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THE ROLE OF ENTERIC GLIA IN OPIOID-INDUCED CONSTIPATION

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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- Kang M, Mischel R, Bhave S, Komla E, Huang C, Dewey W, Akbarali HI (2016) Effects of gastrointestinal bacteria depletion on opioid tolerance and dependence. *Scientific reports* (in review)
- Maguma HT, Datta De D, Bhave S, Dewey WL, Akbarali HI (2014) Specific Localization of β-Arrestin2 in Myenteric Plexus of Mouse Gastrointestinal Tract. *PLoS ONE* 9(8): e103894. doi:10.1371/journal.pone.0103894

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Abstract

Morphine is one of the most widely used drugs for the treatment of pain but its clinical efficacy is limited by adverse effects including persistent constipation and colonic inflammation. Morphine-induced colonic inflammation is facilitated by microbial dysbiosis and bacterial translocation. In this study, we demonstrate that secondary inflammation and persistent constipation are modulated by enteric glia. In chronic morphine treated mice (75 mg morphine pellet/5 days), ATP-induced currents were significantly enhanced in enteric glia isolated from the mouse colon myenteric plexus. Chronic morphine resulted in significant disruption of the colonic epithelium and increased Il-1β in the myenteric plexus. The increase in ATP-induced currents, IL-1β expression and ATP release were also observed in isolated glia treated with lipopolysaccharide (LPS) consistent with bacterial translocation as a potential mediator of chronic morphine-induced inflammation. These effects of LPS were reversed by carbenoxolone, a connexin43 hemichannel blocker. In-vivo treatment with carbenoxolone (25 mg/kg) prevented 1) ATP-induced currents in enteric glia, 2) the decrease in neuronal density, and 3) colonic inflammation in chronic morphine treated mice. Inhibition of connexin43 in enteric glia also reversed morphine mediated decrease in gastrointestinal transit. These findings indicate that bacterial translocation-induced enteric glial activation and inflammation is a significant modulator of morphine-related constipation.

List of abbreviations

GI	Gastrointestinal
OIC	Opioid-induced constipation
MOR	μ-opioid receptor
PAMORA	Peripherally active μ-opioid receptor antagonist
Ach	Acetylcholine
LMMP	Longitudinal muscle myenteric plexus
TLR	Toll-like receptor
LPS	Lipopolysaccharide
PAMP	Pathogen associated molecular patterns
LBP	LPS binding protein
ZO-1	Zona occludens-1
NOS	Nitric oxide synthase
ABX	Antibiotics
DRG	Dorsal root ganglia
ENS	Enteric nervous system
ATP	Adenosine triphosphate
NO	Nitric oxide
VIP	Vasoactive intestinal peptide
GFAP	Glial fibrillary acidic protein
TTX	Tetrodotoxin
GDNF	Glia derived neurotrophic factor

GSNO	S-nitrosoglutathione
STC	Slow-transit constipation
FC	Fluorocitrate
HA	Hemagluttin
ChAT	Choline acetyl transferase
ADP	Adenosine diphosphate
UTP	Uridine triphosphate
EPSPs	Excitatory post synaptic potentials
NMDG	N-methyl-D-glutamine
Cx	Connexin
IBD	Inflammatory bowel disease
TNBS	Trinitrobenzene sulfonic acid
BBG	Brilliant blue G
SW	Swiss webster
H&E	Hematoxylin and eosin
MPE	Maximal possible effect
RMP	Resting membrane potential
I-V	Current-Voltage
TEA	Tetraethyl ammonium
4-AP	4-Aminopyridine
IKdr	Delayed rectifier K^+ currents
EGF	Epidermal Growth Factor
Kir	Inwardly rectifying potassium currents

CBX	Carbenoxolone
DNBS	Dinitrobenzene sulfonic acid
SGC	Satellite glia cells
GIMM	Gastrointestinal motility monitor

Chapter I

Background

Opioids are one of the most widely prescribed drugs for pain management. The use of opioids as effective analgesics has almost doubled over the last 10 years in the clinic. However, efficacy of opioids is largely affected by severe gastrointestinal (GI) adverse effects, including opioid-induced constipation (OIC). Although numerous studies are aimed at studying the mechanisms involved in OIC and developing targets to counteract this side-effect of opioids, highly effective treatment options are still lacking. This dissertation is focused on identifying a potential new mechanism by which gut microbiome-induced enteric glial activation modulates OIC.

1) Adverse effects of chronic morphine use

Opioids refer to a large group of compounds with similar characteristics to that of opium. Morphine is one of the most frequently prescribed opioid analgesic for non-cancer pain (Nelson and Camilleri, 2015) (Grunkemeier et al., 2007) . Due to its strong analgesic and sedative properties, morphine is also used in the treatment of a wide range of other clinical conditions, both acutely and chronically. Morphine is a mainstay of therapy for postoperative / surgery pain in hospitalized patients (Viscusi and Pappagallo, 2012). Besides acute pain management, it is also used to alleviate chronic pain including back pain and osteoarthritis. Morphine along with other opioids are used to relieve moderate to severe pain in cancer patients (Plante and VanItallie, 2010). In spite of strong analgesic properties, several adverse effects are associated with the use of morphine that undermine its clinical utility as an excellent pain reliever. Major side-effects of opioids include addiction, anti-nociceptive tolerance, physical dependence, hyperalgesia, respiratory depression, and gastrointestinal dysfunction (Juurlink and Dhalla, 2012).

A) Opioid-induced constipation

In the GI tract, opioids enhance the amplitude of non-propulsive segmental contractions resulting in constipation as well as abdominal cramps and pain. OIC is the most debilitating gastrointestinal adverse effect in patients on long term opioids (Schaumann, 1955) (Kumar et al., 2014). About 41-81% patients prescribed opioids suffer from constipation. OIC is known to have a major impact on the quality of life of patients and they tend to discontinue opioid therapy due to persistent constipation and the abdominal pain associated with it (Holzer, 2008). The effect of morphine and other opioids on GI inhibition has been extensively studied, however highly effective treatment options for OIC are still lacking.

Conventional treatment strategies for constipation include a regimen of laxatives which combines stimulants (senna or bisacodyl) and stool softeners (surfactants, lubricants, osmotics). In spite of concurrent prescription of laxatives with opioids, decrease in constipation is only seen in less than 50 % of OIC cases (Nelson and Camilleri, 2015). Also, laxatives are associated with other side effects upon chronic use- including nausea, vomiting, diarrhea, and abdominal pain.

Opioids can bind to either μ, δ or κ opioid receptors expressed in the GI tract. Morphine binds to μ-opioid receptor (MOR), which is a seven transmembrane G-protein coupled receptor, predominantly present on enteric neurons. Opioids reduce the firing rate of enteric neurons, which leads to decrease in propulsion and secretion in the GI tract resulting in constipation (North and Williams, 1977) (Wood and Galligan, 2004). Alternative treatments for the management of OIC involve targeting the MOR in the GI tract. PAMORAs (peripherally acting μ-opioid receptor antagonists) target the activation of opioid receptors in the periphery without undermining their central effects. Thus, PAMORAs can reverse the peripheral activity of opioids, such as GI inhibitory effects, while maintaining the analgesic properties. Methyl naltrexone bromide is the

first clinically available peripheral opioid antagonist. It poorly crosses the blood brain barrier and therefore does not antagonize the central effects of opioids. Development of another PAMORA, Alvimopan, was discontinued after it failed Phase III clinical trials owing to its inability to improve the frequency of bowel movement in chronic cancer pain patients. On the other hand, Nalaxogel was found to enhance bowel movements in patients suffering from OIC (Holzer, 2008) (Nelson and Camilleri, 2015). Thus, there have been inconsistent results with respect to the effectiveness of these compounds. Moreover, long term use of PAMORAs is associated with severe cardiovascular side effects (Holzer, 2008) (McNicol et al., 2008).

Other strategies for treatment of OIC include Lubiprostone, a chloride channel activator, that increases fluid secretion and gut motility; Prucalopride, an agonist at 5HT4 receptor, secondarily releases Acetylcholine (ACh) but is not yet approved for the treatment of OIC (Kumar et al., 2014).

Early work by Paton demonstrated the effect of morphine on guinea pig longitudinal muscle myenteric plexus (LMMP) using electrical field stimulation. His studies showed that morphine inhibited longitudinal muscle contractions by decreasing the release of acetylcholine from myenteric neurons. This reduction of neurotransmitter release is due to neuronal hypo-excitability following activation of potassium channels and membrane hyperpolarization upon opioid administration (North, 1976) (North and Williams, 1977). Activation of MOR also causes inhibition of calcium channels and production of adenylate cyclase leading to a decrease in neurotransmitter release.

Recent studies have pointed towards the differences in the effect of morphine on the small and large intestine. While the ileum becomes tolerant to the inhibitory effects of morphine, the colon does not (Ross et al., 2008). This is a major problem in the clinic, since patients on chronic morphine become tolerant to the analgesic effects and are prescribed higher doses to maintain the

analgesic efficacy. However, due to the absence of tolerance development in the colon, these escalating doses further induce GI inhibition leading to persistent constipation. This absence of tolerance development in the colon is attributed to lack of tolerance to circular muscle contractions resulting in enhanced non-propulsive contractions and a typical decrease in peristalsis. The mechanisms underlying this differential tolerance to morphine are complex and not fully understood. Morphine exposure leads to MOR desensitization after phosphorylation by G protein-coupled receptor kinases (GRKs) and the recruitment of β-arrestins. Previous studies by Bohn et al. indicate the importance of β-arrestin 2 in anti-nociceptive tolerance to morphine. They demonstrate that chronic morphine-induced analgesic tolerance is not seen in β-arrestin 2 knockout mice (Bohn et al., 1999). In the GI tract, Kang et al. investigated the role of β-arrestin 2 in differential tolerance to long-term morphine in the ileum and colon. According to their findings, chronic morphine administration produced a decrease in β-arrestin 2 levels in the ileum but not the colon. Thus development of tolerance in the ileum correlates with down-regulation of β-arrestin 2. The colon, which does not produce tolerance to repeated morphine exposure, shows maintained β-arrestin 2 levels (Kang et al., 2012). In accordance with these findings, in the β-arrestin 2 knockout mice, along with the ileum, tolerance to morphine also developed in the colon. Thus, while lack of β-arrestin prevents tolerance development to the central effects, such as analgesia, tolerance was seen in the colon when β-arrestin was knocked out.

In line with the understanding of the β-arrestin pathways, the concept of biased agonists has recently been developed. Biased ligands are opioid agonists that have greater affinity for one of the signaling pathways, either G-protein activation or β-arrestin recruitment. TRV-130 is one such compound with strong bias toward G-protein activation and reduced β-arrestin 2 recruitment than morphine (DeWire et al., 2013) (Soergel et al., 2014). Preliminary studies show that TRV-130

exhibits strong analgesic effects comparable to morphine but lacks the side-effects such as constipation.

B) Chronic morphine-induced bacterial translocation and intestinal inflammation

Together with constipation as a major side-effect in the GI tract, several recent clinical and animal studies have collectively suggested a role of morphine and opioid receptor activation in the development of gut bacterial infections. The GI tract is colonized by 100 trillion commensal micro-organisms, known as gut microbiota, that symbiotically evolve with the host cells. The gut microbiota play an important role in gut hemostasis and in protection of the host from diseases. The commensal micro-organisms inhibit the colonization of pathogenic microbes in the gut lumen by competing for nutrients. The gut microbes are also essential for maintenance of gut epithelial barrier integrity as well as for differentiation and maturation of intestinal immune cells (Nagao-Kitamoto et al., 2016).

Under physiological conditions, there is a symbiotic relation between the gut microbiota and host, which is directed towards the maintenance of host health. However, various factors such as stress, diet and medications are known to perturb this balance leading to bacterial dysbiosis. Furthermore, intestinal bacterial overgrowth combined with a leaky epithelial barrier and impaired immune function leads to translocation of bacteria from the gut lumen to extra-intestinal organs and systemic circular. Bacterial translocation progresses to the development of sepsis and is commonly seen in patients with burn injuries and shock (Kamada et al., 2013).

It is now well established that morphine causes bacterial dysbiosis in the gut lumen and translocation of intestinal bacteria to extra-intestinal organs. The primary evidence of morphine-induced bacterial translocation was noted in early 1990s. At that time, Runkel N and his group were studying the role of prolonged intestinal transit in disrupting intestinal microflora and thereby

causing bacterial translocation. In their study, morphine was used as an agent to produce pharmacological inhibition of GI transit. Interestingly, continuous subcutaneous infusion of 20 mg/kg morphine for up to 24 h in male Sprague-Dawley rats was found to not only slow GI transit but also increase bacterial counts, predominantly of Gram-negative rods, throughout the entire small intestine (duodenum, jejunum, ileum) and cecum (Runkel et al., 1993). Moreover, bacterial translocation to mesenteric lymph node (MLN), liver and spleen was observed in the morphine-treated animals. A similar observation was reported by Hilburger et al. in 1997 in C57BL/6J mice subcutaneously implanted with 75 mg morphine pellets for 24 or 48 hrs. Morphine treatment was found to enhance bacterial colonization of P. mirabilis and enterococci in the peritoneal cavity, spleen and liver and this effect was blocked in the presence of μ-opioid antagonist, naltrexone. Furthermore, morphine-pelleted mice became hyper-susceptible to a sublethal endotoxin challenge and this was reversed by simultaneous implantation of a naltrexone pellet (Hilburger et al., 1997). Recent findings by Meng at al. demonstrate that along with bacterial translocation, mice also exhibit intestinal inflammation after 24 h exposure to morphine (Meng et al., 2013).

The mechanism underlying compromised gut immune function, bacterial translocation and intestinal inflammation following morphine treatment was hypothesized to be mediated by Toll-like receptor (TLR) activation. TLRs are pattern recognition receptors that recognize patterns of microbial origin. Activation of these receptors stimulates downstream signaling pathways leading to release of inflammatory cytokines and chemokines (Takeda and Akira, 2004). TLR signaling is an essential component of innate immune responses in the gut. TLR4, in particular, is activated by lipopolysaccharide (LPS), a component of the cell membrane of gram-negative bacteria. LPS is a pathogen associated molecular pattern (PAMP) that binds to TLR4. LPS is made up of three molecules: 1) the hydrophobic section is composed of Lipid A molecules, 2) the hydrophilic core

polysaccharide chain consists of an inner and an outer core and 3) the hydrophilic O-antigenic oligosaccharide is specific to the bacterial type. The Lipid A molecule contributes to most of the biological effects of LPS and is used in place of LPS in some studies as an agonist of TLR4. LPS binds to LPS binding protein (LBP) which transports LPS to the cell membrane. LBP is a soluble protein. LBP is not absolutely necessary for activation of TLR4 receptor. The LPS-LBP complex further binds to CD14. CD14 exists either in a soluble (sCD14) or a membrane bound (mCD14) form. CD14 facilitates the binding of LPS to the MD-2 (a co-receptor of TLR4) which then forms a complex with TLR4. Binding of LPS results in homo-dimerization of the TLR4 receptor. Binding and recognition of LPS by TLR4, recruits a series of adapter molecules which follow either a MyD88 dependent or MyD88-independent (TRIF dependent) pathway. Activation of the downstream signaling cascade results in the release of the critical pro-inflammatory cytokines and/or type I interferons causing inflammation (Wang et al., 2015). Excessive activation of this pathway is thought to lead to septic shock.

Increased intestinal permeability and inflammation is observed in morphine treated mice due to disrupted localization of two proteins integral for the formation of epithelial tight-junctions, namely occludin and zona occludens-1 (ZO-1). The role of TLRs in morphine-induced bacterial translocation was confirmed using TLR2 and TLR4 knock out mice. Morphine-treated TLR2 and 4 knockout mice demonstrate continuous expression of occludin and ZO-1 and show lower bacterial translocation into MLN and liver (Meng et al., 2013).

Morphine-induced intestinal inflammation remains persistent with chronic morphine treatment. This is surprising because activation of TLRs is characterized by an initial over-shoot of cytokines, which is then followed by a silencing phase, where pro-inflammatory cytokine production is suppressed with time. This is referred to as endotoxin tolerance. However, morphine is known to

abrogate natural endotoxin tolerance, resulting in sustained inflammation. Studies have pointed to a role of micro-RNA miR-146a in mediating endotoxin tolerance. The TLR4 agonist, LPS, enhances miR-146a, which then inhibits certain downstream signaling molecules of TLR4 signaling, leading to decreased inflammation over time. Chronic morphine treatment significantly decreases LPS-induced mir-146a expression in macrophages, which prevents downregulation of IRAK-1and TRAF-6 (targets for miR-146a). IRAK1 and TRAF participate in downstream signaling of all major TLR pathways (except TLR3) and thus contributes to persistent inflammation seen with chronic morphine (Banerjee et al., 2013).

In recent years, several studies have focused on investigating the effect of inflammation on morphine-mediated GI inhibition and resultant constipation. Using acute and chronic intestinal inflammation mouse models, where experimental inflammation was induced using croton oil, Puig et al., demonstrated that increase in intestinal inflammation enhances the potency of a single acute dose of morphine towards inhibition of GI transit (Puig and Pol, 1998). Inflammation also upregulates μ-opioid receptor expression in mouse intestine. Previously, inflammatory cytokines, specifically IL-6, have been shown to modulate the expression of the MOR gene. The mechanism involved in inflammation mediated morphine-induced GI inhibition and increase in MOR expression was further investigated by Pol et al. In their study, nitric oxide, a mediator of numerous physiological processes and inflammatory conditions in the gut, was evaluated as a target. Peripheral gut inflammation due to croton oil was found to enhance nitric oxide levels and in turn increase the expression of MOR in the gut, resulting in impaired gastrointestinal transit. The anti-transit effects of morphine were reversed in mice treated with NOS inhibitors ((NG-nitro-L-arginine-methyl ester hydrochloride (L-NAME)) and (L-N6-(1-iminoethyl)-lysinehydrochloride (L-NIL)) (Pol et al., 2005). These findings collectively establish that the acute anti-transit effects

of morphine are modulated in the presence of experimental inflammation. It is known that chronic morphine treatment enhances inflammation in the gut. This dissertation will evaluate the role of this secondary inflammation produced by chronic morphine on OIC.

C) Effect of inflammation on morphine`s anti-nociceptive properties

As described earlier, chronic use of morphine is associated with central side-effects including tolerance, dependence, withdrawal and hyperalgesia. These adverse effects compromise the efficacy and beneficial properties of morphine as an analgesic. Through many decades, efforts have focused on elucidating the neuronal mechanisms underlying the development of opioid tolerance, dependence and withdrawal. However, with increasing understanding of the role of opioids in altering the resident gut microbial composition and inducing bacterial translocation, some recent studies have been directed towards evaluating the gut-brain axis, specifically the role of gut microbes in modulating morphine`s central effects (Rousseaux et al., 2007) (Theodorou et al., 2014) (Collins and Bercik, 2013). Our current findings demonstrate that depletion of colonic bacteria with a broad-spectrum antibiotic treatment (ABX) prevents many of the pathologic changes occurring in the gut with chronic morphine treatment, including gut epithelial barrier permeability, bacterial translocation, and inflammation. Concurrent with their effect in the GI tract, ABX prevents the development of anti-nociceptive tolerance following chronic morphine treatment. The role of dorsal root ganglion (DRG) neurons in mediating the reversal of opioid-induced analgesic tolerance is now being evaluated. It has previously been reported that nociceptive DRG neurons projecting from the colon are hyper-excitable following chronic morphine treatment, suggesting that these neurons are tolerant to the inhibitory actions of morphine. This is likely due to enhanced inflammation in the colon due to chronic morphine

treatment, which has previously been reported to increase the excitability of sensory neurons projecting to the DRGs (Ross et al., 2012) (Chen et al., 2012). In accordance with these findings, our study shows that DRG neurons isolated from mice chronically treated with morphine are more excitable as seen by their lower threshold potential compared to the placebo group. However, neurons isolated from ABX treated mice chronically treated with morphine continue to respond to morphine, indicating a lack of tolerance. Thus, the reversal in tolerance observed in the DRG neurons was consistent with the behavioral test for anti-nociception. It is therefore likely that gut bacteria-induced colonic inflammation affects the excitability of DRG neurons and modulates the development of tolerance. Studies have shown that morphine causes bacterial dysbiosis and specific changes in the composition of the gut microbes. These changes in the composition of the bacteria might be important in the development of analgesic tolerance.

2) Enteric glia

Previous studies evaluating the effects of chronic morphine on the ENS and mechanisms involved in OIC have mostly focused on morphine`s actions on neuronal excitability. However, there is growing interest about the role of morphine in facilitating bacterial translocation and enhancing related inflammation. This possibly alters persistent constipation commonly seen with chronic morphine use. With respect to potential pathways mediating morphine-induced secondary inflammation, the effect of chronic morphine on enteric glia, also known as immune cells of the ENS, needs to be assessed.

A) Structure and Organization of the Enteric Nervous System (ENS)

The ENS, often referred to as the second brain, is an intrinsic nervous system of the GI tract that extends from the esophagus to anus. It is a division of the autonomic nervous system that provides local control over some important GI functions. The ENS plays a key role in regulating blood flow, secretion, absorption, propulsion and interaction with the immune system of the gut. The ENS is composed of two intrinsic networks: submucosal plexus and myenteric plexus. The submucosal plexus is embedded between the circular muscle and the mucosa, while the myenteric plexus lies between the two muscle layers, longitudinal and circular. The submucosal plexus controls absorption and secretion in the GI tract while the myenteric plexus is essential for regulating the propulsion and motility patterns in the gut (Furness, 2012). Both the plexuses consist of bundles of cell bodies of neurons, termed as ganglia, connected by nerve fibers and surrounded by enteric glia. Thus, the ENS comprises of two main cell types: neurons and glia. Neurons in the ENS have been comprehensively studied with respect to their role in modulating GI functions. Enteric neurons are categorized according to the major neurotransmitter they release as either excitatory (ACh, ATP) or inhibitory (NO, VIP). By means of this neurotransmission, enteric neurons signal to neighboring neurons and glial cells (Furness et al., 2014).

The submucosal and myenteric plexus ganglia consists of cell bodies of three types of neurons: sensory, motor and interneurons. The bolus in the gut lumen activates stretch receptors on enterochromaffin cells lining the lumen and induces them to release serotonin. This in turn activates the nerve endings of sensory neurons, whose cell bodies lie in the myenteric ganglia. Sensory neurons further induce the excitatory motor neurons to cause a contraction behind the bolus and inhibitory motor neurons to cause a relaxation in front of the bolus. These rhythmic

contractions and relaxation initiate a peristaltic movement that pushes the bolus from the oral to anal end (Bayliss and Starling, 1901) (Bornstein et al., 2004) (Kunze and Furness, 1999).

B) Classification of enteric glia

The term enteric glia was coined by Giorgio Gabella in the early 1980`s when enteric glia were classified as a distinct cell type in the enteric ganglia. Several studies are now geared toward understanding the complex interactions between neurons and glia in the ENS (Komuro et al., 1982). With the development of new techniques to classify and study enteric glia, more attention is now drawn towards their role in the physiology and pathophysiology of the gut.

Enteric glia are often compared to astrocytes in the CNS. Morphologically, both cell types have an irregular, stellate structure having networks of highly branched and densely packed projections called gliofilaments (Reichenbach et al., 1992). These processes express robust immunoreactivity for vimentin during development and glial fibrillary acidic protein (GFAP) during adulthood. In spite of similarities between enteric glia and astrocytes, they exhibit remarkable differences with respect to their developmental origin. While CNS astrocytes are derived from precursor cells that line the neural tube, enteric glia are derived from the neural crest and arise from the ectoderm. The first glial precursors appear at E11.5 and glial differentiation occurs in the presence of growth factors produced by gut mesenchyme, after the neuronal progenitors start to differentiate. Notch signaling and Sox10 are important factors known to inhibit enteric neuron differentiation and promote gliogenesis. Glia-derived neurotropic factor (GDNF), on the other hand, induces neuronal and glial differentiation at low concentrations and stimulates solely neuronal differentiation at higher concentrations (Coelho-Aguiar Jde et al., 2015) (Laranjeira and Pachnis, 2009). This

difference in embryonic origin might explain some of the functional variabilities seen between enteric glia and astrocytes.

Enteric glia have a higher expression of GFAP compared to the astrocytes, presumably due to the influence of the gut microenvironment. GFAP is the most commonly used marker for enteric glia (Jessen and Mirsky, 1980). However, since it does not stain the nuclei, it cannot be used for glial quantification. Sox10, SRY-related HMG-box transcription factor, is a better marker for quantification purposes since it stains the glial nuclei (Hoff et al., 2008). The calcium binding protein S100β is another glial marker and is localized to the glial cytoplasm (Ferri et al., 1982). Although most enteric glia express these common glial markers and have similar developmental origin, they consists of distinct subpopulations which might determine their functional heterogeneity.

From the early 1980s to 1990s, researchers attempted to categorize enteric glia using different means of classifications Fig 1. Gabella classified enteric glia based on location (Gabella, 1984). Geoffrey Burnstock and Hanini further divided myenteric/intra ganglionic enteric glia according to their morphology, namely the shape of the cell body and projections (Komuro et al., 1982) (Hanani and Reichenbach, 1994). The four major classes of enteric glia based on location and morphology are: 1) Intra ganglionic/type I- star-shaped enteric glial cells residing within the myenteric and submucosal ganglia that resemble protoplasmic astrocytes 2) Inter ganglionic/ type II- elongated enteric glial cells within inter ganglionic fiber tracts that resemble fibrous astrocytes of white matter in the brain 3) Mucosal/ type III - enteric glial cells with several long-branching projections found below the mucosal epithelial cells 4) Intramuscular/ type IV- elongated enteric glial cells associated with nerve fibers in the circular and longitudinal muscles.

The intra ganglionic (Type I) and inter ganglionic (Type II) enteric glia are in closest proximity to enteric neurons in the ganglia. Initially, enteric glia were only thought to provide physical/mechanical support to the enteric neurons in the ganglia. However, more and more studies are now establishing novel roles of enteric glia in the ENS. Similar to CNS astrocytes, enteric glia play a major role in neuron-glia cross talk and enteric neurotransmission. Stimulation of inter ganglionic neurons has been shown to induce intracellular Ca^{2+} signaling in enteric glial cells, thus demonstrating neuron-glia communication. This was blocked by tetrodotoxin (TTX), a voltage-gated Na^{+} channel blocker (Gulbransen and Sharkey, 2009).

