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THE ROLE OF α3β4* SUBTYPE OF NICOTINIC ACETYLCHOLINE RECEPTORS IN REVERSING OPIOID-INDUCED CONSTIPATION IN MICE

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THE ROLE OF α3β4* SUBTYPE OF NICOTINIC ACETYLCHOLINE RECEPTORS IN REVERSING OPIOID-INDUCED CONSTIPATION IN MICE

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

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Clarification of Contributions

Part of this dissertation has been submitted for publication elsewhere (Gade, A.R; Khan F.; Kang M.; Grider,J.R.; Damaj,M.I.; Dewey,W.L.; Akbarali,H.I. The role of α3β4 nicotinic receptors in reversing opioid-induced constipation in mice)

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List of Abbreviations

nAChRs	Nicotinic acetylcholine receptors
CNS	Central nervous system
ANS	Autonomic nervous system
ENS	Enteric nervous system
MOR	μ-opioid receptor
NO	Nitric oxide
VIP	Vasoactive intestinal peptide
ChAT	Choline acetyltransferase
LMMP	Longitudinal muscle myenteric plexus
ATP	Adenosine triphosphate
EFS	Electric field stimulation
UC	Ulcerative Colitis
AGID	Autoimmune gastrointestinal dysmotility
PCR	Polymerized chain reaction
NOS1	Nitric oxide synthase 1
GFAP	Glial fibrillary acidic protein
GPCR	G-protein-coupled receptors
PKC	Protein kinase C
PKA	Protein kinase C
OBD	Opioid-induced bowel dysfunction
OIC	Opioid-induced constipation

pA	Picoampere
pF	Picofarad
mV	Millivolt
RMP	Resting membrane potential
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
FBS	Fetal bovine serum
EGTA	Ethylene glycol tetraacetic acid
GDNF	Glial cell-derived neurotrophic factor

List of Compounds

Chapter III & IV:

(-)-Nicotine hydrogen tartrate

Adenosine 5′-triphosphate disodium salt hydrate

Chapter V:

Hexamethonium chloride

Mecamylamine hydrochloride

α-conotoxin AUIB

α-conotoxin M II

NS3861

Abstract

THE ROLE OF α3β4* SUBTYPE OF NICOTINIC ACETYLCHOLINE

RECEPTORS IN REVERSING OPIOID-INDUCED CONSTIPATION IN MICE

By Aravind Reddy Gade, MS

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2015

Major Director: Hamid I. Akbarali, Ph.D.
Professor, Department of Pharmacology and Toxicology

Opioids are excellent pain relievers. A major side-effect of chronic opioid treatment is constipation whereas withdrawal following chronic exposure leads to diarrhea and increased gastrointestinal motility. These effects of chronic opioids are mediated by μ-opioid receptors expressed on enteric neurons. Previous studies have shown that chronic opioids enhance sensitivity to nicotine in the gastrointestinal tract. This suggested that prokinetic effects of nicotine mediated through the activation of nicotinic acetylcholine receptors (nAChRs) may be useful in reversing opioid-induced constipation. The goal of this dissertation was to investigate the nAChR subtype expressed on enteric neurons and their role in reversing opioid-induced constipation. The effect of nicotine on small intestinal transit and fecal pellet output were determined in-vivo in morphine-pelleted mice

(75 mg for 4 days). Nicotine-induced currents were measured by whole-cell voltage clamp in isolated adult mouse myenteric neurons treated with morphine over short term (10 mins) and long term (16-20 hrs). Following long term morphine exposure in-vivo (morphine pellet – 4 days), nicotine increased fecal pellet output, and enhanced small intestinal transit. The prokinetic effect of nicotine was not seen in placebo pelleted mice or after acute morphine (10 mg/kg, 30 min). Peak-amplitude of nicotine-induced inward currents in isolated neurons was also enhanced after long-term but not short term exposure to morphine. Nicotine-induced currents were inhibited by mecamylamine (10 μM) and α-conotoxin AUIB (3 μM), suggesting the expression of 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. In conclusion, development of peripherally selective α3β4 partial agonists may be of therapeutic benefit in treatment of chronic opioid-induced constipation.

CHAPTER I

INTRODUCTION

Opiate alkaloids derived from the plant *Papaver somniferum* have been used to treat pain for over thousand years. They are the most commonly prescribed drugs to treat chronic nonmalignant pain in the US (Nelson *et al.*, 2015). In spite of their highly efficacious analgesic properties, side effects such as tolerance, addiction and gastrointestinal adverse effects limit their clinical utility. Gastrointestinal adverse effects include nausea and vomiting, abdominal pain and constipation which are collectively referred to as opioid-induced bowel dysfunction (OBD) (Panchal *et al.*, 2007). Apart from OBD, opioid use during surgeries frequently results in post-operative ileus, a condition where the small intestine is temporarily paralyzed (Lubawski *et al.*, 2008).

In spite of the discomforting side effects, the number of opioid prescriptions is on the rise in the US. The dispensing of opioid analgesics increased from 149 million prescriptions in the year 2003 to 203 million prescriptions in the year 2013 and over 4% of US adults are taking opioids, primarily for noncancer pain (Nelson *et al.*, 2015). The increase in opioid use is encountered with an increase in opioid induced constipation (OIC) cases in the gastroenterology clinics. 41-81 % of the patients on chronic opioids with noncancer pain report problems of OIC (Holzer, 2008). Figure 1 illustrates the increase in opioid usage and opioid treatment admissions during the years 1999-2010

Conventional treatment strategies for constipation such as use of laxatives, stool softeners and dietary fiber are effective in less than 50 % of OIC cases and are associated with adverse side effects (Nelson *et al.*, 2015). Alternate approaches for the treatment of OIC include targeting the site of action of opioids in the periphery without altering its CNS effects.

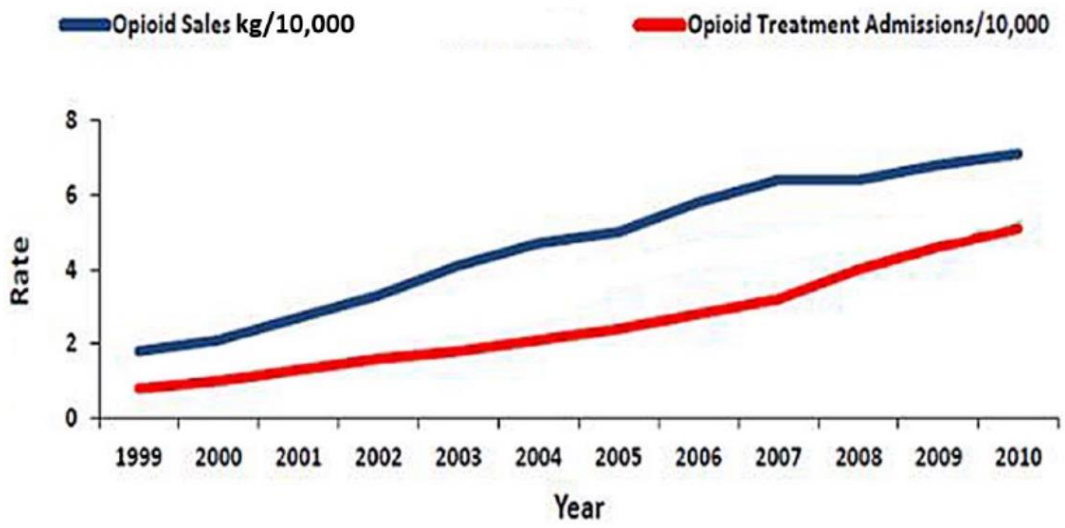

Figure 1: *Rates of opioid sales and opioid abuse treatment admissions during the years 1999-2010. Reproduced from: Ref:http://www.cdc.gov/HomeandRecreationalSafety/pdf/HHS_Prescription_Drug_Abuse_Report_09.2013.pdf*

The analgesic effects of opioids are mediated through the μ-opioid receptors (MOR) in the CNS whereas side effects such as constipation are mediated primarily through the μ-opioid receptors expressed in the periphery. In the periphery, μ-opioid receptors are highly expressed in the GI tract, primarily on the enteric neurons. Opioids decrease the firing of enteric neurons, which leads to

decrease in propulsion and secretion in the GI tract ultimately leading to constipation (Wood *et al.*, 2004).

Peripherally active μ-opioid receptor antagonists (PAMORAs) can reverse the opioid effects in the periphery without altering the analgesic properties. PAMORAs such as methylnaltrexone, alvimopan and nalaxogel induce bowel movements in both OIC and post-operative ileus cases and improves the quality of life of patients on opioids (Holzer, 2008; Nelson *et al.*, 2015). In spite of inducing effective treatment for OIC, long term use of PAMORAs is associated with cardiovascular side effects (McNicol *et al.*, 2008).

Apart from antagonizing opioid receptors, activating other receptor types also help in treating OIC. Activating the $5HT_4$ receptors of enterochromaffin cells and ClC-2 channels of intestinal apical epithelial membrane produce prokinetic effects. Lubiprostone, a ClC-2 channel activator, recently received US Food and Drug Administration (FDA) approval for OIC in noncancer patients. Prucalopride, an agonist at 5HT4 receptor, is currently under clinical trials for OIC (Nelson *et al.*, 2015).

Notwithstanding the prokinetic effects of these drugs, our understanding of the process (es) leading to opioid-induced constipation is still rudimentary. While tolerance develops to many of the effects of opioids following chronic use, opioid-induced constipation does not become tolerant (Galligan *et al.*, 2014). This is largely due to the lack of tolerance development in the colon. One hypothesis is that chronic opioid does not reduce β-arrestin2 levels in the colonic neurons resulting in maintained opioid-induced signaling (Kang *et al.*, 2012; Ross *et al.*,

2008). It is therefore desirable to define targets that can enhance neuronal activity following long-term opioid exposure. The work described in this thesis relates to early findings that chronic morphine induces supersenstivity to nicotine in enteric neurons (Goldstein *et al.*, 1973; Johnson *et al.*, 1978). Since nicotine stimulates gastrointestinal motility, the focus of this study is to determine the mechanism and potential use of nicotinic receptor ligands in opioid-induced constipation.

Nicotinic Acetylcholine receptors (nAChR) are widely expressed in the GI tract, primarily on the enteric neurons (Zhou *et al.*, 2002). Compounds targeting nAChRs are currently in clinical trials for treating GI disorders such as diabetic gastroparesis and irritable bowel syndrome (IBS). (https://www.clinicaltrials.gov/ct2/show/study/NCT01149200).

In the following background section, I provide an overview of the gastrointestinal tract, the role of nicotinic receptors and opioid-induced constipation.

BACKGROUND:

A. Enteric nervous system and GI Motility:

The function of gastrointestinal (GI) tract is governed by central nervous system (CNS) and autonomic nervous system (ANS). ANS includes sympathetic, parasympathetic and enteric nervous system (ENS). The contribution of CNS and ANS differ considerably along the GI tract. The striated muscles of the upper esophagus are innervated directly by the neurons with cell bodies in the medulla oblongata and the smooth muscles of the lower esophagus are innervated by the

enteric neurons of the ENS. In spite of the presence of intrinsic connections, the peristalsis in the esophagus is primarily controlled by the neuronal connections from the CNS. Severing the central connections leads to the complete paralysis of esophagus suggesting a major role played by the CNS. Similarly in the stomach, contractile activity and acid secretion are primarily governed by the CNS through vago-vagal reflexes. However removal of the central connections does not paralyze the stomach suggesting a partial role played by the ENS. In the lower GI tract (small and large intestine), ENS plays an important role in regulating the GI motility, secretion and absorption. Although, CNS has significant innervations in the lower GI tract, severing these connections do not alter the peristaltic movements in the gut. In the lower GI, CNS's control is pronounced primarily near the rectal region where it regulates the voluntary control of defecation. In contrast to CNS, altering the ENS structure or function in the lower GI tract is fatal (Brookes *et al.*, 2013; Furness, 2012; Furness, 2008; Lomax *et al.*, 2010).

The enteric nervous system is composed of two plexii, myenteric and submucosal plexus. While myenteric plexus regulates the motility patterns in the GI tract, mucosal plexus regulate the absorption and secretory functions. The ganglia within each plexus are composed of cell bodies of sensory, motor and interneurons. Based on the type of neurotransmitter released they are classified as excitatory or inhibitory. There are around 30 different neurotransmitters involved in the neurotransmission in the ENS. Acetylcholine and ATP are the major excitatory neurotransmitters whereas NO and VIP contribute to the

majority of inhibitory neurotransmission in the GI tract (Furness, 2012; Grider, 2003). Figure 2 and 3 illustrate the basic organization and localization of enteric nervous system in the GI tract.

The enteric nervous system's role in initiation and propagation of peristalsis in the intestine was first identified by Bayliss and Starling in the small intestine of dog at the end of 19th century (Bayliss *et al.*, 1899). Peristalsis in the wall of intestine is initiated by the stretch receptors expressed on the enterochromaffin cells lining the lumen of intestine. Upon activation of stretch receptors by the luminal contents, enterochromaffin cells release serotonin, which further activates the nerve endings of sensory neurons. Sensory neurons communicate with the neurons in the myenteric ganglia and activate the excitatory motor neurons towards oral side to cause a contraction and inhibitory motor neurons towards anal side to cause a relaxation. The alternate contraction and relaxation in the intestine initiates a peristaltic movement. These movements progress along the wall of intestine by the means of signals sent through interneurons and help in the movement of luminal contents (Bornstein *et al.*, 2004; Grider, 2003; Kunze *et al.*, 1999).

The structure and function of ENS is altered in disease states and by different drugs. In enteric neuropathies such as Hirschsprung's disease, where parts of GI tract have no enteric ganglion cells, the smooth muscle cannot relax and pass the stool. This causes bowel obstruction and leads to complications such as megacolon (De Giorgio *et al.*, 2004). In majority of cases, treatment involves the removal of effected part to prevent death. Similar symptoms are also seen in

Chagas disease, an enteric neuropathy caused due to the infection with protozoan *Typanosoma Cruzi* (da Silveira *et al.*, 2007).

A number of drugs used clinically alter the GI motility through their actions on the enteric neurons. Motilin and erythromycin stimulate the GI motility, in part by activating the cholinergic neurons. Local application of Botulinum toxin reduces the GI motility by inhibiting the release of acetylcholine from the cholinergic neurons. Opioids analgesics induce side effects such as constipation by activating the μ-opioid receptors expressed on enteric neurons. Loperamide, a partial agonist at μ-opioid receptor produces its anti-diarhheal effects through a similar mechanism (Tack, 2000).

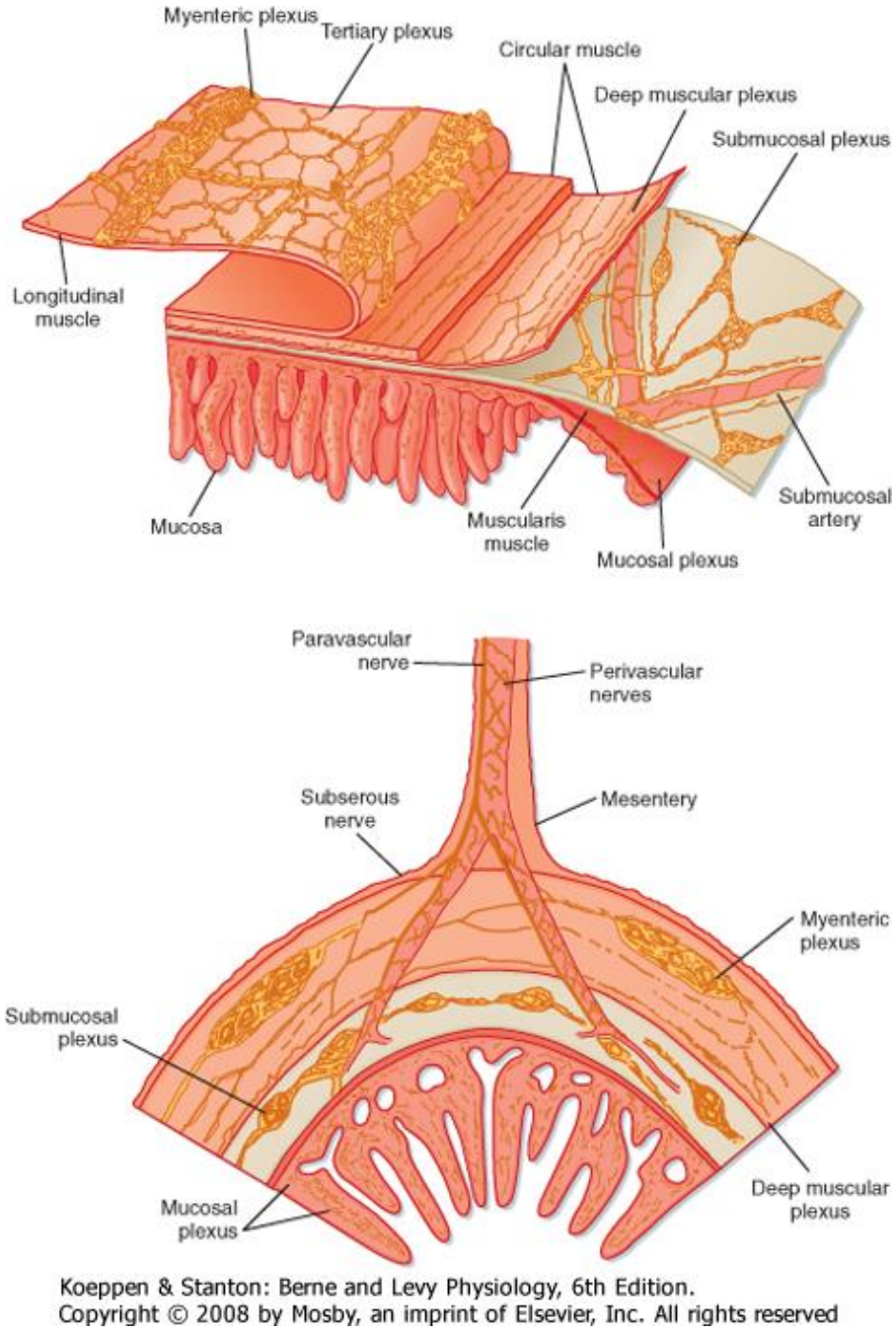

Figure 2: ***Organization of enteric nervous system in the GI tract****. Myenteric plexi are present in between the longitudinal and circular muscle layers whereas the submucosal plexi are localized in the submucosal layer.*

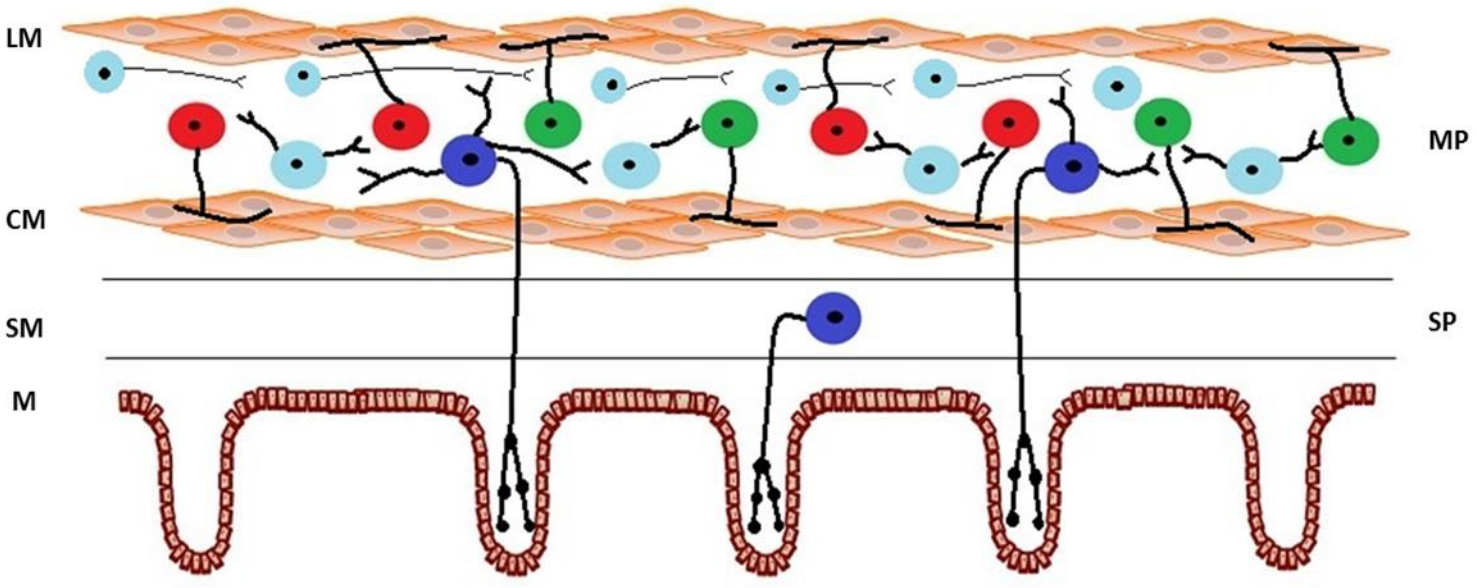

Figure 3: ***A simplified representation of neuronal connections in the Enteric Nervous system.*** *Sensory neurons, Excitatory neurons Inter neurons Inhibitory neurons. LM – Longitudinal Muscle layer, CM – Circular Muscle layer, SM – Submucosa, M – Mucosa, MP – Myenteric Plexus, SP – Submucosal Plexus.*

B. Morphine and GI motility:

Morphine, an opioid analgesic has been used for the treatment of pain for over thousand years. In spite of being a potent analgesic, side effects such as constipation limit its clinical utility. Reports of morphine induced constipation in the literature date back to 1800's. However the mechanism involved was not understood until 1950's, when Paton identified morphine induced suppression of acetylcholine release in an isolated guinea pig ileum preparation (Paton, 1957).

Paton examined the electric field stimulated (EFS) contractions of isolated guinea pig ileum in an organ bath setup. In these experiments, exposure to morphine suppressed the EFS stimulated contractions in the longitudinal muscle of guinea pig ileum (Paton, 1957). In a longitudinal muscle preparation containing the myenteric plexus and longitudinal smooth muscle, EFS stimulates the release of acetylcholine at the neuromuscular junction to induce contractions. Exposure to morphine inhibits the release of acetylcholine resulting in a depression in the EFS induced contractions. In a circular muscle preparation, there is a continuous ongoing release of inhibitory neurotransmitters (VIP and NO) by the inhibitory neurons to maintain the tone of muscle. Application of morphine in such a preparation induces contractions by suppressing the release of inhibitory neurotransmitter (Grider *et al.*, 1987; Wood *et al.*, 2004).

Application of morphine in vivo and in vitro are frequently encountered with decrease in contractile activity and increase in segmental contractions in the intestinal segments leading to suppression of peristaltic propulsion and increase in intraluminal pressure. The decrease in contractile activity is best explained

with the decrease in the acetylcholine release at the neuromuscular junction of longitudinal muscle. Unlike longitudinal muscle, the circular muscle is primarily innervated by the inhibitory neurons and a decrease in the release of inhibitory neurotransmitter release by morphine is therefore expected to induce segmental contractions (Ross *et al.*, 2008) (Grider *et al.*, 1987).

