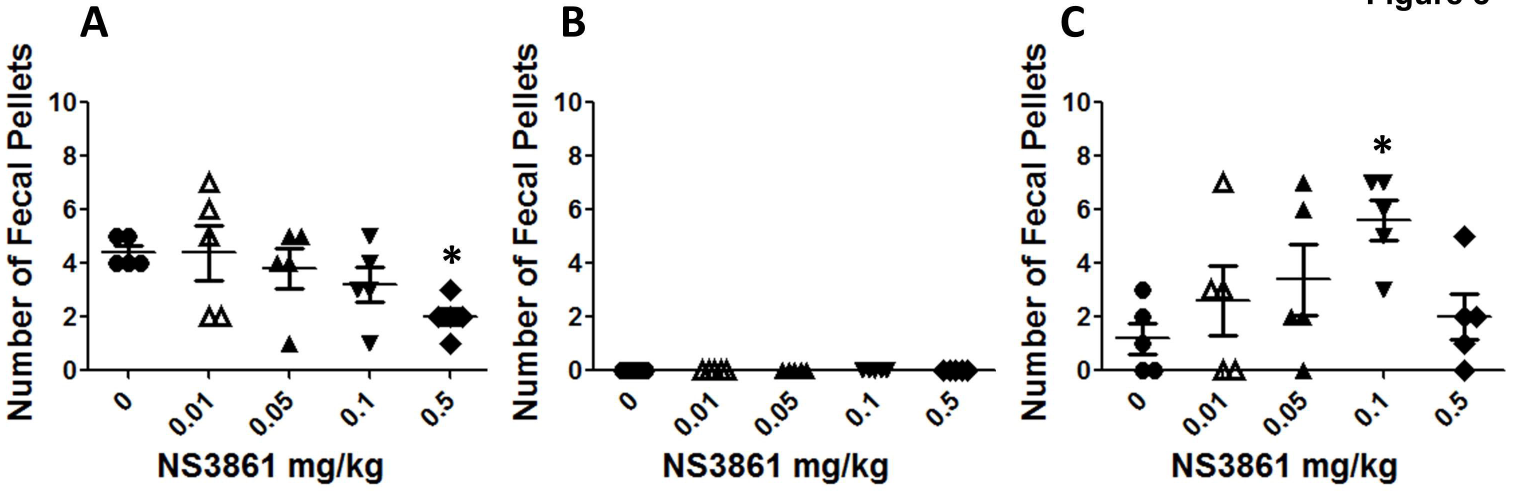
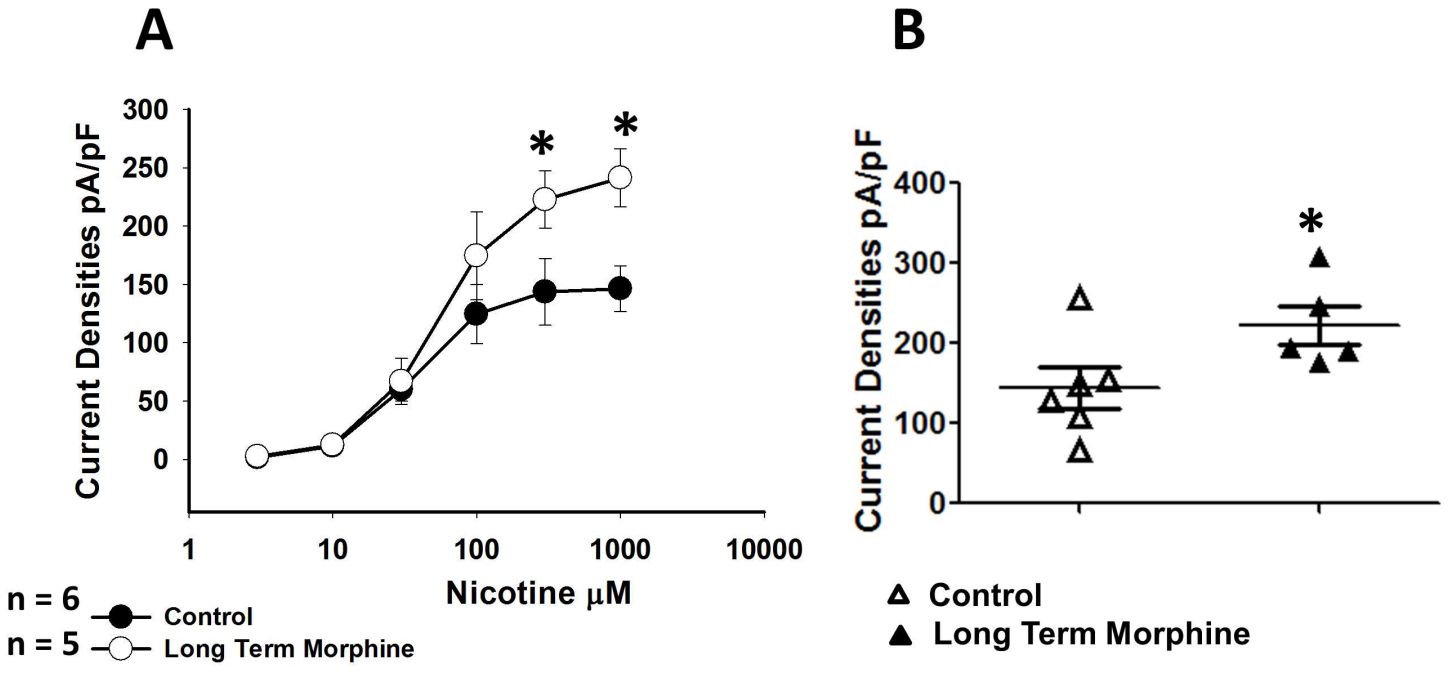
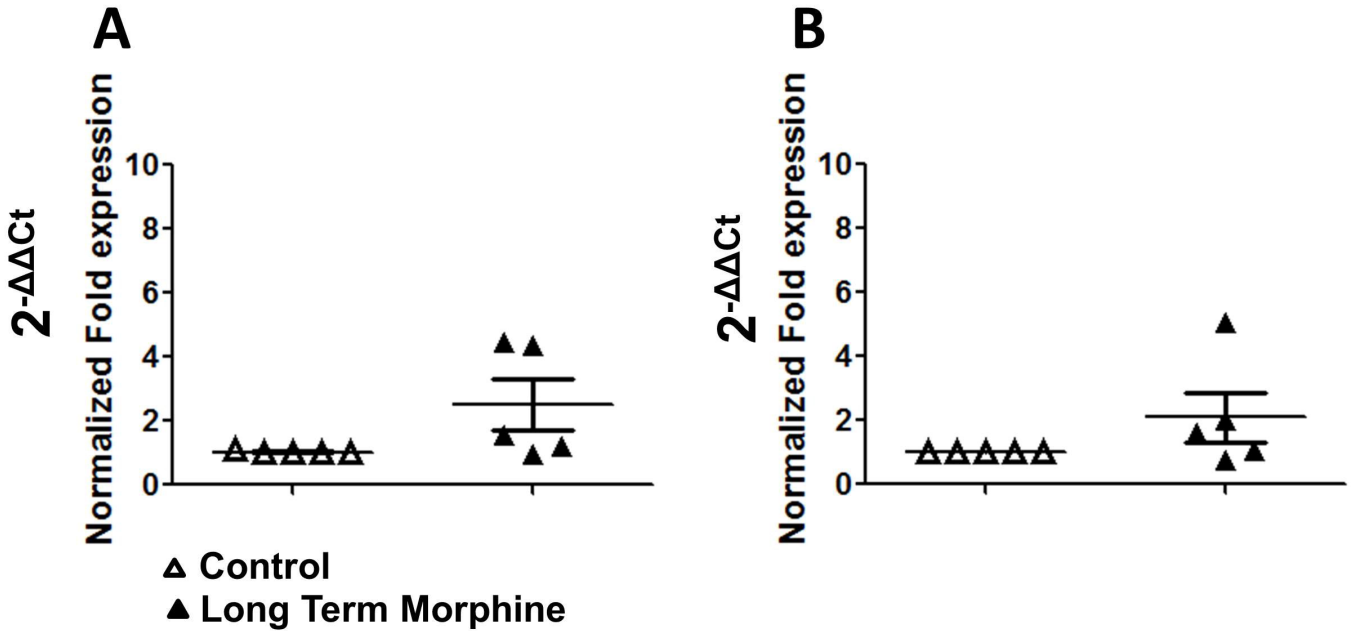

A
B
C
Number of Fecal Pellets
10
8
6
4
2
0
0
0.175
0.350
0.525
1.050
Nicotine mg/kg
*

Figure 8


A
B
C
Number of Fecal Pellets
10
8
6
4
2
0
0
0.01
0.05
0.1
0.5
NS3861 mg/kg
*


A
Current Densities pA/pF
300
250
200
150
100
50
0
1
10
100
1000
10000
Nicotine µM
*
*
n = 6
Control
n = 5
Long Term Morphine
B
Current Densities pA/pF
400
300
200
100
0
*
Control
Long Term Morphine


A
B
2-ΔΔCt
Normalized Fold expression
10
8
6
4
2
0
Control
Long Term Morphine