Intra ganglionic glia provide neurons with anti-oxidants like glutathione (Abdo et al., 2010), growth factors like glia-derived neurotrophic factor (GDNF) (von Boyen et al., 2011)and neurotransmitter precursors such as glutamine synthase and l-arginine, which are essential for the synthesis of nitric oxide (NO), γ-aminobutyric acid (GABA) and glutamate (Nagahama et al., 2001). Enteric glia express cell surface enzymes that can either generate or degrade specific neuroactive substances and neurotransmitters in the extracellular milieu. Eg- Enzyme ectoATPase expressed on glial cells hydrolyzes extracellular ATP and regulates activation of purinergic signaling in enteric neurons (Braun et al., 2004). Enteric glia also play a role in preventing neurotoxicity by buffering the levels of extracellular GABA and potassium ions (Fletcher et al., 2002).

The mucosal or type III enteric glia, which are present directly beneath the epithelium, play a major role in maintaining the gut epithelial barrier integrity and containing the commensal bacteria and other pathogenic microbes within the gut lumen. The mucosal enteric glia influence the differentiation and proliferation of epithelial cells lining the gut lumen by secreting certain growth factors such as pro-epidermal growth factor (proEGF) and transforming growth factor (TGF)-β1,

S-nitrosoglutathione (GSNO), and active lipids such as 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) (von Boyen et al., 2006). *In vivo* models of conditional ablation of mucosal enteric glia (GFAP-positive cells in the gut) demonstrate enhanced epithelial permeability (Cornet et al., 2001). This compromise of gut epithelial barrier integrity can be a direct effect of mucosal glia on epithelial cell differentiation or can be due to an indirect effect of secondary inflammation caused by glial depletion. Similar observations were made when GSNO release by enteric glia was inhibited. GSNO is known to protect intestinal epithelial cells from invasion by Shigella flexneri, an enteroinvasive pathogen that causes dysentery (Flamant et al., 2011).

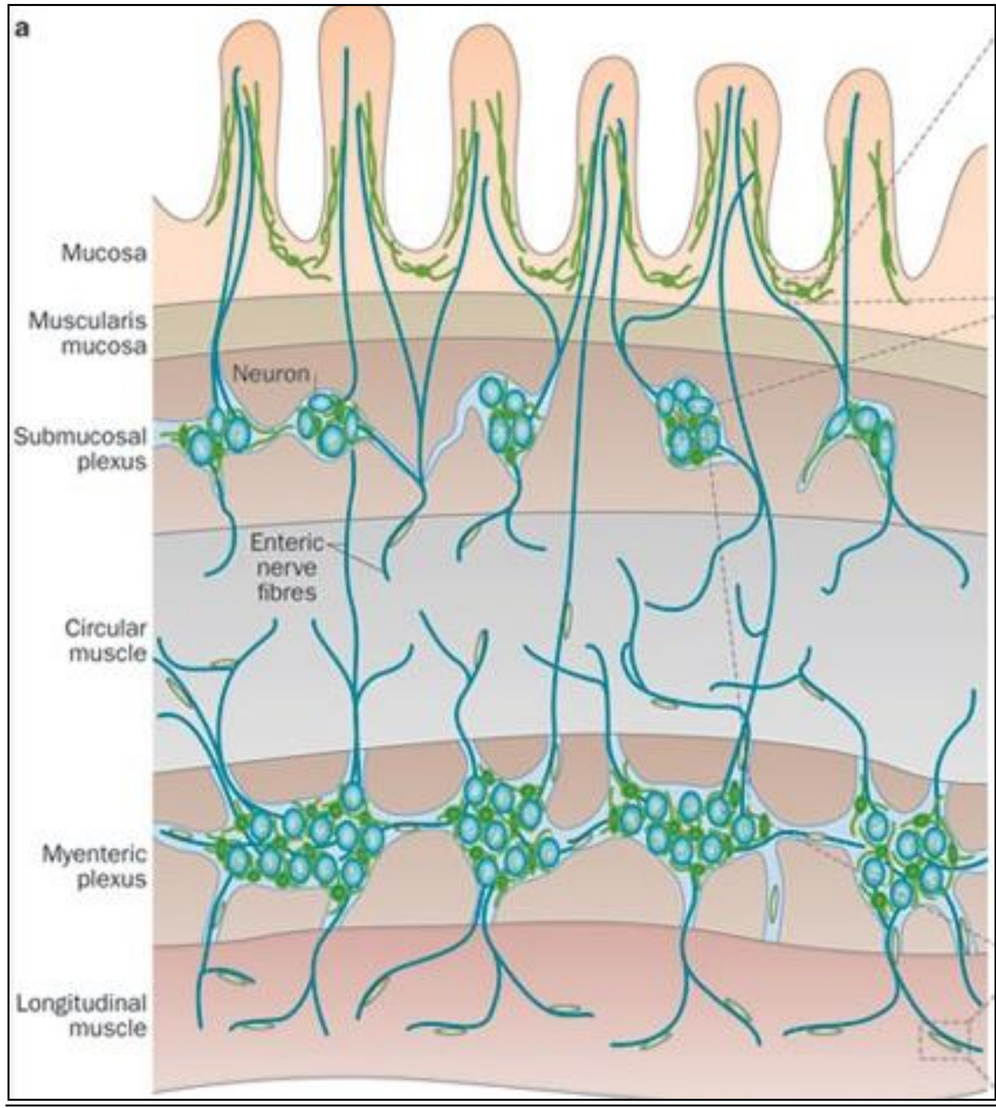


Figure 1: Classification and localization of enteric glial cells (in green). Image modified from (Gulbransen and Sharkey, 2012)

C) Role of enteric glia in GI motility disorders

Indirect evidence points to the role of enteric glia in controlling gastrointestinal motility. In patients with slow-transit constipation (STC), a significant reduction in the number of S-100 positive glial cells was observed in both the submucosal and the myenteric plexus in the terminal ileum. This decrease in glial cells did not seem to affect the morphology, integrity or cell counts of other cell types like enteric neurons, fibroblast-like cells and smooth muscles (Bassotti et al., 2006). Similar studies in animal models utilized pharmacological or genetic approaches for glial ablation. Nasser Y, et al. evaluated the effect fluorocitrate (FC), a widely used gliotoxin, on intestinal motility, secretion, and inflammation. FC increased glial expression of the phospho ERK-1/2 in isolated guinea pig ileal segments. This alteration in glial function by FC resulted in a decrease in small intestinal transit without any effect on colonic transit. Also, decrease in enteric glial numbers was not accompanied by intestinal inflammation (Nasser et al., 2006). Another study by Aube AC, et al. induced glial ablation by injecting activated hemagglutinin (HA) specific CD8+ T cells in transgenic mice expressing HA in glia. They observed that glial disruption led to changes in the proportions of ChAT and NOS positive neurons in the myenteric plexus. Reduction in glial numbers also resulted in slowing of gastrointestinal transit, increased intestinal permeability and inflammation (Aube et al., 2006). A similar decrease in glial and neuron counts was observed in the myenteric plexus of aged rats (26 months old) as compared to adult rats (5-6 months old). This loss of the ENS innervation of neurons and glia in aging animals is thought to be a possible mechanism for the loss of GI function and slowing of GI transit that is observed with aging (Phillips et al., 2004). Altogether, these findings suggest that enteric glia play an important role in regulating gastrointestinal motility.

3) Purinergic signaling in the gut

Several studies have implicated a key role of purine/pyrimidine neurotransmitters and their receptors in the regulation of GI inflammatory conditions as well as motility. Understanding the role of the purinergic pathway in morphine-induced inflammation and constipation might point to novel mechanisms involved in OIC.

A) Classification, localization and functions of purinergic receptors

Purinergic neurotransmitters are purine based molecules such as adenosine, adenosine 5`-diphosphate (ADP), adenosine 5`-triphosphate (ATP) and pyrimidine based molecule uridine 5`-triphosphate (UTP). The concept of ATP as a neurotransmitter was rejected by several scientists since ATP was a well-known intracellular energy source involved in the Krebs cycle and to consider ATP as an extracellular signaling molecules seemed implausible (Burnstock et al., 2010). Separate receptors for these purines and pyrimidines were identified in 1978, almost a decade after the discovery of ATP as a neurotransmitter (Burnstock et al., 1997). This class of receptors is termed as `purinoreceptors` or `purinergic receptors` and they are expressed on the cell surface of a variety of cell types throughout the body. Purinergic receptors are further divided into P1 and P2 subtypes based on agonist potencies at these receptors (Ralevic and Burnstock, 1998).

P1 receptors- These receptors are G-protein coupled receptors that are activated by adenosine. Similar to other G-protein coupled receptors, P1 receptors have a seven transmembrane domain with a cytosolic C-terminus and extracellular N-terminus. P1 receptors are divided into four subtypes: A1, A2A, A2B and A3. Of these, A1 and A3 receptors are coupled with inhibitory Gi G-proteins and activation of these subtypes by adenosine leads to inhibition of downstream adenylate cyclase and decrease in cAMP. Exogenous adenosine is known to inhibit intestinal motility by depressing peristalsis in the small and large intestine, mainly due to stimulation of A1

receptors (Suzuki et al., 1995). A1 receptor activation inhibits the release of the excitatory transmitter, Ach, from the motor neurons that project to the circular and longitudinal muscle layers of the GIT tract, resulting in decreased muscle contraction and reduced peristalsis (Lee et al., 2001) (Nitahara et al., 1995). Some studies have noted the presence of A1 receptors on smooth muscles, which leads to direct inhibition and relaxation of these muscles to alter GI motility. Other studies suggest a role of A1 receptor activation in depressing activity evoked by sensory stimuli and decreasing excitability of the mechanoreceptive and/or chemoreceptive intrinsic sensory (AH/Dogiel type II) neurons. Adenosine mediated A1 receptor activation leads to hyperpolarization of these neurons and suppresses the slow EPSPs (excitatory post-synaptic potentials) and enhances AHPs in these neurons which results in reduced firing (Thomas and Bornstein, 2003). On the other hand, A2A and A2B subtypes are coupled with Gs G-proteins and activation of these subtypes by adenosine results in increase in cAMP. Their role in regulating motility is not fully understood.

P2Y receptors- P2X and P2Y receptors are the two most widely studied subtypes of P2 receptors. P2Y receptors are metabotropic (G-protein coupled) receptors that are activated by ADP, ATP and UTP. Similar to P1 receptors, P2Y receptors are heptathelical, consisting of seven transmembrane domains with a cytosolic C-terminus and extracellular N-terminus. The P2Y receptor family consists of eight members (P2Y1, 2, 4, 6,11,12,13 and 14) that have been cloned in humans. Of these, the P2Y1, 2, 4, 6 and 11 subtypes (P2Y1-like receptors) are coupled with Gq G-proteins and activation of these receptors leads to activation of phospholipase C and generation of inositol phosphates and release of Ca^{2+} store. The P2Y12, 13 and 14 subtypes (P2Y12-like receptors) are coupled with inhibitory Gi G-proteins and activation of these subtypes by their natural ligands results in inhibition of downstream adenylate cyclase and decrease in cAMP.

In the ENS, calbindin positive AH/Dogiel type II myenteric neurons exhibit strong immunoreactivity for P2Y12 receptors in guinea pig intestine. Some calretinin positive neurons also express P2Y2 and P2Y6 receptors while P2Y6 receptors are also present on a small percentage of inhibitory NOS positive neurons. P2Y1 receptors have been identified in the myenteric as well as submucosal plexus and were found to co-localize with some NOS expressing neurons. They are proposed to be important in transmission between interneurons in the descending inhibitory pathway due to their role in evoking slow EPSPs. P2Y1 receptors are also suggested to be involved in sensory transduction in the gut wall. Mechanical deformation in the gut wall leads to release of ATP from the mucosa, which further activates P2Y receptors and evokes action potentials in adjacent myenteric AH/ Dogiel type II neurons.

P2X receptors- P2X receptors are ionotropic receptors (ligand gated ion channels) which when activated by their natural ligand ATP, open non-selective cation channels highly permeable to calcium, sodium and potassium. Several subtypes of P2X receptors (P2X1-7) have been cloned. These receptors form trimeric pores which are either homomeric (eg-P2X7) or heteromeric (eg-P2X2/3). The P2X1 and 3 receptors exhibit much faster desensitization, while P2X2, 4, 5 and 6 receptors desensitize slowly (Burnstock and Kennedy, 2011). P2X7 receptor is unique as compared to the other P2X subtypes. P2X7 requires 10-100 times higher concentration of ATP for activation as compared to other P2X subtypes. Also, activation of the P2X7 receptor leads to an initial opening of non-selective cation channels within a few milliseconds, however prolonged activation by an agonist results in opening of reversible pores that are permeable to certain large molecules up to 900 Da molecular weight, including NMDG (N-methyl-D-glutamine, 195 Da), choline (104 Da), and fluorescent dyes such as propidium (415 Da). Due to this pore forming property, P2X7 receptor is thought to be resistant to desensitization and can participate in positive

feedback signaling, an event that is of considerable interest in inflammation and apoptosis (Diezmos et al., 2016). The exact mechanism for this pore formation is not completely understood. Some studies suggest that the pore results from dilation of the P2X7 channel. However more growing evidence points to the presence of a second pore (eg- pannexin-1 hemichannel) which is activated following binding of ATP to P2X7 receptor. P2X7-mediated Pannexin-1 activation appears to be important for the release of large molecule dyes. This pore forming ability is now also studied in other P2X receptor types including P2X4, 5 and 6.

In the ENS, the AH/Dogiel type II sensory neurons express immunoreactivity for P2X2 and P2X7 receptor subtypes in the myenteric plexus. P2X3 receptors have been found on ascending interneurons while NOS positive neurons, which include either inhibitory motor neurons or descending interneurons mostly express P2X2 receptors. The expression of P2X5 receptors is confined to axons in the myenteric plexus and they are also abundantly present on the submucosal plexus neurons.

P2X receptors are implicated in enteric neurotransmission. Studies show that P2X receptors mediate transmission from descending interneurons to inhibitory motor neurons thus playing a role in the descending reflex pathways in the gut. This function is likely mediated by P2X2 receptors expressed on inhibitory NOS positive motor neurons. In P2X2 receptor knock-out mice, suppression of propulsive reflexes and inhibition of P2-mediated fast EPSPs in myenteric neurons has been noted, however these mice exhibited intestinal transit comparable to controls. Thus, the exact role of these receptors in regulation of GI motility is not completely understood (Bornstein, 2008).

B) Purinergic signaling in enteric glia

Purinergic neuron-glia communication in the ENS was collectively studied by several groups in the early 2000s. Just like enteric neurons, enteric glia too express receptors for purinergic neurotransmitters and respond to purines and pyrimidines. Purines are thought to be important in regulating both basal physiological and pathophysiological functions of enteric glia. In the myenteric plexus of guinea pig colon, it has been suggested that sympathetic nerves release ATP, which then activates enteric glia (Gulbransen et al., 2010). Immunohistochemical studies demonstrate predominant expression of P2X7 (Vanderwinden et al., 2003)and P2Y4 receptors (Van Nassauw et al., 2006) on enteric glia. Further evidence of neuron-glia interactions was provided by Gulbransen et al. where they showed that stimulation of myenteric neurons by ATP elicited increases in intracellular calcium in neighboring enteric glial cells. This activation of Ca waves in enteric glia was thought to be mediated by P2Y4 receptors (Gulbransen and Sharkey, 2009). Findings of an electrophysiology study in a primary enteric neuron–glia culture from mouse suggest that enteric neurons primarily express P2X receptors, while glial cells express P2Y receptors under physiological conditions. Apart from responding to ATP, enteric glia are also involved in the release of ATP via Cx43 hemichannels, which triggers intercellular Ca2+ waves in adjacent glia and neurons. Furthermore, enteric glia express an extracellular surface-bound ectonucleotidase, NTPDase2. This enzyme regulates the extracellular level of ATP following release from nerve terminals or that released by enteric glia itself (Braun et al., 2004).

C) Involvement of purinergic receptors in gut inflammatory conditions

Purinergic receptors, specifically P2X7R, have been largely explored in the context of inflammatory bowel disease (IBD) (Roberts et al., 2012). Some clinical data suggest that there is an upregulation of P2X7 receptor expression in the epithelium and lamina propria of colonoscopy samples of Crohn`s disease patients. P2X7 receptor was found to colocalize with immune cells such as dendritic cells and macrophages. Upregulation of P2X7 receptor expression correlated with increased levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-17, decreased levels of anti-inflammatory cytokine IL-10 and enhanced apoptosis in the epithelium and lamina propria of Crohn`s disease patients (Neves et al., 2014). Similar observations have been made in animal models of colitis. In a rat model of trinitrobenzene sulfonic acid (TNBS) induced colitis, a P2X7 receptor antagonists brilliant blue G (BBG) attenuated the severity of colitis and significantly reduced infiltration by T-cells and macrophages in the lamina propria (Marques et al., 2014). Furthermore, a P2X7 knock out mouse model did not exhibit decreased body weight, infiltration of mononuclear cells and increase in the microscopic damage score that was observed in the control group when colitis was induced using TNBS (Neves et al., 2014).

Like P2X7, other purinergic receptor subtypes are also implicated in IBD pathophysiology. In inflamed colon samples collected at colectomy from patients with active Crohn's disease, the number of P2X3 immunoreactive neurons were found to be markedly higher in the myenteric plexus compared to controls. A validated high-density gene microarray analysis established patterns of gene dysregulation and found an upregulation in purinergic receptor genes (P2X1, P2X4, P2X7, P2Y2, and P2Y6) in a rat TNBS colitis model. These changes in the expression of purinergic receptors were similar to that observed in IBD patients (Guzman et al., 2006). studies

have highlighted the potential of purinergic drugs for the treatment of functional bowel disorders (Burnstock, 2008).

D) The role of connexin hemichannels in GI disorders

Connexin hemichannels are structural precursors of gap junctions abundantly present in the GI tract as well other organ systems in the body. Gap junctions were discovered in 1967 and were studied in liver cells. Almost two decades later, in 1986, the first 2 connexins were cloned from liver cells (Paul, 1986) and these were also found to be present in the stomach of rats. Presently, 21 connexins have been identified in humans and the nomenclature is based on their molecular weight. Connexins have 4 membrane-spanning domains, 2 extracellular loops, a cytoplasmic loop and a cytoplasmic N and C-terminal. By acting as gap junctions between cells, connexins mediate the diffusion of chemical substances, including ATP, cyclic AMP and IP3 (Dbouk et al., 2009) (Decrock et al., 2009) between adjacent cells. More recently, new roles of these connexins are being investigated and studies suggest that connexins also participate in the exchange of small hydrophilic molecules such as nucleotides and ions between the cytosol and the extracellular environment (Alexander and Goldberg, 2003). This function of connexins is analogous to pannexins, which also share a similar structure to connexins. However, connexins and pannexins do not share sequence homology and are genetically unrelated (Baranova et al., 2004). Both these channels have been widely studied in conjunction with purinergic signaling pathways, due to their role in ATP release.

About ten connexin hemichannels have been found to be expressed in the GI tract, mainly in the ileum and colon. Cx26, Cx31, Cx32, Cx36, Cx40, Cx43 and Cx45 are found in both ileum and colon, while Cx37, Cx57 are only present in the small intestine and Cx31 is exclusively present in

the colon. Even in these tissues, specific connexins localize in different layers of the gut wall: epithelium, mucosa, submucosa, muscle layers, enteric neurons and glia. Eg- In humans, in colon tissue, Cx26 and Cx32 exhibit strong immunoreactivity in the intestinal epithelial cells, whereas Cx36, Cx40 and Cx43 are confined to cells in the myenteric plexus. Cx43, in particular, is mainly found in enteric glia and Cx36 in neurons (Diezmos et al., 2016).

Together, these connexin hemichannels play a key role in transfer of intercellular and extracellular signals. Cx26 alters the production of tight junction proteins in the intestinal epithelium and thus controls gut epithelial barrier integrity (Morita et al., 2004). Cx36 colocalizes with NOS^+ neurons in the myenteric plexus of mouse colon. Thus, it participates in inhibitory neurotransmission in the gut. Cx36 knockout mice exhibit spontaneous contractile properties due to absence of this inhibitory nerve transmission (Nagy et al., 2014). Cx43 is the most studied subtype of connexin hemichannels with respect to intestinal inflammation and GI motility. In mouse colon, Cx43 is important for mediating calcium responses in enteric glia, which is a proposed mechanism by which it moderates colonic transit and GI motility (Lurtz and Louis, 2007) (McClain et al., 2014). Furthermore, Cx43 also plays an important role in innate immune control of commensal-microbiota mediated regulation of intestinal epithelial barrier repair. In an ulcerative colitis mouse model, activation of TLR2 was found to enhance Cx43 expression leading to an increase in intercellular signaling in intestinal epithelial cells in that way that regulated gut barrier function.

Aims

The overall goal of this project was to study the effects of chronic morphine on the ENS, specifically enteric glia, in order to expand our understanding of the mechanisms involved in mediating morphine`s adverse effects, namely constipation and analgesic tolerance.

The specific aims of this study were:

Aim 1: To characterize the electrophysiological properties of enteric glia

Aim 2: To study the effect of chronic morphine on connexin/purinergic signaling and associated inflammation in enteric glia

Aim 3: To evaluate the role of connexin hemichannels in morphine-induced constipation and analgesic tolerance

Chapter II

Materials and Methods

Drugs and chemicals: The following drugs and chemicals were used: Lipopolysaccharide/LPS (Sigma-Aldrich, MO), ATP (Sigma-Aldrich, MO), PPADS (Abcam, MA), Carbenoxolone (CBX) (R&D Systems, MN), Gap26 (Anaspec, CA). Morphine sulfate and morphine pellets (75 mg) were obtained from National Institutes of Drug Abuse (Bethesda, MD).

Animals: All the animal protocols were conducted in accordance with the procedures reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University (VCU IACUC). Adult male Swiss Webster mice (SW) (Harlan Sprague Dawley, Inc. Fredrick, MD, USA) weighing about 25-30g and housed five mice per cage in 12/12 light/dark cycle were utilized for this study. Access to food and water was available *ad libitum* throughout the experiments.

Surgical Implantation of Morphine pellets: For chronic morphine administration, SW mice were implanted with 75 mg/kg morphine pellets as previously described (Ross et al., 2008). 2.5 % isoflurane was used to anesthetize the mice after which the hair on the back of their neck was shaved. Povidone iodine 10% and ethanol 70% was used to clean the bare skin. A 1 cm transverse incision was made into the subcutaneous space toward the dorsal flank using sterile surgical instruments and a placebo/morphine pellet was placed distal to the incision. The incision was closed with sterile auto clips. Mice were allowed to recover in their home cages after the surgery and assessed every 48 h for signs of infection and weight loss.

Cell cultures: The rat enteric glial cell line CRL-2690 (ATCC) was cultured in DMEM (4.5 g/l glucose) supplemented with 10% FBS, 1 mM glutamine and penicillin/streptomycin.

Isolation of primary enteric glia from the adult mouse myenteric plexus: Mixed cultures of enteric neurons and glia were isolated as previously described (Smith et al., 2013). After euthanizing the mice, the colon tissue was collected and placed in ice-cold Krebs solution (in mM: 118 NaCl, 4.6 KCl, 1.3 NaH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11 glucose, and 2.5 $CaCl_2$, bubbled with carbogen 95% O_2/5% CO_2). The fecal pellets were flushed out with ice-cold Krebs. The tissue was cut into short segments and threaded longitudinally on a plastic rod. The longitudinal muscle layer along with the myenteric plexus (LMMP) was gently stripped using a cotton-tip applicator. LMMP strips were minced and digested in 1.3 mg/ml collagenase Type II (Worthington) for 1 h, and then incubated in 0.05% trypsin for 7 min. Tissue was triturated and collected using centrifugation after each digestion step. Cells were then plated on laminin (BD Biosciences) and poly-D-lysine-coated coverslips in DMEM (4.5 g/l glucose) supplemented with 10% FBS, 1 mM L-glutamate and penicillin/streptomycin.

Electrical Recordings: Whole-cell patch-clamp recordings were made using an Axopatch 200B amplifier (Molecular Devices). Voltage and current clamp protocols were performed on enteric glial cell line (CRL-2690) and glia isolated from colon LMMP of SW mice on day 1 and 2 after isolation. Cells were plated on coverslips and placed in a perfusion chamber continuously perfused with external solution containing (in mM; 135 NaCl, 5.4 KCl, 0.3 NaH_2PO_4, 1 $MgCl_2$, 5 glucose and 2 $CaCl_2$, pH adjusted to 7.4 using 1M NaOH). Patch electrodes were pulled from borosilicate glass capillaries using Flaming-Brown horizontal puller (P-87; Sutter Instruments, Novato, CA). Resistance of the patch electrodes used was 2-6 MΩ. Patch electrodes 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). In voltage-clamp mode, currents were measured by holding the cell at −60 mV and giving voltage pulses from −100 mV to 50 mV at intervals of 10 mV. In gap-free mode, recordings were

made at a holding potential of −60 mv and by perfusing ATP in the bath for 10-15 secs. In current clamp mode, the presence or absence of action potentials was determined by injecting the cells with current ranging from −0.03 nA to 0.09 nA in 0.01 nA increments.

RT-PCR: Quantitative real time PCR was performed using RNA extracted from the enteric glial cell line (CRL-2690) treated with LPS or morphine, and RNA extracted from the colon tissue collected from SW mice treated with either 75 mg placebo or morphine pellets for 5 days. RNA was isolated using Trizol (Invitrogen) following the manufacturer's protocol. The concentration and purity of RNA samples was quantified using a spectrophotometer (Bio-Rad Smart Spec Plus). Real time PCR was performed with iTaq Universal SYBR Green One-Step Kit (Bio-Rad) using 200 ng total RNA per reaction. 18s was used as an internal control. Expression of each gene was normalized to 18s. All PCR data is plotted as a percentage of maximum normalized gene expression. All experiments were performed in duplicate from 3-6 separate samples.