Morphine's actions in the intestine are mediated through the activation of μ-opioid receptors expressed on the enteric neurons. Electrophysiological recordings reveal that morphine acts by hyperpolarizing the membrane potential of enteric neurons through the activation of K^+ channels and inhibition of Na^+ and $Ca2^+$ channels (Akbarali *et al.*, 2014; Smith *et al.*, 2012; Wood *et al.*, 2004). While the hyperpolarization of the myenteric neurons suppresses the neurotransmitter release at the neuromuscular junction, hyperpolarization of submucosal neuron suppresses the secretion in the intestine. Suppression of secretion along with propulsion ultimately leads to constipation and hard stools.

The effects of morphine are not the same after chronic exposure. Chronic exposure to morphine develops tolerance and dependence in the CNS. Similarly in the GI tract, chronic morphine develops tolerance and dependence to the contractile effects in the ileum but not colon. Previous work done by Ross et al demonstrated that, in a circular muscle preparation, morphine induced contractions are depressed after repeated exposures in the ileum but not colon (Ross *et al.*, 2008). This difference is attributed to the differential downregulation of β-arrestin2 in the ileum and colon following morphine exposure. β-arrestin 2 levels in the ileum are decreased following chronic morphine exposure. However

the levels of β-arrestin2 are not altered in the colon. Moreover, in a β-arrestin 2 knock out animal, tolerance to morphine develops in both ileum and colon (Kang *et al.*, 2012). This implies the important role played by β-arrestin 2 in morphine tolerance.

Enhanced contractility in the GI tract, leading to symptoms such as diarrhea is seen in patients during morphine withdrawal. Similar symptoms are also witnessed in animals induced with precipitated withdrawal by injecting naloxone after prolonged exposure to morphine. The gastrointestinal phenotype of morphine dependent animals is attributed to the modification of the intracellular environment of the enteric neurons leading to enhanced phosphorylation of adenylate cyclase (Chakrabarti *et al.*, 1998), alterations in cholinergic signaling (Neugebauer *et al.*, 2013), enhanced sensitivity to neuronal agonists (Goldstein *et al.*, 1973; Johnson *et al.*, 1978) etc. Targeting these altered signaling mechanisms may help in reversing the withdrawal symptoms. For example, targeting the cholinergic signaling by using nicotinic antagonists are known to decrease morphine withdrawal symptoms.

C. nAChRs and GI motility:

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand gated ion channels expressed primarily in the nervous system. They are highly expressed in the GI tract, primarily on the enteric neurons and contribute to the excitatory neurotransmission. They are cation channels and are permeable to Na^{+} and Ca^{2+}. When activated at resting membrane potential, they allow the flow of ions

across the membrane to depolarize the cell. nAChRs play an important role in regulating the inflammation and motility in the GI tract (Wu *et al.*, 2004).

Nicotine, an agonist at the nAChRs alters different functions of the GI tract in normal and disease conditions. Nicotine has both pro and anti-inflammatory effects in the GI tract (Wu *et al.*, 2004). α7 nAChR containing immune cells are expressed in the GI tract and mediate the anti-inflammatory effects of nicotine. Nicotine acts as an anti-inflammatory agent in the treatment of ulcerative colitis and smokers are reportedly at a lower risk to develop ulcerative colitis (UC) (AlSharari *et al.*, 2013) (Wu *et al.*, 2004). However, it also has pro-inflammatory effects in diseases such as Crohn's where it worsens the symptoms (Thomas *et al.*, 2000). This difference in the actions of nicotine is attributed to the different types of immune cells involved in Crohn's and UC (Thomas *et al.*, 2000). Nicotine also protects the gut barrier during burn injuries by acting at the α7 nAChRs expressed on the epithelial cells (Costantini *et al.*, 2012).

nAChR receptor family in the mammalian nervous system consists of 11 subunits: α2 - α9 and β2 – β4 and the subunit composition can be homomeric or heteromeric (McGehee, 1999; Sargent, 1993). The pharmacological and electrophysiological properties are unique for each subunit combination. Pharmacological and immunohistochemistry studies of nAChR subunits in the enteric neurons of neonatal guinea pig have shown the functional expression of α3, α5, β2, β4 subunits (Zhou *et al.*, 2002). However, the specific role of each of these subunits in nAChR mediated responses in the GI tract is not clear. Figure 4 shows the structure and organization of nAChRs in the membrane.

nAChRs are expressed on the sensory, inter and motor neurons of the myenteric ganglia in the ENS and targeting them alters the motility significantly. Local administration of monoclonal antibodies against α3 subunit decreases the motility in the small intestine of mice suggesting the importance of nAChRs of GI tract in regulating the GI motility (Meeusen *et al.*, 2013). Moreover autoantibodies against α3 nAChR have been shown to cause autoimmune motility disorder such as autoimmune gastrointestinal dysmotility (AGID) (Lennon *et al.*, 2003). Nicotine induces contractions in the isolated ileum tissue by activating the nAChRs expressed on enteric neurons of the myenteric ganglia (Fishlock *et al.*, 1966). It stimulates the GI tract to increase the gastric emptying in smokers (Grimes *et al.*, 1978). Similarly, local application of nicotine patches accelerates motility in the large intestine and decreases the total colonic transit time (Rausch *et al.*, 1998).

Although nicotinic receptors are potential targets for accelerating motility, targeting them for the treatment of motility disorders has gained importance only recently. Compounds targeting the nAChRs are currently under clinical trials to increase GI motility in patients suffering with diabetic gastroparesis and irritable bowel syndrome (https://www.clinicaltrials.gov/ct2/show/study/NCT01149200).

nAChRs may serve as targets for treating motility disorders especially opioid induced constipation as the sensitivity of nAChRs agonists in the intestine is enhanced after prolonged exposure to morphine (Johnson *et al.*, 1978). The changes in cholinergic signaling mediated through nAChRs play an important role in morphine withdrawal and therefore are potential targets to treat morphine withdrawal symptoms (Neugebauer *et al.*, 2013). For example, α3β4* nAChR

antagonists can decrease the central and peripheral symptoms seen during morphine withdrawal (Muldoon *et al.*, 2014). Therefore it of interest to examine the alterations in the activity and expression of these receptors after morphine exposure which may provide an insight into the mechanism of action involved in morphine withdrawal symptoms.

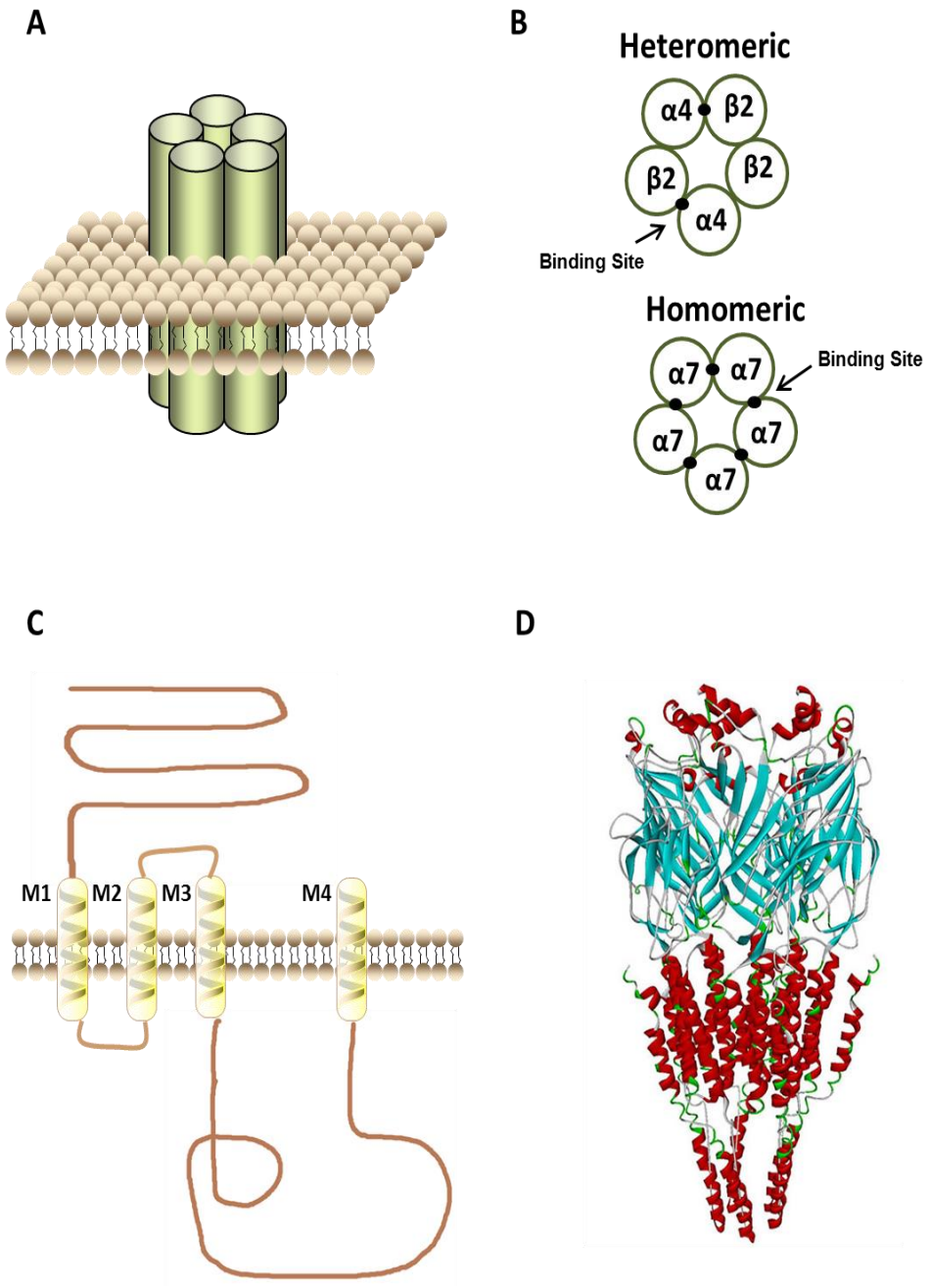

Figure 4: ***Structure and organization of nicotinic acetylcholine receptors*** *A) Nicotinic receptors are comprised of five subunits B) The subunits of nAChR may be homomeric or heteromeric C) Each subunit is made up of four transmembrane domains D) Model structure of α7 containing nAChR from (*http://www.niehs.nih.gov/research/atniehs/labs/ln/icp/index.cfm).

CHAPTER II

MATERIALS AND METHODS:

Materials: 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(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) housed in 12/12 light/dark cycle vivarium were used for the experiments. Mice expressing Tdtomato in cholinergic neurons were developed by crossing B6;129S6-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J (Jax stock no. 007905) X B6;129S6-*Chat*$^{tm2(cre)Lowl}$/J (Jax stock no. 006410). Similarly, mice expressing tdtomato in VIP neurons were developed by crossing STOCK *Vip*$^{tm1(cre)Zjh}$/J (Jax stock no.

010908) X B6;129S6-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J (Jax stock no. 007905). Mice null for the α5 and α7 (Jackson Laboratories), 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. All the 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. Isolated longitudinal-myenteric plexus (LMMP) was then minced and subjected to digestion with collagenase for one hour at 37^{o}C in the water bath. After digestion the tissue was collected using centrifugation and subjected to treatment with trypsin for 7 mins at 37^{o}C in the water bath. After digestion the tissue was mixed with DMEM media containing FBS and antibiotics to neutralize trypsin. Tissue was then collected and washed in the neurobasal media. The washed tissue is passed through a 70 µm sieve to remove any undissipated tissue. The isolated cells were collected through centrifugation 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 the recordings. EPC 10 amplifier (HEKA, Bellmore, NY) was used for recordings. Enteric neurons from Days 1 and 2 after isolation were used for all the patch clamp experiments. Cells were placed in a perfusion chamber 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 micro pipettes (Patch electrodes) 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. Pipettes were 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 giving a series of current injections to the cell in a current clamp mode at resting membrane potential. Nicotine and ATP were perfused into the bath solution. The cells were washed after each nicotine exposure for at least 3 mins before exposing it 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 experiment was performed following the BIO-RAD (Hercules, CA) iTaq Universal SYBR Green One-Step Kit. In order to quantify the mRNA of nAChRs, a standard curve was first plotted using different concentrations of genomic DNA with each primer. The standard curve is 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. Experiments were performed in triplicates from five separate biological samples. 18s rRNA was used as internal control to quantify the normalized fold change. Primers used are listed in table 1.

Gene	Forward Primer	Reverse Primer
α3	5'-CCGCTGTCCATGCTGATGCT-3'	5'-GCCACAGGTTGGTTTCCATG-3'
α4	5'-TGCCGCTCCTGCTGCTCTTA-3'	5'-GCGGACAAGGACCACATCTG-3'
α5	5'-GTTGCCTGAGCTATCCTCTG-3'	5'-GTTGCCTGAGCTATCCTCTG-3'
α6	5'-GACCAGGGAAACCTGCACTC-3'	5'-GATCGGAGACATTCTCCACC-3'
β2	5'-TGCTCCAACTCTATGGCGCT-3'	5'-CACCAGCTCAGAGCCATTAG-3'
β3	5'-CAGGCTTCCTACGGGTCTTC-3'	5'-GGGCGGACACATTTCTGATA-3'

β4	5'-CCCTGCTCCTCGTCTCTCTGT T-3'	5'-TGGAGATGAGCTGGGAGGAG-3'

Table 1: Primers used for the PCR experiments. All the primers are designed using vector NTI®.

Upper gastrointestinal motility by charcoal meal test: Mice were divided into the following groups for the experiment. 1) Control (Placebo-pelleted (4-days)) 2) Acute morphine –treated (Placebo-pelleted + single 10 mg/kg morphine injection) and 3) chronic morphine (75 mg morphine-pelleted (4-days)). Animals were fasted overnight with access only to water and were placed on an elevated mesh to prevent the ingestion of their own feces. On the day of experiment, animals were given charcoal through an oral gavage and were sacrificed thirty mins later and the small intestine was removed and placed on ice. Charcoal was administered immediately after an i.p. injection of saline or nicotine. Charcoal transit was assessed based on the localization of the leading edge of charcoal in the intestine. Small intestinal transit is calculated as a function of whole small intestine length from the pylorus. The following formula was used to calculate small intestinal transit = (Charcoal transit) / (Total length of the small intestine from pylorus to caecum) X 100.

Total fecal pellet output: Fecal pellets were 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 or short term morphine –treated (Placebo-pelleted + single 10 mg/kg morphine injection) and 3) chronic or long term morphine (75 mg morphine-pelleted (4-days)). The nicotine doses were chosen based on the results from small intestine transit assay. Mice in each group were injected with saline or 0.5, 1.0, 1.5 and 3 mg/kg 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 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 GraphPadPrism6 were used for data analysis. All the data were represented in mean ± SEM and scattered plots were used to show the distribution. P value less than 0.05 was considered significant. T-test was used to compare the differences between two different groups. Dose-response to nicotine in charcoal meal test and fecal pellet output assay were analyzed using One-Way ANOVA and fisher's LSD. Dose-response to NS3861 in fecal pellet out assay was analyzed using One-Way ANOVA and tukey kramer post-hoc test. Dose-response to nicotine in patch clamp experiments were analyzed using Two-Way ANOVA and tukey kramer post hoc test. Tests are also mentioned under figure legends. EC50 values were calculated using lease

square linear regression analysis followed by calculation of confidence limits (Bliss, 1967).

Longitudinal Muscle Myenteric Plexus (LMMP) preparation and Immunohistochemistry: Mice were euthanized and the segments of ileum are placed in a dissecting dish containing ice cold PBS. The contents of the intestine were flushed using PBS and the tissue was cut open across the myenteric border using a fine scissors. The sheet of tissue was pinned and stretched on a dissecting plate containing Sylgard. Tissue was then fixed with 4% paraformaldehyde for 4 hrs. After exposure to formaldehyde, tissue was washed three times with 1X PBS and placed under the microscope to strip of the layers of intestine. Using a fine forceps, mucosa, submucosa and circular muscle layers are stripped off and the longitudinal muscle layer is exposed. The isolated LMMP preparation was used for immunostaining or stored in 0.1% sodium azide for future use. Before the immunostaining, tissue was permeabilized with 0.5 % triton x-100 for 1 hr and blocked with 10% normal goat serum for 1 hour. Tissue was then treated with desired primary (Overnight) and secondary antibodies (4 hours) for immunostaining. After the treatment with antibodies, the tissue was transferred onto a glass slide and allowed to dry. A drop of mounting media was placed on the dried tissue and covered off with a cover glass. The cover glass is then sealed off using glue at the corners before visualizing under the microscope.

Immunocytochemistry: Enteric neurons isolated from the mouse ileum and maintained in culture for 4 days were used for immunostaining. Cells were fixed by exposure to 4% paraformaldehyde for half-hour, permeabilized with 0.5 %

triton x-100 for half hour and blocked with 10% normal goat serum for 1 hour. Cells were then treated with desired primary (Overnight) and secondary antibodies (1 hour) for immunostaining. Cells were washed three times with 1X PBS between each step. The antibodies used for immunostaining are listed in table 2.

Antibody (Host)	**Source**	**Concentration**
Primary antibodies		
β-III tubulin (rabbit)	Abcam	1:100
GFAP (mouse)	Chemicon	1:500
Calretinin (goat)	Swant	1:100
Calbindin (mouse)	Swant	1:100
NOS1 (mouse)	Santa Cruz	1:50
MOR (Rabbit)	Alamone Labs	1:500
Secondary antibodies		
Alexa 488 - Conjugate	Life Technologies	1:1000
Alexa 594 - Conjugate	Life Technologies	1:1000

Table 2: Antibodies used for the immunostaining experiments.

CHAPTER III

Effects of morphine on nAChR activity in isolated enteric neurons:

As mentioned earlier, the sensitivities to nicotine and other neuronal agonists are enhanced in the isolated ileum of morphine tolerant guinea pigs (Goldstein *et al.*, 1973; Johnson *et al.*, 1978). The changes in the sensitivities are due to the changes in the cellular functions in the enteric neurons of myenteric ganglia. Fleming et al correlated the super sensitivity of neuronal agonists to the changes in the resting membrane potential of the enteric neurons (Fleming *et al.*, 1988). Microelectrode recordings on the longitudinal muscle myenteric plexus from an isolated ileum of morphine tolerant guinea pig displayed depolarization of enteric neurons (Leedham *et al.*, 1992). These changes were attributed to the alterations in the activity of sodium-potassium pump expressed on the enteric neurons (Kong *et al.*, 1997). However, the effects of morphine on the activity of neuronal receptors are not well understood.

Synaptic transmission in the enteric nervous system is mediated through the G-protein coupled receptors (GPCRs) and ligand gated ion channels. GPCRs mediate the neurotransmission through a slow multi step process that includes the binding of ligand to the receptor followed by activation of intracellular signaling leading to alteration of ion channel function. They primarily mediate the slow excitatory and inhibitory postsynaptic potentials in the enteric nervous system. In the case of ligand gated ion channels, ligand binds directly on the channel complex leading to the alteration of channel activity ultimately leading to the transmission of signal. This usually results in a fast transmission when

compared to GPCRs and therefore ligand gated ion channels mediate the fast excitatory post synaptic potentials in the enteric nervous system.

Nicotinic acetylcholine receptors and P2X receptors are ligand gated ion channels widely expressed in the GI tract and mediate a variety of functions. In the myenteric plexus, they mediate the majority of excitatory neurotransmission. Electrophysiological studies using microelectrodes have shown that by using nicotinic and P2X antagonists, EPSPs (excitatory post synaptic potentials) are blocked in more than 70% of the neurons tested in the myenteric plexus (Nurgali *et al.*, 2003). These receptors are co-expressed and are assumed to be present on the sensory, motor and interneurons of the ganglia and mediate the enteric neurotransmission (Galligan *et al.*, 2004). Moreover these receptors are also known to interact with each other allosterically (Zhou *et al.*, 1998).

ATP and Acetylcholine (ACh), the endogenous ligands at P2X and nAChRs respectively are co-transmitters in the ENS (Decker *et al.*, 2009; Zhou *et al.*, 1998). P2X receptors when activated by ATP allosterically inhibits the ACh induced responses at nAChRs. Moreover, the co-expression of P2X receptors in HEK cells is associated with an inhibition of nAChR activity. This allosteric inhibition is shown to be mediated through the formation of a heteromeric complex between P2X and nAChRs (Decker *et al.*, 2010).

Isolated enteric neuronal culture presents a unique opportunity to study the alterations in the activity of neuronal receptors. In the present study, we examined the effects of nicotine and ATP, agonists at nAChRs and P2X receptors respectively on isolated enteric neurons from LMMP after long term

morphine exposure to get a better insight into the mechanism involved in the super sensitivity.

3A. Characterization of enteric neurons in culture:

The myenteric plexus is a combination of different types of neurons. They can be sensory, inter or motor neurons. Based on the type of neurotransmitter released they can be inhibitory or excitatory neurons. While acetylcholine contributes to the majority of excitatory neurotransmission, VIP and NO contribute to the majority of inhibitory neurotransmission (Kunze *et al.*, 1999). Acetylcholine releasing (cholinergic) and NO releasing (nitrergic) neurons are localized based on the presence of their synthesizing enzymes ChAT (Choline acetyl transferase) and NOS1 (Neuronal Nitric oxide synthetase1) respectively. Whereas the VIP neurons (vipergic) are localized by immunostaining for the neurotransmitter itself. The chemical coding of neurons in the myenteric ganglia has been well characterized in the small intestine of guinea pigs and mice (Sang *et al.*, 1996; Sang *et al.*, 1998). The goal of the present study was to determine if this chemical coding is recapitulated in the isolated enteric neuron culture. In order to test that, we compared the chemical coding of neurons in the culture with that of the whole tissue myenteric plexus using immunostaining techniques.

Results:

Chemical coding of neurons from the enteric neuronal culture:

Previous work from our lab has demonstrated the expression of both neurons and glia in the isolated cells from the LMMP of ileum (Smith *et al.*, 2013). Figure 5 shows the expression of neuronal marker β-III tubulin and glial marker GFAP in the LMMP and isolated neurons from mouse ileum. In order to localize the expression of cholinergic and vipergic neurons in the myenteric ganglia, we used transgenic mice expressing a florescent protein tdtomato in cholinergic or vipergic neurons. The genetic design of these mice was achieved by inserting the sequence for tdtomato in the promotor region of ChAT (a protein expressed exclusively in cholinergic neurons) or VIP using a cre-lox recombination (Genotype of the animals in the methods section). Tdtomato was expressed in the LMMP preparation and isolated enteric neurons from both the transgenic mice, suggesting the expression of ChAT and VIP on the cells in the culture (Fig 6A & B). In order to examine the expression of the nitrergic neurons, we stained the myenteric ganglia with the antibody for NOS1 (nitric oxide synthase 1), the enzyme responsible for synthesis of nitric oxide in the neurons. The immunostaining experiments revealed the expression of nitrergic neurons in both, LMMP and isolated neurons (Fig 7A).

