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Antinociceptive tolerance to morphine is driven by colonic inflammation and mediated by peripheral opioid receptors

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Antinociceptive tolerance to morphine is driven by colonic inflammation and mediated by peripheral opioid receptors

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

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ABX	antibiotic cocktail (streptomycin, neomycin, vancomycin, metronidazole, and ampicillin)
AC	adenylyl cyclase
β-arr2	β-arrestin2
°C	degrees Celsius
cAMP	cyclic adenosine monophosphate
CD	Crohn's disease
CNS	central nervous system
DNA	deoxyribonucleic acid
DOR	δ-opioid receptor
DRG	dorsal root ganglion
ED50	effective dose 50
ENS	enteric nervous system
FITC	fluorescein isothiocyanate
g	gram(s)
GI	gastrointestinal
GPCR	G protein-coupled receptor
H&E	hematoxylin and eosin
hr	hour(s)

i.p.	intraperitoneal
IBD	inflammatory bowel disease
IL	interleukin
kg	kilogram(s)
KOR	κ-opioid receptor
LPS	lipopolysaccharide
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
MOR	μ-opioid receptor
MP	morphine pellet
MPE	maximum possible effect
mRNA	messenger ribonucleic acid
MTNX	methylnaltrexone
NAP	17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[(4'pyridyl) acetamido] morphinan
μL	microliter(s)
N	sample size (animals)
OIC	opioid-induced constipation
PAMORA	peripherally active μ-opioid receptor antagonist
PAMP	pathogen-associated molecular patterns
PNS	peripheral nervous system
PP	placebo pellet

qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
s.c.	subcutaneous
sec	second(s)
SEM	standard error of the mean
SW	swiss webster
TLR	toll-like receptor
TNF	tumor necrosis factor
TNBS	2,4,6-trinitrobenzenesulfonic acid
UC	ulcerative colitis

Abstract

ANTINOCICEPTIVE TOLERANCE TO MORPHINE IS DRIVEN BY COLONIC INFLAMMATION AND MEDIATED BY PERIPHERAL OPIOID RECEPTORS

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

Virginia Commonwealth University, 2019

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Opioids are powerful analgesics. Despite their high efficacy for the management of moderate to severe pain, their clinical utility is limited due to the occurrence of adverse effects. The main problem associated with opioid use is the differential rate of tolerance development to the various pharmacological effects of opioids, with tolerance to respiratory depression occurring at a slower rate than analgesic and euphoric effects. The development of analgesic tolerance, where the efficacy of the drug progressively diminishes with repeated administration, requires higher doses of the drug to achieve a maximum effect. Reports have implicated inflammation as a major driver of analgesic tolerance development. With surmounting evidence that the prototypical opioid, morphine induces pro-inflammatory cytokine release in the brain, spinal cord, and gastrointestinal tract, a question arises of whether pro-inflammatory cytokine release in the gut as a result of chronic morphine treatment is paralleled with the development of morphine antinociceptive

tolerance. This dissertation investigated the rate at which antinociceptive tolerance to various doses of morphine developed to a different degree in the presence of colonic inflammation. Using a mouse model, colonic inflammation was induced with 2,4,6-Trinitrobenzenesulfonic acid (TNBS) and then the mice were pelleted with 25 mg, 50 mg (2x25), or 75 mg morphine pellet. Antinociceptive tolerance to morphine was determined in a warm-water tail-immersion assay upon an administration of a morphine challenge dose (10 mg/kg). Inflammatory cytokine expressions and protein levels were measured from whole colon using qPCR and ELISA, respectively. Morphine antinociceptive tolerance was significantly enhanced in the presence of colonic inflammation in a dose and time dependent manner. With a daily injection of 0.5 mg/kg peripheral opioid receptor antagonist 6β-N-heterocyclic substituted naltrexamine derivative (NAP), mice pelleted with 25 mg, 50 mg (2x25), or 75 mg morphine pellets were tested on day 5, 4, or 3, respectively. Tolerance to morphine as well as the enhanced tolerance observed in the presence of colonic inflammation was prevented with daily NAP treatment. However, NAP did not block morphine-induced or TNBS-induced inflammation. Collectively, our findings indicate that inflammation is a major modulator of morphine antinociceptive tolerance and peripheral opioid receptors may be responsible for mediating antinociceptive tolerance.