Primers used are listed in table

Rat	P2X4 F	5'-CCCTGGCTACAACTTCAGATAC-3'
Rat	P2X4 R	5'-TCGTCCACAAAGGACACATAC-3'
Rat	P2X7 F	5'-AATGAGTCCCTGTTCCCTGGC-3'
Rat	P2X7 R	5'-AATACACACGGTGGCCAAACC-3'
Rat	Cx43 F	5'-CTGTACTTGGCTCACGTGTTCTAT-3'
Rat	Cx43 R	5'-CGTGGGAGTTGGAGATGGTGC-3'
Rat	IL-6 F	5'-CCTGGAGTTTGTGAAGAACAACT-3'

Rat	IL-6 R	5'-GGAAGTTGGGGTAGGAAGGA-3'
Rat	TNFα F	5'-TGTCGATGCCTGAGTGGAT-3'
Rat	TNFα R	5'-AGGGAGGCCTGAGACATCTT-3'
Rat	IL-1β F	5'-CACCTTCTTTTCCTTCATCTTTG-3'
Rat	IL-1β R	5'-GTCCTTGCTTGTCTCTCCTTGTA-3'
Mouse	IL-1β F	5'-CCTGAACTCAACTGTGAAATGC-3'
Mouse	IL-1β R	5'-CGAGATTTGAAGCTGGATGC-3'

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

Immunocytochemistry: Mixed primary cultures isolated from colon LMMP of SW mice were plated on coverslips and cultured for 3-5 days. Cells were fixed in 4% formaldehyde for 30 min. Cells were permeabilized with 0.01% Triton X-100 in PBS for 30 min and blocked with 10% goat serum for 1 h. Cells were incubated with the primary antibody overnight at 4°C. Cells were washed with PBS and incubated with the secondary antibody for 1 h at room temperature. Primary antibodies used were as follows: astrocytic marker GFAP (mouse, Millipore, 1:500), β-III tubulin (1:1000), μ-opioid receptor MOR (rabbit, Alomone, 1:500). Secondary antibodies used were as follows: Alexa-594 Dye (anti-mouse, Molecular Probes, 1:1000), Alexa-594 Dye (anti-rabbit, Molecular Probes, 1:1000) and Alexa-488 Dye (anti-rabbit, Molecular Probes, 1:1000). Images of immunofluorescence labeling were taken with an Olympus Fluoview Confocal Microscope and software (version 5.0).

Determination of neuronal density: Whole-mount LMMP preparations of colonic myenteric plexus were prepared by removing the mucosal layers and circular muscle layers as previously described (Maguma et al., 2014). Colon LMMP preparations were fixed in 4% formaldehyde overnight. Tissues were then permeabilized with 0.5% triton x 100 for 1 h before immunostaining. Tissue were blocked with 10% goat serum for 1 h and treated with anti-Hu (mouse, Invitrogen, 1:100) primary antibody overnight at 4ºC followed by Alexa 488 (anti-mouse, Molecular Probes, 1:1000) secondary antibody for 4 h. Images of the myenteric ganglia were taken under the confocal microscope. ImageJ software was used to analyze the neuronal packaging density in the myenteric ganglia. The surface area of the ganglia was calculated using the ImageJ software and the number of neurons per mm^2 were counted. At least ten ganglia were counted from each animal. All the studies were performed in a double blinded fashion.

Histology: The colon tissue was removed from SW mice pelleted with either placebo or 75 mg morphine pellets for 5 days and given i.p. injections of saline or CBX (25mg/kg or 50 mg/kg) on day 3 and 4. The fecal pellets were flushed out from the colon with Krebs. The tissue was cut open longitudinally and embedded in OCT for cryo-sectioning. 16-µm sections were cut, fixed in 4% paraformaldehyde and stained with hematoxylin and eosin (H&E).

ATP Assay: The rat enteric glial cell line (CRL-2690) was plated on a 24-well plate and treated with LPS (0-100 µg/ml) for 1 h with/without CBX or PPADS. After 1 h exposure to LPS ± Cx43/P2X antagonists, 100 µL aliquots of supernatant were collected from these cells and placed into a 96 well plate. ATP concentration in the supernatants was measured by a luciferase-based assay using an ATPLite™ Kit (Perkin Elmer, Waltham, MA) according to the manufacturer's instructions. Luminescence in the aliquots was measured using a microplate reader and ATP concentrations were calculated based on a standard curve.

Fecal pellet output assay: To measure whole GI transit, the fecal pellet output assay was performed in non-fasted mice pelleted with either placebo or 75 mg morphine pellets for 5 days and given i.p. injections of saline or CBX (25mg/kg) on day 3 and 4. On day 5 which was the test day, mice received a subcutaneous saline or 2/10 mg/kg morphine challenge. 20 min after the challenge injection, mice were placed into individual experimental cages. The number of fecal pellets expelled were recorded every 30 mins. All the studies were performed in a double blinded fashion.

Measurement of whole GI transit assay with red carmine dye: To measure whole GI transit, non-fasted mice were pelleted with either placebo or 75 mg morphine pellets for 5 days and given i.p. injections of saline or CBX (25mg/kg) on day 3 and 4. On day 5 which was the test day, mice received an oral gavage of 150 µl of non-absorbable carmine red dye 6% w/v in 0.5% carboxy methyl cellulose. Mice were placed into individual experimental cages. The time from gavage to appearance of the red dye in the fecal pellets was noted as total transit time. A cut-off time was set at 6 h. All the studies were performed in a double blinded fashion.

Tail-Flick Assay: The warm-water tail-flick test was utilized to measure somatic pain. The tail-flick response was elicited by immersing the distal 2-3 cm of the tail into a warm water bath maintained at 52 ± 0.1 °C. Latency time was measured as the time from the onset of tail immersion in the water bath to withdrawal of the tail. A 10-s maximal cut-off time was established to prevent tissue damage. The withdrawal latency time measured before administration of a morphine challenge was considered as baseline latency. The test withdrawal latency time was measured 20 min after a 10mg/kg subcutaneous acute morphine injection. Anti-nociception was quantified as the percentage of maximal possible effect (%MPE), which was calculated as %MPE = [(test

latency-baseline latency) / (10-baseline latency)] × 100. The %MPE value was calculated for each treatment group using up to 10 mice per group.

Data Analysis: Statistical analysis was performed using Graph Pad Prism 6 (GraphPad Software, Inc.). Parametric data were compared using Student's t-test. Multiple group comparisons were made by one-way ANOVA analysis followed by Tukey`s post-hoc test. The results are expressed as the mean value with SEM. *P value <0.05 was considered significant.

Chapter III

Characterization of the electrophysiological properties of enteric glia

Enteric glia, just like CNS astrocytes, are considered as electrically non-excitable cells, as they do not fire action potentials. However, recent advances in this field show that enteric glial cells, initially thought as quiescent cells, are constantly sensing changes to their microenvironment and responding to it. With growing interest in understanding the electrophysiological properties of these cells, studies suggest that enteric glial cells exhibit unique biophysical and functional electrical properties. This signaling likely mediates the bidirectional communication between glia and neurons in the ENS.

Some of the initial studies that demonstrated the electrophysiological properties of neurons or glia in the ENS, utilized either LMMP preparations or isolated cells from guinea pig intestine (Rugiero et al., 2002) (Clerc et al., 1998). The major drawback of the LMMP preparation is that the presence of a connective tissue layer over the ganglia and muscle movements underneath makes the use of patch electrodes difficult. These issues were overcome in the isolated neuron-glia cultures but their studies were confined to guinea pigs (Hanani et al., 2000). Previously, our lab has developed a methodology to isolate primary glia from the ileum and colon of adult mouse myenteric plexus (Smith et al., 2013). This technique provides the advantage of utilizing these cultures for electrophysiological or biochemical experiments in mice and further extending them to transgenic mouse models.

Properties of primary glial cells

The aim of the present study was to characterize the expression of ion channels and to investigate the electrophysiological properties of enteric glia. Like CNS astrocytes, enteric glia too express a

variety of ion channels for the movement of ions across the plasma membrane. These ion channels play an important role in regulating membrane potential and intracellular ion concentrations. Among them, K^+ channels are the most studied due to their involvement in maintaining the resting membrane potentials (RMP) (Farber and Kettenmann, 2005).

To study the electrophysiological properties of enteric glia, mouse primary glial cultures were utilized. Although electrical properties of enteric neurons have been previously studied, much less is known about membrane properties of enteric glia. In this study, whole cell patch-clamp technique was used to study the electrophysiological properties of enteric glia in culture. The primary isolated cultures of LMMP consists of both enteric glia and neurons from mouse colon Fig 2. Enteric glia were distinguished from neurons based on their inability to elicit action potentials with a positive current injection in current-clamp mode. On the other hand, neurons elicited action potentials since they are excitable cells and also displayed inward sodium currents upon depolarization in voltage-clamp mode. Inward currents were absent in glial cells Fig 3.

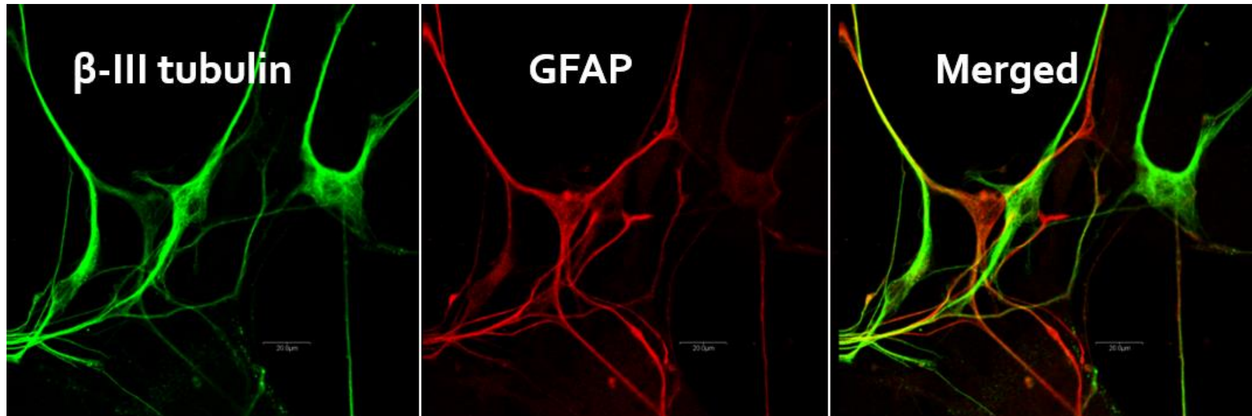


Figure 2: Mixed culture of enteric neurons and glia. Co-immunostaining of neuronal marker β-III tubulin and astrocytic marker GFAP in primary cultures isolated from the LMMP of adult SW mouse colon (Smith et al., 2013).

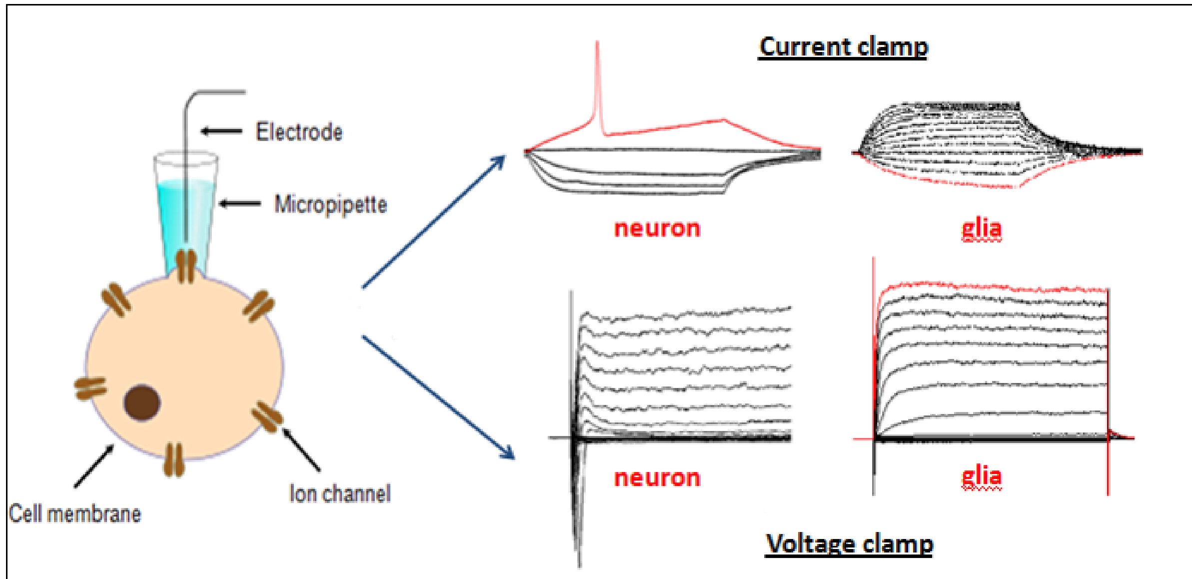


Figure 3: Identification of neurons and glia using whole-cell patch clamp technique. Neurons elicited action potentials in current-clamp mode and exhibited inward sodium currents upon depolarization in voltage-clamp mode. Action potentials and inward currents were absent in enteric glial cells.

To characterize the electrophysiological properties of enteric glia, patch-clamp recordings in the whole cell configuration were made from 16 glia, which were identified based on their inability to elicit action potentials. Whole-cell currents were activated in cells held at −60 mV by applying 200 ms voltage steps ranging from −100 mV to 50 mV at intervals of 10 mV. Input resistance was measured from a holding potential of –60 mV and was 3271 ± 708 MΩ. The resting membrane potential of these cells was −33 ± 3.6 mV, which was comparable to that reported by other groups (Hanani et al., 2000). The average cell size of these cells was 16.82 ± 3.2 pF Fig 4.

The isolated mouse primary glia exhibited two distinctive profiles. 4/16 glia displayed a passive response to hyper and depolarizing potentials with a linear I-V relationship. These cells lacked time and voltage-activated currents and are conventionally considered as `passive´ Fig 5. Previous studies have described the presence of astrocytes with linear I-V curves from spinal cord slices of neonatal rats (Chvatal et al., 1995) as well as glial cells in hippocampal slices of 10–12 day old mice (Steinhauser et al., 1992). Passive currents have also been reported by Hanini et al. in enteric glia from isolated myenteric ganglia of guinea pig small intestine. They observed that these passive currents could be as a result of cell-cell coupling since gap junction blocker, octanol, inhibited these currents (Hanani et al., 2000). Another study hypothesized the contribution of the two-pore domain K^+ channel isoforms, TWIK-1 and TREK-1 in passive conductance observed in CNS astrocytes (Zhou et al., 2009).

The remaining 12/16 glia demonstrated outward currents in response to depolarizing voltage steps and the I-V relationship showed strong outward rectification. These outward currents showed no inactivation during depolarizing voltage steps. The kinetics of these currents imply that these are delayed rectifier currents. They are known to be activated at –40 mV and increase in amplitude as the cell depolarizes. Classical potassium channel blockers TEA and 4-aminopyridine (4-AP)

partially abolished the outward currents suggesting that these sustained outward currents are delayed rectifier K^+ currents (IKdr) Fig 6. TEA is a K^+ channel blocker that inhibits several different types of K^+ channels by binding within the ion conduction pathway on either the intracellular or extracellular side of the cell membrane. In the present study, TEA was utilized as an extracellular inhibitor where it was perfused through the physiological solution in the bath for 5 mins before applying depolarizing potentials to activate the K^+ channels. Binding of external TEA to K^+ channels requires the presence of an aromatic residue at site 449, preferably phenylalanine or tyrosine. TEA cation simultaneously interacts with four aromatic side chains (one from each subunit) from the channel pore to inhibit K^+ conductance (Lenaeus et al., 2005).

4-AP too exhibits concentration-dependent inhibition of four types of voltage-activated K^+ channels, including delayed rectifier potassium currents. Some theories suggest that 4-AP binds to both open and inactivated channels. 4-AP poorly binds to closed channels but once it binds it remains trapped in closed channels because it does not unbind from the channels at hyperpolarized potentials. Extracellular 4-AP blocks K^+ channels from the intracellular side of the membrane by crossing the membrane in its non-ionized form and then binding to the channel in its ionized form (Choquet and Korn, 1992).

Previous findings in myenteric plexus explants demonstrated that enteric glia from neonatal guinea pigs also displayed similar voltage- and time-dependent outwardly rectifying K^+ currents that diminished when the K^+ ion in the internal pipette solution was replaced by cesium, a cation that is impermeable through potassium channels. These currents were also blocked in the presence of TEA in the external solution (Broussard et al., 1993).

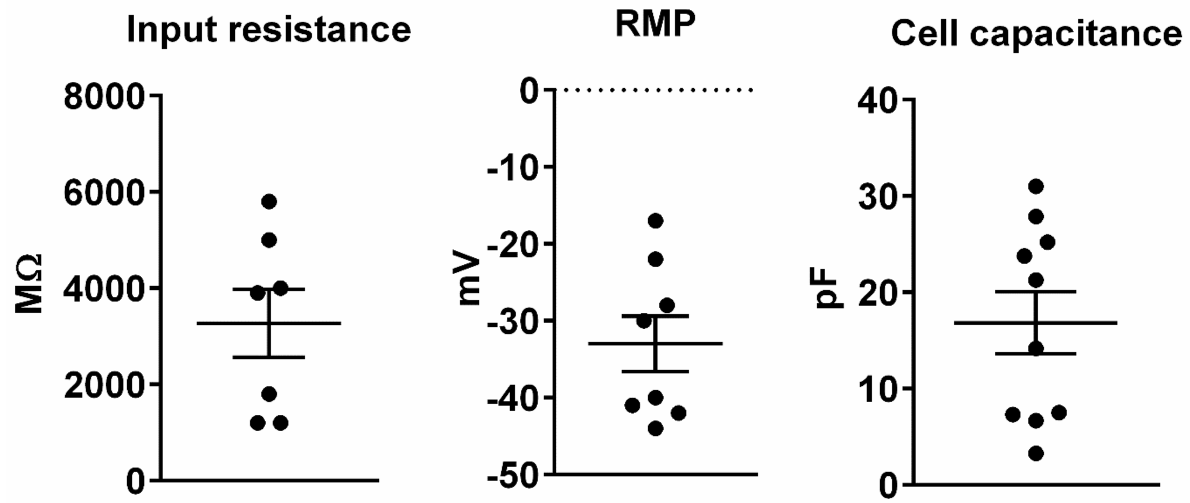


Figure 4: Passive properties of primary enteric glia. Input resistance, resting membrane potential and cell size measured in primary glia isolated from the LMMP of adult SW mouse colon.

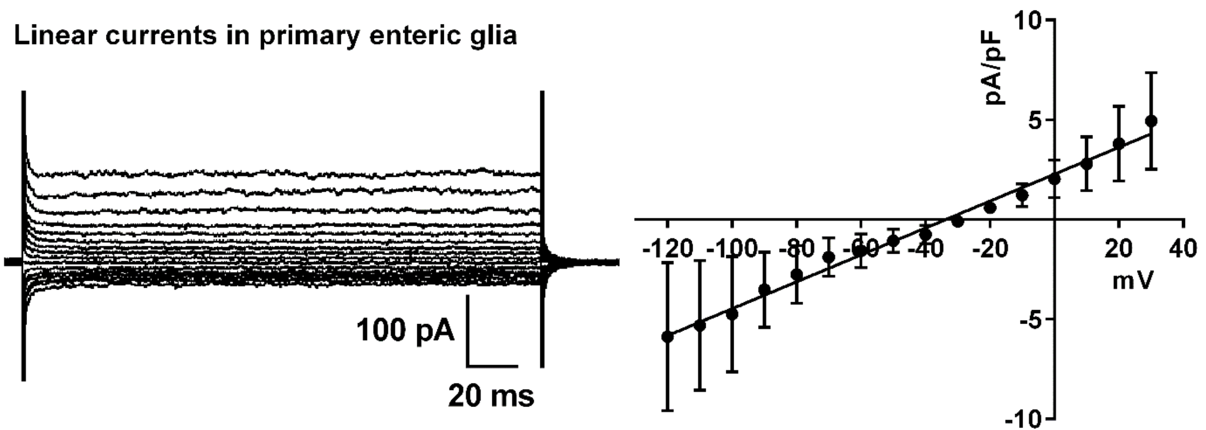
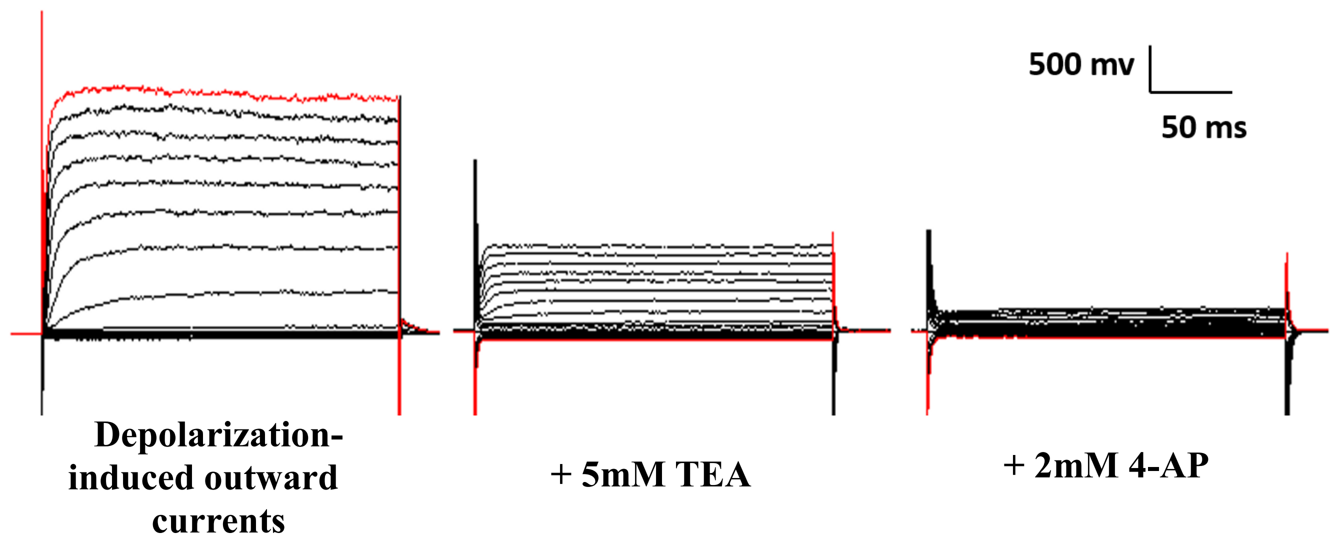


Figure 5: Whole cell current profiles of primary enteric glia (linear currents). Representative voltage-clamp recording and I-V relationships for passive linear currents recorded from cells held at –60 mV and given pulses from –120 to +30 mV with 10 mV increments.



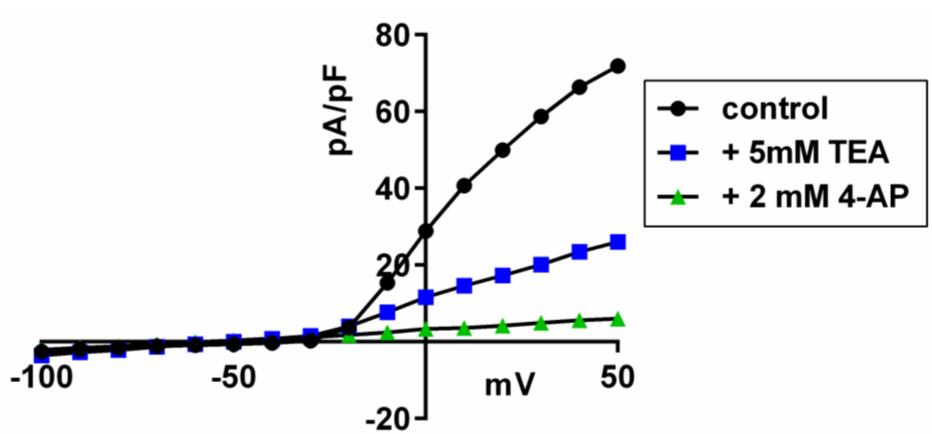


Figure 6: Whole cell current profiles of primary enteric glia (IKdr). Representative voltage clamp recording for delayed rectifier potassium currents recorded from cells held at –60 mV and given pulses from –100 to +50 mV with 10 mV increments. I-V curve showing the effect TEA and 4-AP on delayed rectifier potassium currents.

Properties of enteric glial cell line

In addition to primary glial cultures, an enteric glial cell line was used to confirm and extend findings in primary enteric glia. The rationale for the use of a glial cell line is that it has not been possible to isolate purified populations of primary enteric glia from the myenteric plexus. The presence of contaminating, non-glial cell types (neurons and smooth muscle) might potentially confound the interpretation of some findings, especially in the biochemical assays. We therefore characterized the enteric glial cell line and compared its electrophysiological properties to the primary glial cells. The rat enteric glial cell line CRL-2690 is generated from enzymatically dissociated LMMP preparations of the jejunum of adult male Sprague-Dawley rats. According to their procedure, the isolated enteric glial cells were first purified and then transformed. Supernatants from the virus-producing cell line GPE-egfr-neu were used for infecting the glial cells. The vector egfr/neu is a hybrid gene that codes for the extracellular domain of the human EGF receptor (Epidermal Growth Factor Receptor) and the intracellular tyrosine kinase domain of the proto-oncogene c-neu. The glial cells were allowed to grow in media consisting of EGF. Thus, binding of EGF to the extracellular domain of EGFR resulted in activation of the intracellular tyrosine kinase domain of c-neu inducing a mitogenic impulse. In this way, retroviral gene transfer was used to establish this cell line. Importantly, the glial cell line displays strong morphological and physiological similarities to primary enteric glia, as well as robust GFAP, S-100 and vimentin immunoreactivity also comparable to primary enteric glia (Ruhl et al., 2001).

The rat enteric glial cell line exhibited similar passive membrane properties as the primary glial cells. The resting membrane potential of these glial cells was –32 ± 3.14 mV and their average cell size was 24.91 ± 2.59 pF Fig 7.

The rat enteric glial cell line presented two unique membrane current profiles as compared to the primary glia. One was characterized by the presence of hyperpolarization activated inward currents known as inwardly rectifying potassium currents (Kir). This comprised of 9/40 cells. Kir channels are involved in many physiological processes due to their ability to flux K^+ ions near the K^+ equilibrium potential. Thus, these channels help maintain the resting membrane potential at hyperpolarized values. To further characterize these currents, their sensitivity to external Ba^{2+} ions was assessed. 100 μM $BaCl_2$ abolished the inwardly rectifying currents with no effect on the outward currents depicting the presence of Kir currents Fig 8. Ba^{2+} is a commonly used K^+ channel inhibitor. Channel block by external divalent cations, including Ba^{2+}, occurs due to its binding to a deeper site located approximately half-way within the membrane electrical field as opposed to the block by external monovalent cations that interact with the shallow site that barely senses the membrane electric field (Alagem et al., 2001). Ba^{2+} can fit into the K^+ channel selectivity filter, but its charge causes it to bind too tightly. Thus, Ba^{2+} blocks the conductance of K^+ ions through the channel (Jiang and MacKinnon, 2000). The E125 residue on K^+ channel facilitates the entry of Ba^{2+} into the deep pore region and T141 residue stabilizes the Ba^{2+} ion in its binding site in the pore (Alagem et al., 2001). Previous studies in enteric glia from isolated myenteric ganglia of guinea pig small intestine also demonstrated the presence of these Ba^{2+} sensitive Kir channels (Hanani et al., 2000).