In order to examine the presence of sensory neurons in the isolated culture, we studied the immunoreactivity of the calcium binding protein, Calbindin. Previous reports have demonstrated the expression of calbindin exclusively in the sensory neurons of myenteric plexus (Furness *et al.*, 1988). Immunostaining with the

antibody for calbindin showed the expression in both LMMP as well as isolated neurons, suggesting the presence of sensory neurons in LMMP and culture (Fig 7B).

Similarly, to examine the presence of motor neurons in the isolated culture, we studied the immunoreactivity of the calcium binding protein, Calretnin. Calretnin was previously shown to be expressed exclusively in the motor neurons of the myenteric plexus (Brookes *et al.*, 1991). In the present study we used an antibody against calretnin in the LMMP and isolated neurons. Calretinin stained positive in the LMMP and isolated culture suggesting the expression of motor neurons in the isolated culture (Fig 7C).

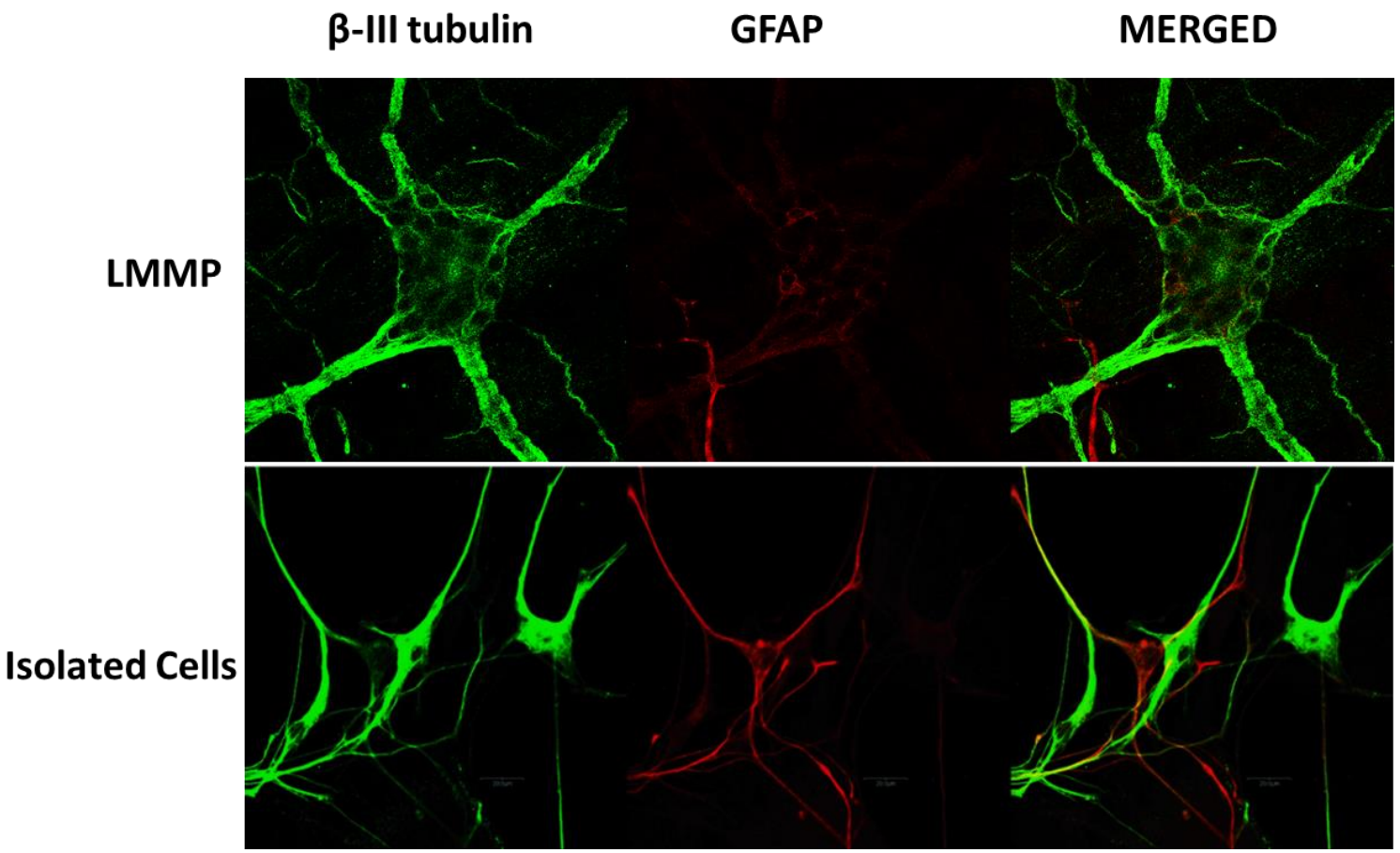

Figure 5: Neurons and Glia in myenteric plexus: *Costaining of neuronal marker β-III tubulin and glial marker GFAP in the LMMP and isolated neurons from mouse ileum. Image from (Smith et al., 2014)*

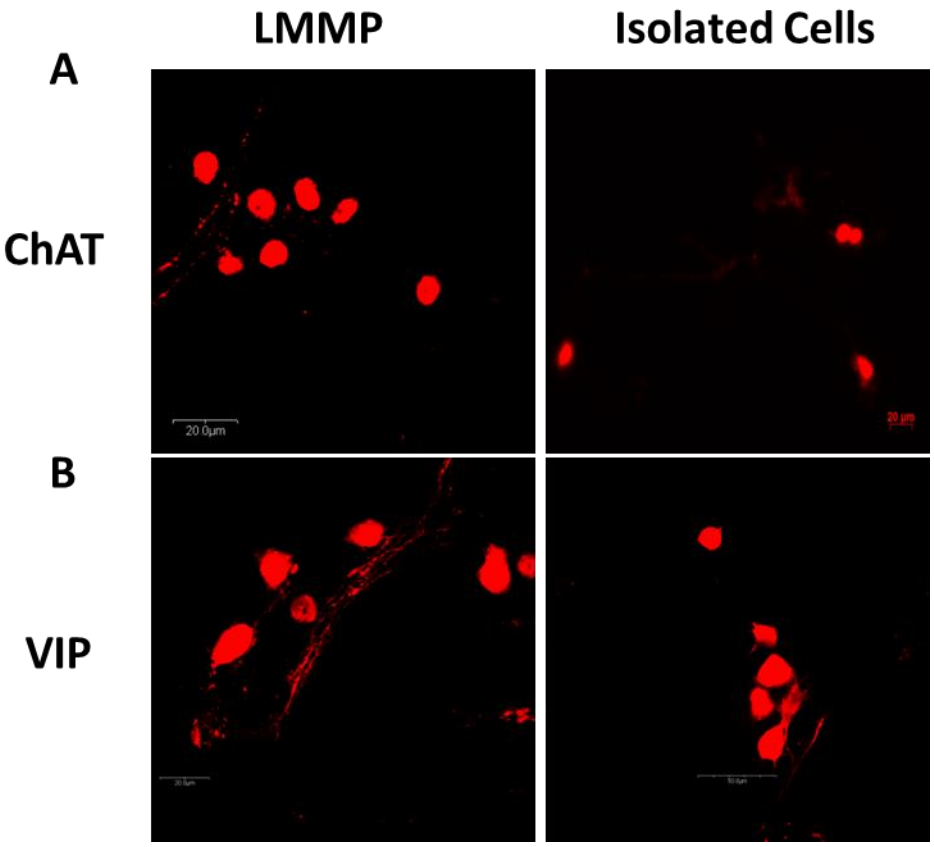

Figure 6: Chemical coding of enteric neurons - 1: *A) ChAT positive and B) VIP positive neurons in the LMMP and isolated neurons from the ileum of mice.*

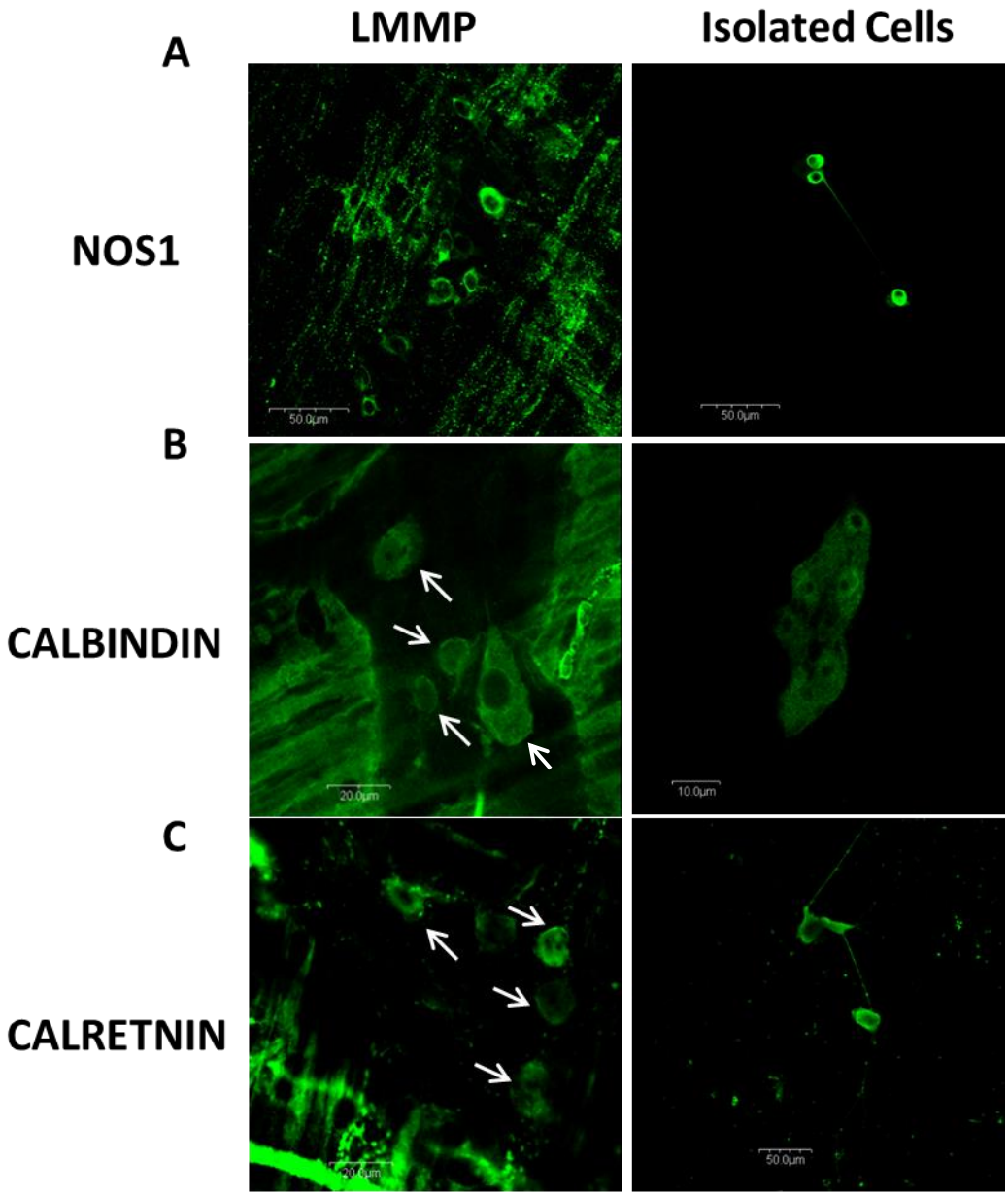

Figure 7: Chemical coding of enteric neurons – 2. *A) NOS1, B) Calbindin and C) Calretinin immunopositive neurons in the LMMP and isolated neurons from the ileum of mice.*

Colocalization of the neurotransmitters in the enteric neurons of myenteric plexus:

Previous reports have shown a high colocalization between the VIP and NOS1 expressing neurons and a low colocalization between NOS1 and ChAT or VIP and ChAT in myenteric ganglia of mice (Sang *et al.*, 1996). In order to compare the co-localization of neurotransmitters in LMMP and isolated cells from ChAT and VIP mice, we stained them with the antibody targeting NOS1. The immunohistochemistry from these experiments revealed the co-localization of subpopulation of nitregic neurons with VIP neurons (Fig 8). However there was no co-localization seen between cholinergic neurons and the nitrergic neurons (Fig 9). The co-localization pattern was similar in the isolated culture suggesting that the phenotype of the neurons remained intact after the isolation.

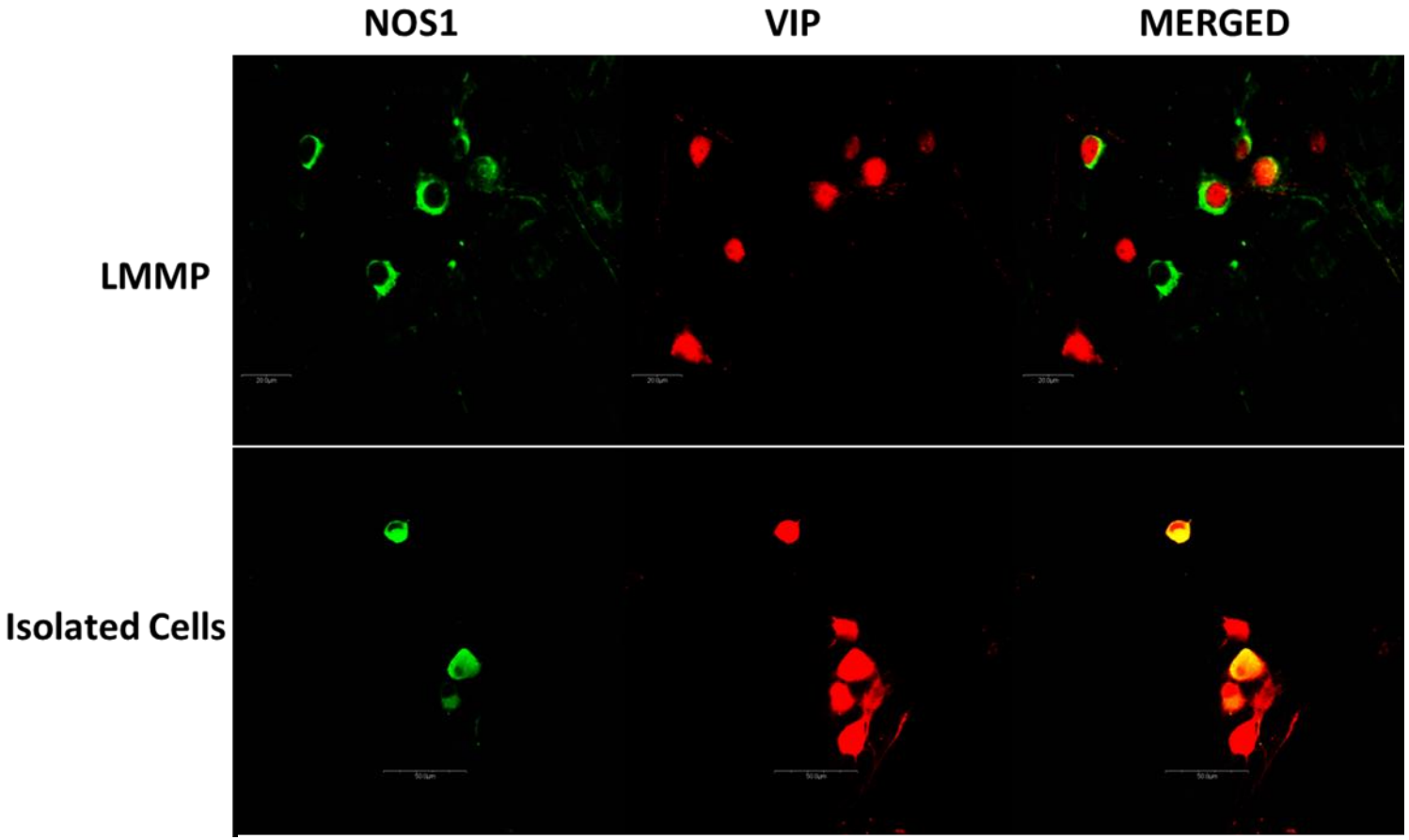

Figure 8:Colocalization of nitrergic and vipergic neurons: *Costaining of NOS1 immunopositive neurons with VIP neurons in the LMMP and isolated cells from the ileum of mice. Yellow immunostaining indicates colocalization.*

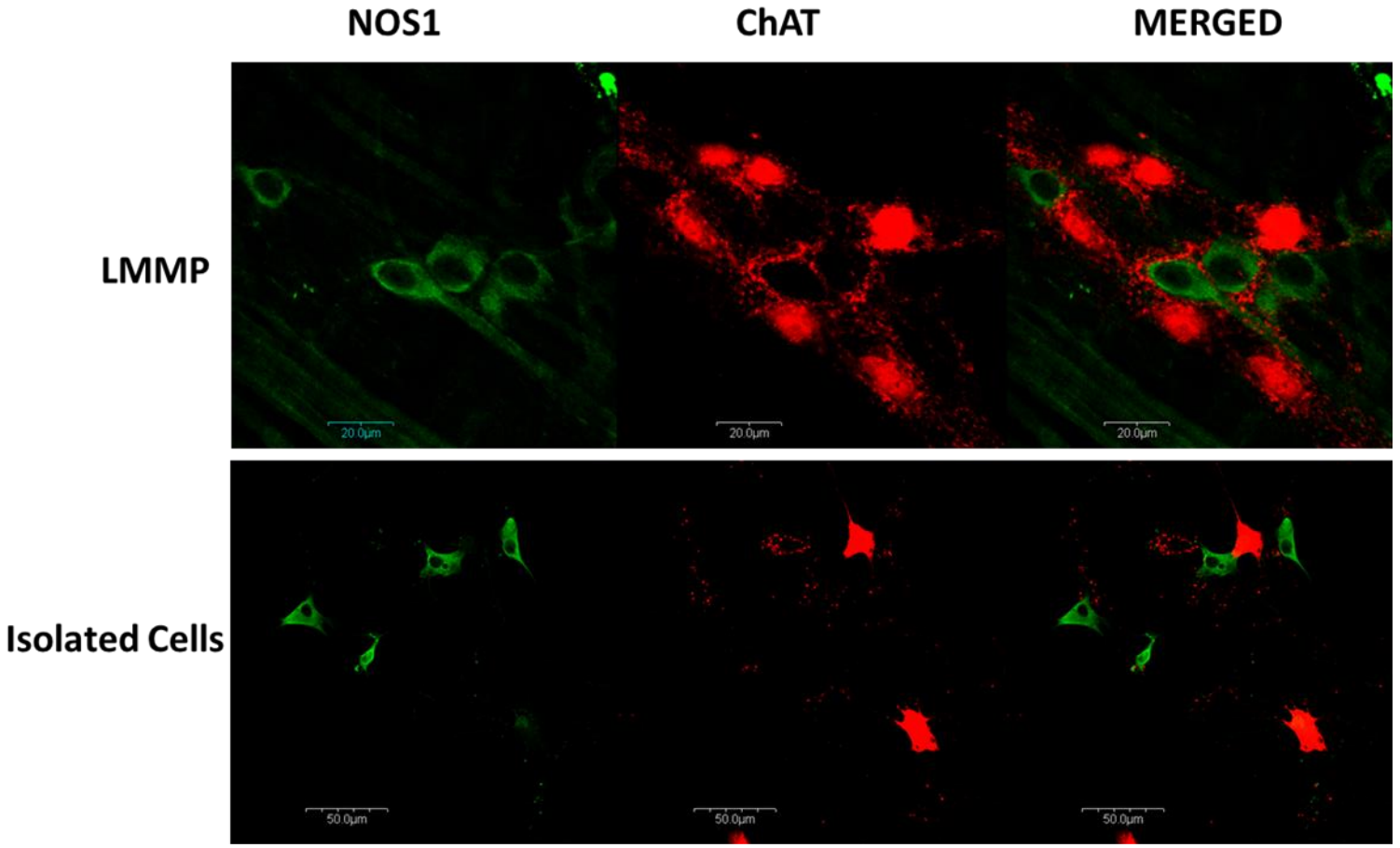

Figure 9: Colocalization of nitrergic and cholinergic neurons*. Costaining of NOS1 immunopositive neurons with ChAT neurons in the LMMP and isolated cells from the ileum of mice. No colocalization seen.*

Localization of μ-opioid receptors on enteric neurons:

Opioids effects in the GI tract are mediated through the activation of the μ-opioid receptors (MOR) expressed on the enteric neurons. However, the specific localization of MORs in the subtypes of neurons is not known. Lack of a specific antibody targeting the MOR has been a major limitation in the field in terms of studying the localization of these receptors. Several studies have reported the non-specificity of the MOR antibodies available in the market (Niwa *et al.*, 2012; Smith *et al.*, 2012). However the antibodies used in these studies were raised against the c-terminal of the MOR. In the present study we performed immunostaining of MOR with the antibody targeting the extracellular 22-38 amino acid epitope of N-terminus of the receptor and examined the localization and chemical coding of MOR expressing neurons at the whole tissue and isolated enteric neuron level.

Figure 9 shows the expression of MOR in subpopulation of neurons in the myenteric ganglia. The antibody also stained positive in the isolated enteric neuron culture.

Co-immunostaining of MOR with NOS1 revealed a low level of colocalization observed between the nitrergic neurons and MOR in both whole tissue as well as isolated enteric neuron level (Fig 11).

Colocalization between MOR and ChAT showed a significant co-localization at both the whole tissue as well as in the isolated cells suggesting a high level of colocalization between these cells (Fig 12).

Colocalization was also examined between MOR and VIP. Images from whole tissue did not show any colocalization. However, the colocalization in the isolated enteric neuron level still needs to be performed (Fig 13).

These data suggest that the MOR receptors are expressed in isolated enteric neurons, primarily in the cholinergic neurons.