CHAPTER 1

Introduction

I. History of Opioids

The history of opiates dates back to 3400 BC where opium poppy plants (*Papaver somniferum)* were first cultivated in the lower Mesopotamia region, modern-day Iraq and Kuwait (Brownstein, 1993). They were named "Hul Gil" or "Joy Plant" by the Sumerians who were aware of the plant's euphoric abilities. The euphoric properties of opium poppy plants were found in the poppy juice (opium latex), which contained the naturally occurring analgesic alkaloid compounds called opiates (Booth, 1996).

The cultivation of opium has spread throughout history from ancient Greece to ancient Egypt and it was extensively used for pain relief, anesthesia during surgery, insomnia, and even to pacify crying children. Though opium was used for a variety of ailments among ancient societies, its active ingredient was not known until 1805 when a German scientist, Friedrich Sertürner isolated the first active alkaloid from opium (Brownstein, 1993). Sertürner labeled this substance "morphium" after the Greek god of dreams, Morpheus, because of the substance's somniferous properties. "Morphium" is better known today as morphine. Morphine is the most abundant psychoactive alkaloid compound found in opium followed by codeine. The oral bioavailabilty of morphine is quite poor when consumed by mouth (20-40%). Thus following the invention of hyperdermic needle in 1853, morphine was used intravenously during minor surgical procedures; giving rise to the clinical use of opioids.

Morphine became the most prolific drug used during the U.S. Civil War (1861 – 1865) and as a result, many soldiers became addicted to the point where the term "soldier's disease" was coined to refer to post-war morphine addiction. Due to its abusive liability, scientists sought

to find a "non-addictive" substitute for morphine. In 1874, heroin, an acetylated form of morphine was first made from morphine as a safer replacement by the English chemist Alder Wright. Unaware of its addictive properties, heroin was marketed as a morphine substitute and a cough suppressant by the German pharmaceutical company Bayer in 1898 (Hosztafi, 2001). As a result, this gave rise to heroin addiction in the United States and Western Europe.

By the end of the 19th Century and early 20th Century, the U.S.'s focus was to end the non-medical use of opioids, which prompted Congress to pass the Opium Exclusion Act in 1909, banning the importation of opium for smoking purposes. Soon after, the Harrison Narcotics Tax Act of 1914 was passed, imposing a nominal tax on the distribution and sale of heroin, opiates, and cocaine. A few years later after morphine became a controlled substance under the Act, oxycodone and hydrocodone, other prospective candidates emerged. Oxycodone was developed by German scientists at the University of Frankfurt in 1916 and hydrocodone in 1920. Scientists had high hope for these two new substances since they were not derived from morphine but rather from two other alkaloids found in opium, thebaine and codeine. The idea behind the development of these "semi-synthetic" compounds, oxycodone and hydrocodone, was that they would be devoid of the addictive properties of morphine and heroin but retain their analgesic effects. They were quickly proven wrong; they were just as addictive as morphine, but with a superior oral bioavailability (oxycodone – 60-87% and hydrocodone – 70%) than morphine.

By mid-20th century, abuse was on the rise when oxycodone combined with aspirin was marketed as Percodan after its approval in 1950 by the U.S. Food and Drug Administration (FDA), who was given the responsibility to oversee the safety of food, cosmetics, and drugs in order to be sold as safe products. From the early 1960s, the abuse of prescription opioids became a major concern in the U.S. In 1970, the Control Substance Act (CSA) was passed to regulate the

manufacturing, importation, possession, use and distribution of certain prescription narcotics or opioids. The Act consolidated all regulated substances under a federal law implemented by the Drug Enforcement Agency (DEA) into five different "schedules" based upon the substance's safety and potential for abuse or addiction, and medicinal values. This was the official "War on Drugs" in the U.S. executed by President Richard Nixon.