The other 31/40 cells exhibited fast activating and inactivating outward currents in response to depolarizing potentials. These are commonly known as transient outward potassium currents and showed pronounced outward rectification in their I-V relationship Fig 9. The rapid component of current decay is attributed to the presence of these channels. These currents are usually activated at depolarizations above −40 mV.

Despite differences in their current profiles, similarities were observed between the primary glia and the cell line in the current clamp mode. Both cell types exhibited passive responses upon current injections implying that they are non-excitable cells Fig 10.

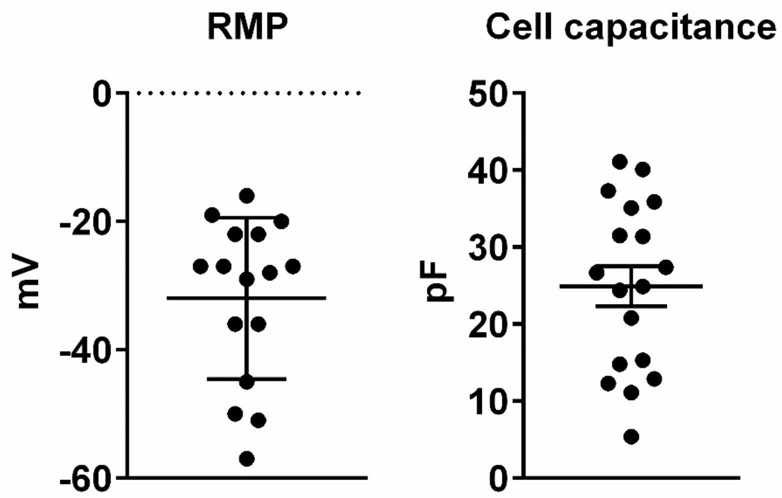


Figure 7: Passive properties of enteric glial cell line: Resting membrane potential and cell size measured in rat enteric glial cell line CRL-2690.

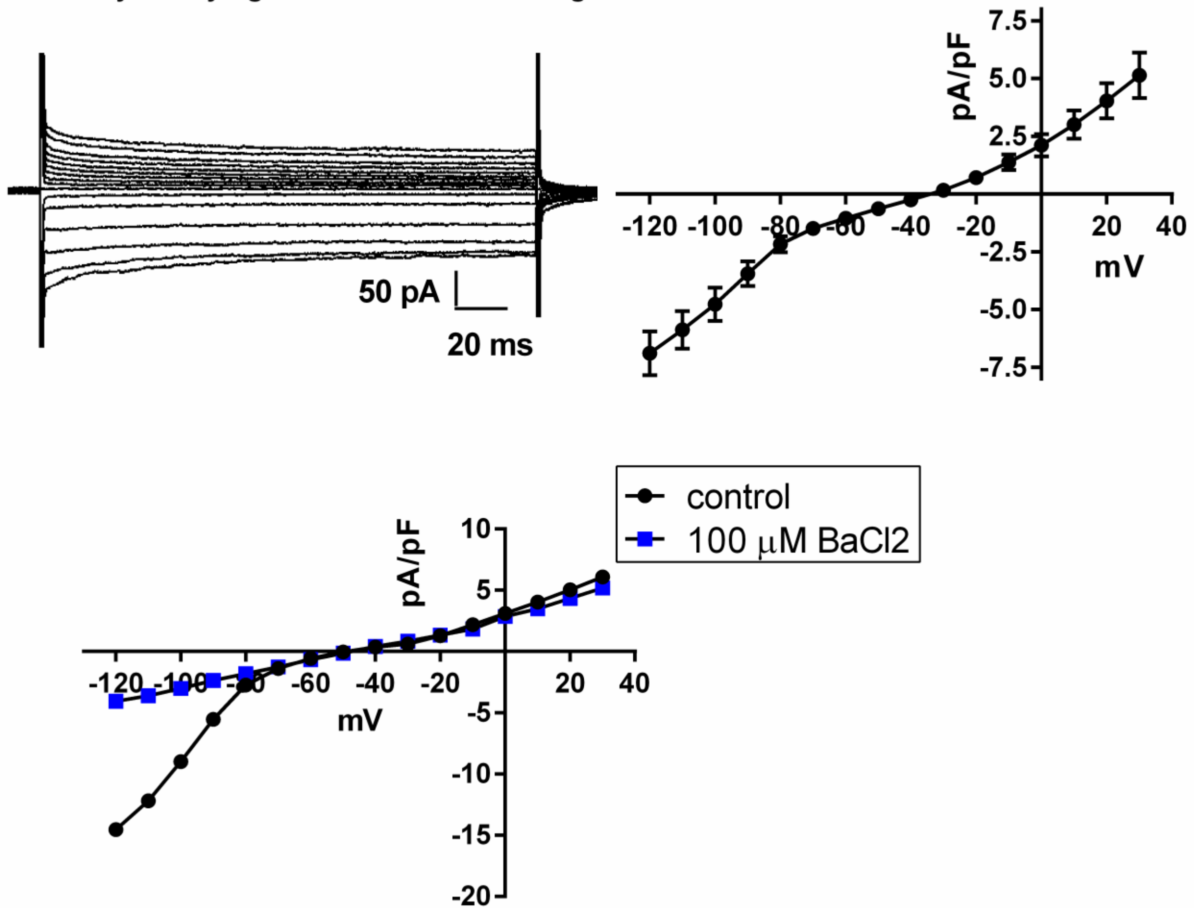


Figure 8: Whole cell current profiles of rat enteric glial cell line (Kir). Representative voltage clamp recording for inwardly rectifying potassium currents recorded from cells held at −60 mV and given pulses from –120 to +30 mV with 10 mV increments. I-V curve showing the effect $BaCl_2$ on inwardly rectifying potassium currents.

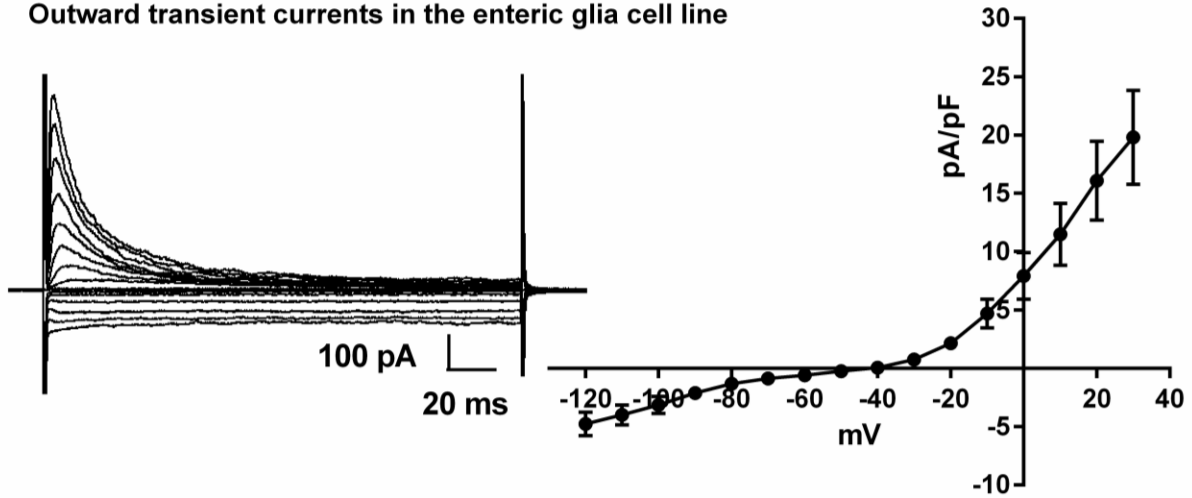


Figure 9: Whole cell current profiles of rat enteric glial cell line (transient outward K^+ currents). Representative voltage clamp recording and respective *I-V* relationships transient outward potassium currents recorded from cells held at −60 mV and given pulses from –120 to +30 mV with 10 mV increments.

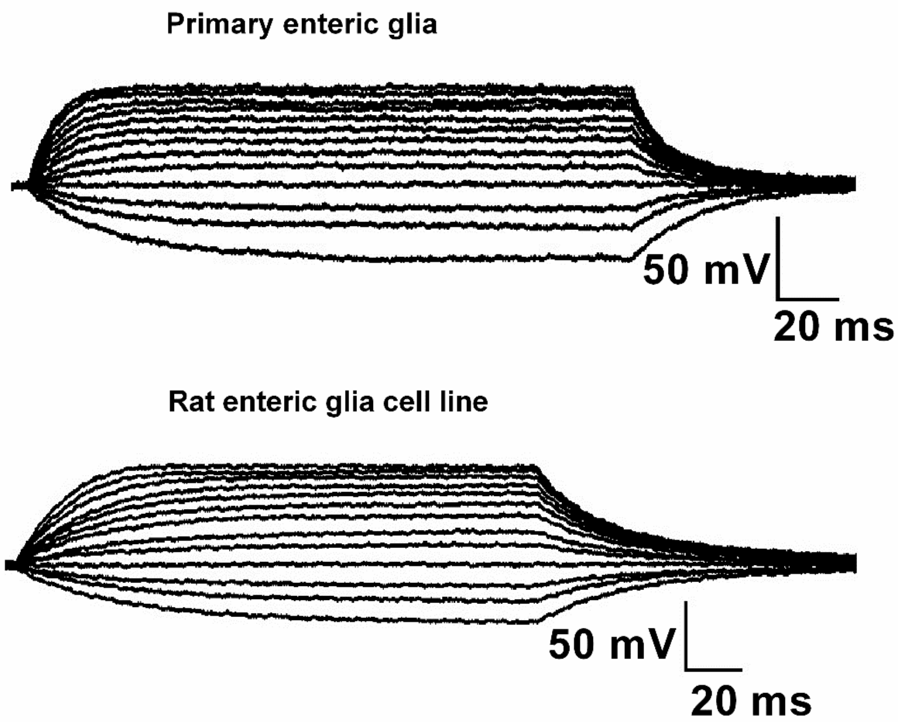


Figure 10: Representative traces of primary enteric glia and enteric glial cell line in the current clamp mode.

Summary

- The present study provides the first evidence of electrophysiological properties of primary enteric glia isolated from adult mouse myenteric plexus and compares them to the rat enteric glial cell line.
- Our findings demonstrate that a majority of the mouse glia express delayed rectifier potassium currents similar to those from neonatal guinea pig myenteric plexus explants.
- In comparison to the mouse primary glia, the rat enteric glia presented at least two voltage dependent K^+ channels- transient outward K^+ channels and inwardly rectifying K^+ channels.
- In spite of differences in the expression of voltage gated potassium channels, both the glial cell line and the primary enteric glia exhibited comparable RMPs. None of the glia elicited action potentials, confirming that they are non-excitable cells.

Chapter IV

Effect of chronic morphine on purinergic signaling and associated colonic inflammation in enteric glia

As described earlier, purinergic signaling is the hallmark of inflammation. Upregulation of purinergic receptors and their activity is implicated in many GI inflammatory diseases. To identify the role of enteric glia in chronic morphine-induced inflammation in the colon, purinergic activity was measured in these cells. In this study, the whole-cell patch clamp technique was utilized to evaluate the activation of purinergic P2X receptors. P2X receptors are activated by their native ligand, ATP. These receptors have 3 ligand-binding sites, which when occupied by ATP, produces a conformational change that allows for the opening of non-specific cation channels. In many instances, the influx of Ca^{2+} through P2X channels is thought to be the major contributor towards activation of a downstream physiological response. P2X receptors have Ca^{2+} permeability equal to or greater than that of the 100-fold more abundant Na^{+} ions. Although the precise mechanism of ion selection is unknown, studies suggest that three polar residues (T336, T339 and S340) just extracellular to the gate are critical for interaction with Ca^{2+} (Egan and Khakh, 2004).

The gating of P2X receptors comprises of an activation phase (inward current induced by agonist application), a desensitization phase (slowly developing decay in the presence of agonist) and a deactivation phase (resulting from rapid decay of current upon removal of agonist). All the P2X receptor subtypes exhibit differences in their gating properties. P2X receptor 1 and 3 rapidly activate and desensitize, whereas P2X receptor subtypes 2 and 4 demonstrate slower desensitization profiles. The gating of P2X7 receptor is more complex because prolonged activation of P2X7 subtypes enhances the conduction of larger molecules along with smaller cations (Coddou et al., 2011).

ATP induces inward currents in enteric glia in mice chronically treated with morphine: To examine the effect of chronic morphine-induced inflammation in the colon on activation of enteric glia, ATP- induced inward currents were measured in these cells. Purinergic receptor activity was measured in primary glia isolated from the colon LMMP of SW mice pelleted with either 75 mg morphine pellets or placebo pellets for 5 days. Our immunohistochemistry data shows co-localization of P2X4 receptors with the astrocytic marker GFAP, illustrating the presence of these purinergic receptors on enteric glial cells Fig 11. Upon assessing the passive properties of these glial cells, we found no significant difference in the average resting membrane potential (RMP) of enteric glia from the colon LMMP of morphine-pelleted mice (–36.07 ± 3.72 mV, n=13) compared to glia of placebo-pelleted mice (–33.86 ± 1.67 mV, n=7) Fig 12A.

To define the functional presence of purinergic receptors in enteric glia, whole cell patch clamp studies were performed in gap-free mode. P2X receptors were activated by perfusing 1 mM ATP in the bath for 10-15 secs. ATP induced inward currents with peak amplitude of 1.03 ± 0.49 pA/pF (n=8) in the enteric glia isolated from placebo-pelleted mice. The peak amplitude of ATP-induced inward currents increased to 7.36 ± 1.97 pA/pF (n=13) in morphine-pelleted mice (p=0.023) Fig 12B. These findings suggested that ATP-induced currents were significantly enhanced by chronic morphine treatment.

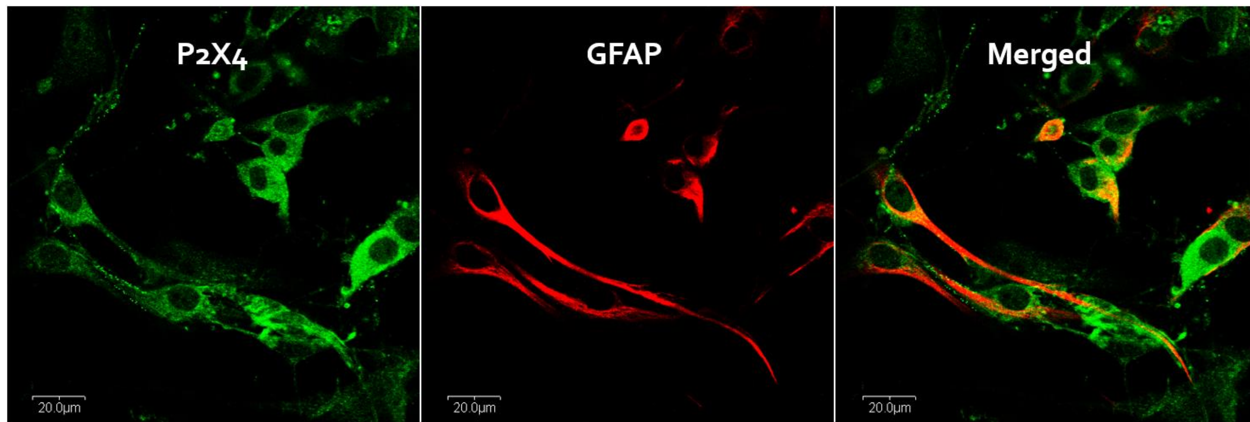


Figure 11: Expression of P2X4 in the ENS. Co-immunostaining of purinergic P2X4 receptors and astrocytic marker GFAP in primary cultures isolated from the LMMP of adult SW mouse colon.

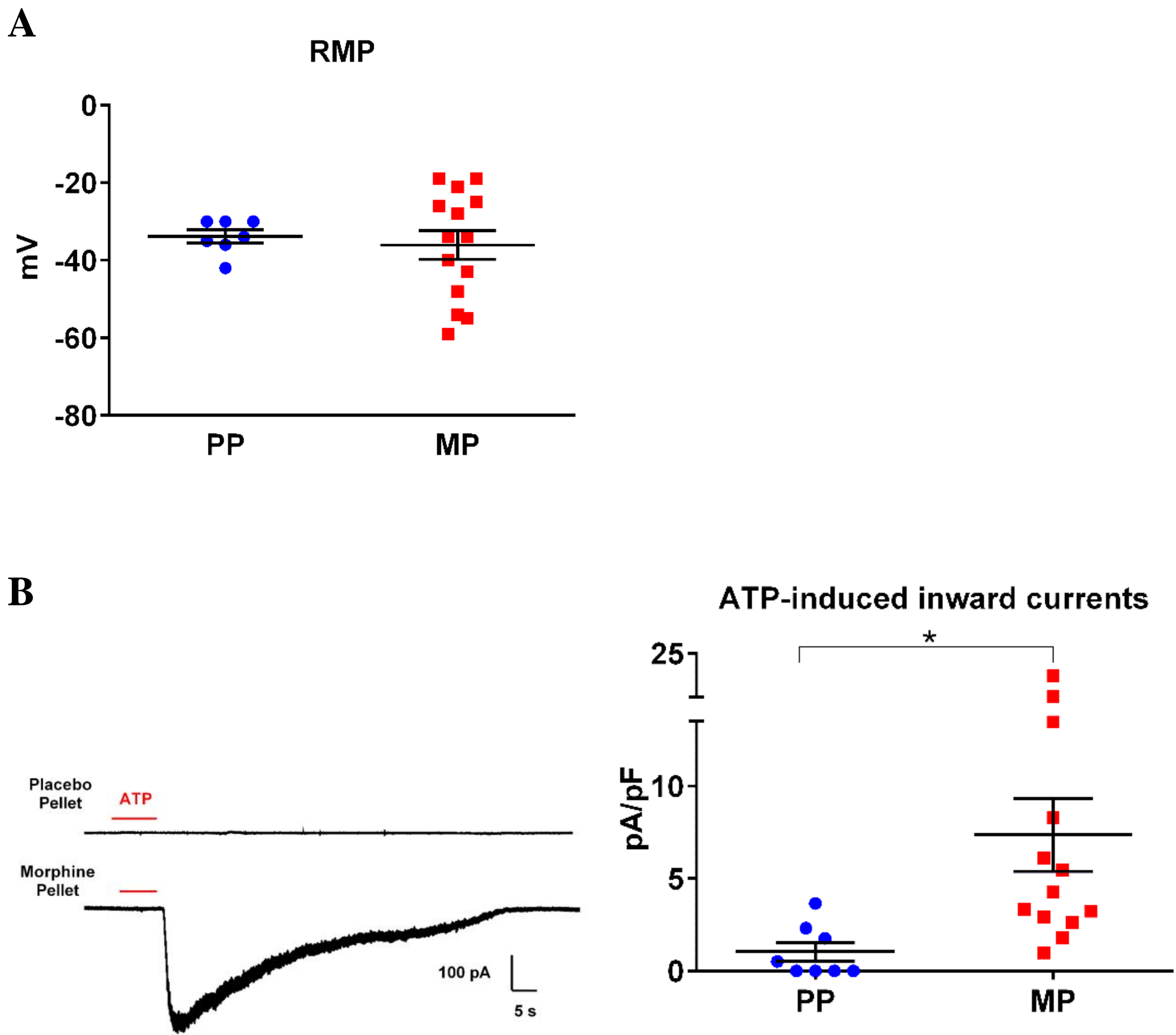


Figure 12: ATP induces inward currents in enteric glia in morphine treated mice. A) RMP and B) Peak amplitude of inward current induced by 1 mM ATP in enteric glia from the colon LMMP of placebo (n=8, N=5) and morphine (n=13, N=8) pelleted mice. *P < 0.05 by Student's t-test.

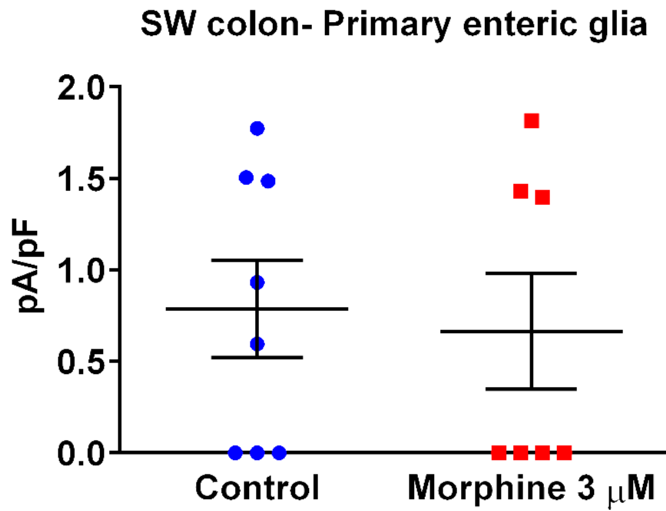
***In vitro* morphine treatment does not enhance ATP-induced inward currents in enteric glia**: To test whether the enhancement of purinergic receptor activity in enteric glia from morphine-pelleted mice was a direct effect of morphine on the µ-opioid receptors (MOR), ATP currents were measured from enteric glia pre-treated with 3 µM morphine for 16 h after cell isolation.

In the primary glia, there was no significant difference in the peak amplitude of ATP-induced inward current in morphine treated glia (0.66 ± 0.31 pA/pF, n=7) compared to the untreated control glia (0.79 ± 0.26 pA/pF, n=8) in primary cultures isolated from the colon LMMP of SW mice (p=0.7685) Fig 13A. The control and morphine treated primary glia also exhibited comparable RMPs (-33 ± 3.6 vs -33.6 ± 2.34 mV) Fig 13B.

Similarly, in the enteric glial cell line CRL-2690, there was no significant enhancement in the peak amplitude of ATP-induced inward current in morphine treated glia (2.89 ± 0.95 pA/pF, n=10) compared to the untreated control glia (1.31 ± 0.28 pA/pF, n=11) (p=0.1404) Fig 14A. The control and morphine treated primary glia exhibited comparable RMPs (-32 ± 3.14 vs -34.22 ± 4.26) Fig 14B. These findings indicate that the enhanced ATP-induced currents from morphine-pelleted mice are not due to direct effects of morphine on enteric glia.

Consistent with lack of direct effects of morphine on enteric glia, MOR was found to co-localize only with neuronal marker β-III tubulin but not with the astrocytic marker GFAP Fig 15.

A



B

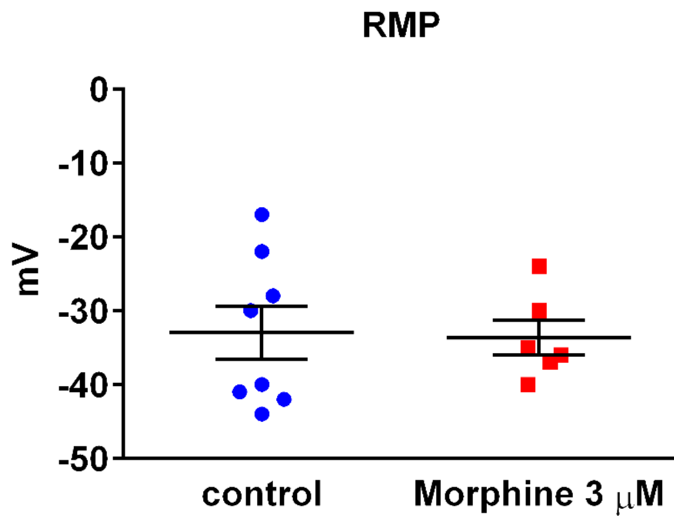
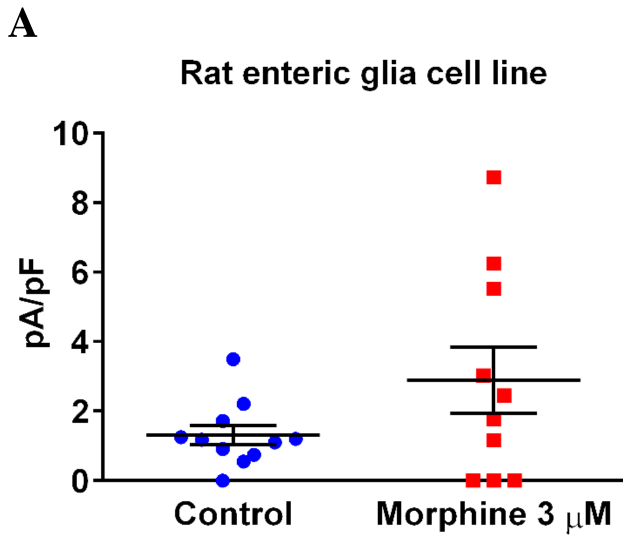


Figure 13: *In vitro* morphine treatment did not alter ATP-induced inward currents in primary enteric glia. A) Peak amplitude of inward current induced by 1 mM ATP and B) RMP measured in control (n=8) and 16 h morphine treated enteric glia (n=7) isolated from the colon LMMP of SW mice.

A



B

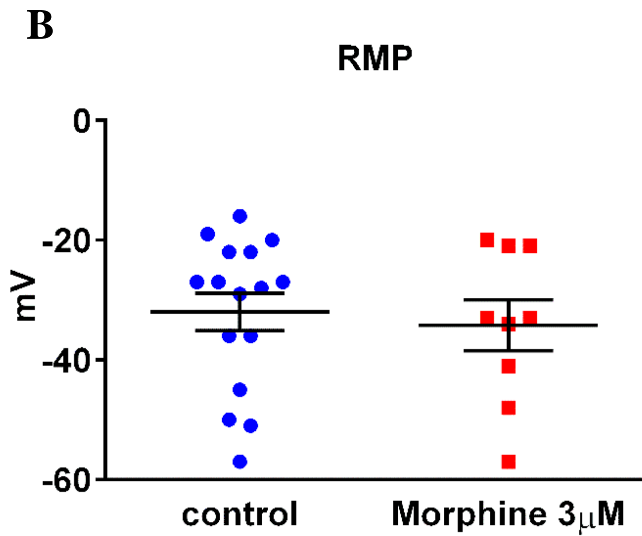


Figure 14: *In vitro* morphine treatment did not alter ATP-induced inward currents in rat enteric glial cell line. A) Peak amplitude of current induced by 1 mM ATP and B) RMP measured in control (n=11) and 16 h morphine treated (n=10) cells from the enteric glial cll line**.**

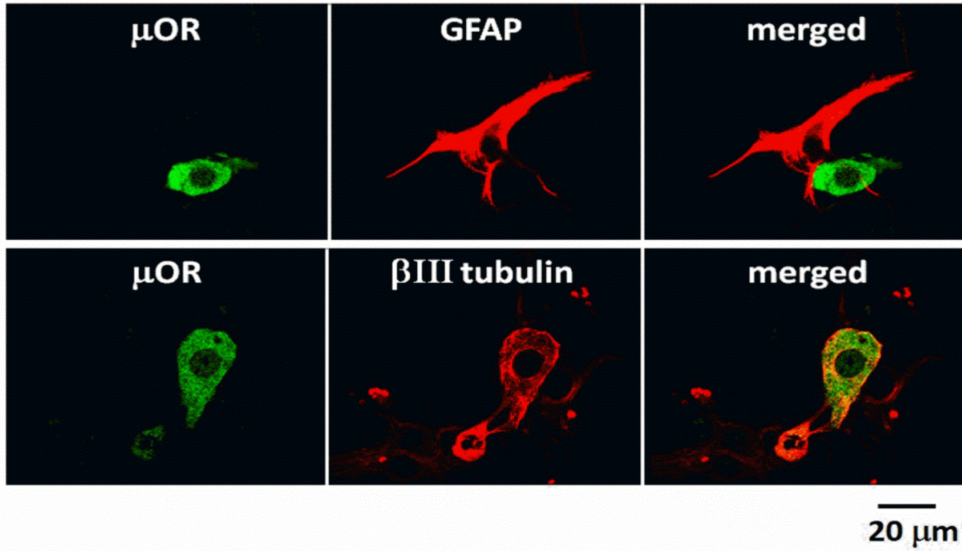


Figure 15: Distribution of MOR in the primary cultures of neurons and glia isolated from the colon LMMP of SW mice. Co-immunostaining of MOR and astrocytic marker GFAP/neuronal marker β-III tubulin in primary cultures isolated from the LMMP of adult SW mouse colon.