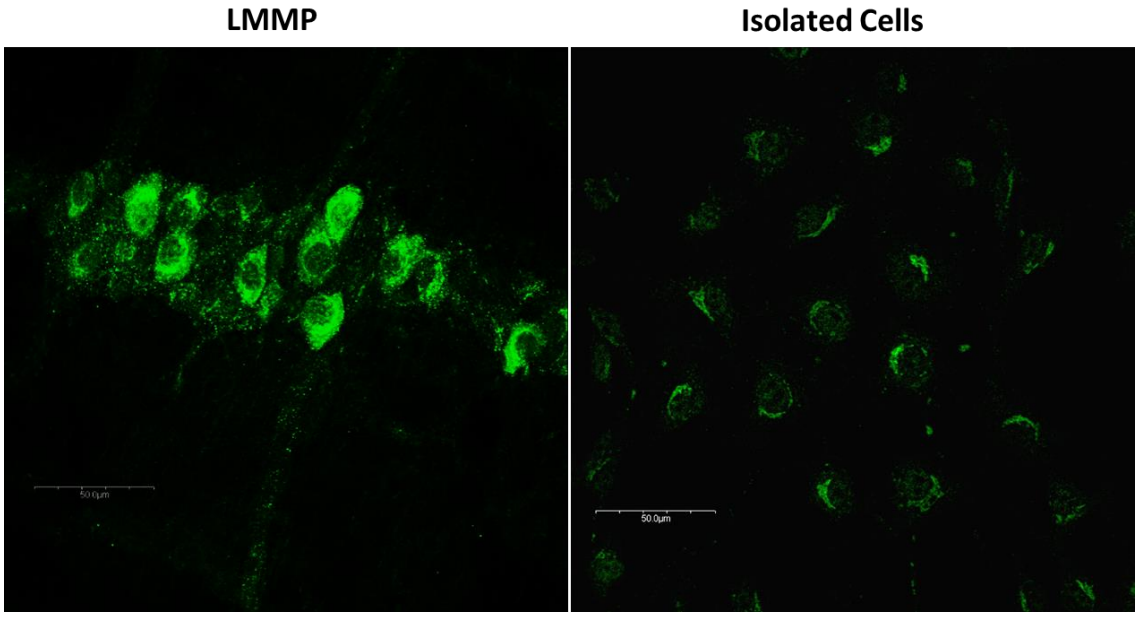

Figure 10: μ-opioid receptors on enteric neurons. *MOR immunopositive neurons in the LMMP and isolated neurons from the ileum of mice.*

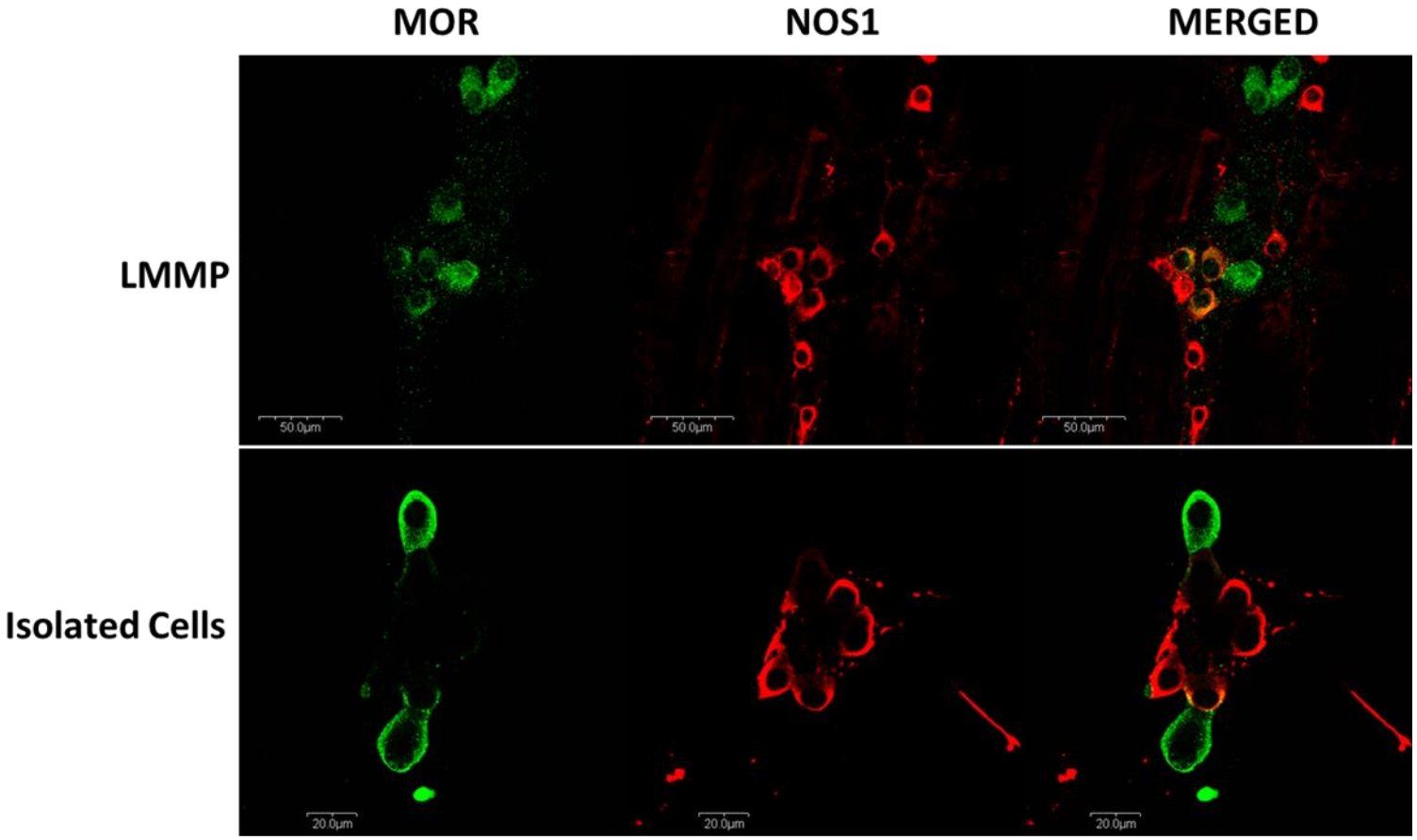

Figure 11: Colocalization of MOR in nitrergic neurons. *Costaining of MOR immunopositive neurons with NOS1 immunopositive neurons in the LMMP and isolated cells from the ileum of mice. Yellow immunostaining indicates colocalization.*

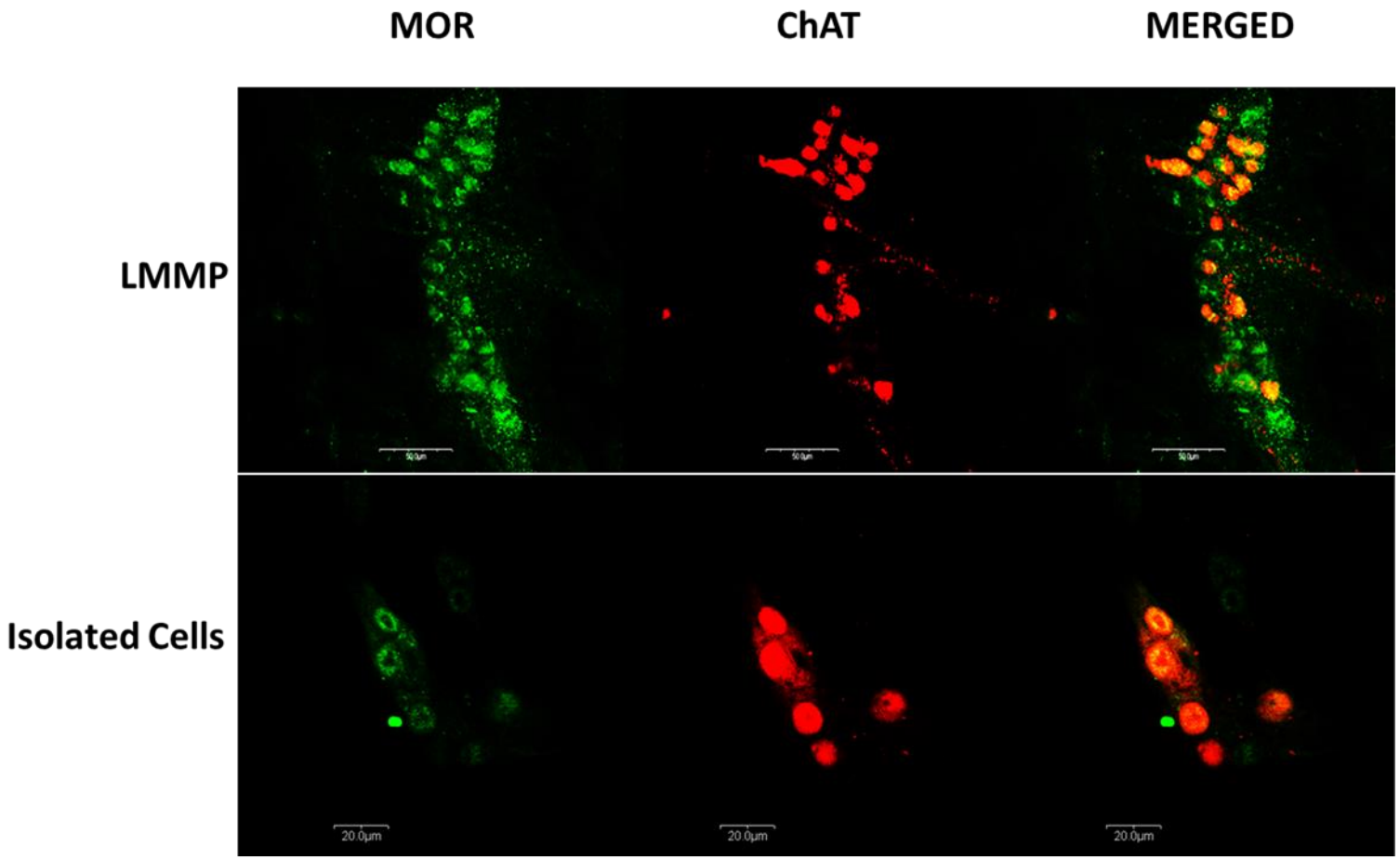

Figure 12: Colocalization of MOR in cholinergic neurons: *Costaining of MOR immunopositive neurons with ChAT +ve neurons in the LMMP and isolated cells from the ileum of mice. Yellow immunostaining indicates colocalization.*

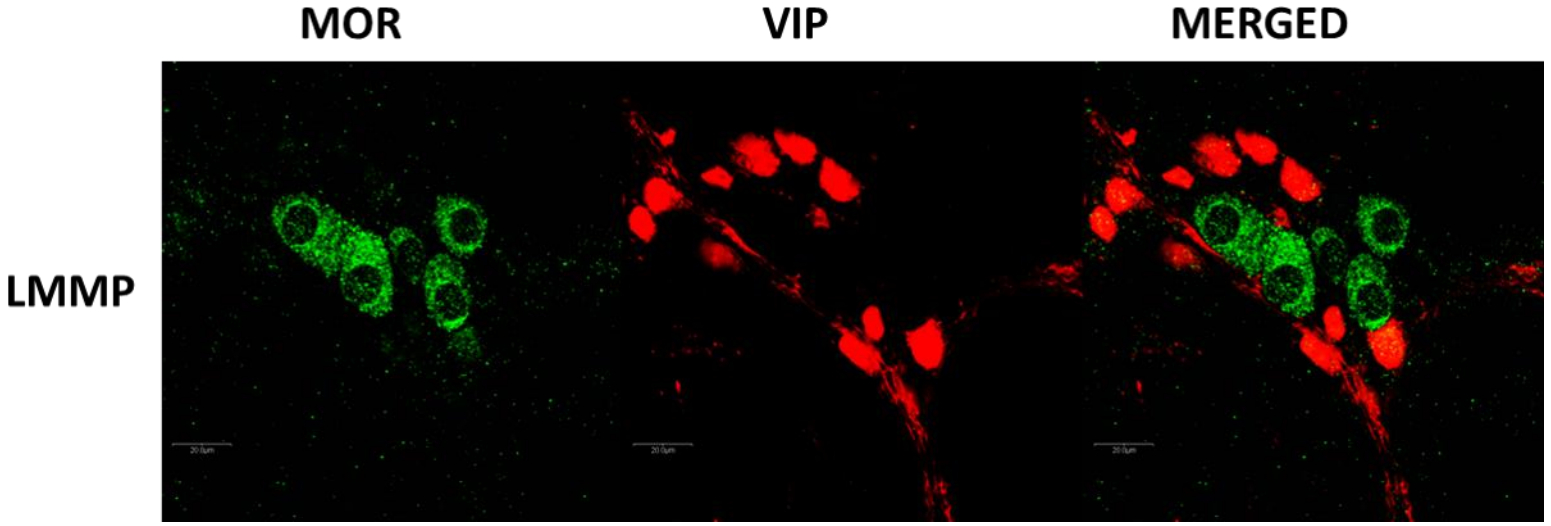

Figure 13: Colocalization of MOR in vipergic neurons. *Costaining of MOR immunopositive neurons with VIP neurons in the LMMP from the ileum of mice. No colocalization seen.*

Electrophysiological properties of Isolated Enteric neurons:

Isolated enteric neurons provide a unique opportunity to study the electrophysiological properties and alterations in the membrane receptor function. Previous reports from our lab have shown that the primary enteric neurons isolated from the myenteric plexus are composed of a mixture of neurons and glia (Smith *et al.*, 2013; Smith *et al.*, 2014). We used patch-clamp technique to study the electrophysiological properties of the neurons in the culture. Neurons in the culture were identified based on their ability to elicit action potentials in a current-clamp mode. Cells exhibiting action potentials also displayed inward currents when depolarized in voltage-clamp mode. Action potentials and inward currents were absent in non-neuronal cells which are presumably glia (Fig 14).

Enteric neurons isolated from the transgenic ChAT and VIP mice will help in understanding the properties of the cholinergic and VIPergic neurons in the culture. ChAT neurons and VIP neurons are identified under the microscope based on the expression of florescent protein, tdtomato. Cells expressing tdtomato from enteric neuronal culture of ChAT and VIP mice elicit action potentials and inward currents similar to neurons from the control swiss webster mice suggesting that they are neurons (Fig 15).

We performed a preliminary study to compare the electrophysiological properties of ChAT and VIP neurons. The resting membrane potentials were not significantly different among the ChAT +ve and ChAT –ve neurons. The resting membrane potential of ChAT +ve and ChAT –ve neurons is -49.4 ± 2.6 mV (n=5)

and -46 ± 1.7 mV (n=5) respectively (Fig 16A). However the rheobase, the minimum amount of current required for eliciting an action potential were significantly higher in ChAT +ve neurons. The rheobase in ChAT +ve and ChAT –ve neurons are 23 ± 5.8 and 3 ± 1.2 pA respectively (Fig 16B). This suggests that the excitability of cholinergic neurons is lower compared to the other neurons in the culture. Only three VIP +ve neurons were tested which didn't show any significant difference with the ChAT +ve neurons in terms of resting membrane potential and rheobase. However, the data from VIP -ve neurons, which serve as controls, has not been obtained yet.

Overall, this preliminary study gave us an insight into the distinct electrophysiological properties of cholinergic and VIP neurons in the enteric nervous system. Future studies examining more biophysical and pharmacological properties such as function of ion channels and actions of different drugs will help in understanding the role of these neurons in executing different functions of the GI tract.

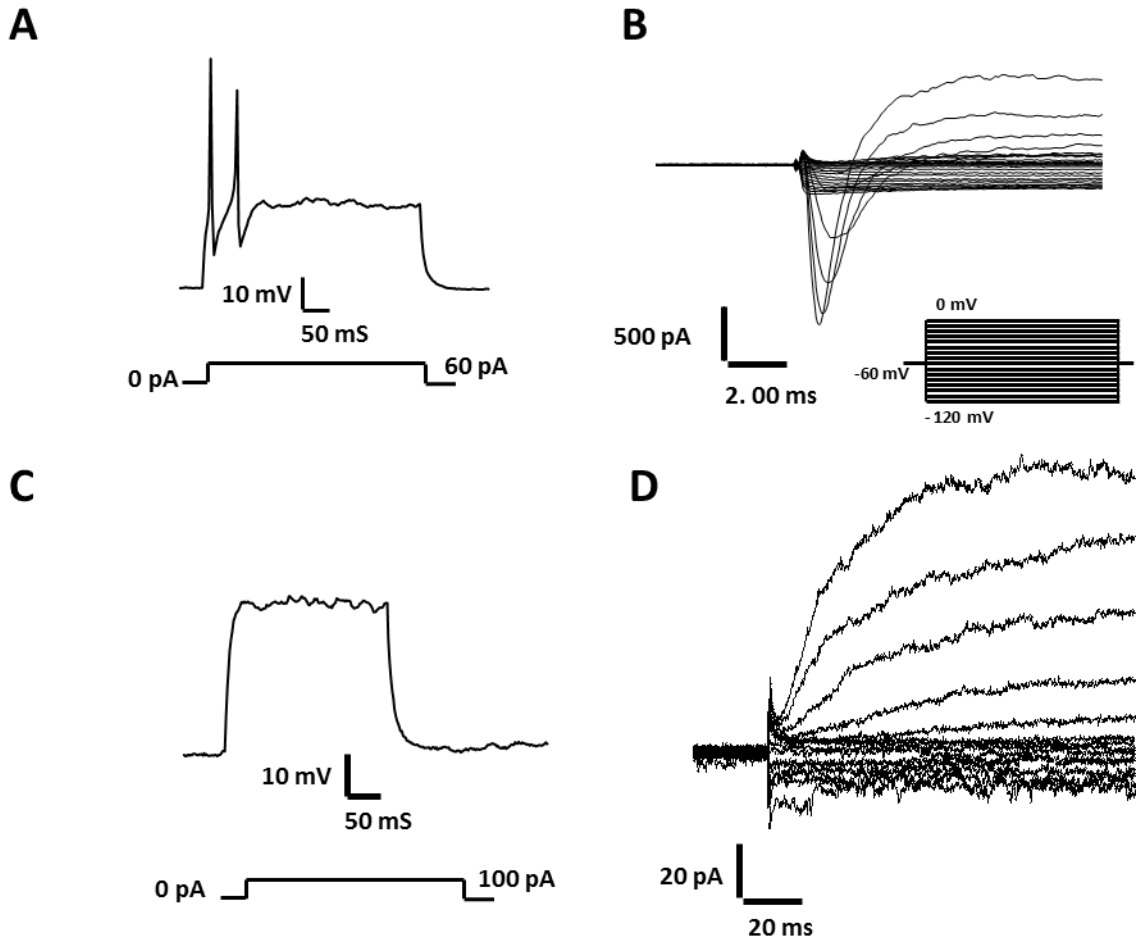

Figure 14: Electrophysiology of enteric neurons: *A) Current clamp recording from a neuron showing an action potential with a current stimulus of 60 pA C) Current clamp recording from a glial cell showing no action potential with a current stimulus of 100 pA. B) Whole-Cell voltage clamp recordings from neurons showing inward sodium current induced upon giving a series of voltage steps from -120 mV to 0 mV. D) Whole-cell voltage clamp recording from a glial cell without any inward currents.*

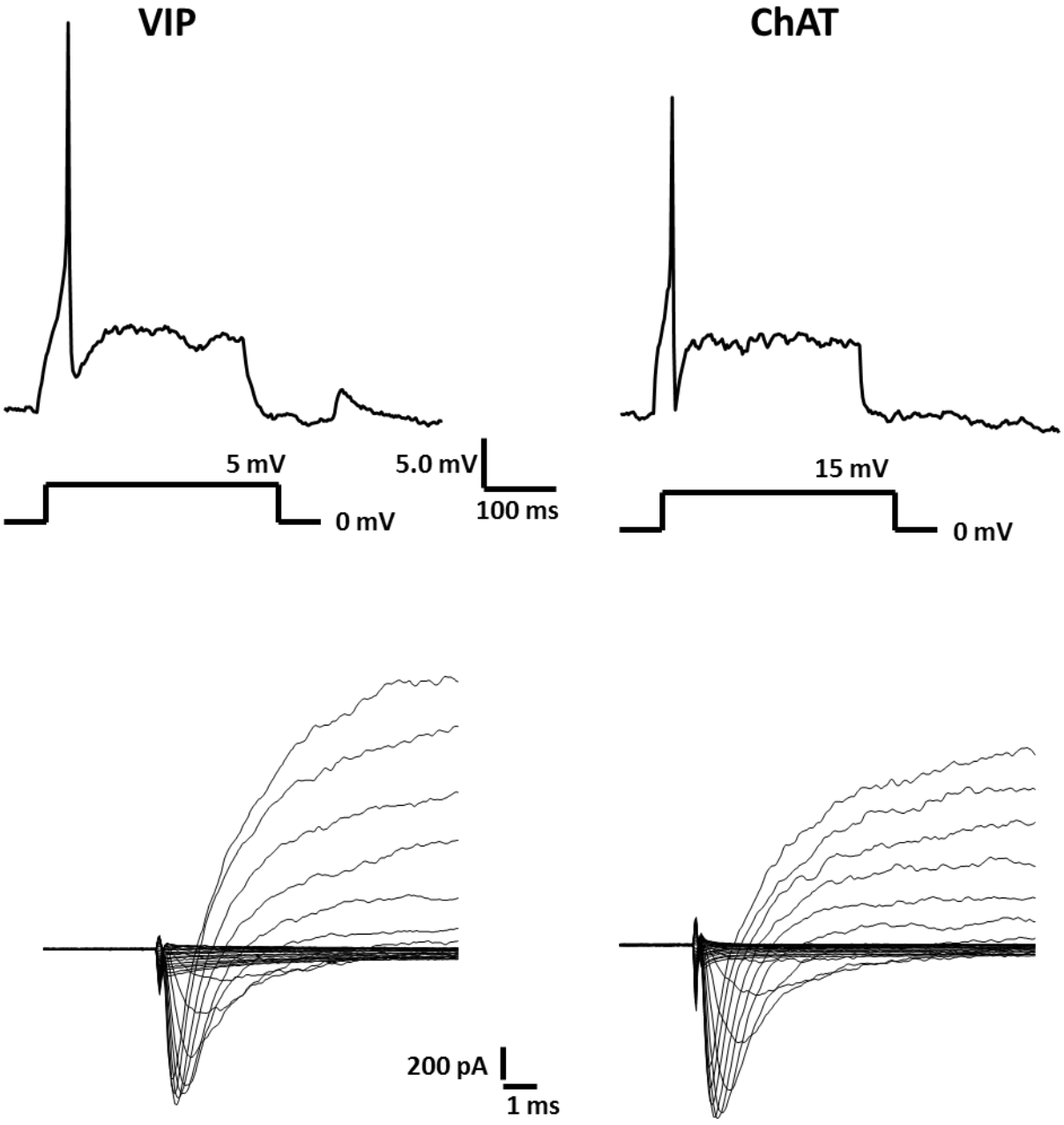

Figure 15: Electrophysiology of cholinergic and vipergic neurons: *Electrophysiological properties of VIP (n=3) and ChAT (n=5) neurons.*

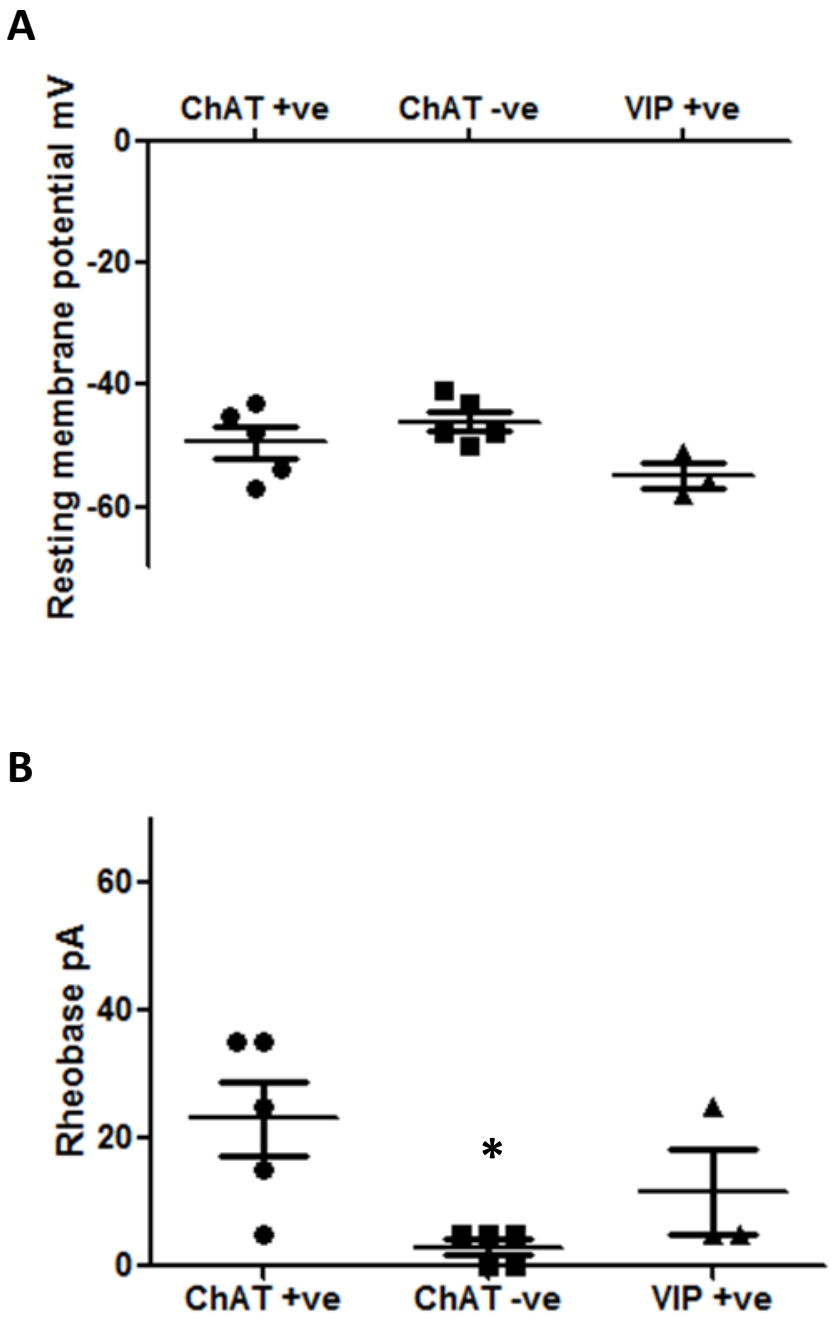

Figure 16: Excitability properties of cholinergic and vipergic neurons: *A) Resting membrane potentials of neurons from ChAT +ve, ChAT –ve and VIP +ve neurons B) Rheobase of ChAT +ve, ChAT –ve and VIP +ve neurons.*

Summary 3A:

This study examined the chemical coding and electrophysiological properties of isolated enteric neurons in culture. The important findings from this study are as follows.