Over the next few decades, the need for pain management increased and opioids were prescribed for almost all types of pain. In 1996, the concept of pain was introduced as the "fifth vital sign" by the American Pain Society (Campbell, 1996). The goal was to raise awareness that patients suffering from pain were undertreated. Soon after, the Veterans Health Administration developed a strategy on how to manage pain (Booss, 2000) and the Joint Commission on Accreditation of Healthcare Organizations emphasized a frequent assessment of pain in all patients on a 10-point scale (Phillips, 2000). This led to the overprescription of opioid medications for the management of chronic pain. Due to the diminished control of pain resulting from the development of analgesic tolerance and opioid-induced hyperalgesia (OIH), patients escalated their drug intake and became addicted to the prescription opioids, giving rise to the "prescription opioid epidemic or opioid crisis" in the U.S.

According to the U.S. Centers for Disease Control and Prevention (CDC), the rate of opiate-related death has increased by 72 percent in just one year from 2014 to 2015. The National Institute on Drug Abuse (NIDA) estimated an approximate of 2.1 million people abusing prescription opiate in the U.S. In March 2016, the CDC issued a "Guideline for Prescribing Opioids for Chronic Pain" to provide recommendations for primary care physicians on how to approach the treatment of chronic pain based on thorough assessment of the risk and harm of opioids, selection of the appropriate opioid, and when to initiate opioid use for chronic pain treatment

(Dowell *et al.*, 2016). Though the "opioid epidemic" may be a long-term problem in the U.S., the new guidelines provide a guide as to how to minimize prescription opioid abuse with continuous efforts from physicians in implementing the recommendations.

II. Pharmacology of Opioids

i. Opioid Receptors

Opioids bind to the opioid receptors that are expressed in the central nervous system (CNS) and the peripheral nervous system (PNS). Analgesia produced by opioids is primarily mediated through μ-opioid receptors (MOR) in the CNS. Other classical opioid receptors include δ-opioid receptors (DOR) and κ-opioid receptors (KOR). All three opioid receptors (μ, δ, and κ) belong to the superfamily of seven-transmembrane-spanning G protein-coupled receptors (GPCRs) and are genetically encoded by OPRM1, OPRD1, and OPRK1 genes, respectively (Chang and Cuatrecasas, 1979; Chang *et al.*, 1979; Evans *et al.*, 1992; Thompson *et al.*, 1993; Wess, 1997). Opioid receptors are $G_{i/o}$-coupled receptors, exhibiting a hallmark characteristic of an extracellular N-terminal domain, seven hydrophobic transmembrane helical domains connected by three hydrophilic extra/intra-cellular loops, and an intracellular carboxy (COOH) terminal tail. All three classical opioid receptors have approximately 60% conserved homology (Mansour *et al.*, 1995; Ninković and Roy, 2013). The greatest homology is found in the transmembrane domains as well as in the third intracellular loop; whereas, the greatest diversity is found in the amino (NH_2) and carboxy (COOH) termini as well as in the extracellular loops (Mansour *et al.*, 1995).

GPCRs belong to the largest family of signaling proteins and are fundamentally important for physiological functions through detection of various agonists such as hormones, neurotransmitters, paracrine agents, and more (Tuteja, 2009; Ninković and Roy, 2013; Stevens *et al.*, 2013; Venkatakrishnan *et al.*, 2013). With their complex signaling mechanism, upon binding of agonists or their association with other regulatory proteins, GPCRs assume different conformations from the resting states by post-translational modifications. MORs, KORs, and DORs are targets for various pain medications and are the most studied antinociceptive GPCRs (Tuteja, 2009; Stevens *et al.*, 2013; Venkatakrishnan *et al.*, 2013). The expression of opioid

receptors varies from tissue to tissue both centrally and peripherally. In the CNS, MORs are primarily found throughout the cortical regions, DORs are found in the spinal interneurons, KORs are predominantly found in the entorhinal cortex as well as in the cerebellum and the spinal cord. In the PNS, all three opioid receptors can be found in the gastrointestinal tract, reproductive organs of both sexes as well as in the lungs, kidneys, and adrenal glands (Wittert *et al.*, 1996). Furthermore, MORs are not only expressed on primary sensory neurons (Scherrer *et al.*, 2009), but also in the periaqueductal gray (PaG), the spinal cord, and in the dorsal root ganglia (DRG), where their activation by opioids mediate antinociceptive effects. Additionally, MORs can be found on cells of the immune systems such as macrophages, T cells, and β cells (Chuang *et al.*, 1995) and their activation can either result in the suppression of the immune system or the production of inflammatory cytokines.