LPS enhances P2X receptor activity in enteric glia: Since *in vitro* morphine treatment did not activate enteric glia and enhance ATP currents, we hypothesize that *in vivo* chronic morphine-induced enhancement of purinergic activity and associated inflammation in enteric glia is indirectly mediated by bacterial translocation. As described earlier, bacteria can activate TLRs found on a variety of cell types in the gut wall, including epithelial cells, immune cells, neurons and glia. Studies by Meng et al. showed that chronic morphine (75 mg morphine pellet for 1 day) induces bacterial translocation across the intestinal epithelial barrier and this effect of morphine is not seen in TLR2 and TLR4 knockout mice. These findings pointed towards contribution of Gram-positive and Gram-negative bacteria in mediating morphine-induced bacterial translocation. Recent findings in our lab utilizing the 5 day chronic morphine pelleted mice have demonstrated that while there is a decrease in non-pathogenic Lactobacillales, there is an increase in Enterobacteriales, which is an order that consists mostly of pathogenic Gram-negative bacteria, including E. Coli, Enterobacter, Salmonella and Proteus. Therefore, to determine the effect of this morphine-induced gut bacterial dysbiosis and translocation on activation of enteric glia, lipopolysaccharide/LPS (a product of Gram-negative bacteria) was utilized in the following experiments.

ATP currents were measured from enteric glia pre-treated with LPS for 16 h. In the enteric glial cell line CRL-2690, the peak amplitude of ATP-induced inward current was 0.94 ± 0.22 pA/pF (n=17) in control/untreated glia, 3 ± 1.14 pA/pF (n=10) in glia treated with 1 µg/ml LPS, 4.73 ± 1.51 pA/pF (n=8) in glia treated with 10 µg/ml LPS and 7.89 ± 1.38 pA/pF (n=10) in glia treated with 100 µg/ml LPS. This enhancement of ATP-induced inward current produced by 10 µg/ml LPS was blocked when cells were perfused with 500 nM PPADS, a non-specific P2X blocker, in the bath (peak current amplitude: 0.44 ± 0.22 pA/pF, n=5). Similar inhibition of ATP currents was

seen upon pretreatment of 10 µg/ml LPS treated glial cells with 50 µM CBX (CBX), a Cx43 gap junction blocker (peak current amplitude: 0.67 ± 0.33 pA/pF, n=6) Fig 16. These findings demonstrated that LPS dose-dependently enhanced purinergic receptor activity in the enteric glial cell line and this increase in ATP currents was mediated by Cx43 hemichannels and P2X receptors. CBX (3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate) is a small molecule inhibitor, glycyrrhetinic acid derivative with a steroid-like structure and is a component of licorice plant. In spite of its nonspecific actions in inhibiting pannexins, 11β-hydroxysteroid dehydrogenase, NMDA receptors and GABA-A receptors, CBX is the most commonly used connexin hemichannel antagonist (Connors, 2012).

In the primary glial cultures, ATP significantly enhanced inward currents from 1.6 ± 0.84 pA/pF (n=9) in the control glia to 31.34 ± 10.87 pA/pF (n=7) in glia isolated from the colon LMMP of SW mice and treated with 10 µg/ml LPS. The role of Cx43 hemi channels in enhancement of ATP currents in primary glia was confirmed by using a more specific antagonist, Gap26. The connexin mimetic peptide Gap26 is derived from amino acids 64–76 on the first extracellular loop of Cx43 hemichannel (Evans et al., 2012). The increase in ATP-induced inward current produced by 10 µg/ml LPS was blocked upon pretreatment with 20 µM Gap26 (peak current amplitude: 1.28 ± 1.12 pA/pF, n=5) Fig 17.

Further, to address whether LPS-induced enhancement of purinergic receptor activity in enteric glia was due to an increase in P2X receptor expression, P2X4 and P2X7 mRNA expression was measured in RNA extracted from the enteric glial cell line (CRL-2690) after exposure to LPS (0-100 µg/ml) for 1 h. LPS dose-dependently upregulated both P2X4 and P2X7 mRNA expression in enteric glia as compared to the untreated/control glia Fig 18A, B. Unlike LPS, *in vitro* morphine alone too did not upregulate P2X4 and P2X7 mRNA expression in enteric glia compared to the

untreated control glia when measured in RNA extracted from the enteric glial cell line (CRL-2690) after exposure to 3 μM morphine for 16 h Fig 19.

LPS enhanced ATP currents in both the primary glia cultures as well as the rat glial cell line but did not modify other passive properties of these glia, namely RMP and outward potassium currents. All primary glia treated with 10 μg/ml LPS expressed outward potassium currents (IKdr). LPS did not change the I-V relationship of IKdr as seen by similar outward current magnitude and reversal potential Fig 20. Similarly, LPS, did not affect the transient outward K^+ currents in enteric glial cell line Fig 21.

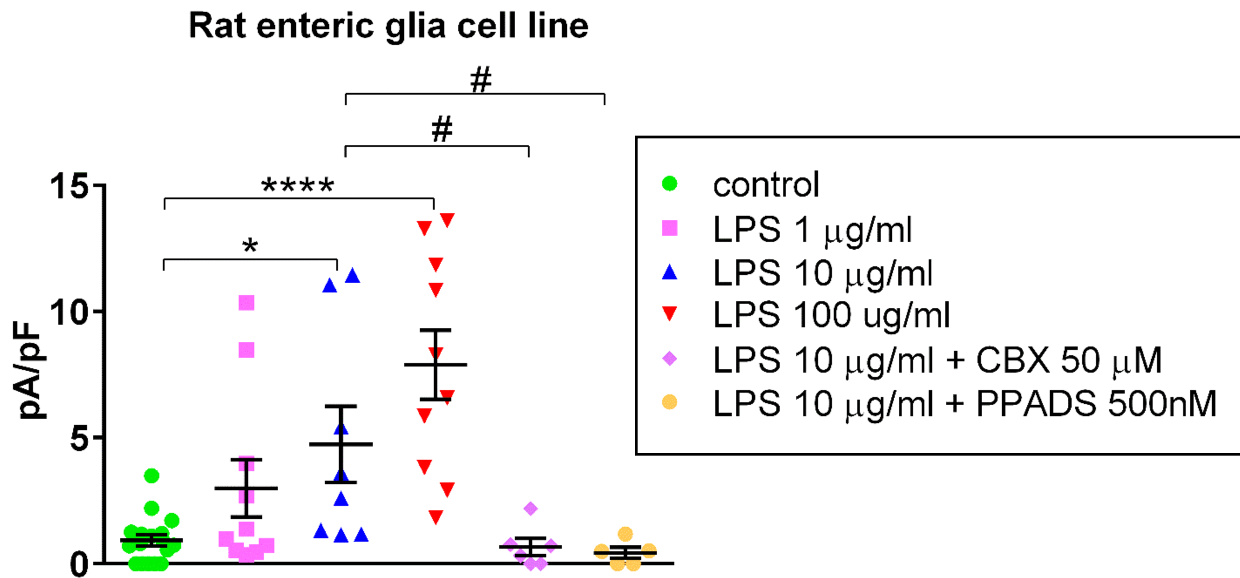


Figure 16: LPS enhances P2X receptor activity in primary enteric glia. Peak amplitude of current induced by 1 mM ATP in the enteric glial cell line treated with LPS for 16 h with or without CBX or PPADS. *P < 0.05, ****P < 0.0001 vs control by one-way ANOVA (Tukey`s post-hoc). # P < 0.05 vs LPS 10 μg/ml by one-way ANOVA (Tukey`s post-hoc).

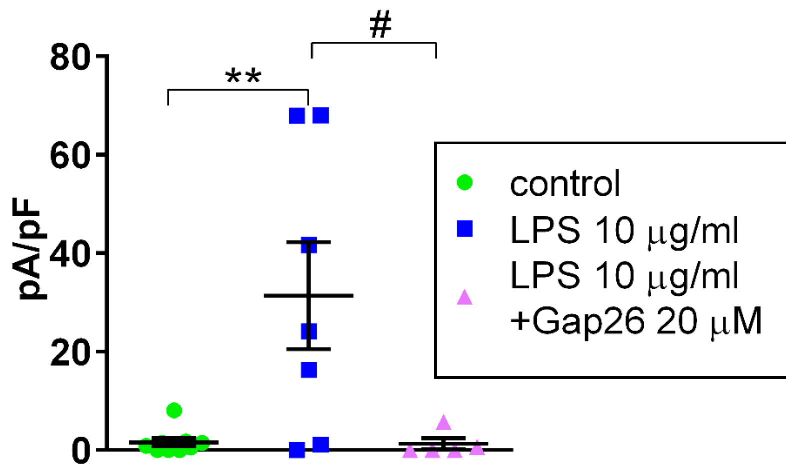


Figure 17: LPS enhances P2X receptor activity in rat enteric glial cell line. A) Raw trace displaying inward currents induced by 1 ATP. B) Peak amplitude of current induced by 1 mM ATP in enteric glia isolated from colon LMMP of SW mice and treated *in vitro* with LPS for 16 h in the presence or absence of Gap26. $^{**}P < 0.01$ vs control by one-way ANOVA (Tukey`s post-hoc). $^{\#}$ $P < 0.05$ vs LPS 10 µg/ml by one-way ANOVA (Tukey`s post-hoc).

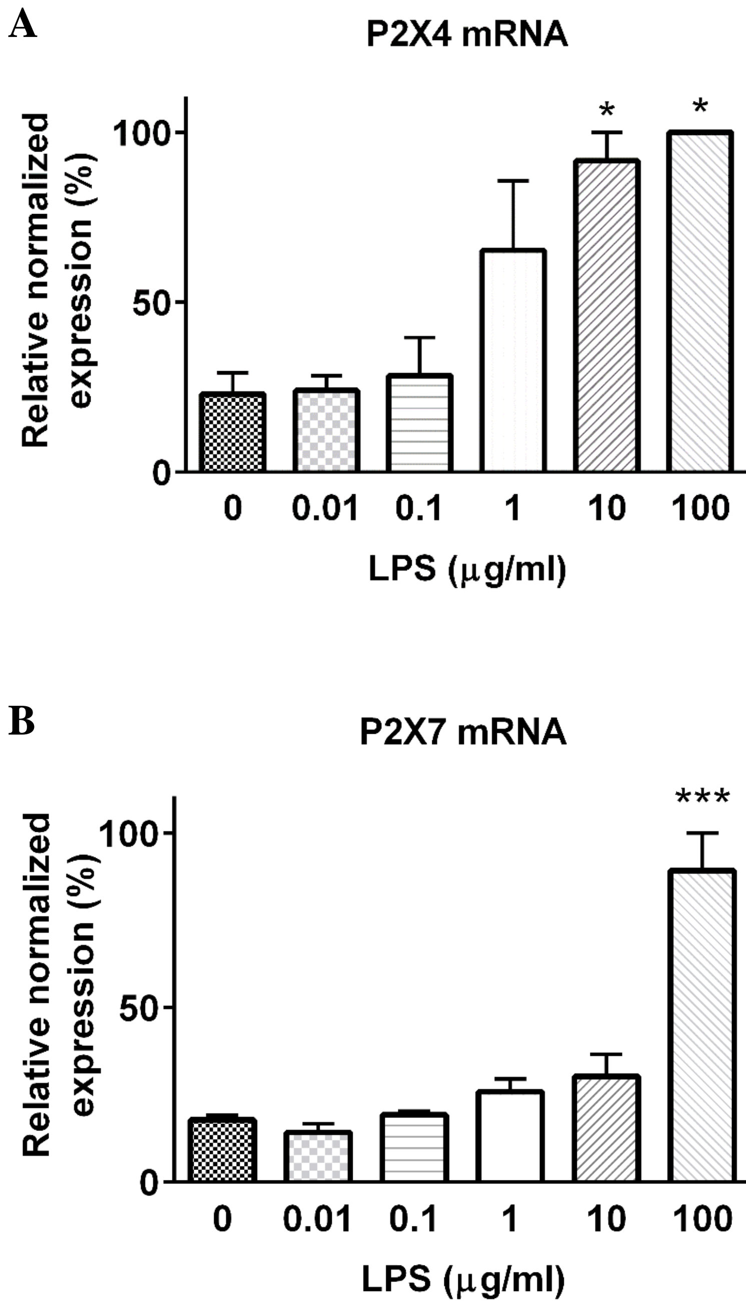
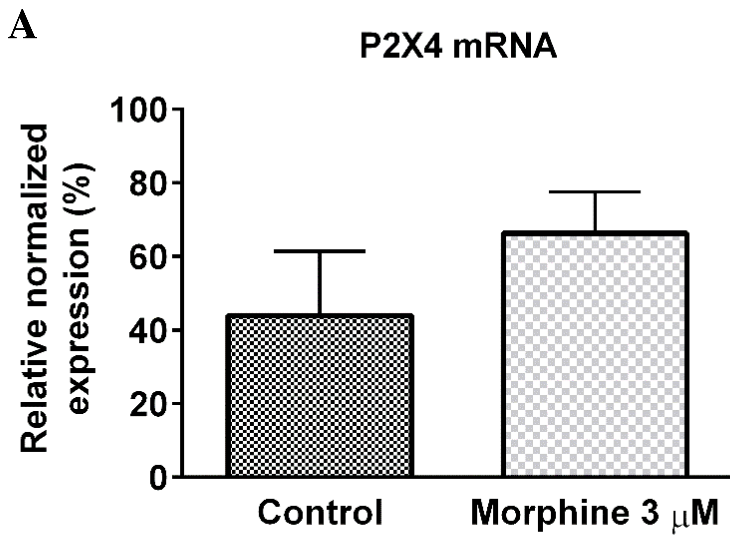


Figure 18: Effect of LPS on purinergic receptor expression in enteric glia. A) P2X4 and B) P2X7 mRNA expression measured in RNA extracted from the enteric glial cell line after exposure to LPS for 1 h (N=6). *P < 0.05, ***P < 0.001 vs control by one-way ANOVA (Tukey`s post-hoc).



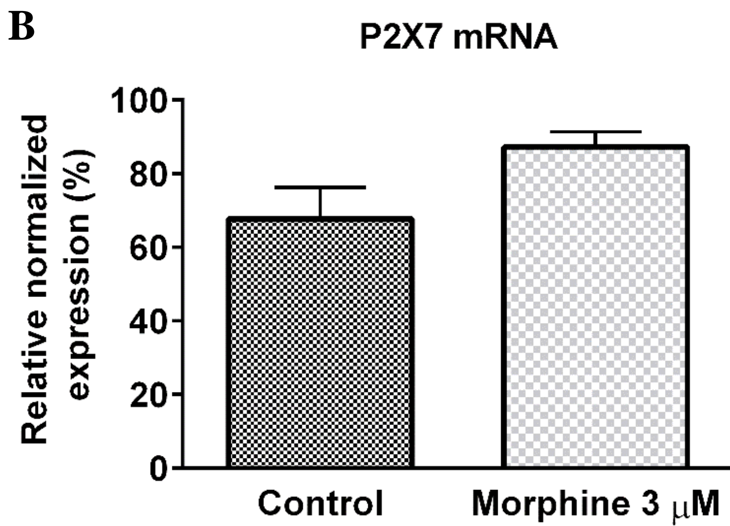
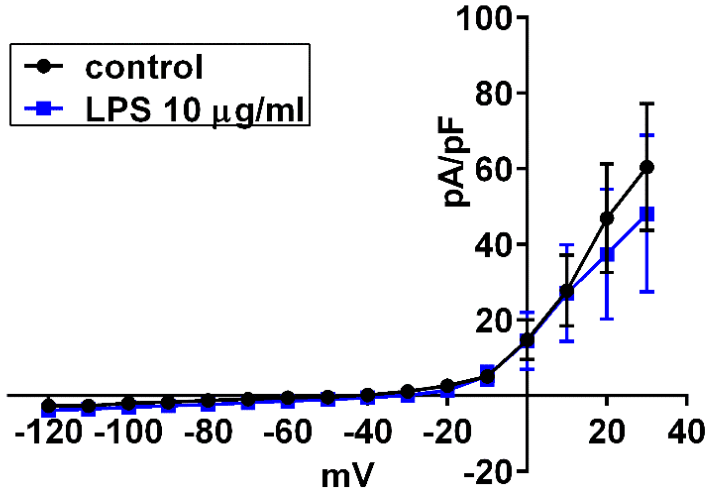


Figure 19: Effect of morphine on purinergic receptor expression in enteric glia. A) P2X4 and B) P2X7 mRNA expression measured in RNA extracted from the enteric glial cell line after exposure to 3μM morphine for 16 h (N=6).

A



B

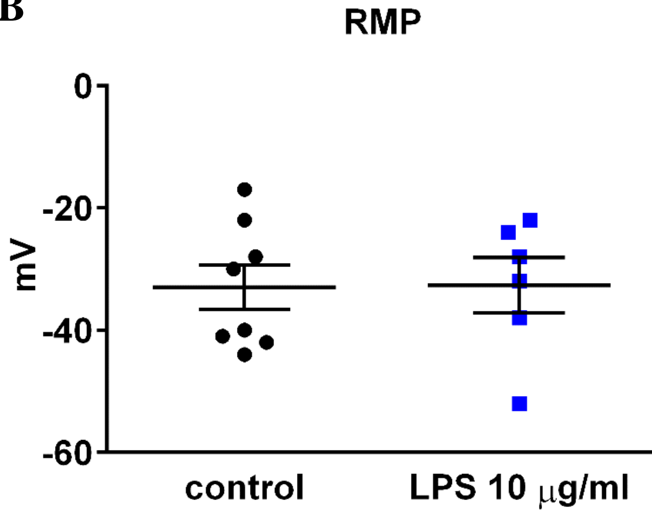
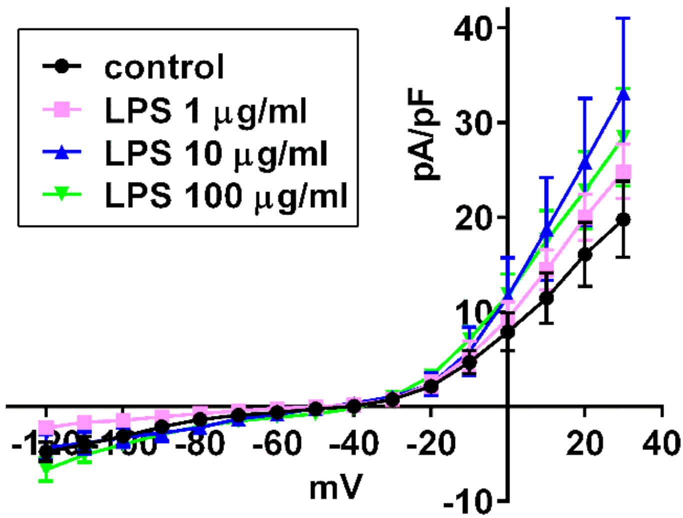


Figure 20: Effect of LPS on outward currents in primary enteric glia. A) I-V curve showing the effect LPS on delayed rectifier potassium currents and B) RMP in primary enteric glial cells.

A



B

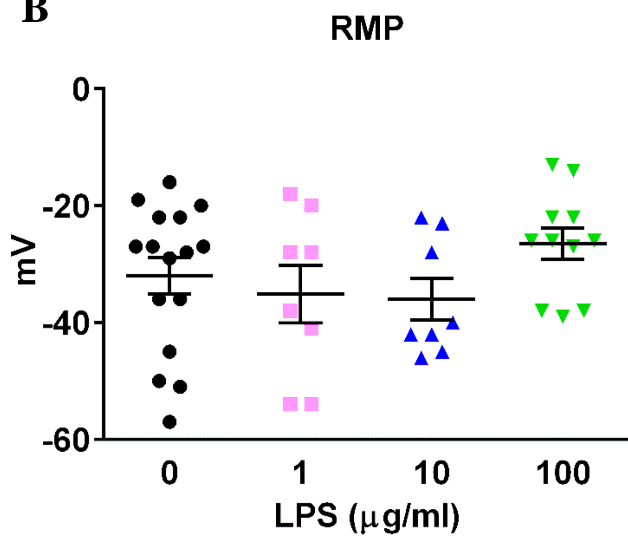


Figure 21: Effect of LPS on outward currents in rat enteric glial cell line. A) I-V curve showing the effect LPS on transient outward potassium currents and B) RMP in rat enteric glial cell line.

LPS enhances the release of ATP in enteric glia via connexin 43 hemichannels: To evaluate the effect of LPS on cell damage, the level of ATP was measured in the condition media from the enteric glial cell line (CRL-2690) exposed to LPS for 1 h using the ATPLite™ Kit (Perkin Elmer, Waltham, MA). This assay kit is based on the production of light by the reaction of ATP released in the cell culture media with externally added luciferase enzyme and d-luciferin substrate. Therefore, increase in ATP release due to cell damage is proportional to light emitted and can be measured using a luminometer. Our findings showed that there was a significant increase in extracellular ATP levels on treatment with 100 µg/ml LPS compared to the control/untreated enteric glia Fig 22. Also, Cx43 mRNA expression was measured in RNA extracted from the enteric glial cell line (CRL-2690) after exposure to LPS (0-100 µg/ml) for 1 h. RT-PCR data showed that LPS dose-dependently upregulated Cx43 mRNA expression in enteric glia as compared to the untreated/control glia Fig 23. Moreover, the increase in ATP release produced by 100 µg/ml LPS was partially blocked with 50 µM CBX (Cx43 antagonist) but not with 500 nM PPADS (a non-selective P2X antagonist). These findings established the importance of Cx43 hemichannels in the release of ATP from enteric glia.

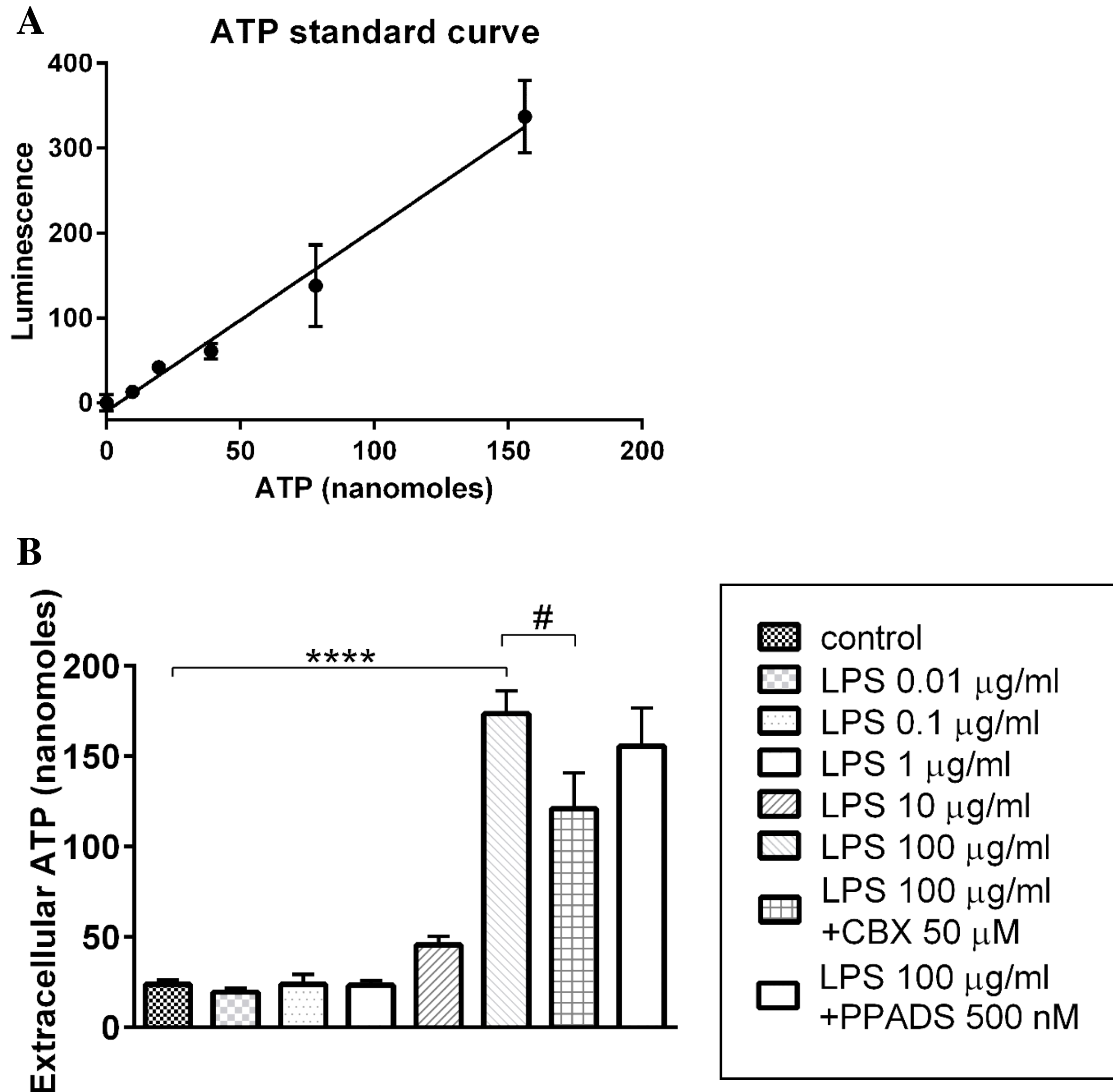


Figure 22: LPS induced the release of ATP in enteric glia is mediated by connexin 43 hemi channels. A) ATP standard curve and B) ATP concentration measured in the supernatants collected from the enteric glial cell line treated with LPS for 1 h in the presence and absence of CBX and PPADS. ****$P < 0.0001$ vs control and $^{\#}P < 0.05$ vs LPS 100 µg/ml by one-way ANOVA (Tukey`s post-hoc).