1) Isolated enteric neurons are a mixture of excitatory and inhibitory neurons
2) The chemical coding of isolated enteric neurons are representative of the neurons at the whole tissue level.
3) Isolated enteric neurons in culture can be identified based on the action potential elicited in patch clamp mode.
4) μ-opioid receptors are primarily expressed in the cholinergic neurons.
5) The electrophysiological properties of cholinergic neurons are distinct from the non-cholinergic neurons in the culture.

3B. Effects of nicotine and ATP on isolated enteric neurons:

Results:

Long term exposure to morphine enhances nicotine induced excitability:

Previous reports have suggested that the enhanced nicotine induced contractions in long term morphine treated guinea pigs may be mediated due to depolarization of enteric neurons (Kong *et al.*, 1997; Leedham *et al.*, 1992). We have previously shown that overnight treatment with morphine (3 µM) results in tolerance/dependence which can be functionally examined as enhanced excitability upon precipitated withdrawal similar to neurons treated with morphine overnight (Smith *et al.*, 2014).

In the present study we examined the effects of nicotine on resting membrane potential, depolarization and excitability properties in the enteric neurons after overnight exposure to morphine (16-20 hrs) (3 µM). The resting membrane potential and the effects of nicotine were assessed on these cells in a current clamp mode. In contrast to the earlier findings, the resting membrane potentials were not significantly different in enteric neurons treated with long term morphine. The 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 µM) did not significantly affect the resting potential -48.3 ± 1 mV; (n=42) (Fig 17A). 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 17B nicotine at 1 µM slightly depolarized control

neurons by 2.2 ± 0.3 mV (n=5), however with long-term morphine, the depolarization (3.8 ± 1.2 mV (n=8)) resulted in spontaneous action potentials in three out of eight cells while action potentials were not generated in cells not treated with morphine upon application of 1 μM nicotine. Furthermore, the amplitude of depolarization induced by 3 μM nicotine increased significantly in morphine treated neurons. At 3 μM nicotine, the depolarization in control cells was 9.7 ± 1.2 mV (n=5) and 16.1 ± 2.2 mV (n=8) in long term morphine treated cells (Fig 17C). These data suggested that the cells were significantly more sensitive to nicotine following long-term morphine treatment.

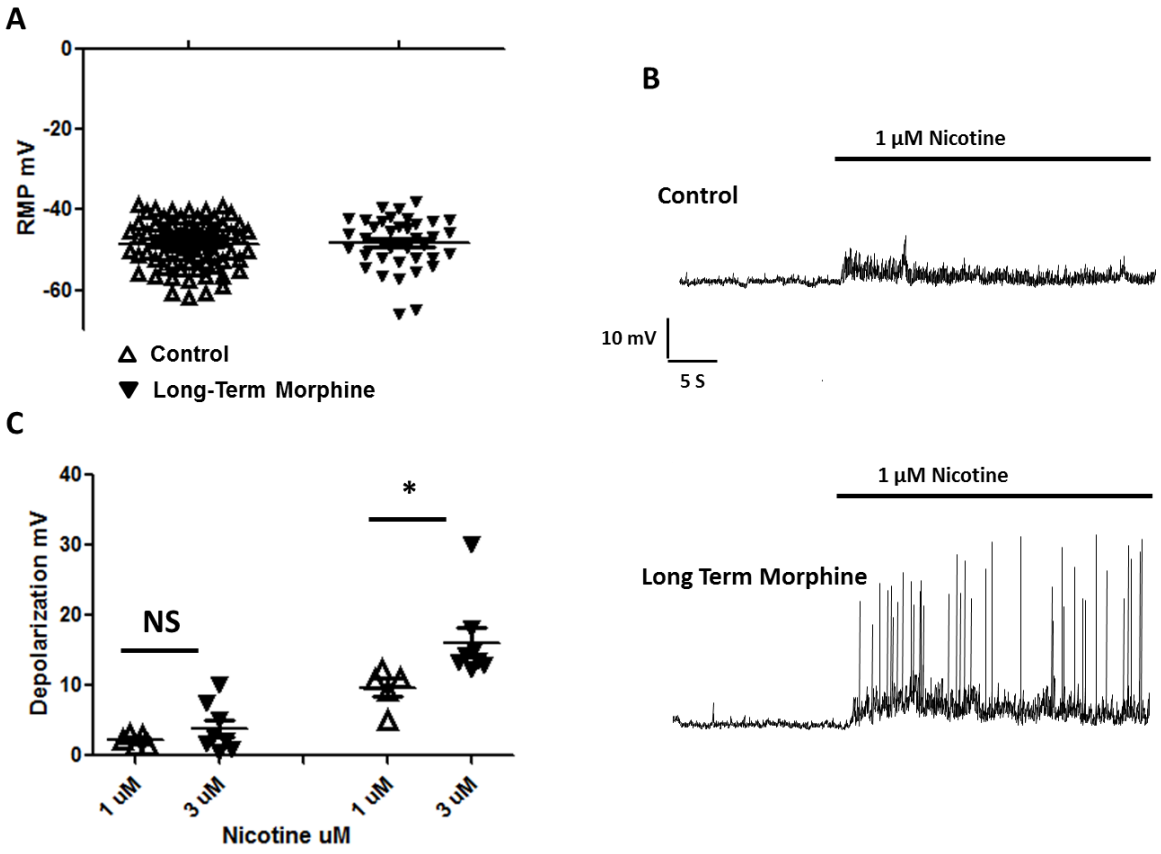

Figure 17: Long term exposure to morphine enhanced the nicotine induced excitability: *A) Resting membrane potentials of neurons from control (n=73) and long term morphine group (n=42) in a current clamp mode. B) Raw traces showing the depolarization induced by the 1 µM nicotine in control and after 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*

Long term but not short term exposure to morphine enhances nicotine induced currents:

Enhancement in nicotine induced excitability may be mediated through an increase in the nAChR mediated currents. To study the short term and long term effects of morphine on nAChRs mediated currents, enteric neurons were treated with 3 µM morphine for a period of 10 mins in the bath or over-night for a period of 16-20 hours in culture. nAChR activity was assessed on these cells using nicotine as an agonist in a whole-cell voltage clamp mode. Cells were held at -60 mV and the peak amplitude of inward currents were plotted against the nicotine concentration (Fig 18). The dose-response curve displayed an increased nAChR activity in cells from prolonged exposure but not in the short exposure group when compared to the control drug naïve group. 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 19A). The amplitude of maximal currents induced by a single individual exposure to 1 mM nicotine was 139.4 ± 34.3 pA/pF in control and 228 ± 14.7 pA/pF after long-term exposure to morphine (Fig 19B). Though there was an increase in the amplitude of nicotine induced currents at high concentrations there was no difference seen in the EC 50 values among all the three groups suggesting a shift in the efficacy but not potency of nicotine post prolonged exposure to morphine.

In order to further check if these effects of morphine are mediated through the µ-opioid receptors, we used naloxone, an antagonist at MOR. 1 µM of naloxone was added to the cells 30 mins prior to adding morphine and left overnight (16-20

hrs) in the culture. Cells treated with 1 μM Naloxone alone overnight were used as controls. The currents induced by 1 mM nicotine were not significantly changed in the cells treated with naloxone and morphine when compared to the controls. This suggested that the effects of morphine are mediated through the activation of μ-opioid receptors.

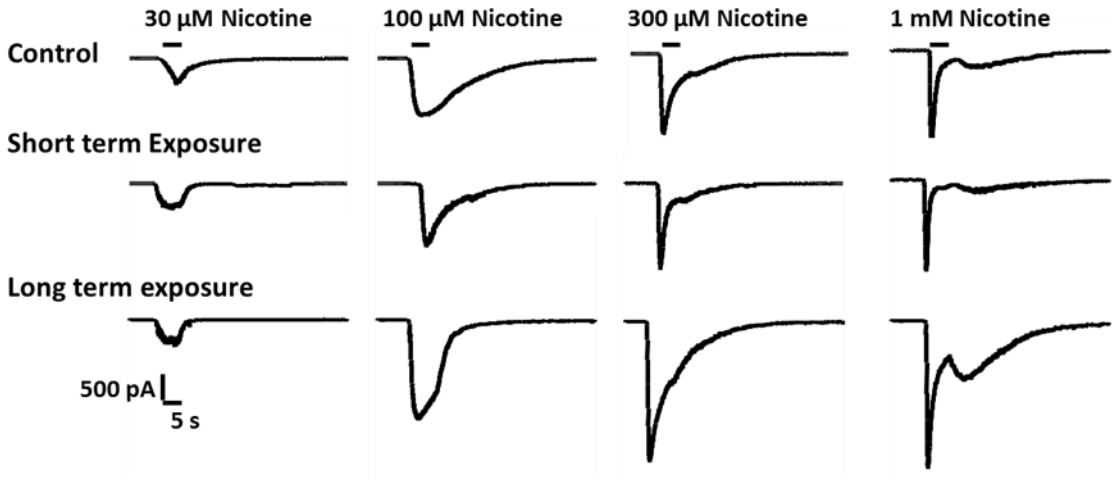

Figure 18: Short term but not long term exposure to morphine enhances nicotine induced currents - 1*: Rawtraces of recording from neurons with different concentrations of nicotine in control drug naïve, short term morphine treated and long term morphine treated enteric neurons.*

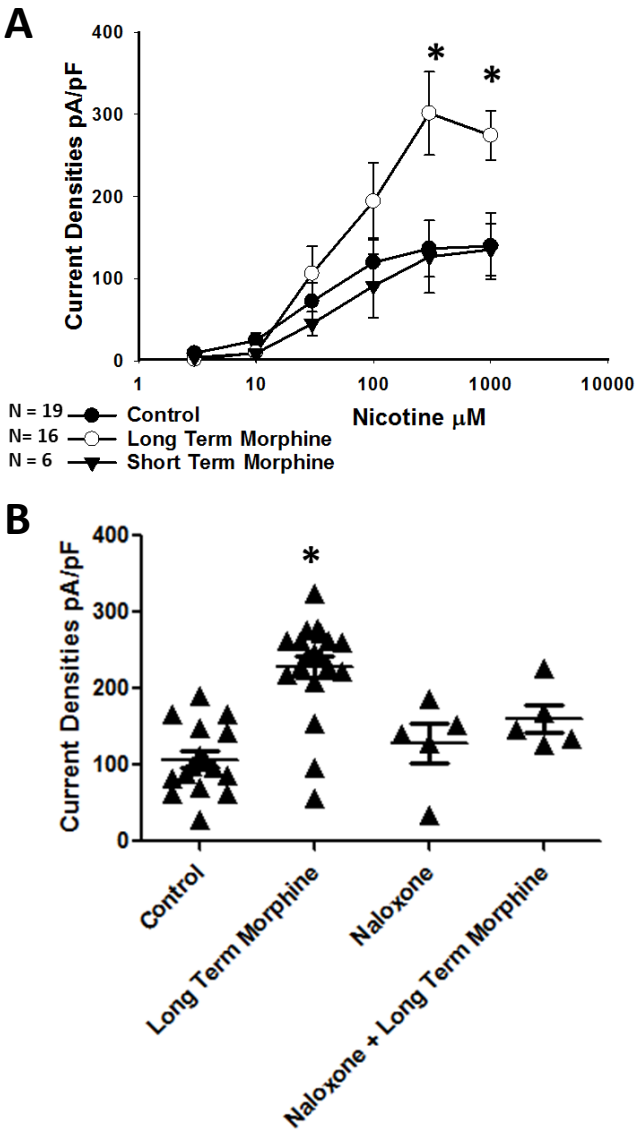

Figure 19: Short term but not long term exposure to morphine enhanced nicotine induced currents - 2 *A) Concentration-response curves of peak currents at different concentrations of nicotine. Two-Way ANOVA. Tukey kramer Post Hoc test. *P < 0.05 B) Scattered plot showing the distribution of data of currents 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). One-Way ANOVA. Tukey kramer Post Hoc test. *P < 0.05*

Long term exposure to morphine did not alter ATP induced current:

The activity of nAChRs is dependent on their interaction with the P2X receptors. P2X receptors when activated are known to alter the acetylcholine induced currents in the enteric neurons. Decrease in membrane expression of P2X receptors increases the nAChR activity and vice versa (Decker *et al.*, 2010; Zhou *et al.*, 1998).

In order to test if the increase in efficacy of nicotine is mediated through the change in P2X receptor activity, we examined the effects of long term morphine exposure on P2X receptor mediated currents. 1mM ATP, an agonist at P2X receptor induced inward current at a holding potential of -60 mV (Fig 20A). ATP induced currents were examined in cells exposed to long term morphine. There were 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 20B).

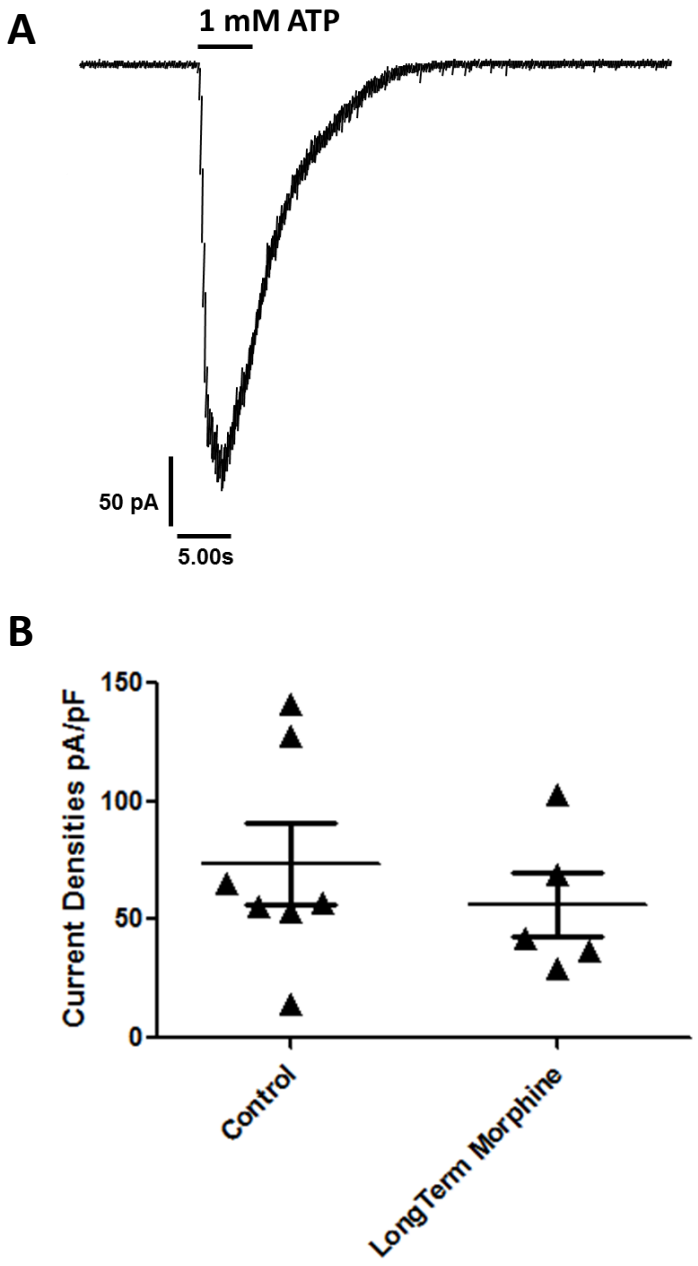

Figure 20: Long term exposure to morphine did not alter ATP induced current*: A) Raw trace displaying the inward current induced by 1 mM concentration of ATP. B) Amplitude of current induced by 1 mM ATP in control (n=7) and long term morphine treated cells (n=5). Student's t-test. *P < 0.05*

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 at higher doses in cells treated with long term morphine. 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 21A). The amplitudes of currents induced by a single exposure to 1 mM nicotine were 148 ± 28.5 pA/pF in control and 222 ± 24.4 pA/pF in long term morphine treated cells respectively (Fig 21B). This suggested that the increase in efficacy of nicotine following long term exposure to morphine is not associated with or mediated through the morphine dependence.

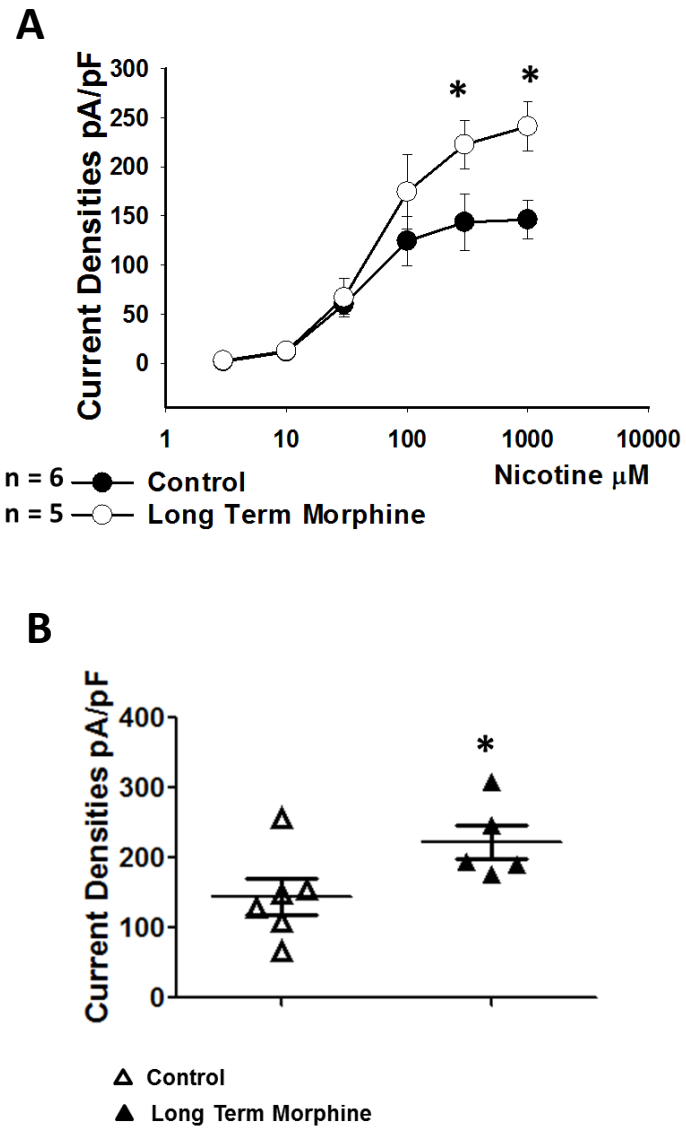

Figure 21: Nicotine induced currents in neurons isolated from mouse colon*: A) Concentration response curves of nicotine induced currents in neurons isolated from mouse colon. B) Scattered plot showing the distribution of data peak inward currents induced by 1 mM Nicotine in control (n=6) and long term morphine treated cells (n=5). Two-Way ANOVA. Tukey kramer Post Hoc test. *P < 0.05*

Summary 3B:

This study examined the effects of morphine on nAChR and P2X receptor activity. The important findings are as follows.

1) Long term exposure to morphine does not alter the resting membrane potential of the isolated enteric neurons.
2) Long term but not short term exposure to morphine enhances the nicotine induced currents in the enteric neurons.
3) Long term exposure to morphine does not alter the ATP induced currents in the enteric neurons.
4) Increase in nicotine induced currents is not associated with morphine dependence.

CHAPTER IV

Effects of nicotine on opioid-induced constipation in mice

Nicotine, the active ingredient of cigarette smoke alters the GI motility in different ways. Short-term application of nicotine through transdermal nicotine patches decreases the total colonic transit time (TCTT) suggesting an increase in the motility in healthy volunteers. However the reverse is seen after long term application (Rausch *et al.*, 1998). The possible explanation is due to the desensitization of the receptors. Similarly the total transit time after smoking in smokers and non-smokers is significantly high suggesting a decrease in the motility (Meier *et al.*, 1995). However, the role of nicotine in these effects is not clear as cigarette smoke contains around hundred different constituents. Effects of cigarette smoking on GI motility are in contrast to the personal experiences reported by smokers that bowel movements are induced by smoking a cigarette in the morning.