ii. Opioid Receptor Functions

Activation of all opioid receptors by opioids results in a series of guanine nucleotide-binding protein (G protein) cascade events. For example, when morphine binds to the receptor, it stimulates a conformational change in the receptor by interacting with a G protein inside the cell. A subsequent exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the $G\alpha_{i/o}$ subunit follows (Qin *et al.*, 2011; Stewart and Fisher, 2015) and causes the G protein to dissociate into $G\alpha_{i/o}$ and $G\beta\gamma$ subunits. This initiates a series of kinase-dependent events to induce analgesia at the pre/post-synaptic sensory neurons (in the PNS) and in the CNS.

The G_α subunit is divided into subclasses such as $G\alpha_s$ (stimulatory), $G\alpha_i$ (inhibitory), $G\alpha_o$ (inhibitory), and $G\alpha_q$ (stimulatory). Upon dissociation of the G protein into Gα and Gβγ, on one hand, the $G\alpha_i$ subunit interacts with adenylyl cyclase (AC) to inhibit the functional activity of cAMP/PKA pathway (Kurose *et al.*, 1983), while the Gαo subunit blocks voltage-gated calcium

(Ca^{2+}) channels (Hescheler *et al.*, 1987). On the other hand, the Gβγ subunit 1) inhibits voltage-gated Ca^{2+} channels by blocking Ca^{2+} influx at the pre-synaptic neurons (Schroeder *et al.*, 1991; Moises *et al.*, 1994). This prevents excitatory neurotransmitter (i.e. glutamate) release from the pre-synaptic vesicles (Yoshimura and North, 1983; Glaum *et al.*, 1994; Kohno *et al.*, 1999). 2) Gβγ subunit activates GIRK channels (Torrecilla *et al.*, 2002, 2008; Sadja *et al.*, 2003) to promote potassium (K^+) efflux from the post-synaptic terminals. This results in the hyperpolarization of the post-synaptic neurons (inhibitory postsynaptic potential or IPSP) which then prevents firing of action potentials (Schulman, 1981; North and Williams, 1983; Yoshimura and North, 1983). Additionally, Gβγ subunit was also shown to promote the recruitment of GRK2/3, activate the MAPK cascade via PI3K-/c-Src kinase pathway (Polakiewicz *et al.*, 1998; Williams *et al.*, 2001), as well as activate the PLC/PKC and CaMKII pathways (Raehal *et al.*, 2011). Collectively, the activation of MORs on the pre- and post-synaptic neurons by opioids produces a synergistic action to reduce neuronal firing in order to decrease pain perception (Yoshimura and North, 1983; Glaum *et al.*, 1994; Kohno *et al.*, 1999).

In the gastrointestinal tract, opioids mediate their effects by binding to MORs that are expressed on the enteric neurons. Activation of MORs in the enteric nervous system (ENS) by an opioid receptor agonist leads to the inhibition of the enteric neuron function resulting in constipation. The conformational changes of the GPCR that take place in the ENS when an opioid agonist binds to the MOR is similar to those in the CNS, in terms of coupling to the Gαi/o and Gβγ subunits. However, in the GI tract the inhibition of neuronal activity results in alteration of both secretory functions and muscle contractility which is attributable to the modulation of ionic conductances such as activation of GIRK and inactivation of Ca^{2+} (Akbarali and Dewey, 2017). In addition, the inactivation of Na^+ channels in the enteric neurons upon morphine administration

resulting in decreased neuronal firing and excitability has been demonstrated as one of morphine's effects on ion channels. Voltage clamp studies from our laboratory conducted in isolated myenteric neurons from mouse ileum have found that morphine inhibits tetrodotoxin-resistant (TTX-R) sodium channels thus increasing the threshold at which action potentials fire, decreasing the amplitude of action potentials, and preventing the ability to fire subsequent action potentials (Smith *et al.*, 2012, 2014). More recently, our laboratory has also demonstrated that morphine acutely inhibits TTX-R Na^+ channels in mouse dorsal root ganglia (DRG) neurons (Mischel *et al.*, 2018). These findings confirm morphine's action not only in the ENS, but also in the DRG, which serves as primary "relay stations" between the PNS and the CNS.