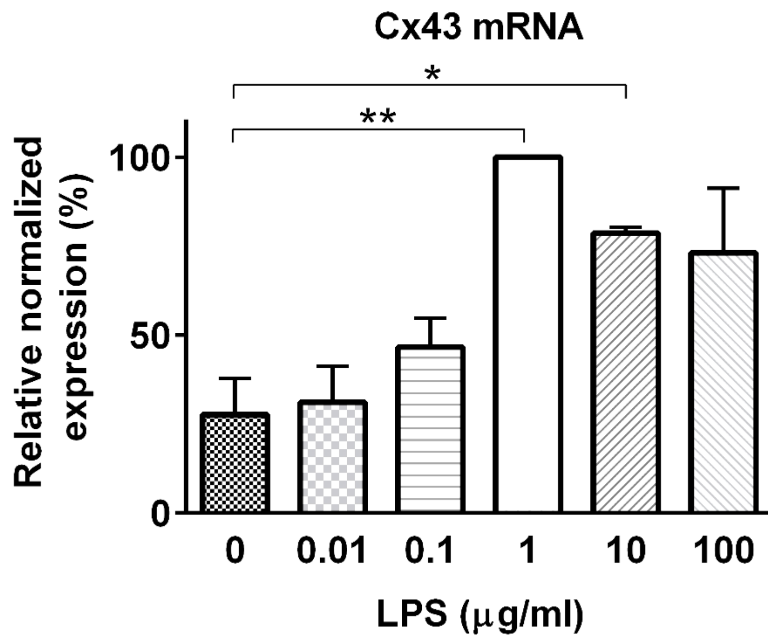


Figure 23: Effect of LPS on Cx43 hemichannel expression in enteric glia. Cx43 mRNA expression measured in RNA extracted from the enteric glial cell line after exposure to LPS for 1 h (N=6). *P < 0.05, **P < 0.01 vs control by one-way ANOVA (Tukey`s post-hoc).

LPS enhances the expression of inflammatory cytokines in enteric glia: To study the effect of LPS-induced enhancement of purinergic receptor activity on production of inflammation in enteric glia, IL-6, TNFα and IL-1β mRNA expression was measured in RNA extracted from the enteric glial cell line (CRL-2690) after exposure to LPS (0-100 μg/ml) for 1 or 16 hrs. RT-PCR data showed that LPS upregulated IL-6 mRNA expression in enteric glia after 1 h exposure as compared to the untreated/control glia. The upregulation of IL-6 produced by 10 μg/ml LPS was partially blocked by CBX as well as PPADS Fig 24A. A similar trend in IL-6 expression was seen after 16 h exposure to LPS Fig 24B. A different profile was observed with TNFα, where short term LPS exposure (1 h) did not alter TNFα mRNA expression but upregulation was seen after 16 hr exposure (Guedia et al., 2016) Fig 24C. On the other hand, IL-1β showed a similar trend to IL-6 mRNA expression Fig 24D. These findings suggested that LPS-induced enhancement of pro-inflammatory cytokines are mediated by Cx43 hemi-channels and P2X receptors.

To evaluate the role of TLR4 in LPS-induced enhancement of inflammatory cytokines in enteric glia, IL-6 mRNA expression was measured in RNA extracted from the enteric glial cell line (CRL-2690) after exposure to LPS (0-10 μg/ml) for 16 hrs in the presence of a TLR4 antagonist, OxPAPC (30 μg/ml). Consistent with our earlier findings, RT-PCR data indicated that LPS upregulated IL-6 mRNA expression in enteric glia after 16 h exposure as compared to the untreated/control glia. OxPAPC showed a trend towards reduction of IL-6 mRNA upregulation seen with 0.1 and 1 μg/ml LPS. Interestingly, contrasting observations were made when OxPAPC was used along with 10 μg/ml LPS. OxPAPC markedly enhanced the increase in IL-6 mRNA expression seen with 10 μg/ml LPS. These findings suggested that antagonism of TLR4 had two-fold effects on low and high concentration LPS-induced enhancement of pro-inflammatory cytokines Fig 25.

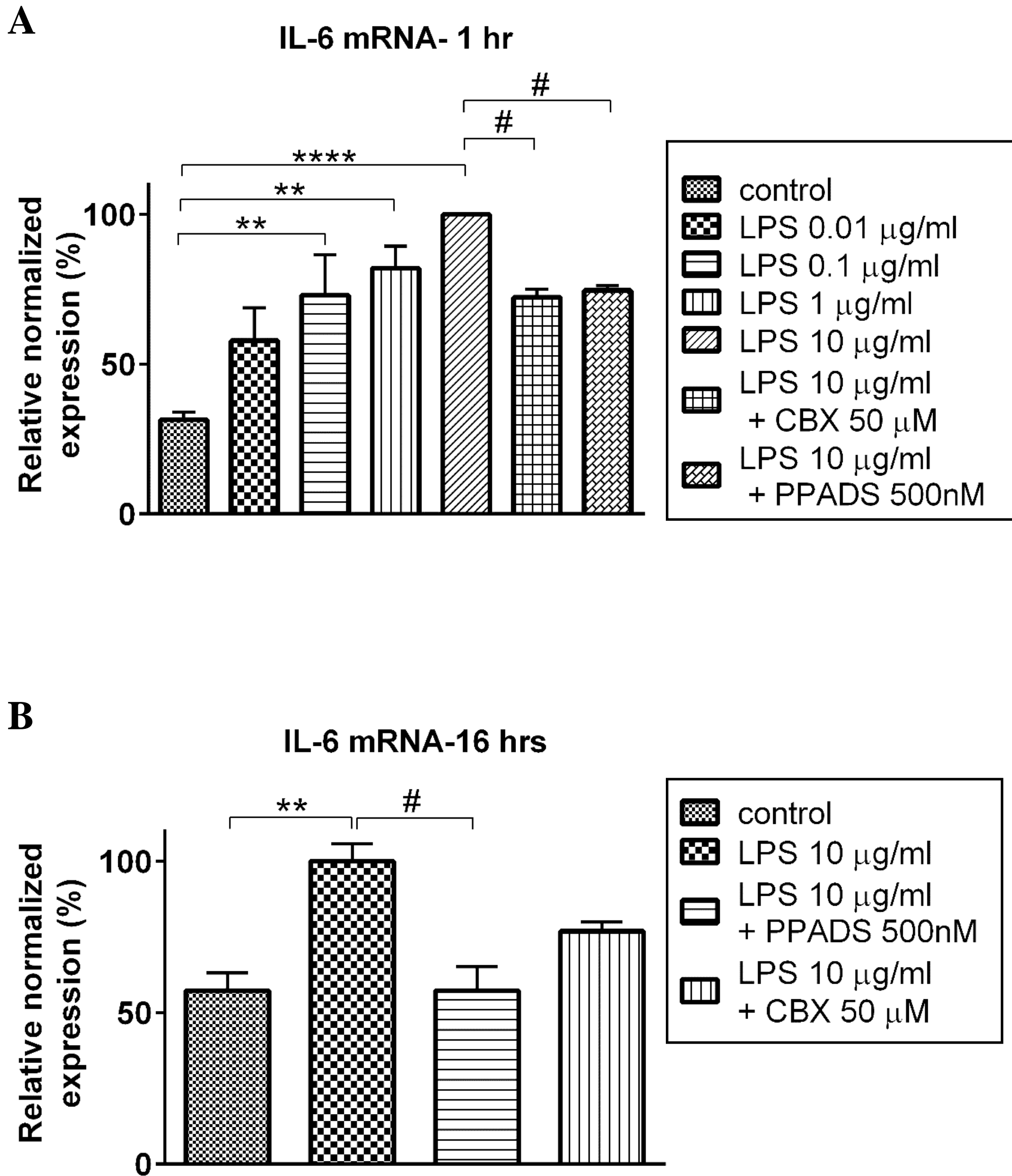


Figure 24: LPS induces expression of inflammatory cytokines in enteric glia is mediated via the Cx43/P2X pathway. IL-6 mRNA expression measured in RNA extracted from the enteric glial cell line after exposure to LPS for A) 1 h and B) 16 h respectively (N=6). $^{**}P < 0.01$, $^{****}P < 0.0001$ vs control and $^{\#}P < 0.05$ vs LPS 10 μg/ml by one-way ANOVA (Tukey`s post-hoc).

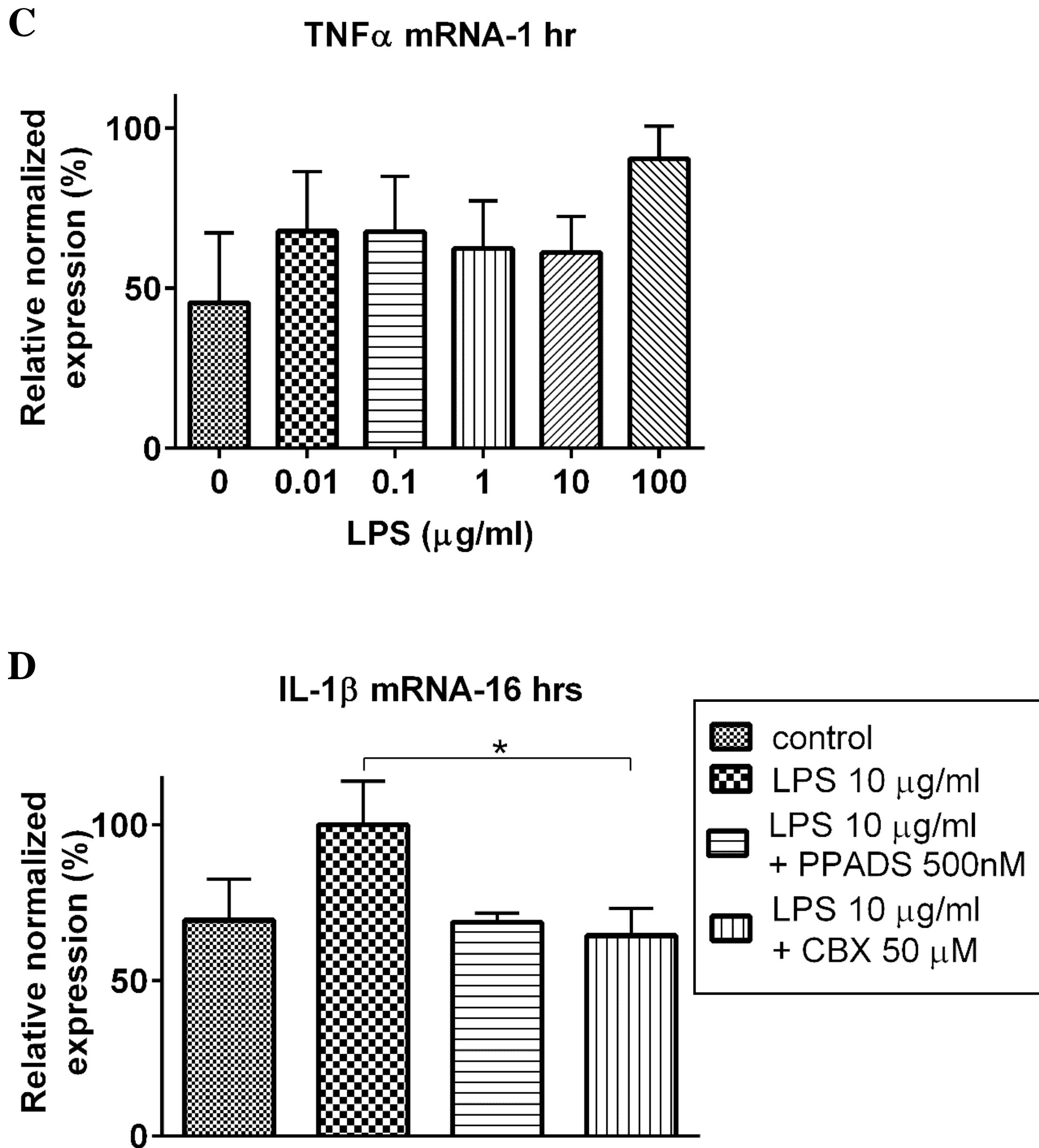


Figure 24: LPS induces expression of inflammatory cytokines in enteric glia is mediated via the Cx43/P2X pathway. C) TNFα mRNA expression measured in RNA extracted from the enteric glial cell line after exposure to LPS for 1 h (N=6). D) IL-1β mRNA expression measured in RNA extracted from the enteric glial cell line after exposure to LPS for 16 h (N=6). $^{\#}P < 0.05$ vs LPS 10 μg/ml by one-way ANOVA (Tukey`s post-hoc).

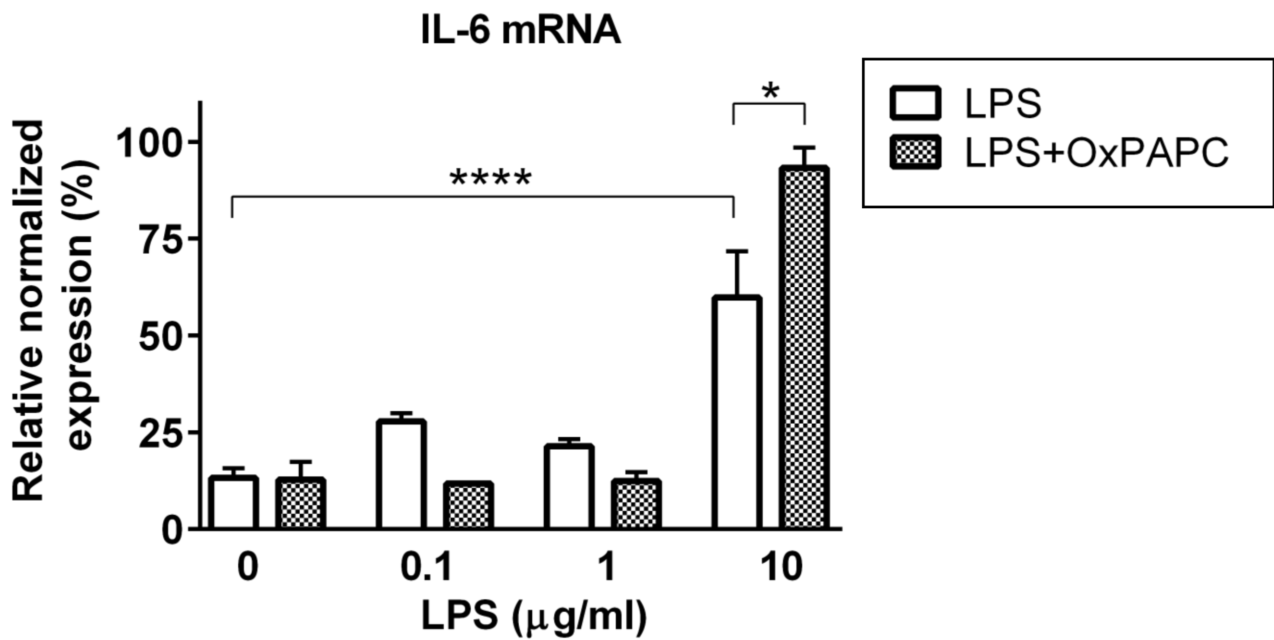


Figure 25: Effect of TLR4 antagonist, OxPAPC, on LPS-induced expression of inflammatory cytokines in enteric glia. IL-6 mRNA expression measured in RNA extracted from the enteric glial cell line after exposure to LPS for 16 h and co-treated with 30 μg/ml OxPAPC or vehicle (N=2-8). ****$P < 0.0001$ vs control and *$P < 0.05$ vs LPS 10 μg/ml by one-way ANOVA (Tukey`s post-hoc).

CBX attenuates chronic morphine-induced inflammation and barrier disruption: To further test our hypothesis that *in vivo* chronic morphine-induced enhancement of purinergic activity and associated inflammation is indirectly mediated by bacterial translocation, cytokine expression was measured in presence of CBX. Mice were pelleted with either a placebo or 75 mg morphine pellet for 5 days and given i.p. injections of saline or CBX (25mg/kg) on day 3 and 4. RNA was isolated either from the whole colon tissue or specifically from colon LMMP from these mice. RT-PCR data indicated that there was an upregulation of IL-1β upon chronic morphine treatment as compared to placebo treatment. 25 mg/kg CBX prevented chronic morphine-induced inflammation but also showed a trend towards reducing basal IL-1β expression in placebo treated mice Fig 26.

In the presence of CBX, ATP did not enhance inward currents in the enteric glia from morphine pelleted mice (peak current amplitude: 0.9393 ± 0.5150, n=5) as compared to the placebo pelleted mice (peak current amplitude: 0.6481 ± 0.6481, n=6). Thus, CBX prevented the enhancement of purinergic receptor activity in chronic morphine treated mice Fig 27.

Histological analysis of H&E stained colon samples demonstrated disorganization of the epithelium and disruption of crypts in the mucosal layer of the gut wall with chronic morphine exposure vs the placebo counterparts. Moreover, CBX treatment appeared to protect the morphine-induced barrier compromise resulting in a more intact gut wall structure. CBX did not have any effect on the epithelial organization in the placebo group Fig 28.

Previous reports have demonstrated a decrease in neuron packing density in the myenteric plexus of colon in a DNBS (dinitrobenzene sulfonic acid) model of colonic inflammation (Brown et al., 2016). In order to test if morphine-induced inflammation causes neuronal death, neuronal

packaging density in the myenteric plexus of the placebo and morphine pelleted animals was calculated. A significant decrease in the neuronal density was seen in the myenteric plexus of the mice pelleted with morphine for 5 days when compared to the placebo pelleted animals (p=0.0003). The average neuronal density was 3601 ± 183 and 2665 ± 125 cells per mm^2 respectively in placebo and morphine pelleted animals. Morphine induced neuronal loss was significantly reversed when the animals were treated with 25 mg/kg CBX (p=0.02). The average neuronal density was 3356 ± 237 cells per mm^2 in morphine pelleted animals treated with CBX Fig 29.

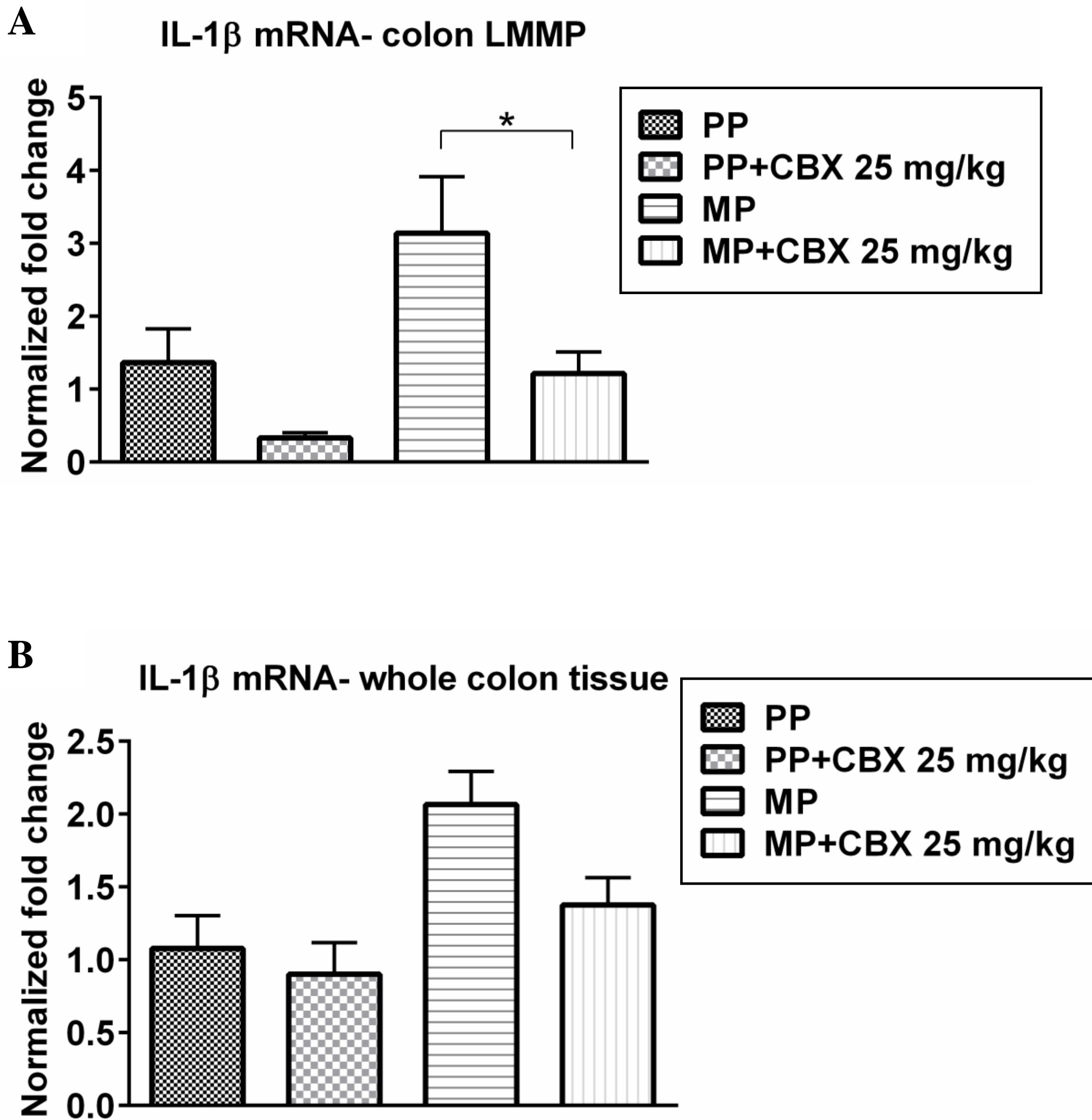


Figure 26: CBX mitigates chronic morphine-induced inflammation in the colon. IL-1β mRNA expression in A) whole colon tissue and B) colon LMMP isolated from mice pelleted with either placebo or 75 mg morphine pellets for 5 days and given i.p. injections of saline or CBX (25 mg/kg) on day 3 and 4. (N=6). *P < 0.05 by one-way ANOVA (Tukey`s post-hoc).

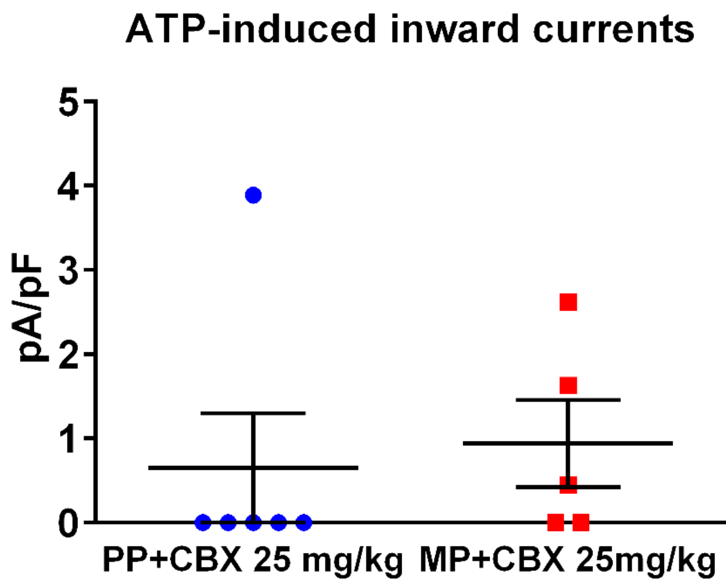


Figure 27: CBX alleviates chronic morphine-induced enhancement of purinergic activity in enteric glia. Peak amplitude of current induced by 1 mM ATP in enteric glia isolated from the colon LMMP of placebo (n=6, N=3) and morphine (n=5, N=3) pelleted mice given i.p. injections of CBX (25 mg/kg).

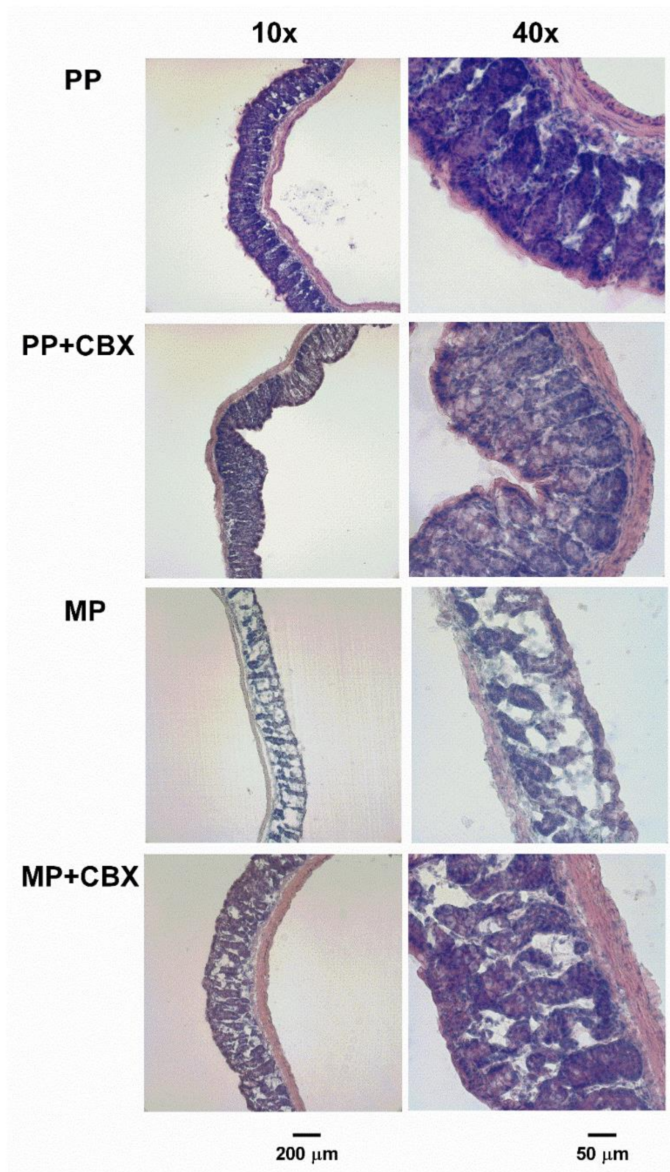


Figure 28: Histological analysis of colon cross sections. Representative H-E staining sections from the colon of mice pelleted with either placebo or 75 mg morphine pellets for 5 days and given i.p. injections of saline or CBX (25 mg/kg) on day 3 and 4.