Nicotine is an agonist at the nicotinic acetylcholine receptors (nAChRs). Activation of the receptor following application of nicotine leads to the depolarization of cell membrane. In neurons, this leads to an increase in excitability and enhances the release of neurotransmitter. In a longitudinal muscle preparation of small intestinal segment, nicotine induces contractions through the activation of the excitatory neurons of myenteric ganglia. In a circular muscle preparation, it causes a relaxation through the activation of the inhibitory

neurons (Fishlock *et al.*, 1966; Romano, 1981). Additionally, it also increases the amplitude of the rhythmic contractions of the intestinal muscle and makes them more regular (Alvarez, 1936).

Although nicotine was proven to have prokinetic effects in the GI tract, it has not been used or tested for the treatment of motility disorders. Opioid induced constipation is a commonly encountered motility disorder in the clinics. It is associated with a decreased contractility and secretion in the intestine. As mentioned earlier nicotine at lower doses increases the contractility in the intestine and moreover the sensitivity to nicotine is higher in intestines of opioid treated animals. Therefore the efficacy of nicotine in inducing motility might be higher in the patients who are on opioids. In the present study, we tested the effects of nicotine on acute and chronic morphine induced constipation in mice.

Results:

Effects of nicotine on small intestinal transit in morphine treated mice:

Previous reports from our lab have shown that short term and long term exposure to morphine significantly decrease the small intestinal transit in mice. To determine the effects of nicotine on opioid-induced decrease in motility, small intestine transit was measured in mice treated with acute morphine or chronically implanted with morphine pellet. In placebo pelleted mice (control), the distance traversed by charcoal gavage was 74.7 ± 7 % of the length of the intestine at 30 mins post-gavage. A single acute injection (i.p.) of nicotine did not significantly affect the transit at 0.175, 0.350 and 0.525 mg/kg, however it significantly

decreased the transit at the highest dose (1.05 mg/kg) to 49.3 ± 4 %. A single subcutaneous injection of 10 mg/kg morphine in placebo pelleted animals significantly decreased the distance for the leading edge of charcoal to 24.9 ± 2.9 % of the total intestine. Nicotine did not affect the distance traveled at any of the doses tested (0.175, 0.350, 0.525 mg/kg) in the acute morphine treated mice. In mice pelleted with 75 mg morphine for a period of four days, small intestinal transit was 31.5 ± 2.2 % of the total length. There was a significant increase with 0.525 mg/kg nicotine and a trend towards reversing small intestinal transit at lower doses in this group (Fig 22). However, there was no significant increase after administration of a higher dose of nicotine 1.050 mg/kg.

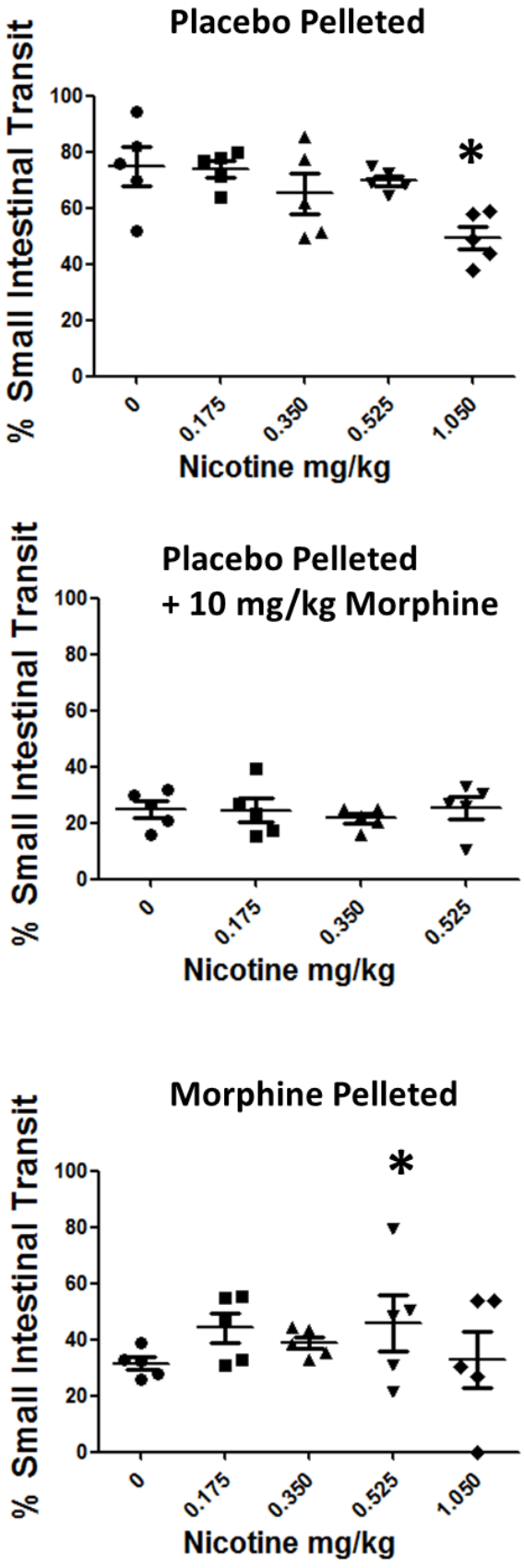

Figure 22: Effects of nicotine on small intestinal transit*: Scatter plot displaying the % distance moved by charcoal after a single I.P injection of saline or different concentrations of nicotine in Placebo pelleted mice, Placebo pelleted mice receiving 10 mg/kg morphine and Morphine Pelleted mice (N = 5 in each group) One-Way ANOVA. Fisher's LSD. *P < 0.05*

Effects of nicotine on whole gastrointestinal transit in morphine treated mice:

Previous reports from our lab have shown that morphine decreases the fecal output in mice (Fitting *et al.*, 2015). To examine the effect of nicotine on gastrointestinal transit, the total fecal pellets expelled were recorded after 30 mins administration of nicotine. In placebo-pelleted mice, the number of pellets (measured between 30 min – 60 min interval after injection) following saline injection was 5 ± 1.1 pellets. A single acute injection (i.p.) of nicotine significantly decreased the number of fecal pellets expelled with increasing doses (0.175, 0.350, 0.525 and 1.050 mg/kg). In mice treated with acute morphine (10 mg/kg), there was complete inhibition of pellet expulsion during the first hour and nicotine did not stimulate pellet expulsion. However, in morphine pelleted animals, nicotine 0.175 mg/kg significantly enhanced the number of fecal pellets expelled to 3.3 ± 1. There was however, no significant differences seen with other nicotine doses tested (0.350, 0.525 and 1.050) (Fig 23). These data suggested that nicotine can stimulate GI transit within a narrow range of doses in chronically morphine-treated mice.

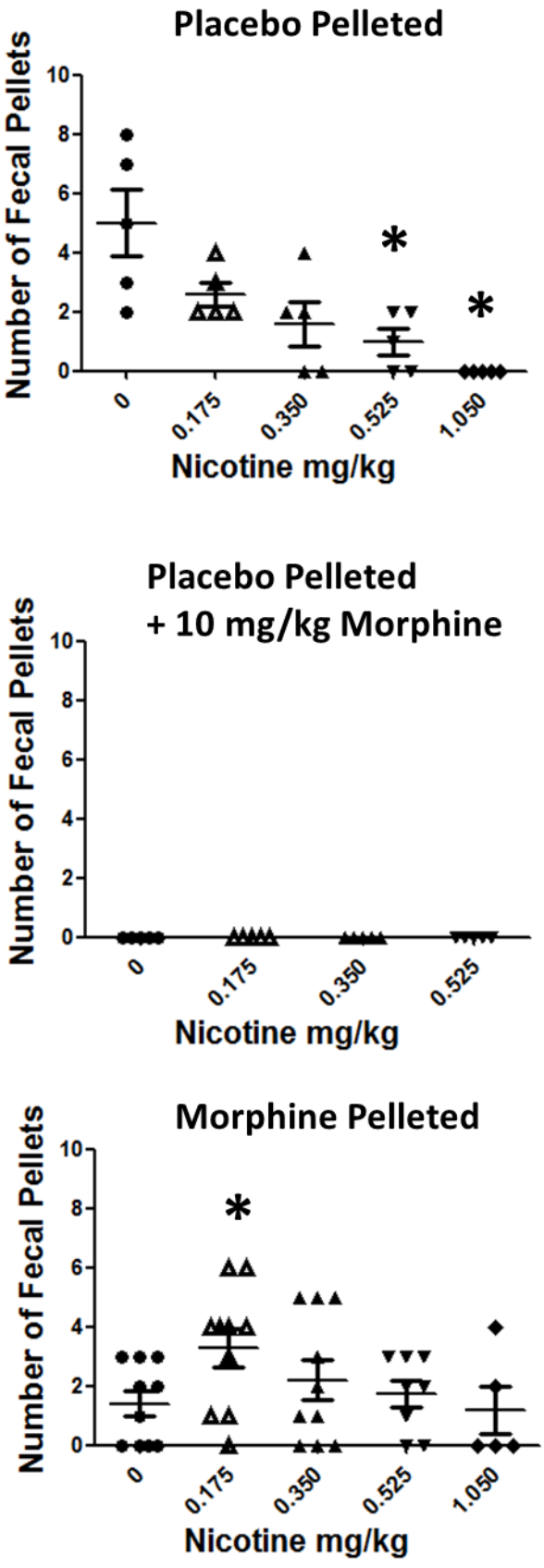

Figure 23: Effects of nicotine on fecal output *(30 mins – 60 mins): Scatter plot displaying the number of fecal pellets expelled after a single I.P injection of saline or different concentrations of nicotine in Placebo pelleted mice, Placebo pelleted mice receiving 10 mg/kg morphine and Morphine Pelleted mice (N = 5-10 in each group) One-Way ANOVA. Fisher's LSD. *P < 0.05*

Summary:

This study examined the effects of nicotine on GI motility in morphine treated mice. The important findings are as follows.

1) Nicotine did not alter the small intestinal motility and fecal pellet output at lower doses but significantly decreased the motility at higher doses.
2) Nicotine reversed the decrease in small intestinal motility and fecal pellet output after long term but not short term morphine exposure.

CHAPTER V

THE ROLE OF α3β4 NICOTINIC RECEPTORS IN REVERSING MORPHINE-INDUCED CONSTIPATION

Previous chapter discussed the role of nicotine in reversing opioid-induced constipation. Although nicotine is a stimulatory drug in the GI tract, it only had a partial effect in a narrow dose range in reversing chronic opioid-induced constipation. This may be attributed largely to the activation of other nAChRs by nicotine expressed on other tissues. Therefore identifying and specifically targeting the receptor mediating nicotine's effects in the GI tract may be helpful in effectively reversing the opioid-induced constipation.

As described earlier, nicotine induces contractions in the intestine through the activation of nicotinic acetylcholine receptors expressed on the enteric neurons (Fishlock *et al.*, 1966). Identifying the subtype of receptors mediating the nicotine's effects on the enteric neurons will help in effectively targeting these receptors.

Nicotinic acetylcholine receptors are pentameric ligand gated ion channels. They are either homomeric or heteromeric and are made up of different subtypes of subunits. The subunit composition is different all over the body. The pharmacological and electrophysiological properties of the different combinations of the subunits are unique and therefore help in identification and specifically targeting the receptor (Luetje *et al.*, 1991).

In the nervous system, nicotinic receptors are composed of eleven separate subunits α2 – α9 and β2 - β4 subunits (McGehee, 1999; Sargent, 1993). Zhou et

al examined the immunohistochemical, pharmacological and electrophysiological properties of nAChRs in the enteric neurons isolated from the myenteric plexus of neonatal guinea pigs (Zhou *et al.*, 2002). Immunohistochemical studies had demonstrated the expression of α3, α5, α7 and β2 subunits in the enteric neurons. Similarly, pharmacological studies revealed the functional expression of α3, β4 and β2 but not α7 subunits based on the sensitivities to subtype specific agonists and antagonists. However the composition of the receptors mediating the nicotine or acetylcholine induced responses is not known.

In the present study, we used genetic and pharmacological approach to identify the contribution of each of these subunits and subunit combinations mediating the nicotine induced responses in the enteric neurons. We then specifically targeted the identified subunit to examine its role in reversing opioid-induced constipation and also studied the changes in the expression of subunits after chronic morphine exposure.

5A. α3β4 subunit is the major nAChR expressed in the mouse enteric neurons:

Results:

mRNA expression of nAChRs in the LMMP of ileum:

Most of the literature on nAChRs and enteric neurons has been reported from the neonatal guinea pigs and nothing is known about the nAChR expression in the mouse myenteric plexus. In the present study, we examined the relative expression of the subunits by quantifying the mRNA levels of nAChRs in the LMMP isolated from the ileum of mice. We first plotted a standard curve with ct values obtained with different concentrations of genomic DNA for each primer. We then compared the ct values obtained from the sample tissue extracted from the LMMP of mouse ileum for each primer in the standard curve. Based on where the ct value of sample falls in the standard curve, we calculated the mRNA expression of each of the subunits (Fig 24). Data from qPCR demonstrated the expression of nAChRs in the order of α3 > β2 > β4 > α5 > α4 > β3 > α6 subunits (Fig 25). These data are in agreement with earlier data from the neonatal guinea pig suggesting the expression of α3, β2, β4 and α5 subunits in the enteric neurons of myenteric plexus (Zhou *et al.*, 2002). However, these data may not reflect the functional expression of these subunits in the neurons.

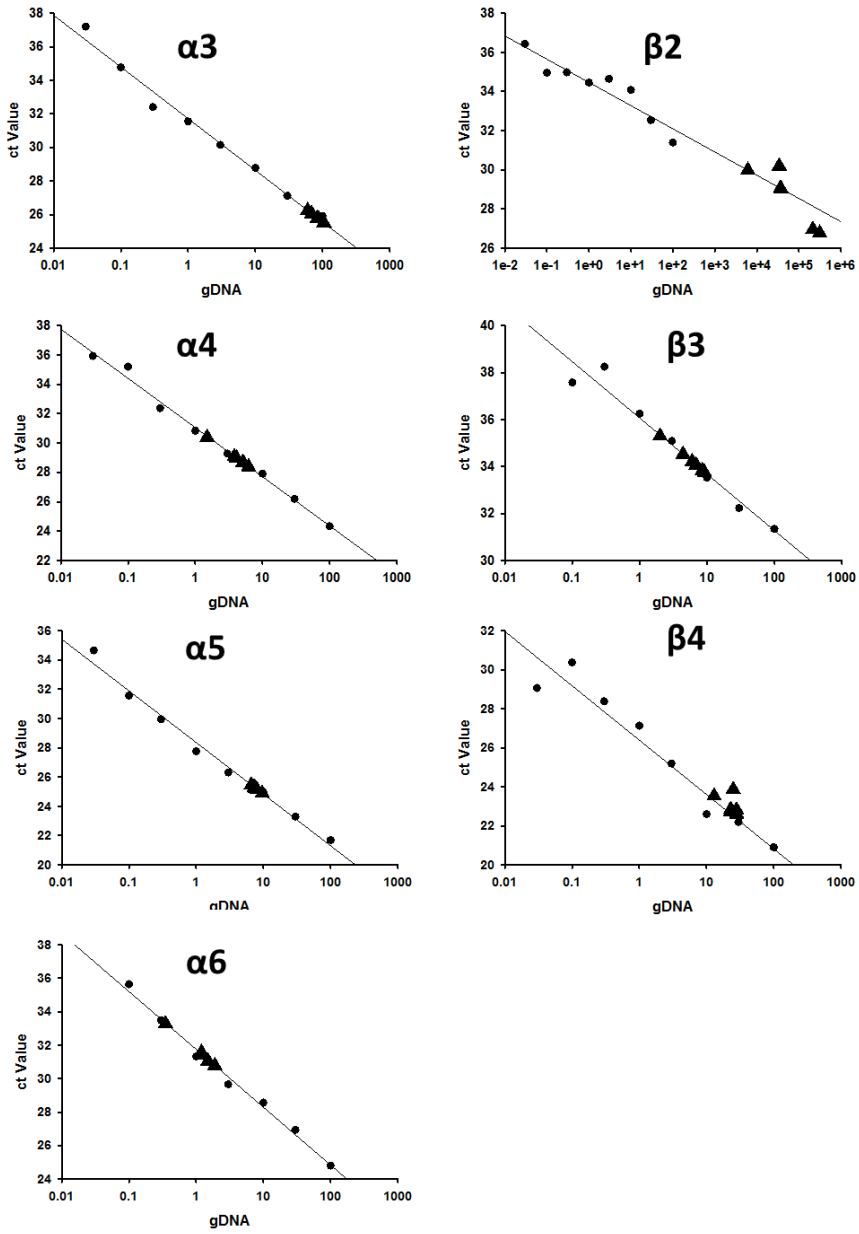

Figure 24: mRNA expression of nAChRs in LMMP -1: *Graphs displaying the ct values obtained with different concentrations of DNA* (●) *in the presence of different nAChR primers.* (▲) *indicate the ct values of samples obtained from RNA of LMMP tissue obtained from ileum of mice (n=5-6).*

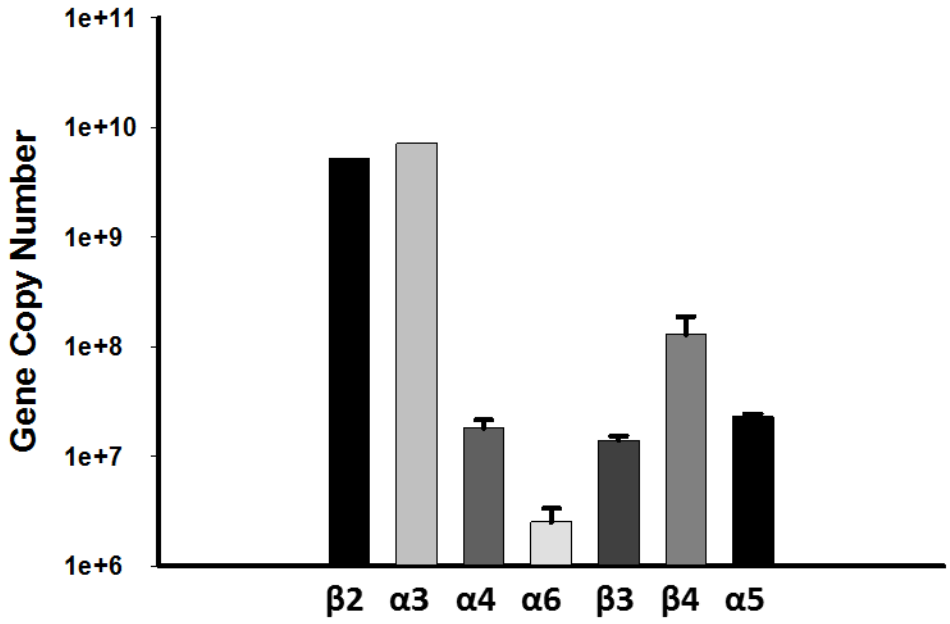

Figure 25: mRNA expression of nAChRs in LMMP -2: *Bar graph displaying the gene copy number of samples obtained from RNA of LMMP tissue with different nAChR primers.*

Effects of nicotine on enteric neurons from nAChR knock out mice:

In order to further examine the functional role of these subunits in the enteric neurons, we used a genetic approach. Enteric neurons were isolated from nAChR subunit specific knock out mice and the activity of nicotine was measured. Enteric neurons isolated from C57BL/6J mice are used as controls as they formed the background strain for all nAChR knock-outs. Figure 26 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 27). 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.

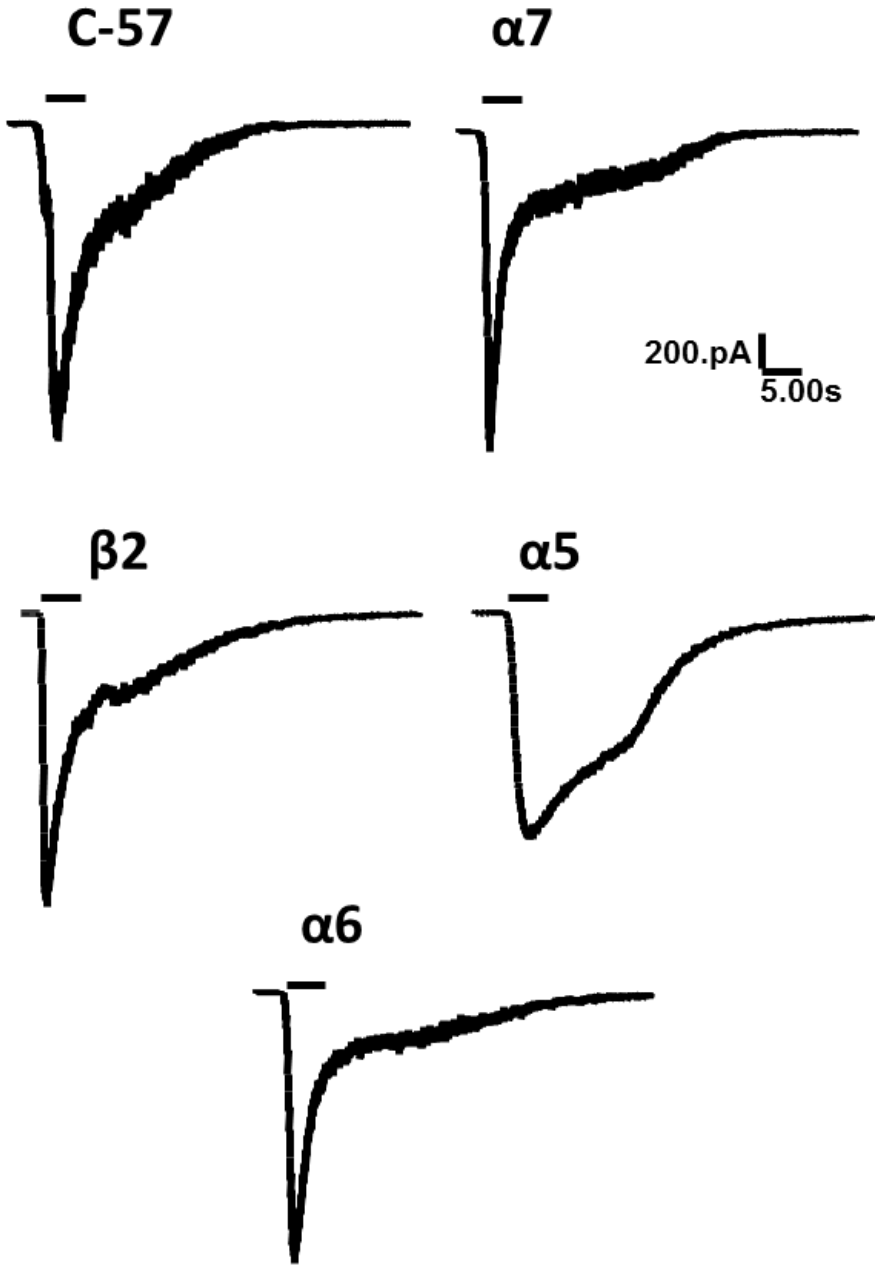

Figure 26: Nicotine induced currents from nAChR knock out mice - 1: *Raw traces of currents induced by 0.3 mM nicotine from neurons isolated from LMMP of α7, c-57, α5, α6 and β2 K.O mice.*