iii. Endogenous opioid peptides

Opioid receptors are not only activated by exogenous opioids but also by endogenous opioids. The endogenous opioid peptides are derived from three opioid protein precursors: pro-enkephalin, pro-opiomelanocortin, pro-dynorphin, by a process of proteolytic cleavages. By the mid-1970s to the late 1980s, about 20 or more endogenous opioid peptides were discovered and they were classified under three major families: enkephalins (Hughes *et al.*, 1975), endorphins (Bradbury *et al.*, 1976; Li *et al.*, 1976), and dynorphins (Goldstein *et al.*, 1981). The enkephalins are small pentapeptides (containing five amino acid peptides) existing in two forms: leucine enkephalin and methionine enkephalin and are formed by the cleavage of pro-enkephalin. The cleavage of pro-opiomelanocortin (POMC) gives rise to β-lipotropin, which in turn produces the 31 amino acid peptide β-endorphin. Two other forms of endorphins include α-endorphins and γ-endorphins, whose exact roles within the body have yet to be characterized. Lastly, dynorphins arise from the precursor protein pro-dynorphin. There are many forms of dynorphins including

dynorphin A, dynorphin B, and α/β-neo-endorphin and they all range from 10 to 17 amino acids (Goldstein *et al.*, 1979, 1981; Day *et al.*, 1998).

Through receptor binding assays, it was found that the enkephalins were associated with DORs, the endorphins had a high affinity for both MORs and DORs, and the dynorphins were selective for KORs (Hook *et al.*, 2008). Although the endogenous peptides exhibited different receptor binding affinity (Hawkins *et al.*, 1989; Schiller *et al.*, 1989, 1992; Dooley *et al.*, 1995), they all possessed a conserved enkephalin sequence at the N-terminus (Tyr–Gly–Gly–Phe–Met/Leu) with differing C-terminal extension. This is thought to promote binding affinity of the peptides to the MORs, KORs, or DORs as well as their susceptibility to degradation by extracellular proteases (Weber *et al.*, 1983). Other types of endogenous opioid peptides which do not fall under the aforementioned categories are Nociceptin/orphanin FQ and endomorphins. Nociceptin/orphanin FQ is a 17 amino acid peptide that is derived from pro-nociceptin and has an affinity for the ORL receptor which is encoded by the opioid receptor-like 1 (OPRL-1) gene (Mollereau *et al.*, 1994). The endomorphins are tetrapeptides containing NH_2 groups and have an affinity for MORs. There are two forms of endomorphins: endomorphin-1 and endomorphin-2, whose precursor has not yet been identified (Gu *et al.*, 2017).

The localization of endogenous opioid peptides was first thought to be restricted only to the brain, but it was later found that they have a widespread distribution throughout the body with unique precursor transcript distribution patterns. The highest endogenous opioid peptides concentrations found in humans, monkeys, and rodents are in the hypothalamus (Gramsch *et al.*, 1979; Abrams *et al.*, 1980; Hisano *et al.*, 1982). The POMC transcripts are found in the CNS in the region of the arcuate nucleus and in the pituitary gland with their fibers projecting to the areas of the limbic system and the brain stem (Akil *et al.*, 1984). β-endorphins are also found peripherally

in the dorsal root ganglion (DRG) and in the cells of the immune system. Pro-enkephalin and enkephalins expressions are found centrally in the cortex; whereas, peripherally they are found in the spinal cord, gastrointestinal tract, adrenal medulla, as well as macrophages (Watson *et al.*, 1981; Rittner *et al.*, 2005). Lastly, pro-dynorphin and dynorphins are produced not only in the hypothalamus and the posterior pituitary, but also in the gut and the immune cells (Watson *et al.*, 1981; Rittner *et al.*, 2005).