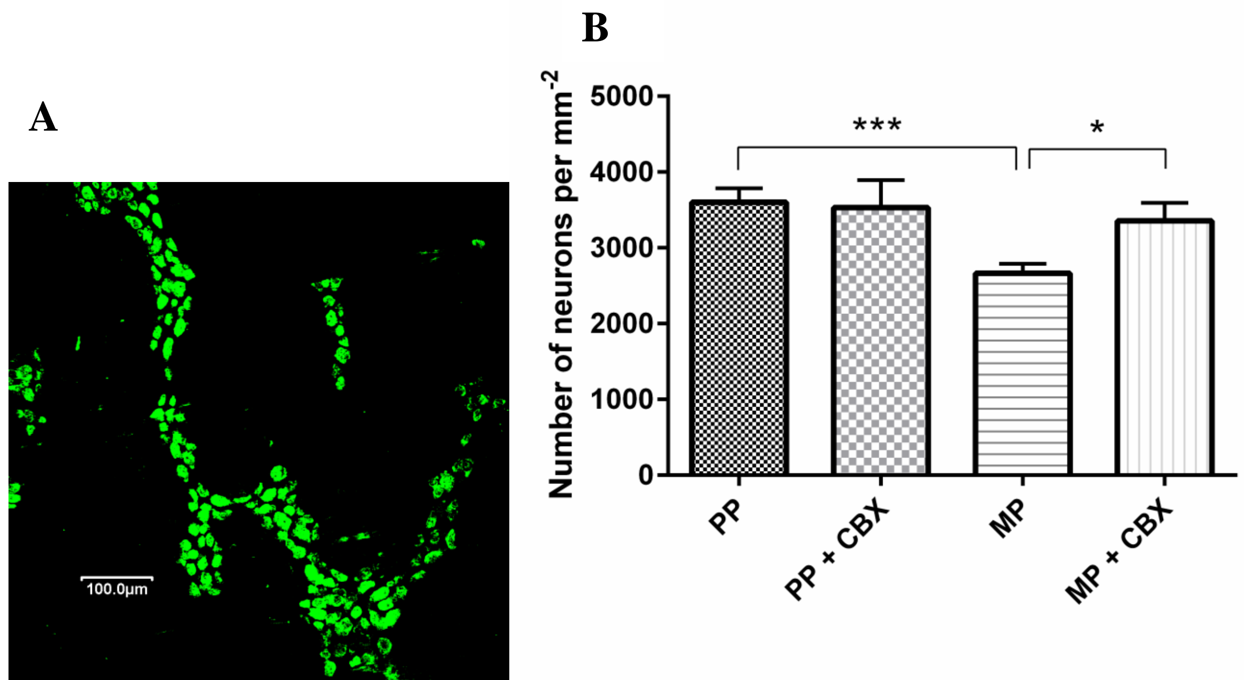


Figure 29: CBX reverses morphine-induced neuronal loss in the myenteric plexus. A) Representative image of neuronal marker Hu staining the colon myenteric plexus. B) Total neuronal density in the colon myenteric plexus in mice pelleted with either placebo or 75 mg morphine pellets for 5 days and given i.p. injections of saline or CBX (25 mg/kg) on day 3 and 4 (N=2-4). *P < 0.05 and ***P < 0.001 by one-way ANOVA (Tukey`s post-hoc).

Summary

This study examined the effect of chronic morphine on P2X receptor activity and associated inflammation in enteric glia. The major findings of this chapter are-

- *In vivo* chronic morphine exposure leads to 1) significant disruption of the colonic epithelium 2) enhanced purinergic signaling/ATP currents in enteric glia and 3) increased IL-1β expression in the myenteric plexus.
- *In vitro* morphine treatment does not enhance purinergic signaling in enteric glia.
- The increase in ATP-induced currents, cytokine expression and ATP release was observed in enteric glia directly treated with LPS, consistent with bacterial translocation as a potential mediator of chronic morphine-induced inflammation. These effects of LPS were reversed by CBX, a Cx43 hemichannel blocker.
- *In vivo* treatment with CBX (25 mg/kg) prevented 1) ATP-induced currents in enteric glia, 2) the decrease in neuronal density, and 3) colonic inflammation in chronic morphine treated mice.

Discussion

This study demonstrates that enteric glia are importantly involved in mediating chronic morphine-induced intestinal inflammation. Alterations in purinergic signaling are widely implicated in many gastrointestinal inflammatory conditions (Roberts et al., 2012) (Antonioli et al., 2013). Purinergic receptors are expressed on a variety of cell types in the GI tract including neurons, glia, ICCs, and smooth muscles. In the present study, we demonstrated that *in vivo* chronic morphine treatment results in increased ATP-induced inward currents in enteric glia.

Enhanced purinergic signaling in enteric glia was not a direct effect of morphine, since morphine alone did not significantly increase the ATP currents and did not upregulate P2X4 and P2X7 mRNA expression in enteric glia *in vitro*. This is not entirely surprising since, unlike CNS astrocytes (Ruzicka et al., 1995) (Turchan-Cholewo et al., 2008), enteric glia do not express MOR. *In vitro* morphine treatment showed a slight trend towards enhancement of ATP current and P2X receptor expression in the enteric glial cell line but not in the primary glial cultures. This can be attributed to the dissimilarities in the types of glial cells found in different species. The lack of enhanced purinergic signaling by *in vitro* exposure of enteric glia to morphine also suggests that morphine, at the concentration used in this study i.e. 3 μM, does not activate TLR4 in enteric glia. Additional dose-response experiments would be necessary to determine the effect of morphine on TLR4 activation in enteric glia. Previous studies have indicated that morphine activates TLR4 at a concentration much higher than that required to activate MOR (Farzi et al., 2015). That morphine does not activate TLR4 in enteric glia differs from findings in CNS astrocytes (Wang et al., 2012) (Hutchinson et al., 2007). It has been demonstrated that CNS glial activation and induction of immune responses via a TLR4 dependent pathway dampens the analgesic effect of opioids. Opioids were predicted to bind to the LPS-binding pocket of MD-2, a co-receptor of TLR4, leading to an inflammatory response that was lower in intensity as compared to LPS itself. Further studies are required to assess differences in morphine-TLR4 interaction in enteric glia vs CNS glia.

Studies by Meng et al. have previously shown that chronic morphine (24h) results in breakdown of epithelial barrier via a TLR2/ TLR4 pathway. We now establish that the bacterial translocation due to chronic morphine exposure results in an increase in purinergic signaling that may be important for initiating an inflammatory response within the gut wall. We found that the TLR4 agonist, LPS, which is a product of the cell membrane of Gram negative bacteria, activates enteric

glia by altering purinergic signaling in these cells. A number of studies have focused on P2Y1 and P2Y4 as key receptors for purinergic activation in the ENS (Gulbransen and Sharkey, 2009). However, Boesman et al. found that CRL-2690 enteric glial cell line does not express P2Y1 receptors, implying the involvement of other P2 receptors in enteric glial purinergic signaling (Boesmans et al., 2013). In the present study, we show that, under basal conditions, enteric glia have a low expression of P2X4 and P2X7 mRNA and small ATP-induced inward currents. An inflammatory state produced by LPS resulted in enhancement of ATP-induced inward currents in enteric glia. This increase in purinergic receptor activity was mediated by P2X receptors since ATP-induced inward currents were blocked in the presence of PPADS, a non-selective P2X receptor antagonist. LPS also enhanced P2X4 and P2X7 mRNA expression in enteric glia. Furthermore, PPADS also decreased LPS-induced pro-inflammatory cytokine production in enteric glia. Complementary to these findings, Vazquez-Villoldo et al. showed that, in the CNS, there was an increase in P2X4 responses upon treatment with LPS, as well as in other neuro-inflammatory conditions such as multiple sclerosis and experimental autoimmune encephalomyelitis in activated microglia from rat spinal cord (Vazquez-Villoldo et al., 2014). Together, these findings suggest that activation of TLR4 enhances P2X receptor activity in immune cells, which in turn triggers an inflammatory response.

In enteric glia, the release of a number of neurotransmitters, including ATP, is regulated by gap junctions. Connexins are gap junctions that permit diffusion of ions and small molecules and further participate in purinergic activation of enteric glia. Of the 21 different types of connexins, the distribution of Cx43 in different layers of the stomach, ileum, and colon has been widely characterized. In the ENS of mouse colon, Cx43 is specifically expressed in enteric glia throughout the myenteric plexus but not in enteric neurons. Our findings suggest that LPS upregulates Cx43

expression in enteric glia and Cx43 hemichannels mediate the release of ATP. The Cx43 antagonists- CBX and Gap26 blocked LPS-induced ATP currents in the enteric glial cell line and in glial primary cultures, respectively. There is ambiguity about the specificity of CBX for Cx43 inhibition and several studies imply that this drug might also be acting on other connexin subtypes. Nevertheless, it is still advantageous for the purposes of this study since Gap26, a more specific blocker of Cx43 hemichannels, had a comparable effect on inhibiting ATP currents as CBX. Moreover, CBX reduced LPS-induced pro-inflammatory cytokine production by enteric glia. Since CBX did not completely block ATP release and inflammation, this would imply the involvement of other pathways for ATP release such as pannexin-p2x7 receptors or exocytosis. Analogous to our data pointing to the role of Cx43 in LPS-induced inflammatory cytokine production, Brown et al`s studies also indicate the importance of enteric glial Cx43 in ENS pathophysiology (Brown et al., 2016). As per their findings, transgenic mice in which Cx43 is conditionally ablated in glia show reduced inflammation and decreased neurotoxicity in a DNBS colitis model.

To determine the role of TLR4 in LPS-induced pro-inflammatory cytokine production, oxidized phospholipid OxPAPC was used as a TLR4 antagonist in this study. Several studies have collectively reported that OxPAPC competitively inhibits the binding of LPS to either soluble proteins such as LBP and CD14, or to the cell-associated protein, MD-2, thus preventing the assembly of LPS with these TLR4 accessory proteins (Erridge, 2009). Paradoxically, OxPAPC has also been shown to activate TLR4 and induce secretion of IL-8 and monocyte chemoattractant protein-1 (MCP-1) in human endothelial cells and monocytes (Miller et al., 2005). OxPAPC has been identified to enhance cytokine production by lung macrophages and induce lung injury via a TLR4-TRIF mediated pathway (Imai et al., 2008). Thus, in spite of the wide use of OxPAPC as a

TLR4 antagonist, there is ongoing debate as to whether OxPAPC is predominantly pro-inflammatory or anti-inflammatory in nature. In the present study, we observed that OxPAPC prevented IL-6 mRNA expression induced by 0.1 and 1 µg/ml LPS-treated enteric glial cells. However, contradictory action of OxPAPC was seen when combined with 10 µg/ml LPS, where OxPAPC further augmented the pro-inflammatory effect of 10 µg/ml LPS. The exact mechanism for these differential effects of OxPAPC need to be further investigated. Our data shows that 10 µg/ml LPS-induced IL-6 mRNA expression was blocked by CBX and PPADS, but not by OxPAPC. Collectively, our findings suggest that this lack of inhibition of high-concentration LPS-induced IL-6 mRNA expression by OXPAPC might be due to activation of an additional connexin-P2X dependent inflammatory pathway.

In the present study, the role of bacterial translocation (LPS) mediated activation of the connexin–purinergic pathway was further investigated in chronic morphine-induced inflammation and constipation. CBX prevented chronic morphine mediated enhancement of ATP currents in enteric glia and alleviated pro-inflammatory cytokine expression in the colon. Furthermore, our findings demonstrated that chronic morphine reduced neuronal survival in the myenteric plexus. This effect is most likely mediated by glial activation of Cx43 and the resultant inflammatory state, since CBX enhanced neuronal survival in morphine-pelleted mice. Loss of myenteric neurons has previously been noted in other models of gut inflammation. An indiscriminant loss of several different types of myenteric neurons, including ChAT^{+}, NOS^{+} and VIP^{+} neurons, was observed in a guinea pig TNBS colitis model. This decrease in neuronal survival was associated with infiltration of neutrophils in the colon (Linden et al., 2005). Reduction of enteric neuronal and glial cell population in myenteric and submucosal plexus was noted in necrotizing enterocolitis (NEC), a disease occurring in premature infants (Wedel et al., 1998). Furthermore, inflammation-induced

neuronal loss has been proposed to be mediated by activation of a neuronal signaling complex comprised of P2X7 receptors and pannexin–1 (Panx1) channels (Gulbransen et al., 2012).

Limitations and future directions

Identification of specific P2X receptor subtypes: To study the involvement of purinergic P2X receptors in morphine/LPS induced enhancement of ATP responses in enteric glia, PPADS was used in this study. However, PPADS is a non-specific blocker of P2X receptors and only one effective concentration of this drug was tested. To identify the role of particular P2X subtypes, specific agonists and antagonists can be utilized. Apart from ATP, the other commonly used P2X agonists are α, β methylene ATP (α, β-met ATP), 2' (3')-O-(4 benzoyl-benzoyl)-ATP (BzATP) and 2-methyl thio ATP (2-metSATP). Different P2X subunits manifest dissimilar sensitivity to these purinergic agonists. Eg- BzATP is frequently designated as a selective agonist at P2X7 while 2-metSATP is more selective at P2X3 receptors. Similarly, there are also specific antagonists for P2X subtypes. Eg- TNP-ATP (P2X1 and 3), 5-BDBD (P2X4) and new experimental AZD compounds (P2X7). There are also knockout mouse models available for most P2X receptor subtypes. One of the major issues with targeting and knocking out a specific P2X receptor subtype is that it might induce upregulation of other P2X receptors as a compensatory mechanism (Goloncser and Sperlagh, 2014). Also while using specific subtype antagonists, it is important to consider that P2X subunits interact with each other to form functional hetero multimers (eg- P2X2/3, P2X4/6, P2X1/5, P2X2/6, P2X1/4, P2X1/2, and P2X4/7) that have dissimilar pharmacological and biophysical properties as compared to individual homo multimers (North and Surprenant, 2000) (Khakh et al., 2001).

Characterization of ATP currents: ATP is an agonist of P2X receptor and is known to desensitize various P2X receptor subtypes at different rates. In the present study, we measured inwards currents in enteric glia after a single exposure to 1 mM ATP. Our data showed that there was a dose-dependent increase in efficacy upon exposure of glial cells to LPS and after *in vivo* morphine treatment. This increase in efficacy correlated with upregulation of P2X mRNA expression. The sensitivity of the glial P2X receptors to ATP can be further tested by evaluating the dose-effect relationship of ATP in activating P2X currents. Our findings demonstrated that multiple exposures to ATP leads to irreversible desensitization resulting in reduced currents. This phenomenon has previously been observed by several groups (Visentin et al., 1999). Thus, in this study, multiple concentrations of ATP were not tested in a single cell. Testing several concentrations of ATP can also help to understand the desensitization patterns of P2X receptor subtypes since high ATP concentrations are known to exhibit a less pronounced desensitization due to P2X7 receptor opening as compared to low ATP concentrations which rapidly desensitize the P2X receptor. The issue of receptor desensitization can be partly overcome by using a fast perfusion system that delivers the agonist in very close vicinity of the cell and prevents long term exposure of the cells to the compound when it is perfused in the entire bath and removed by gravity-based outflow.

Direct effects of LPS on P2X receptors: Since LPS is a widely recognized agonist of TLR4, the effects of LPS in enhancing P2X receptor expression and purinergic activity in enteric glia was assumed to be mediated through TLR4. Accordingly, it was observed that TLR4 antagonist OxPAPC blocked 0.1 and 1 µg/ml LPS-induced enhancement of IL-6 mRNA expression in enteric glia. However, a few studies have reported that residues 573–590 of $P2X_7$ share strong amino acid

homology with the LPS binding site of LBP. This conserved domain is at the C-terminal end of P2X7 and does not interfere with residues important for ATP binding (Denlinger et al., 2001) (Alves et al., 2014). The involvement of direct interaction between LPS and P2X7 can be further tested using TLR4 knock-out models.

Immunostaining using μ-opioid receptor antibody: In this study, to determine the expression and localization of MOR in the ENS, neurons and glia, MOR antibody from Alomone labs was utilized. There has been a lack of specific MOR antibodies in the past (Niwa et al., 2012). The MOR antibody used in this study is claimed to be more specific and is directed to amino acid residues 22-38 of the extracellular N-terminus of MOR. Our data show that MOR is expressed on neurons and co-localizes with β-III tubulin, but is not found on enteric glia as seen by absence of co-localization with glial marker GFAP. Furthermore, other findings in our lab suggest the presence of MOR on specifically cholinergic and nitrergic neurons in the ENS. However, the specificity of this antibody still needs to be validated using either a μ-opioid receptor knock out mouse model, recombinant expression systems or si-RNA approaches. Also, since studies have demonstrated that inflammation upregulates MOR expression in the gut, it would be important to test this antibody in enteric glia isolated from chronic morphine pelleted mice.

Morphine concentration: Previous reports from our lab demonstrated that acute exposure of enteric neurons to 3 μM morphine decreases neuronal excitability and chronic/overnight exposure produces dependence at single cell level. In the present study, the same concentration of morphine (3 μM) was used and its effect on activation of enteric glial cells was tested. Although, 3 μM did not enhance purinergic activity in enteric glia possibly due to lack of MOR expression on enteric

glia, higher concentrations of morphine need to be assessed for TLR4 activation. 3 μM morphine is representative of the concentration of the morphine achieved *in vivo* in mice upon subcutaneous surgical implantation of a 75 mg morphine pellet. Upon morphine pelleting in mice, serum morphine concentrations of more than 4 μM are observed within 4-6 hr and morphine concentration progressively decreases to 1- 2 μM on day 5 after the surgery (Zhang et al., 2011) (Hill et al., 2016).

Chapter V

The role of connexin hemichannels in chronic morphine-induced constipation and analgesic tolerance

Chronic morphine causes microbial dysbiosis and promotes bacterial translocation leading to exaggerated inflammation, especially in the colon. The next aim of this study was to determine the role of this secondary colonic inflammation in modulating two important adverse effects of chronic morphine- persistent constipation and analgesic tolerance.

CBX significantly reduces opioid-induced constipation

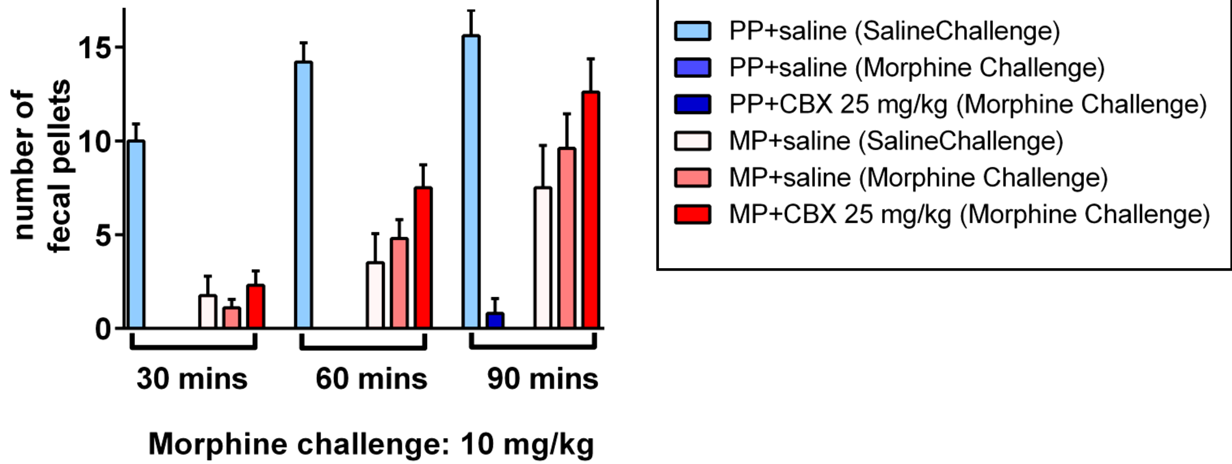
As described earlier, chronic use of morphine leads to GI dysfunction with OIC being the most pronounced (Schaumann, 1955) (Kumar et al., 2014). GI motility is regulated predominantly by the ENS, that consists of neurons and glia (Brookes, 2001). Morphine acts on μ-opioid receptors expressed on enteric neurons to decrease neuronal excitability and this has been considered to be the primary mechanism leading to constipation (Paton, 1997) (North and Williams, 1977). Interestingly, with repeated administration, tolerance does not occur to the constipating effects of chronic morphine, resulting in persistent constipation (Ross et al., 2008). Several recent studies have collectively described that, along with constipation, chronic morphine also causes immune dysregulation, thus increasing susceptibility to infection and initiates a secondary inflammatory response (Roy et al., 1998). Thus, we hypothesized that continued constipation may be exaggerated by secondary inflammation arising from bacterial translocation. In the present study, we evaluated the effect of sustained secondary inflammation upon chronic morphine exposure as one of the crucial factors contributing to persistent constipation. The previous chapter described a possible

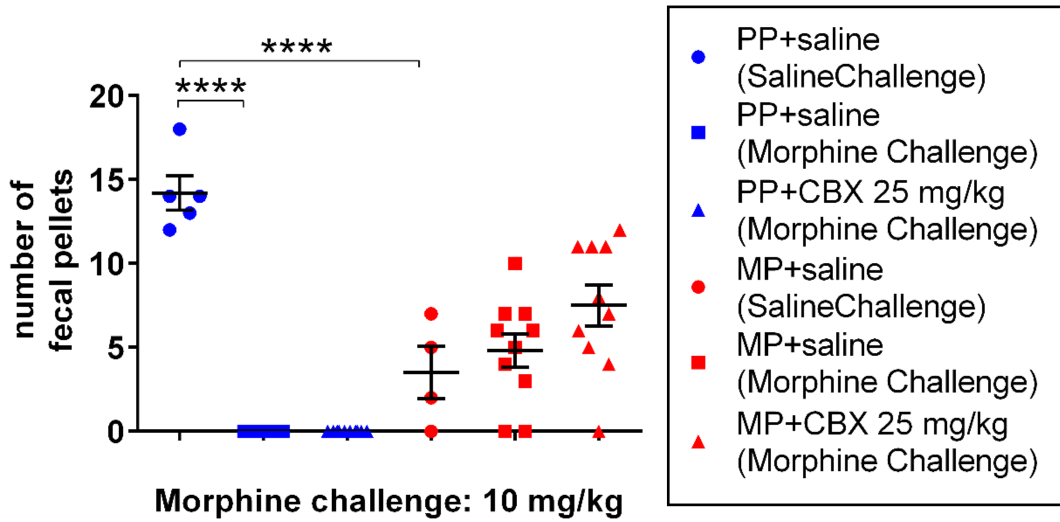
contribution of enteric glia and activation of the connexin-purinergic pathway in mediating chronic morphine-induced inflammation in the gut. To establish the effect of bacterial translocation-induced inflammation and decreased neuronal survival on motility, we utilized CBX, which in our previous studies was shown to decrease morphine-induced inflammation in the colon.

To assess whole GI motility, total fecal pellet output and transit time were measured in non-fasted mice. In an initial set of experiments, a 10 mg/kg subcutaneous acute morphine challenge or saline challenge was given to mice pelleted with placebo or 75 mg morphine pellets for 5 days and treated with/without CBX. The number of fecal pellets expelled by these mice was counted every 30 mins. A similar trend in fecal pellet numbers was observed at each of the time points measured. The data for the 60 mins time point is plotted as a scatterplot. There was a significant decrease in the number of fecal pellets in placebo pelleted mice given a morphine challenge vs a saline challenge. 25 mg/kg CBX did not alter the number of fecal pellets following a morphine challenge in the placebo pelleted mice. Mice pelleted with 75mg morphine pellets developed constipation as seen by their reduced fecal pellet output. 25 mg/kg CBX partially increased the fecal pellet output in the morphine pelleted mice but did not exhibit full reversal of constipation Fig 30A.

In another set of experiments, a 2 mg/kg subcutaneous acute morphine challenge was tested in mice. As expected, there was a decrease in the number of fecal pellets expelled by placebo pelleted mice given a morphine challenge vs a saline challenge. 25 mg/kg CBX demonstrated a trend toward further inhibiting the fecal pellet expulsion in placebo pelleted mice after a morphine challenge. Mice pelleted with 75mg morphine pellets developed constipation as seen by their reduced fecal pellet output. 25 mg/kg CBX slightly enhanced the fecal pellet output in the morphine pelleted mice after 60 mins but did not exhibit full reversal of constipation Fig 30B.

Additionally, in another assay to determine GI motility, whole intestinal transit was measured using carmine red dye. Since carmine red dye is not absorbable from the lumen of the gut, the dye was administered to the mice by oral gavage and the time of first observance of carmine red in the expelled fecal pellets was noted as the total transit time. Chronic morphine significantly slowed GI transit as compared to placebo pelleted mice (171.9 ± 7.6 vs 77.11 ± 6.07 mins, $p < 0.0001$). CBX enhanced intestinal motility in morphine treated mice as indicated by significantly decreased transit time (114.2 ± 17.14 mins, $p=0.0044$) without affecting motility in the control animals (80.9 ± 8.5 mins) Fig 31.


A
Fecal pellet output assay
number of fecal pellets
15
10
5
0
30 mins
60 mins
90 mins
Morphine challenge: 10 mg/kg
PP+saline (SalineChallenge)
PP+saline (Morphine Challenge)
PP+CBX 25 mg/kg (Morphine Challenge)
MP+saline (SalineChallenge)
MP+saline (Morphine Challenge)
MP+CBX 25 mg/kg (Morphine Challenge)


Fecal pellet output
****
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number of fecal pellets
20
15
10
5
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Morphine challenge: 10 mg/kg
PP+saline (SalineChallenge)
PP+saline (Morphine Challenge)
PP+CBX 25 mg/kg (Morphine Challenge)
MP+saline (SalineChallenge)
MP+saline (Morphine Challenge)
MP+CBX 25 mg/kg (Morphine Challenge)

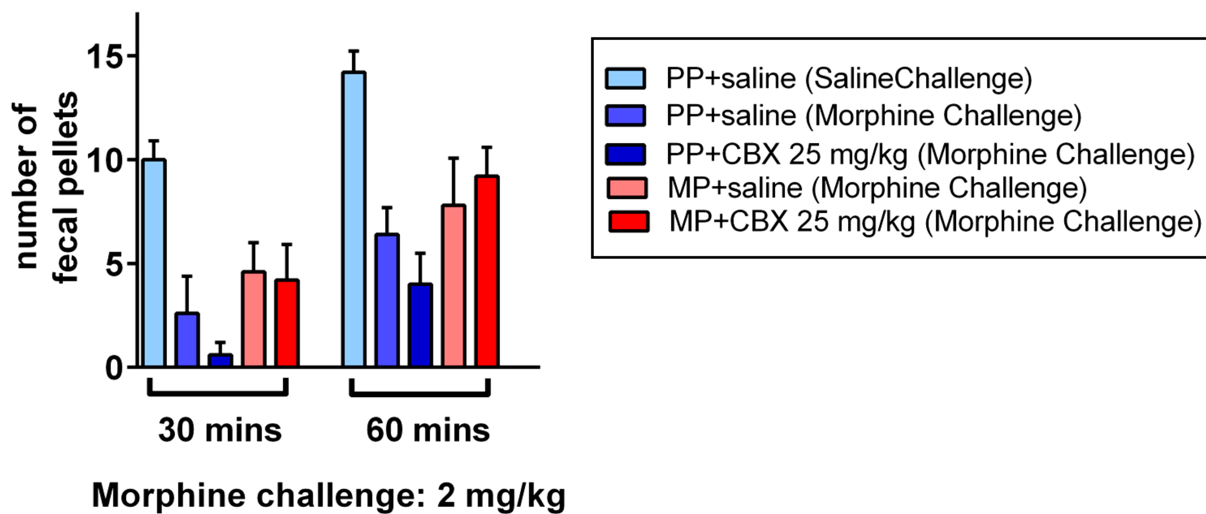

Figure 30: CBX reduces chronic morphine-induced constipation in fecal pellet output assay. Total number of fecal pellets expelled counted in mice pelleted with either placebo or 75 mg morphine pellets for 5 days and given i.p. injections of saline or CBX (25mg/kg) on day 3 and 4 after a saline or A) 10 mg/kg and B) 2 mg/kg morphine challenge. ****P < 0.0001 by one-way ANOVA (Tukey`s post-hoc).