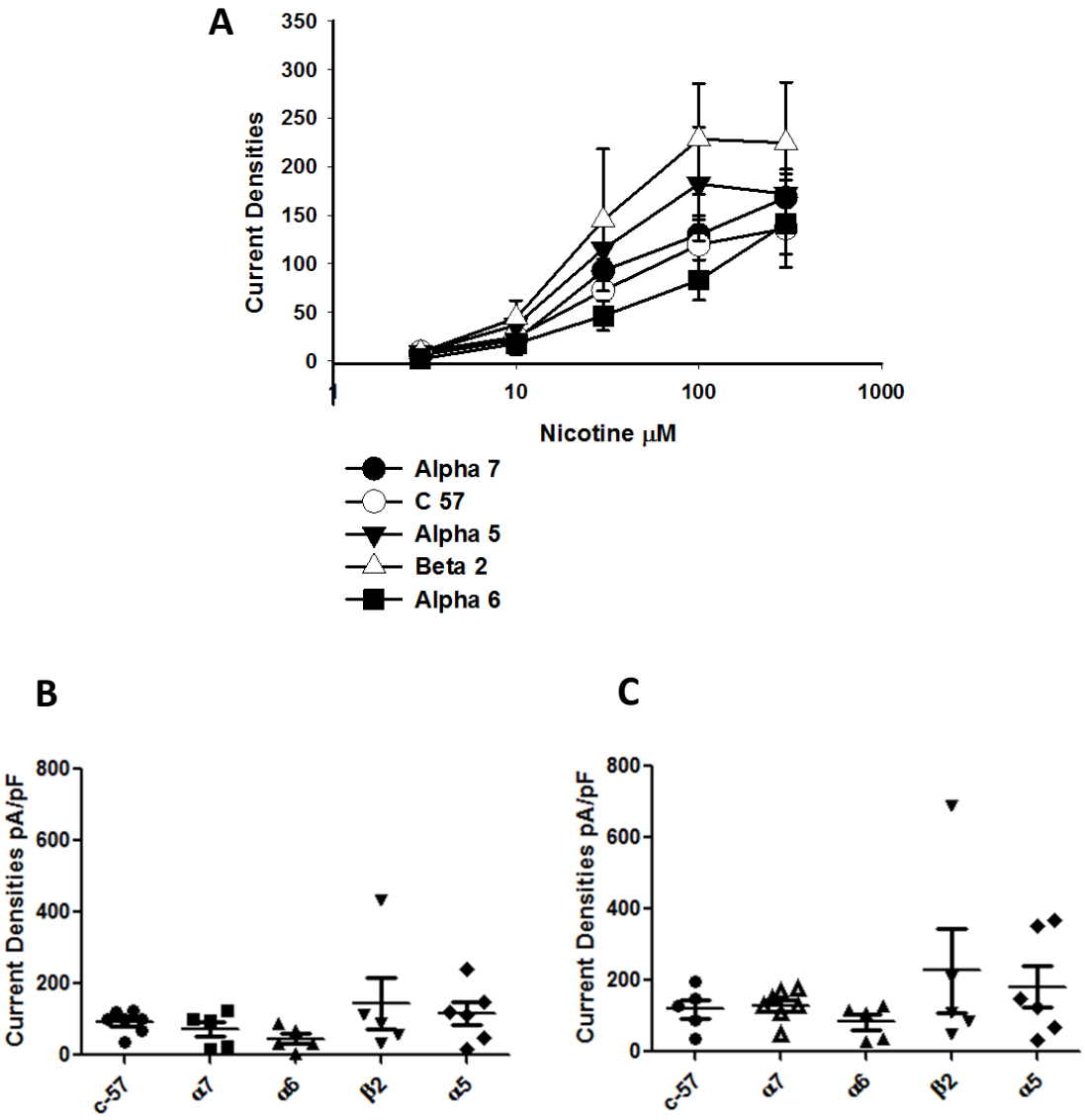

Figure 27: Nicotine induced currents from nAChR knock out mice – 2: *A) Concentration response curves of nicotine induced currents in cells from neurons isolated from LMMP of α7, c-57, α5, α6 and β2 K.O mice. Two-Way ANOVA. Tukey kramer Post Hoc test. *P < 0.05. Scatter plot displaying the amplitude of currents induced by B) 30 μM and C) 100 μM concentrations of nicotine in enteric neurons isolated from C-57, β2 K.O, α5 K.O, α6 K.O and α7 K.O mice*

Pharmacological properties of nAChRs from isolated enteric neurons:

As mentioned earlier, the pharmacological properties of different combination of the subunits are unique. In order 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. Mecamylamine (10 μM) and hexamethonium (10 μM) significantly blocked the nicotine induced currents at concentrations previously reported to be more preferential for α3β4 receptors (Fig 28). 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 containing receptor(Harvey *et al.*, 1997; Luo *et al.*, 1998)(Harvey *et al.*, 1997; Luo *et al.*, 1998)(Harvey *et al.*, 1997; Luo *et al.*, 1998)(Harvey *et al.*, 1997; Luo *et al.*, 1998)(Harvey *et al.*, 1997; Luo *et al.*, 1998)(Harvey *et al.*, 1997; Luo *et al.*, 1998) (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 (Harvey *et al.*, 1997) (Fig 29). These findings suggested that the nicotine induced currents in the enteric neurons may be primarily mediated through the α3β4* nAChR receptors with possible co-expression of α5 subunit modulating receptor desensitization.

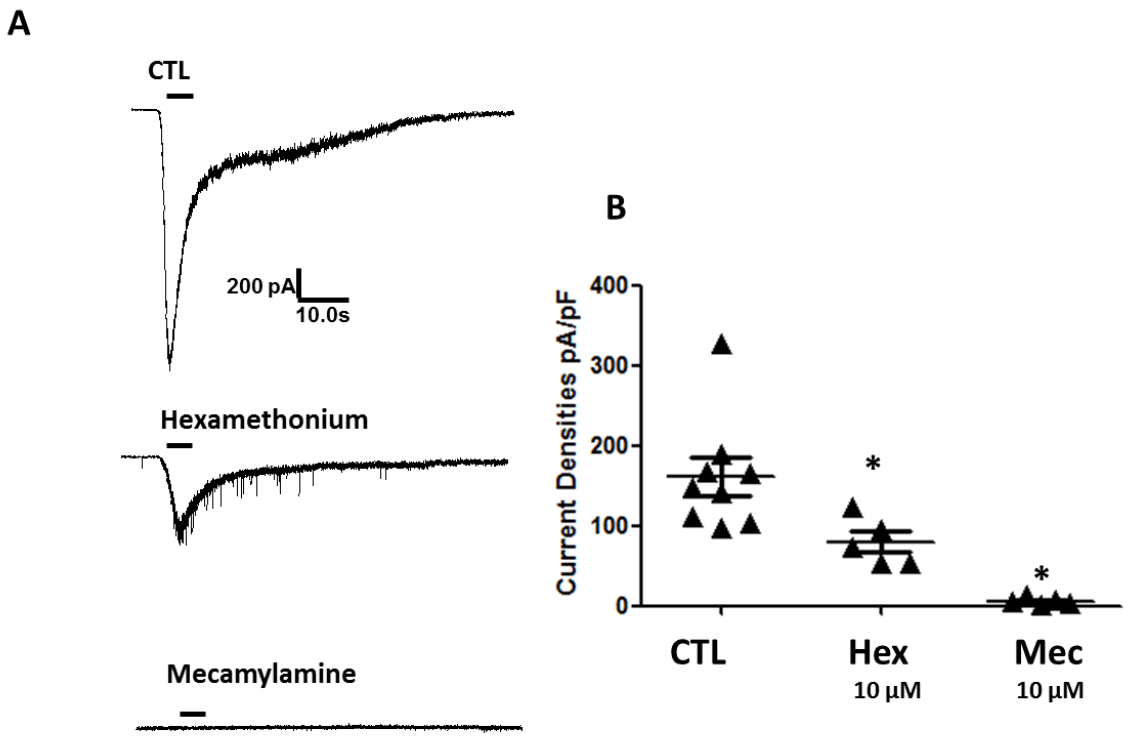

Figure 28: Hexamethonium and mecamylamine block the nicotine induced currents in neurons isolated from mouse LMMP: *A) Raw traces of nicotine induced currents in control cells and in the presence of 10 µM hexamethonium (n=5) and 10 µM mecamylamine (n=5) B) Scatter plot showing the distribution of the amplitude of current in control and in the presence of 10 µM hexamethonium and mecamylamine.*

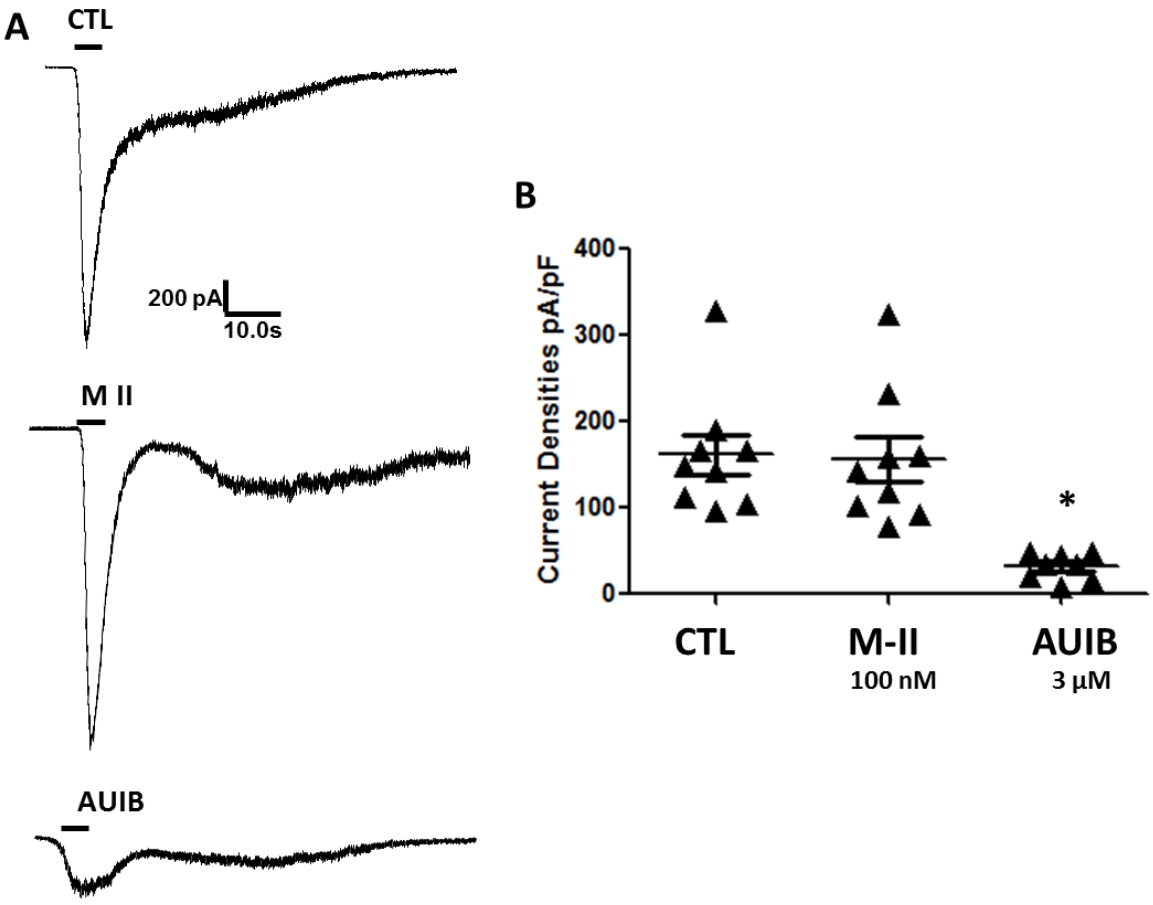

Figure 29: α-Conotoxin AUIB but not M-II block the nicotine induced currents in neurons isolated from mouse LMMP: *A) Raw traces of nicotine induced currents in control cells and in the presence of 100 nM M-II and 3 µM AUIB. B) Scatter plot showing the distribution of the amplitude of current in control and in the presence of 100 nM M-II (n=9) and 3 µM AUIB (n=8)*

Summary 5A:

This study identified the subunit mediating the nicotine induced responses in the enteric neurons isolated from the myenteric plexus of mouse ileum.

1) qPCR studies demonstrated the mRNA expression of nicotinic acetylcholine receptors in the order α3 > β2 > β4 > α5 > α4 > β4 > α6 which are in agreement with earlier studies from neonatal guinea pigs
2) The amplitude of nicotine induced currents were not significantly different in enteric neurons isolated from α5, α6, α7 and β2 knock out mice
3) The desensitization pattern of enteric neurons from α5 knock out mice were delayed compared to controls and other knock outs suggesting its involvement in regulating the desensitization
4) Pharmacological studies using subtype specific antagonists demonstrated that the nicotine induced responses in the enteric neurons are mediated primarily through the α3β4 subtype of nAChRs

5B. Effects of NS3861 on GI motility:

In chapter three, we demonstrated that the efficacy of nicotine is enhanced in enteric neurons after chronic morphine exposure. Similarly, in chapter four, we demonstrated that nicotine partially reverses morphine induced constipation in chronic but not acute morphine treated animals. In the data presented from the earlier section of this chapter, we demonstrated that the nicotine induced responses in the enteric neurons were primarily mediated through α3β4 nAChRs. Therefore in the present section, we sought to identify the role of α3β4 receptor in reversing the morphine induced constipation by using NS3861, a partial agonist at the α3β4 nicotinic acetylcholine receptor. Previous reports from heterologously expressed nAChRs in oocytes have demonstrated a higher affinity and partial agonist properties for NS3861 at α3β4 subtype of nAChRs. However, at relatively higher doses it also displayed full agonist properties at α3β2 nAChRs (Harpsoe *et al.*, 2013).

Results:

In the present study we examined the effects of different doses of NS3861 in reversing opioid-induced constipation by evaluating the gastrointestinal motility. In placebo pelleted mice, the number of pellets expelled following a single saline injection were 4.25 ± 0.25. NS3861 did not significantly alter the number of fecal pellets expelled at 0.01, 0.05, 0.1 mg/kg when given i.p. However, a significant decrease was seen at a dose of 0.5 mg/kg. In mice treated with acute morphine (a single injection of 10 mg/kg morphine), there was complete inhibition of pellet expulsion and NS3861 did not stimulate pellet expulsion. However, in the

morphine pelleted mice, where the number of fecal pellets expelled was significantly lower at 1.2 ± 0.58, the total fecal pellet expelled increased in a dose-related manner with significant increase seen at 0.1 mg/kg NS3861 (5.6 ± 0.75). This suggested that NS3861 significantly enhanced the motility in opioid-induced constipation after chronic but not acute administration of morphine (Fig 30). These effects are lost at a higher dose of NS3861 which may be due to the activation of α3β2 receptors expressed on the other tissues.

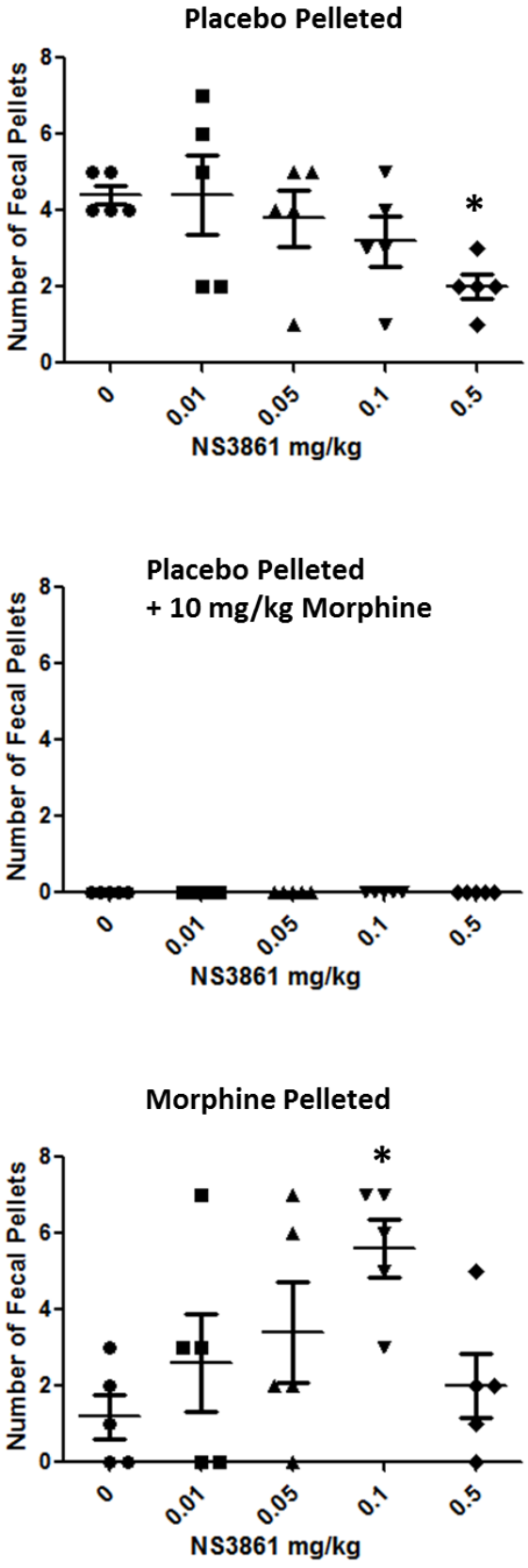

Figure 30: Effects of NS3861 on fecal output: *Scatter plot displaying the number of fecal pellets expelled after a single I.P injection of saline or different concentrations of NS3861 in Placebo pelleted mice, Placebo pelleted mice receiving 10 mg/kg morphine and Morphine Pelleted mice (N = 5 in each group) One-Way ANOVA. Tukey Kramer post-hoc test. *P < 0.05*

Summary 5B:

This study examined the effects of NS3861 on gastrointestinal motility. The important findings are as follows

1) Single acute administration of NS3861 did not alter the motility in control animals at lower doses. However, it significantly decreased the motility at a higher dose
2) Single acute administration of NS3861 reversed the morphine induced constipation in a dose-related manner after chronic but not acute exposure to morphine.
3) Effects of NS3861 in opioid induced constipation were not effective at higher doses suggesting off-target effects or desensitization of the receptors.

5C. α3β4 mRNA expression is not altered after prolonged exposure to morphine:

Previous reports in the literature have demonstrated an enhancement in the nicotine induced responses in the small intestine of morphine tolerant guinea pigs (Goldstein *et al.*, 1973; Johnson *et al.*, 1978). In chapter three, our data suggested that the enhanced response to nicotine may be mediated through an increase in efficacy of nicotine in the enteric neurons. Similarly, in chapter four, we have shown that nicotine partially reversed morphine induced constipation after chronic but not acute exposure to morphine. On the same lines, NS3861, a α3β4 specific partial agonist also reversed morphine induced constipation after chronic but not acute exposure to morphine. This suggested that the enhanced activity of nicotinic receptors mediating the nicotine and NS3861 induced motility occurs after chronic 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 mRNA expression of α3 and β4 subunits in LMMP from mice pelleted with either a placebo or 75 mg morphine for four days. The normalized fold changes of mRNA tested using α3 and β4 primers show a trend in increase with variable data. However, there was no significant increase of both the subunits after chronic exposure to morphine (Fig 31).

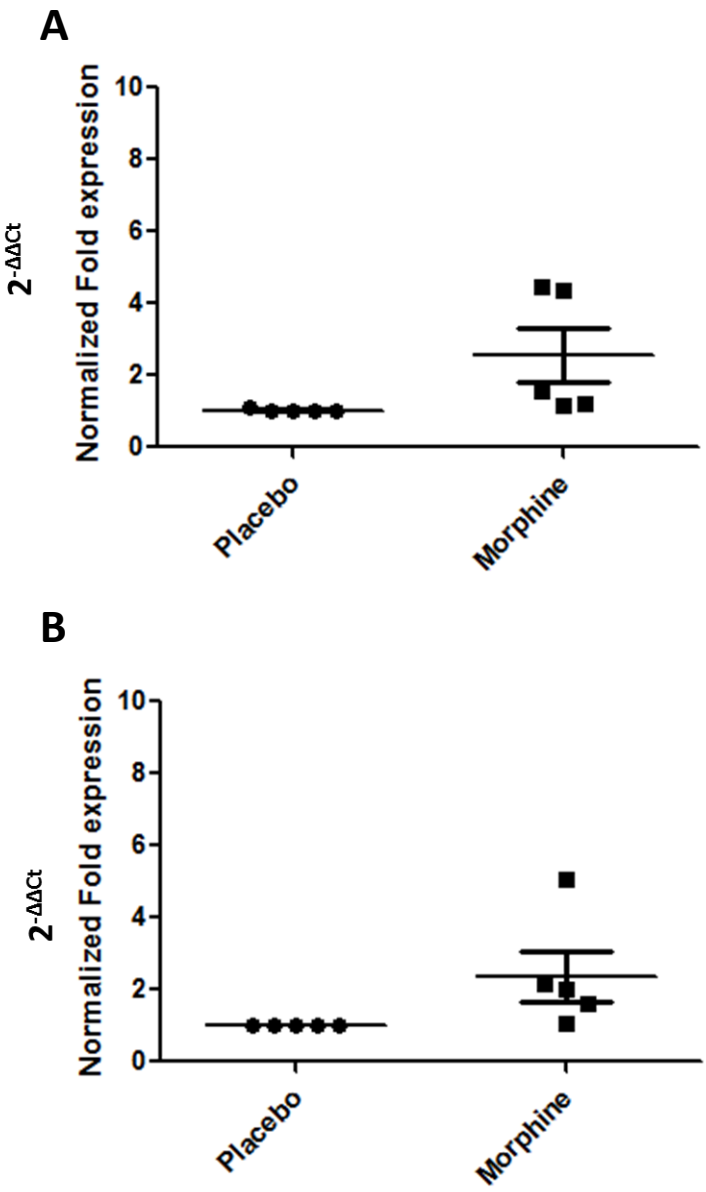

Figure 31: α3β4 mRNA expression is not altered after prolonged exposure to morphine: *Normalized fold change of the mRNA of A) β4 and B) α3 subunits in placebo pelleted (PP) and morphine pelleted (MP) animals. (N=5) Student's t-test *P < 0.05*

Summary 5C:

This study examined the changes in the transcription of α3 and β4 genes by quantifying the mRNA levels. The normalized fold change of mRNA displayed an increasing trend. However, there is no significant change in the mRNA levels compared to the controls. This suggests that the enhanced response to nicotine after chronic morphine exposure may not be mediated through the increase in the transcription of α3 or β4 subtype of nAChRs.