iv. Exogenous opioids

Exogenous opioids include natural opioid alkaloids (morphine, codeine), semi-synthetic opioids (heroin, oxycodone), and synthetic opioids (fentanyl, methadone). All the exogenous opioids have similar chemical and pharmacological properties to morphine, in terms of mediating effects via MORs, DORs, and KORs. The metabolism of opioids primarily occurs in the liver where enzymes are available to promote phase 1 metabolism (modification reactions) and phase 2 metabolism (conjugation reactions) (Smith, 2009). Phase 1 metabolism promotes oxidation/ hydrolysis of drugs involving the cytochrome P450 (CYP) enzymes while phase 2 metabolism conjugates drugs to hydrophilic substances, such as glucuronic acid, sulfate, glycine, or glutathione in order to be excreted from the body (Smith, 2009). Morphine metabolism primarily occurs by phase 2 glucuronidation via UDP-glucuronosyl transferase-2B7 (UGT2B7) yielding two major metabolites: morphine-3-glucuronide (M3G- accounting for 60% of the metabolites produced) and morphine-6-glucuronide (M6G - accounting for 6-10% of the metabolites produced) (van Dorp *et al.*, 2006). Other minor routes of morphine metabolism include *N*-demethylation to normorphine or normorphine 6-glucuronide, diglucuronidation to morphine-3, 6-diglucuronide, and formation of morphine ethereal sulfate (Smith, 2009). Although M3G accounts for the majority of the

metabolites produced, it exhibits no analgesic efficacy. In contrast, M6G has a high affinity for the MOR with potent analgesic effects (van Dorp *et al.*, 2006; Dahan *et al.*, 2008). Exogenous opioids that undergo phase 1 metabolism include but are not limited to fentanyl, methadone, codeine, and oxycodone. The CYP3A4 and CYP2D6 enzymes are responsible for the metabolism of both methadone (Ferrari *et al.*, 2004) and oxycodone (Smith, 2009), whereas, the primary metabolism of fentanyl is via CYP3A4 (Labroo *et al.*, 1997) and of codeine is CYP2D6 (Smith, 2009). Consequently, the metabolism of opioids using CYP enzymes have a potential for drug-drug interactions (Smith, 2009).

III. Adverse effects of chronic morphine

i. Tolerance

Tolerance is defined as a reduction in pharmacological response after a repeated or prolonged administration of a drug. Alternatively, tolerance can also be defined as the need to increase drug intake in order to achieve a maximum response. Tolerance observed in humans can be seen from days to weeks, whereas, it can be seen in animal models ranging from hours to days. The mechanism underlying opioid tolerance is still elusive, albeit it is widely accepted that there are different types of tolerance to opioid effects occurring at a different degree, at a different rate. For example, tolerance to the analgesic effects of opioids develops relatively faster than tolerance to opioid-induced miosis, respiratory depression, and constipation (Dumas and Pollack, 2008; Hayhurst and Durieux, 2016; Hill *et al.*, 2016). The rate of tolerance even differs in one region of the GI tract to the other. For instance, tolerance in the distal part of the small intestine (ileum) occurs rapidly whereas tolerance in the colon develops very slowly, if at all (Ross *et al.*, 2008). Due to the wide range of degrees of tolerance, it makes it difficult to determine the exact mechanism that drives the development of tolerance. Nevertheless, investigators have proposed mechanistic pathways that contribute to the development of tolerance involving adenylyl cyclase, receptor trafficking, intracellular kinases, and β-arrestin 2.

Binding of an opioid to its receptor signals through Gi/Go, thus inhibiting the stimulation of adenylyl cyclase (AC) enzyme to catalyze adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). The first evidence of the involvement of protein synthesis in morphine tolerance was proposed in the 1960's (Way *et al.*, 1968; Cox and Osman, 1969). Later, Collier and Roy demonstrated a biochemical mechanism involving the cyclic AMP system in which they treated morphine-exposed cells with naloxone and observed an increased stimulation of cAMP

(Collier and Roy, 1974; Collier, 1980). They then concluded that tolerance involves a compensatory upregulation of the adenylyl cyclase enzyme in the presence of chronic morphine exposure in order to return cAMP levels back to basal levels despite the continued inhibition by morphine. Their findings were consistent with the dependence of tolerance on protein synthesis proposed by Way and Cox. (Way *et al.*, 1968; Cox and Osman, 1969).