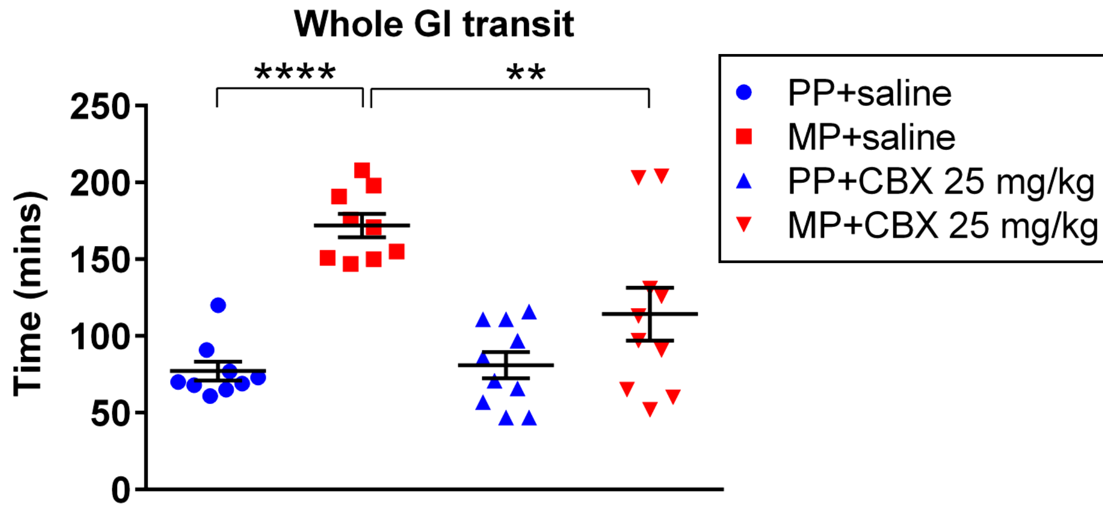

Figure 31: CBX reverses chronic morphine-induced slowing of GI transit. Whole GI transit time in mice pelleted with either placebo or 75 mg morphine pellets for 5 days and given i.p. injections of saline or CBX (25mg/kg) on day 3 and 4 measured after an oral gavage of carmine red dye (N=9-10). **P < 0.01, ****P < 0.0001 by one-way ANOVA (Tukey`s post-hoc).

CBX reverses chronic morphine-induced analgesic tolerance

Although morphine is one of the most widely prescribed drugs for moderate to severe pain clinically, chronic use of morphine is associated with the development of analgesic tolerance, thus limiting its efficacy as an effective pain reliever. Recent findings in our lab indicate that the gut microbiome is an important modulator of the analgesic responses of morphine. When an antibiotic cocktail was used to deplete the microbes in the gut lumen, reversal of analgesic tolerance was observed in mice chronically treated with morphine. Antibiotic administration also prevented the increases in gut permeability, mucosal destruction and colonic inflammation induced by chronic morphine. To further investigate the downstream mechanism underlying the role of gut microbe mediated inflammation in reversal of morphine-induced analgesic tolerance, CBX was utilized as an agent to decrease colonic inflammation. Our data showed that placebo pelleted mice responded to an acute 10 mg/kg morphine challenge and exhibited 83.75 ± 6.8 % MPE. 25 mg/kg CBX did not alter the analgesic effect of the morphine challenge in the placebo pelleted mice (92.9 ± 7.1 % MPE). Morphine pelleted mice developed analgesic tolerance as seen by their lack of response to the morphine challenge injection exhibiting 16.59 ± 4.1 % MPE. Furthermore, morphine pelleted mice that were given i.p. injections of CBX now responded to the morphine challenge (75.35 ± 11.36 % MPE). Interestingly, the MP+CBX group also demonstrated slightly higher baseline tail flick latencies as compared to the other three groups. Collectively, these findings suggested that CBX reversed chronic morphine-induced analgesic tolerance in the tail flick test Fig 32.

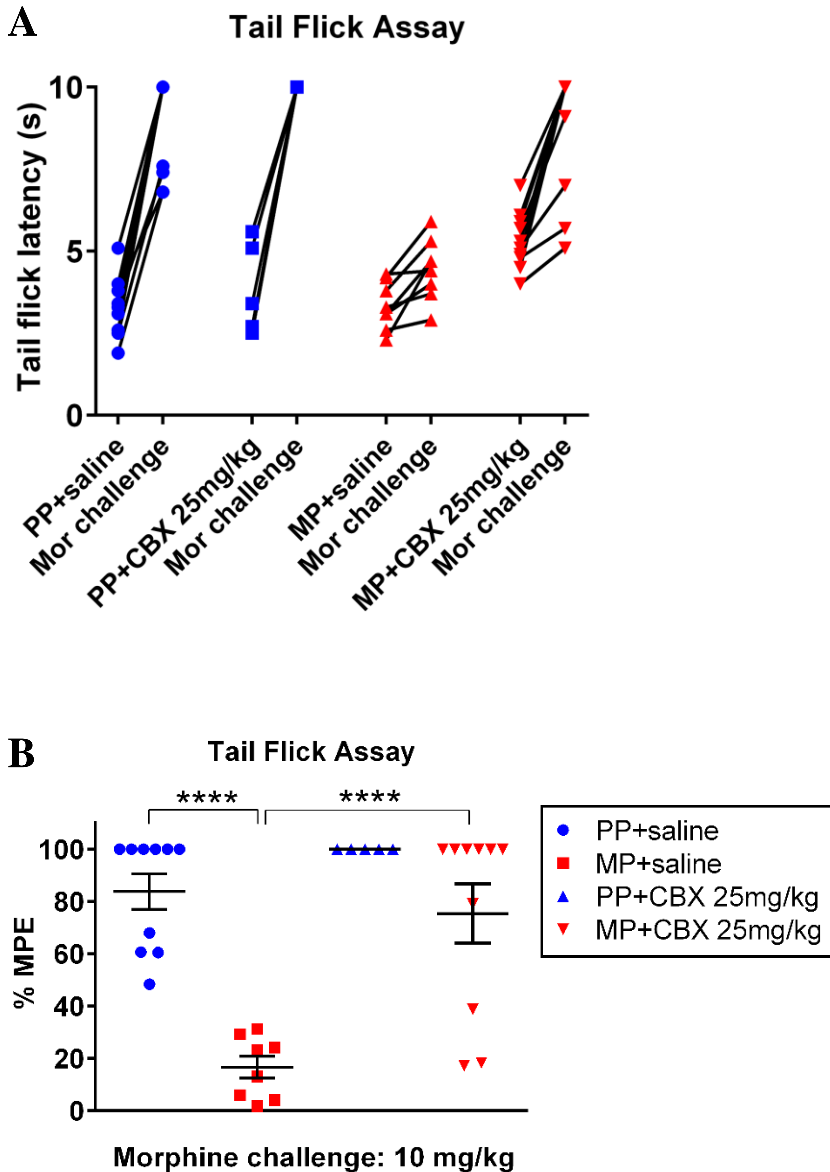

Figure 32: CBX reverses chronic morphine-induced analgesic tolerance in tail-flick test. A) Tail flick latencies in secs and B) The percentage of maximum possible effect (%MPE ± SEM) measured in a tail-flick test for analgesic tolerance in mice pelleted with either placebo or 75 mg morphine pellets for 5 days and given i.p. injections of saline or CBX (25mg/kg) on day 3 and 4 after a 10 mg/kg morphine challenge (N=5-10) ****$P < 0.0001$ by one-way ANOVA (Tukey`s post-hoc).

Discussion

Our present findings illustrate that Cx43 inhibition by CBX not only reduces colonic inflammation but also reverses constipation in chronic morphine treated mice. Two assays were utilized to assess GI motility- the fecal pellet output assay and the carmine red dye to determine transit time. In the first assay, the number of fecal pellets expelled in response to a challenge dose of saline/2 or 10 mg/kg morphine was measured. Morphine challenge dose-dependently inhibited the number of fecal pellets expelled in placebo-pelleted mice re-establishing that acute morphine administration inhibits GI motility. CBX showed a trend toward further reducing the fecal pellet output following 2 mg/kg acute morphine treatment. The exact mechanism underlying acute morphine-CBX interaction is unknown. On the other hand, the GI transit assay using carmine red dye was performed in the absence of any morphine challenge. In this assay, CBX did not affect transit time in placebo-pelleted animals. Contradictory observations were made by McClain et al., where they reported that glial specific Cx43 knockout mice displayed slower colonic transit times in comparison to wild type mice due to blunted Ca^{2+} responses in enteric glia (McClain et al., 2014). Their study indicated the importance of Cx43 in maintaining the physiologic functions of enteric glia and in mediating GI motility. Our findings show that the dose the CBX used in the present study (25 mg/kg) was insufficient to alter motility at basal conditions but inhibited motility in mice treated with acute morphine. Furthermore, CBX demonstrated an opposite effect in mice chronically treated with morphine, where it decreased whole GI transit time and enhanced fecal pellet output. These findings highlight the binary role of Cx43 inhibition in physiologic and pathophysiologic conditions. This is in accordance with other studies that demonstrate that, in a basal/physiologic state, Cx43 inhibition in enteric glial cells blunts glial network activity and neuronal-glia interactions (McClain et al., 2014). Conversely, in context of pathological conditions arising in a DNBS model of colitis, Cx43 inhibition had a protective effect. Mice with a conditional

ablation of Cx43 hemichannels in glia were resistant to the neurotoxic effects of *in vivo* gut inflammation (Brown et al., 2016).

Another important observation made in the present study was that along with decreasing constipation, i.p injections of CBX also reversed analgesic tolerance in a tail-flick test in morphine-pelleted mice. This finding is well supported by previous studies demonstrating that development of morphine anti-nociceptive tolerance is reversed by another Cx43 antagonist, Gap26 (Shen et al., 2014). Interestingly, they injected Gap26 intrathecally into mice to determine the role of spinal astrocytic Cx43 in mediating morphine anti-nociceptive tolerance. However, in our study, CBX was given i.p to the mice. Considering evidence that CBX does not cross the blood brain barrier when given i.p (Leshchenko et al., 2006), this might rule out the role of spinal astrocytic Cx43 in morphine analgesic tolerance in our study. These findings suggest that peripheral gap junction inhibition, most likely occurring in enteric glia or satellite glial cells (SGC) in the dorsal root ganglia (DRG), and decrease in inflammation either at the level of the gut or DRGs, might be playing a role in enhancing morphine`s CNS effects and reversing tolerance. It has been previously reported that gut inflammation in a DNBS colitis model augmented cell coupling between SGCs and neurons in the DRG, resulting in neuronal hyper excitability and leading to visceral pain (Huang et al., 2010). This suggests that gap junction blockers can act as analgesics by targeting the inflammation-induced changes in DRG glia. Whether reversal of morphine-induced analgesic tolerance is mediated by DRG neurons or enteric glia remains to be determined.

In summary, the findings of this study imply a role of gut bacteria mediated glial activation induced colonic inflammation in modulating the two important adverse effects of chronic morphine-constipation and analgesic tolerance.

Limitations and future directions

GI motility assays: In this study, the fecal pellet output assay and the whole GI transit assay were performed to measure GI motility. Both these assays assess whole GI transit. Consistent with our observation, previous findings in our lab have shown that morphine dose-dependently decreases the fecal pellet output in mice (Fitting et al., 2015). However, the major drawback of this experiment is that the total food intake for each animal was not controlled. Also, the fecal pellets were counted every 30 mins which makes this assay less sensitive as compared to the carmine red dye assay for measuring whole GI transit. This might explain some of the variability seen in the data. Nevertheless, our data showed that chronic morphine decreased the number of fecal pellets expelled and this effect was partially reversed by CBX. Other measures of whole fecal pellet output that can be evaluated in this assay are fecal pellet weight and water content. Apart from whole GI transit assessment, the effect of morphine and CBX can be evaluated specifically in the ileum or colon *ex vivo* in the organ bath or GIMM set up. Ileal transit can also be measured using a charcoal meal assay and colonic transit by bead expulsion assay. It is well known that chronic morphine has differential effects in the ileum and colon. It would be further important to study the role of CBX in modulating morphine`s effects in specific gut segments.

Cx43 antagonists and knock out mouse models: In this project, CBX was used as a Cx43 inhibitor in both *in vivo* and *in vitro* experiments. However, many studies in the literature point to the non-specific and off-target effects of CBX. Hence, we used Gap 26, a more specific Cx43 antagonist, in order to confirm some of the findings seen with CBX. In line with CBX, Gap 26 blocked LPS-induced enhancement of ATP currents in enteric glia when used *in vitro*. The major limitation of using Gap 26 was that, being a peptide mimetic, it could not be administered

systemically to mice for *in vivo* experiments due to probable degradation by proteases in the systemic circulation. Thus, in spite of being a more specific Cx43 antagonist, we were unable to test the effect of Gap26 on morphine-induced enhancement of purinergic signaling and inflammation in the colon. Due to similar effects demonstrated by CBX and Gap26 *in vitro*, we considered CBX as a good tool for the purpose of this study.

The role of Cx43 in morphine-induced colonic inflammation and constipation can be further evaluated using knock out mouse models. Although homozygous Cx43 knockout mice ($Cx43^{-/-}$) die in the early postnatal period with cardiac defects (London, 2004), mice with a conditional Cx43 deletion in astrocytes are commonly available (Liebmann et al., 2013) (Theis et al., 2004) and can be utilized to study the effect of Cx43 deletion on enteric glial activation and resultant inflammation upon exposure to morphine/LPS.

Assessment of analgesic tolerance: Our findings demonstrated that i.p injections of CBX reverse analgesic tolerance in a tail-flick test in morphine-pelleted mice. The main question to be addressed in future studies is whether morphine`s analgesic effects are mediated by the gut microbiome and their downstream activation of connexin-purinergic signaling in enteric glia. To evaluate the role of enteric glial Cx43 in modulating analgesic tolerance, enteric glia specific Cx43 knock out models need to be used. Since CBX does not cross the blood brain barrier when given i.p (Leshchenko et al., 2006), this implicates a role of peripheral gap junction inhibition, most likely occurring in enteric glia or satellite glial cells (SGC) in the dorsal root ganglia (DRG) in reversal of analgesic tolerance. The effect of Gap26 on inhibition of enteric glial Cx43 in morphine-pelleted mice could not be tested since i.v or i.p administration of peptides leads to their rapid degradation in circulation. GFAP promoter driven Cx43 knock out mice were also not pertinent to this study

since this model is a global Cx43 knock out in all cells expressing GFAP, including CNS astrocytes and satellite glia in the dorsal root ganglia. Thus, this model will be inadequate to determine the contribution of enteric glia in mediating analgesia. Moving forward, a new Sox10 driven Cx43 knock out mouse model is being developed. Since Sox10 is mostly expressed by peripheral enteric glial cells and central oligodendrocytes (Kuhlbrodt et al., 1998), this model might prove to be a better representative of enteric glial gap junction inhibition as compared to GFAP promoter directed Cx43 inhibition.

Conclusions and perspectives

Taken together, these studies have analyzed the effects of chronic morphine-induced bacterial translocation on enteric glia. The adult human gut contains about 100 trillion microorganisms that constitute the gut microbiome. These gut microbiota are important in maintaining normal gut functions, which include preserving gut epithelial integrity, fighting against pathogenic micro-organisms and mediating inflammatory responses. Our findings show that modifications in the gut microbiome following chronic morphine exposure increases enteric glial activation by modulating the connexin-purinergic pathway Fig 33. These effects of morphine on the ENS, together with an increase in pro-inflammatory cytokine release, could account for decrease in GI motility observed in morphine-pelleted mice. These findings correlate with the development of persistent constipation observed in individuals on long-term morphine treatment. The present study points to one of the possible mechanisms leading to OIC and highlights the role of enteric glial activation of connexin-purinergic pathways in mediating chronic morphine`s effects in the GI tract.

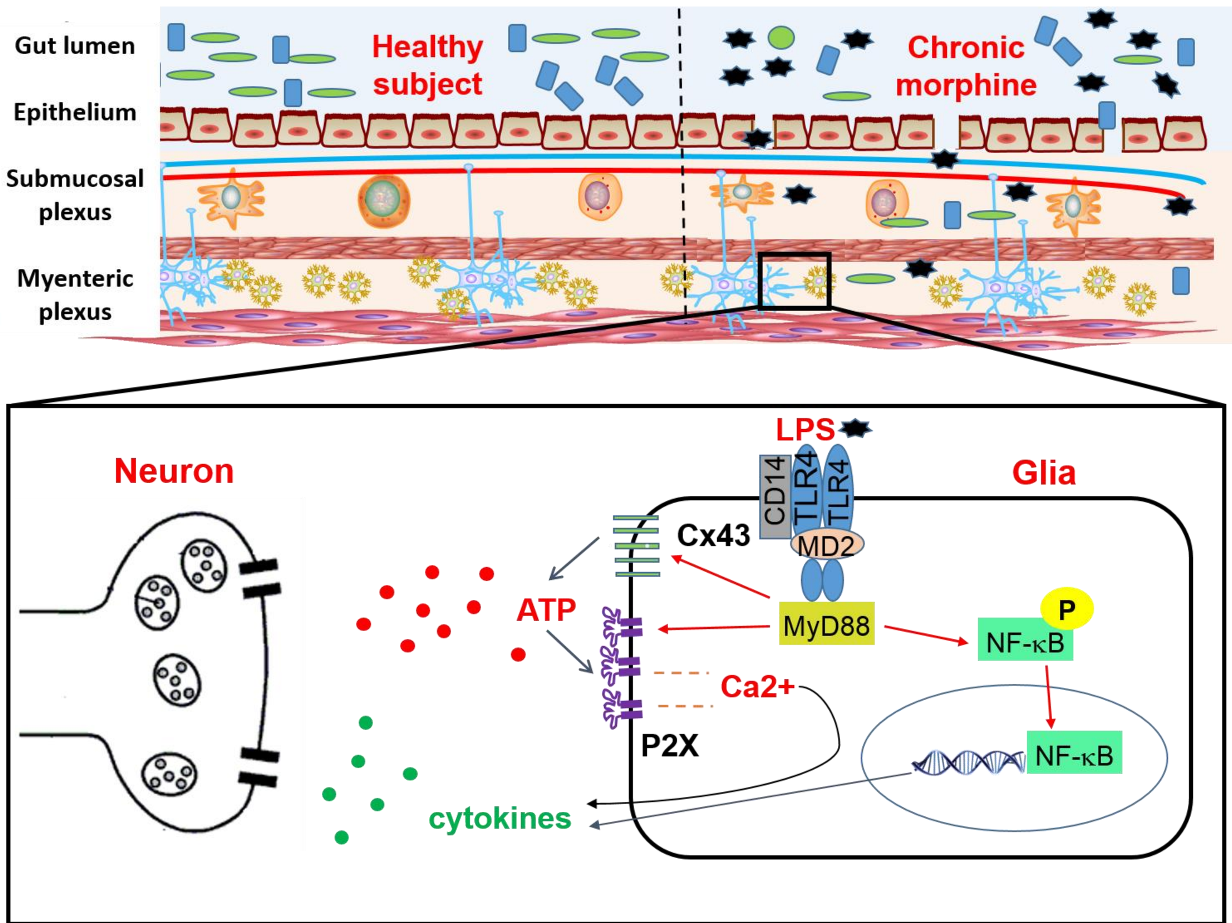

Figure 33: Schematic representation of morphine mediated changes in the gut and activation of connexin-purinergic pathway in enteric glia.

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EDUCATION

VIRGINIA COMMONWEALTH UNIVERSITY Richmond, VA

PhD Candidate in Pharmacology and Toxicology (GPA: 3.9/4.0) 2012 - Present
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MUMBAI UNIVERSITY Mumbai, India

Bachelor of Science in Pharmacy (GPA: 3.9/4.0) 2006 - 2010

RESEARCH

Doctoral Research 2012 - Present

- Investigating the role of gut microbiome and activation of enteric glia in chronic morphine-induced intestinal inflammation, constipation and analgesic tolerance - Conducted with *Dr. Hamid Akbarali*

Masters Research 2010 - 2012

- Investigated synergy between ribonucleotide reductase inhibitors and ganciclovir to inhibit human cytomegalovirus replication *in vitro* - Conducted with *Dr. Michael McVoy*

TECHNICAL SKILLS

- **Electrophysiology -** Whole cell patch clamp on isolated primary cultures of neurons and glia
- **Cell isolation and culture -** Isolation of enteric neurons and glia from adult mouse ileum and colon; Worked with- Rat enteric glia cell line CRL2690, Human MRC-5 fibroblasts CCL-171, Human ARPE-19 epithelial cells CRL-2302
- **Molecular biology -** RNA isolation, PCR, Western blot, ELISA, siRNA techniques, Luciferase based ATP release assay, GFP and luciferase based viral yield reduction assay, cytotoxicity assay, MTT assay
- **Histology -** Tissue fixation and cryo-sectioning, Immunohistochemistry, Cell staining with DAPI, hematoxylin and eosin
- **Microscopy -** Confocal microscopy
- **Gastrointestinal motility assays -** Fecal pellet output assay, Charcoal assay, Bead expulsion assay, *In vitro* Gastrointestinal motility monitor (GIMM)
- **Behavioral assays -** Tail flick test, Acetic acid stretch assay
- **Statistical tools -** Prism, JMP, MacSynergyII

PUBLICATIONS

- **Bhave S**, Elford H, McVoy M. (2013) Ribonucleotide reductase inhibitors hydroxyurea, didox, and trimidox inhibit human cytomegalovirus replication in vitro and synergize with Ganciclovir.*Antiviral Res.*100(1):151-158
- Maguma HT, Datta De D, **Bhave S**, Dewey WL, Akbarali HI (2014) Specific Localization of β-Arrestin2 in Myenteric Plexus of Mouse Gastrointestinal Tract. *PLoS ONE* 9(8): e103894. doi:10.1371/journal.pone.0103894
- **Bhave S**, Gade A, Kang M, Dewey W, Akbarali HI (2016) The role of P2X receptors and connexin hemichannels in chronic morphine induced intestinal inflammation and anti-nociceptive tolerance. (manuscript in preparation)
- Guedia J, Brun P, **Bhave S**, Fitting S, Kang M, Dewey WL, Hauser KF, Akbarali HI (2016) HIV-1 Tat exacerbates lipopolysaccharide-induced cytokine release via TLR4 signaling in enteric nervous system. *Scientific reports* 5;6:31203
- Kang M, Mischel R, **Bhave S**, Komla E, Huang C, Dewey W, Akbarali HI (2016) Effects of gastrointestinal bacteria depletion on opioid tolerance and dependence. *Scientific reports* (in review)

EXPERIENCE

VIRGINIA COMMONWEALTH UNIVERSITY Richmond, VA

- **Guest Lecturer**, Drug Biology, Department of Biology 2015
- **DNA Sequencing Technician**, DNA Core Facilities 2011 - 2012
- **Tutor**, Biochemistry, Office of Special Services for Student 2011
- **Adjunct Faculty**, Biology Lab, Department of Biology 2011

FRANCO INDIAN PRIVATE LTD Mumbai, India

- **Research Trainee**, Quality Control and Assurance Department 2009

LEADERSHIP POSITIONS

- **Communications Chair**, Graduate Student`s Association 2014 -2015
- **Secretary**, PharmTox Student Organization 2014 -2015

AWARDS

- Thesis/Dissertation Assistantship Award, VCU Spring/Summer 2012
- Graduate School Travel Grant, VCU 2013
- Graduate Student Travel Award, ASPET - Experimental Biology meeting 2014 and 2015
- Charles C. Clayton award (recognizing achievement at a middle stage of PhD program), VCU 2014
- Graduate School scholarship for contribution to the GSA executive board, VCU 2015
- Phi Kappa Phi Award, VCU School of Medicine 2016
- ASPET`s trainee award for industrial visit to Janssen, PA 2016

PROFESSIONAL SOCIETIES

- American Association for the Advancement of Science (AAAS)
- American Society for Pharmacology and Experimental Therapeutics (ASPET)
- Phi Kappa Phi Honor Society
- Who`s who among students in US Universities and Colleges honor society

CONFERENCE PRESENTATIONS

- Oral Presentation at 93rd annual meeting Virginia Academy of Sciences 2015
  'The role of Toll-like receptor 4 in enteric glia'
- Poster presentation at Experimental Biology 2015
  'The role of Toll-like receptor 4 in enteric glia'
- Poster presentation at Experimental Biology 2014
  'Role of b-arrestin 2 and GRK5/6 in the development of differential tolerance to morphine in colon and ileum'
- Oral Presentation at the 3rdVirginia Regional Herpesvirus Symposium 2012
  'Investigating Synergy between ribonucleotide reductase inhibitors and CMV antivirals'

SERVICE

- Served as a Co-chair for VCU Health System's Health Fair and Clinic 2014
- Served as a Judge for Metro Richmond STEM fair 2014
- Served as a Chair for Animal and Health Sciences division, Virginia Junior Academy of Sciences (VJAS) 2014
- Served as a Youth Ambassador for HandsOn Greater Richmond Volunteering Organization 2010 – 2013

REFERENCES

Available upon request