CHAPTER VI

Discussion:

Overall, the studies described in this dissertation were focused on identifying a potential target to reverse opioid-induced constipation. The findings suggest that activation of α3β4 subtype of nAChR reverses chronic morphine induced constipation. nAChRs are highly expressed on the enteric neurons and an α3β4 specific partial agonist provides a prokinetic effect following chronic but not acute morphine exposure.

The role of nicotine in reversing morphine induced constipation:

Previous reports have shown an increase in potency of nicotine induced contractions following long term exposure to morphine in the isolated segments of guinea pig ileum (Goldstein *et al.*, 1973; Johnson *et al.*, 1978). Nicotine is an agonist at the nAChRs and is known to increase motility. We hypothesized that nicotine would have an enhanced effect in terms of inducing motility after long term exposure to morphine. We tested this hypothesis by examining the effects of nicotine on GI motility after short term and long term exposure to morphine. Short term (single 10 mg/kg morphine i.p.) and long term (75 mg morphine pellet – 4 days) exposure to morphine resulted in reduced small intestinal transit and fecal pellet output in mice as previously reported (Fitting *et al.*, 2015; Ross *et al.*, 2008). Nicotine in a narrow dose-range increased both the small intestinal transit as well as fecal pellet output in chronic but not acute morphine treated mice (Chapter IV). To test if these enhanced effects are a result of an enhanced

activity of nAChRs of enteric neurons we measured nicotine induced currents in single neurons (Chapter III). Moreover, the narrow dose range by which nicotine enhanced gastrointestinal motility after long term morphine treatment suggested that the action of nicotine at the other nAChR subtypes may negate the stimulatory effects. Therefore it was important to identify the subtype of nAChR mediating the nicotine induced responses. It is also likely that at these higher doses, both central actions as well as possible desensitization of the receptors prevent nicotine mediated stimulation of peristalsis.

Long term exposure to morphine enhances nicotine induced currents:

The enhanced potency of nicotine following long term exposure to morphine in the small intestine of guinea pig is attributed to the depolarization of membrane potential in enteric neurons (Kong *et al.*, 1997). Microelectrode recordings from enteric neurons of myenteric ganglia suggested that the depolarization of enteric neurons from chronic morphine treated guinea pigs is mediated through changes in the sodium potassium pump activity. However, in the present study, membrane potential of isolated single enteric neurons is not altered after long term exposure to morphine (16-20 hrs) in culture. We hypothesized that the enhanced activity of nAChRs expressed in enteric neurons contributes to the enhanced sensitivity to nicotine induced contractions in ileum.

Previous reports in the literature have used microelectrode recordings from the myenteric plexus in an intact tissue preparation to measure the activity of neurons (Kong *et al.*, 1997; Leedham *et al.*, 1992; Taylor *et al.*, 1988). Although this preparation provides an excellent platform to study the excitability properties

of neurons, they may not reflect the activity of receptors as drug induced effects might be influenced by the connections from neighboring cells in the tissue. Therefore an isolated neuronal cell preparation would be ideal to study the activity of receptors expressed on enteric neurons.

Previous reports from our lab described a technique to isolate neurons from the myenteric plexus of mouse ileum (Smith *et al.*, 2012; Smith *et al.*, 2013). Enteric neurons in the myenteric plexus are a heterogeneous population of neurons as they differ in the functional and neurochemical properties (Brookes *et al.*, 1991; Furness *et al.*, 1988; Sang *et al.*, 1998). Although the LMMP tissue contains different types of neurons, the composition of the isolated neurons is not known. In the present study we examined the chemical coding of the isolated neurons using immunostaining techniques. These experiments demonstrated the expression of heterogeneous population of the neurons including sensory, motor, inhibitory and excitatory neurons in the isolated culture which are representative of the neurons from the whole tissue (Chapter III). A very low co-localization has been observed between excitatory cholinergic and inhibitory nitrergic neurons and high co-localization between inhibitory nitrergic and vipergic neurons which was in agreement with previous reports from LMMP of mouse ileum (Sang *et al.*, 1996; Sang *et al.*, 1998). Moreover, the expression of μ-opioid receptors has been primarily localized in the cholinergic and a subpopulation of nitrergic but not vipergic neurons. This is in agreement with previous reports suggesting the involvement of cholinergic neurons in mediating the effects of opioids in the ileum

(Paton, 1957). However, the specificity of the μ-opioid receptor antibody needs to be established.

In order to examine the changes in the nAChR activity following long term exposure to morphine, the isolated enteric neurons were treated in culture with 3μM morphine overnight (16-20 hours). We performed whole-cell patch clamp experiments on the isolated enteric neurons and tested the nicotine induced responses to assess the activity of the nAChRs. The concentration response relation displayed an increase in the efficacy but not potency of nicotine following long term but not short exposure to morphine (Chapter III). This suggested that the enhanced responses to nicotine in the intestine of morphine tolerant animals may be mediated through an increase in nicotine induced currents at the single enteric neuron level. Therefore nicotine's effects on reversing morphine induced constipation may be mediated through the activation of these receptors.

The role of α3β4 nAChR in reversing morphine induced constipation:

Identifying the subunit composition of nAChRs allows us to specifically target the receptor using subtype specific ligands. Data from chapter III and IV suggested that targeting the specific subtype of nAChR expressed on the enteric neurons may reverse the opioid induced constipation in mice. In the present study we approached to identify the subtype of nAChR 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 in

agreement with previous reports from neonatal guinea pig ileum (Zhou *et al.*, 2002) (Chapter V).

Mouse models provide a unique opportunity to study the function of nAChRs by using subtype specific knock out mice. 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 may not be 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 also be a potential subunit modifying the inactivation of the nicotine induced currents (Chapter V). 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).

In order to further examine if the subunit composition is mediated through the α3 subtype of nAChR we used a pharmacological approach. Hexamethonium (10 µM), a non-selective antagonist significantly decreased the nicotine induced currents. Mecamylamine (10 µM), a nicotinic antagonist, completely abolished the nicotine induced currents at a concentration highly specific for α3β4 (Papke *et al.*, 2010). Similarly, 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). However, α-Conotoxin M II (100

nM) at a concentration highly specific for α3β2 did not change the nicotine induced currents (Chapter V). Collectively, these data suggested 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. Although there was a high mRNA expression of β2 subunit in the LMMP of mouse ileum, there seems to be no functional role played by this subtype of nAChR in the enteric neurons.

Since it was determined that the α3β4 subtype of nAChR mediates nicotine induced currents in enteric neurons, we next tested if the activation of these receptors can reverse opioid-induced constipation (Harpsoe *et al.*, 2013). α3β4 subtype of nAChRs are expressed in different parts of the body including the brain. They are ganglionic receptors and mediate different funcions of the sympathetic nervous system. Activation of these receptors using α3β4 specific agonist may overexcite them and alter the functions of sympathetic nervous system. Therefore, using a partial agonist is a better strategy in activating these receptors as they avoid over excitation of the receptors. Partial agonists can act as functional agonists in the absence of endogenous agonist and as antagonists in the presence of excess agonist. Since morphine is known to decrease the release of endogenous neutransmitter acetylcholine by the enteric neruons, partial agonist can act as functional agonists to activate these neurons.

In the present study we tested the effects of NS3861, an α3β4 specific partial agonist on GI motility. Fecal pellet output assay performed using NS3861 displayed an enhancement in the number of fecal pellets expelled in long term

but not short term morphine treated mice (Chapter V). This suggests that the α3β4 subtype of nAChR provides a potential target to reverse chronic morphine induced constipation. However, the effects of NS3861 were lost at higher doses. This may be attributed to the actions of NS3861 at α3β4 or α3β2 receptors in the brain.

Although both the nicotine and NS3861 reversed constipation after chronic morphine treatment, it is not clear if these effects are mediated through the activation or the desensitization of the receptor. Blocking the effects of nicotine and NS3861 using α3β4 specific antagonists such as mecamylamine would help in identifying such mechanisms.

Mechanism of action:

Previous reports in the literature and the results from the present studies have demonstrated an enhancement in the nAChR activity following long term exposure to morphine both in vivo and in vitro. However, the mechanism involved in this enhancement is not understood.

Long term exposure to morphine is associated with changes in the intracellular environment of the enteric neurons which further leads to dependence (Gintzler *et al.*, 2006). Naloxone, an antagonist at μ-opioid receptor enhances the excitability of isolated enteric neurons from ileum of mice after overnight exposure to 3 μM morphine suggesting the induction of precipitated withdrawal or morphine dependence. However, naloxone was ineffective after overnight exposure to morphine in enteric neurons isolated from colon, suggesting that

dependence is absent in these cells (Smith *et al.*, 2014). In order to test if the morphine's effects on nicotine induced currents are associated with dependence, we tested the effects of long term morphine in neurons isolated from colon. Concentration response relation with nicotine showed an increase in nicotine induced currents with an increase in efficacy but not potency (Chapter III). This suggested that the enhanced nAChR activity may not be associated with morphine dependence.

ATP and acetylcholine, endogenous agonists at the P2X and nAChRs are co-transmitters and contribute to the majority of neurotransmission in the GI tract (Zhou *et al.*, 1998). Moreover, the activity of nAChRs is regulated by P2X receptors as they are known to be expressed in closed proximity and form heteromers (Decker *et al.*, 2009; Decker *et al.*, 2010). However, in the present study, ATP induced currents were not significantly changed after long term exposure to morphine (Chapter III). This suggested that the effects of morphine are specific for nAChRs and P2X receptors are not involved in increased efficacy of nicotine.

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. However, the experiments done in the present study showed no significant differences in the mRNA levels of α3 and β4 subtype of nAChRs in the LMMP of placebo and morphine pelleted

mice (Chapter III). Previous reports have shown that by phosphorylating or altering membrane trafficking, nAChR activity can be enhanced (Govind *et al.*, 2009; Walsh *et al.*, 2008; Wecker *et al.*, 2010). Increased nAChR numbers is also reported in brains of morphine dependent mice (Neugebauer *et al.*, 2013). Further studies will be required to examine protein expression levels of nAChR after chronic morphine in enteric neurons.

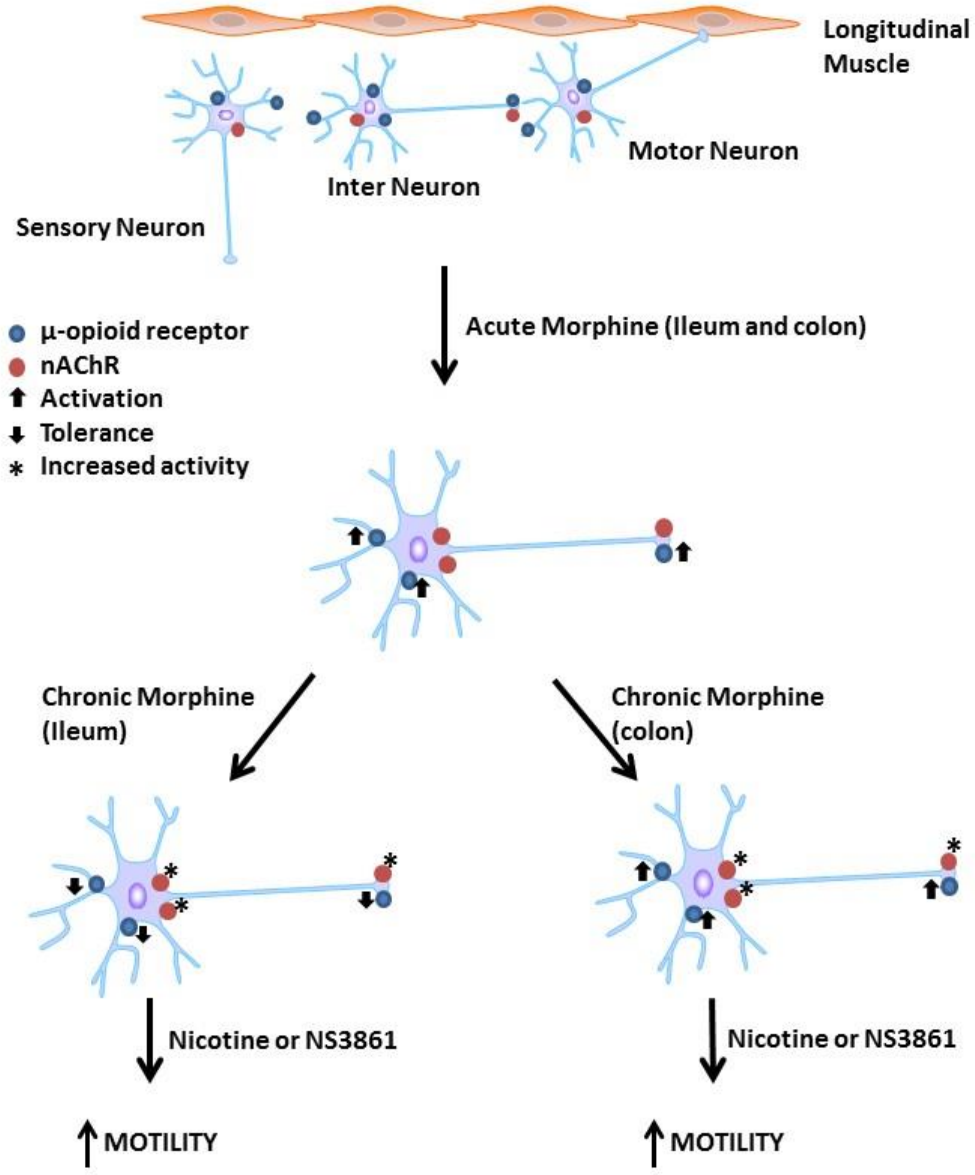

Figure 32: *Model displaying the changes in the activity of μ-opioid receptors and nAChRs after actute and chronic exposure to morphine in enteric neurons of ileum and colon.*

Limitations:

Just like any other study, the experiments described in this dissertation also have limitations. Specific limitations from each chapter are listed below.

Chapter III:

Immunostaining using μ-opioid receptor antibody: There is a lot of debate in the literature over the specificity of antibodies used against μ-opioid receptors (Niwa *et al.*, 2012; Smith *et al.*, 2012). The data from the present study suggested the presence of μ-opioid receptors on cholinergic and nitrergic neurons. This was in agreement with previous reports where it was demonstrated that morphine acts by inhibiting the release of acetylcholine at neuromuscular junction of longitudinal muscle in the small intestine (Paton, 1957). However, the specificity of this antibody is yet to be determined using μ-opioid receptor knock out mice.

Direct effects of morphine on nAChRs: Although the effects of morphine in the present study were shown to be mediated thorugh the mu-opioid receptors, there is also a possibility of these receptors binding directly to the nAChRs. Previous reports have demonstrated the binding of morphine to different subtypes of nAhRs at higher concentrations (Talka *et al.*, 2013). The involvement of direct binding of morphine to nAChRs in the enteric neurons can be tested using mu-opioid receptor knock-out animals.

Concentration response to nicotine: Nicotine is an agonist at nAChR and is known to rapidly desensitize the receptor. Multiple exposures to nicotine are

known to irreversibly desensitize the receptor resulting in reduced currents (Zhou *et al.*, 2002). Concentration response experiments in the present study with nicotine involved multiple exposures. In order to confirm that the results from concentration responses are showing an increase in efficacy, we also performed a separate experiment where we treated our cells with a single exposure to 1 mM nicotine. In both the cases, an increase in nicotine induced currents was seen after long term exposure to morphine.

Previous studies have used specialized systems controlled by a picospritzer (General valve) to deliver nicotine directly to the vicinity of the cell in the bath solution (Zhou *et al.*, 2002). These systems control the delivery of drug through a pressure pulse and avoid over exposure of nicotine to the cells. This helps in preventing desensitization of the receptors. However, in the present study we perfused the drug directly into the whole bath solution using a gravity flow. Although the receptors are more prone to desensitization in this procedure, it is more representative of a physiological exposure.

Analysis of nicotine induced currents: Exposure to higher concentrations of nicotine produced rebound currents apart from the peak currents inducating the rapid recovery of channels from the blocked state. Plotting the total change in charge after exposure to nicotine will include the responses of peak currents and rebound currents. However in the present study nicotine induced currents were assayed by plotting only the peak currents of the raw traces as the rebound currents were found to be proportional to the peak currents.

Morphine concentration: Previous reports from our lab demonstrated that acute exposure to 3 µM morphine decreases the excitability of neurons and long term exposure leads to tolerance and dependence in the single enteric neurons. Although 3 µM concentration of morphine is considered high, it is still representative of the concentration of the morphine in vivo in mice, as surgical implantation of a morphine pellet is associated with an increase in serum morphine concentration to 4 µM on the day of surgery which progressively decreases to a concentration of 2 µM on day 5 after the surgery (Zhang *et al.*, 2011).

Effects of morphine on enteric neurons were examined by exposing the cells to morphine in culture. However, it is also important to study the effects of morphine exposure in vivo. The drawback of performing such an experiment is that the neurons isolated from animals should be allowed to settle down overnight in culture before performing any functional studies. Isolated cells might lose the phenotype acquired after morphine treatment in vivo during the overnight settling period. Therefore, an alternate method that can allow us to use the cells immediately after isolation would help to study the effects of morphine exposure in vivo.

Chapter IV:

Fecal pellet output assay: Previous reports from our lab have shown that morphine dose-dependently decreases the fecal pellet output in mice (Fitting *et al.*, 2015). The main drawback with this experiment is that the amount of food consumed by the mice is not controlled. This may be the reason for higher

variability seen in the data. In spite of this drawback, mice still showed a dose-dependent decrease in the fecal pellet output after administration of morphine suggesting the credibility of this assay (Fitting *et al.*, 2015). However, controlling the amount of food consumed by the animals might increase the sensitivity of this assay and decrease the variability.

Chapter V:

Knock out models: nAChR subunit specific knock out mice were used to identify the subunit mediating nicotine induced currents in enteric neurons. The drawback with this experiment was that, C57BL/6J mice and not wild type littermates were used as controls. However, nicotine induced currents in the knock out models were not significantly different among each other and when compared to C57BL/6J mice. We believe that this information is enough to suggest that the nicotinic receptor subtypes tested here are not the candidate subunits mediating nicotine induced currents in the enteric neurons.

Experiments with antagonists: In order to evaluate the subtype of nAChR expressed on the enteric neurons, we used subtype specific antagonists to block nicotine induced currents. However, only a single effective concentration of antagonists was tested instead of a concentration response to avoid multiple exposures to nicotine.

Future Studies:

Peripherally selective α3β4 partial agonist: α3β4 subtype of nAChRs are expressed in both the central and peripheral tissues. Activation of the receptors in the brain may negate the stimulatory effects in the periphery. Therefore, development of peripherally selective α3β4 partial agonists may be of therapeutic benefit in treatment of chronic opioid-induced constipation.

Effects on propulsive motility: Although nicotine and NS3861 enhanced the fecal pellet output in mice after long term morphine exposure, their actions on the propulsive motility is not known. Gastrointestinal motility monitor (GIMM) is an excellent devise designed to study the propulsive motility in isolated segments of intestine. Effects of nicotine and NS3861 can be evaluated on isolated ileal and colon segments using this devise.

Mechanism of action: Long term exposure to morphine is associated with alterations in the activity of kinases such as PKC and PKA (Dalton *et al.*, 2005; Narita *et al.*, 1994). Moreover, post-translational modifications of nAChRs such as phosphorylation mediated through PKC were shown to enhance the activity of receptor (Wecker *et al.*, 2010). Therefore, examining the changes in the level of phosphorylation or changes in the activity of kinases such as PKC or PKA may help in understanding the mechanism of action involved in changes seen after long term exposure to morphine.

Role of cholinergic and vipergic neurons in morphine's effects: Electrophysiological properties of cholinergic and vipergic neurons from the

myenteric plexus can be examined using transgenic mouse models as described in the chapter III. Identifying these properties will allow us to understand the physiological and drug induced effects mediated through the cholinergic and vipergic neurons in the GI tract. Moreover, immunostaining experiments also revealed the expression of μ-opioid receptors in cholinergic and nitrergic but not vipergic neurons. These findings can be further confirmed by testing the effects of short term and long term exposure to morphine on cholinergic and vipergic neurons. These experiments will help us in understanding the subtype of enteric neurons involved in mediating the morphine's effects in the GI tract.

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Doctor of Philosophy, Pharmacology, 08/2012 - 12/2015 (Expected)
Virginia Commonwealth University (VCU), Richmond, Virginia
Dissertation: nAChRs as targets to reverse morphine induced constipation

Master of Science, Pharmacology, 08/2010 - 05/2012
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Dissertation: Hydrogen sulfide as an allosteric modulator of ATP sensitive potassium channels in colonic inflammation

Bachelor of Pharmacy, 09/2006 - 05/2010
Kakatiya University, Warangal, India

Publications:

Kang M, Hashimoto A, **Gade AR**, Akbarali HI. Interaction between hydrogen sulfide-induced sulfhydration and tyrosine nitration in the KATP channel complex. *American journal of physiology. Gastrointestinal and liver physiology* 308(6):G532-9 (2015)

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Awards:

"ASPET Graduate Student Travel Award", Experiential Biology meeting 2015, Boston, MA

Winner - Best Poster Award, Medical Sciences Section, Virginia Academy of Science (VAS) meeting, VCU, 2014

Second Place - "Graduate Student Best Abstract Award", ISTCP section, ASPET, Experimental biology meeting 2014, San Diego, CA

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Teaching:

Training and mentoring junior graduate students and summer undergraduate fellows with different techniques used in the lab, use of lab instruments and helping them with presentations.

Abstracts: Poster Presentation: Gade AR, Damaj I, Dewey WL, Akbarali HI. Chronic but not acute exposure to morphine enhances nAChR mediated responses in enteric neurons. Experimental Biology (2015)

Poster Presentation: Gade AR, Dewey WL, Akbarali HI. Chronic morphine enhances nicotine responses in single enteric neurons. Virginia Academy of Science (VAS) Meeting, VCU (2014)

Poster Presentation: Gade AR, Dewey WL, Akbarali HI. Chronic morphine enhances nicotine responses in single enteric neurons. Experimental Biology (2014)

Poster Presentation: Gade AR, Akbarali HI. Electrophysiological characterization of purinergic receptors in enteric neuron-glia culture. Experimental Biology (2013)

Poster Presentation: Gade AR, Kang M, Akbarali HI. Hydrogen sulfide as an allosteric modulator of ATP-sensitive potassium channels in colonic inflammation. Experimental Biology (2012)

Poster Presentation: Gade AR, Kang M, Akbarali HI. Hydrogen sulfide as an allosteric modulator of ATP-sensitive potassium channels in colonic inflammation. Watts Day Symposium, VCU (2012)

Professional Organizations: 2011-present American Society for Pharmacology and Experimental Therapeutics (ASPET)

Virginia Academy of Sciences, 2014-present

Service: (2014-15) Served as the vice-chair for the medical science section, Virginia academy of science (VAS)

Served as the vice-president for the Pharmacology and Toxicology student organization (PTSO), VCU (2014-15)

Served as the student government association (SGA) representative for the Pharmacology and Toxicology student organization (PTSO), VCU (2013-14)