Another aspect of the underlying mechanism for opioid tolerance is receptor trafficking. Just like other membrane proteins, MORs also undergo extensive trafficking. The components of MORs trafficking include desensitization and downregulation (internalization/ endocytosis, degradation, and reduced gene expression). Desensitization of MORs involves uncoupling of the receptor from its G-protein, followed by phosphorylation of the receptor with intracellular kinases such as G protein receptor kinases (GRKs), protein kinase C (PKC), protein kinase A (PKA), and c-Jun N-terminal kinases (JNKs) (Williams *et al.*, 2013). During the downregulation process, the number of functional receptors available on the cell is reduced due to internalization of the receptors, degradation and/or reduced biosynthesis of receptors (Tsao and von Zastrow, 2000; Williams *et al.*, 2013). The extent of internalization of the receptors depends on the type of opioid agonist occupying the receptor. Studies have demonstrated that morphine does not cause internalization of the MORs, whereas other mu agonists such as [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) do (Hashimoto *et al.*, 2006). The lack of MOR internalization by chronic morphine suggests that tolerance development occurs by alternative pathways. Some studies suggest that morphine tolerance may be primarily driven by receptor desensitization caused by PKC/PKA-mediated phosphorylation (Bailey *et al.*, 2006, 2009; Hull *et al.*, 2010). On the other hand, tolerance produced by DAMGO was shown to be mainly driven by GRK phosphorylation and β-arrestin2 recruitment (Stafford *et al.*, 2001). Nevertheless, the understanding of opioid

tolerance gets more complicated as studies demonstrate different MOR trafficking patterns based on MOR splice variants. For example, morphine or DAMGO administration was shown to internalize MOR-1C in vivo, whereas, MOR-1 failed to be internalized by morphine but was successfully internalized by DAMGO (Keith *et al.*, 1998; Abbadie *et al.*, 2001). Furthermore, other studies have found differences among MOR-1 splice variants trafficking, with MOR-1B, MOR-1D, and MOR-1E showing more degradation than MOR-1 itself, suggesting that receptor trafficking is dependent on the alternative splicing of the receptor C terminus (Tanowitz *et al.*, 2008; Pasternak and Pan, 2013).

Another proposed mechanism for opioid tolerance is the recruitment of β-arrestin2. The primary function of β-arrestin proteins is to desensitize or "arrest" active GPCRs (DeWire *et al.*, 2007). Upon binding of β-Arr2 to the phosphorylated receptor, a steric hindrance is created occluding the binding site for G-protein, thus preventing its activation. This results in recruitment of scaffolding proteins: clathrin and clathrin adaptor proteins AP2, to promote receptor internalization via coated pits, subjecting the receptor to two fates: degradation (in the lysosomes) or recycling (back to the cell membrane). This canonical pathway is shown to play important roles in morphine desensitization and tolerance. Indeed, morphine exposure to β-Arr2 knockout mice prevents desensitization of MORs (Bohn, 1999) and delays the development of antinociceptive tolerance in the tail-immersion assay (Bohn *et al.*, 2002).

In addition to GRK-mediated phosphorylation events, several other intracellular kinases have been linked to opioid tolerance, for example PKC, PKA, CaMKII, and JNK/MAPKs (Liu and Anand, 2001; Koch and Höllt, 2008). The majority of studies supporting these claims were focused on the reversal of morphine tolerance using kinase inhibitors. For example, using PKC inhibitors such as Gö-6976, Gö-7874, Bisindolylmaleimide I HCl, and sangivamycin were shown

to attenuate morphine antinociceptive tolerance (Smith *et al.*, 1999; Javed *et al.*, 2004; Hull *et al.*, 2010). Additionally, the PKA inhibitor, KT-5720 also prevented the development of antinociceptive tolerance to morphine, in both radiant heat tail flick test as well as in the hot plate test (Bernstein, 1997; Javed *et al.*, 2004). The reversal of morphine tolerance was also observed using inhibitors of CaMKII (Fan *et al.*, 1999; Tang *et al.*, 2006), MAPKs Raf (Tumati *et al.*, 2010), ERK (Chen *et al.*, 2008), and JNK (Chen *et al.*, 2008; Melief *et al.*, 2010). Other compounds that were also implicated in the reversal of morphine antinociceptive tolerance include antibiotics/vancomycin, gap junction inhibitors, astrocyte inhibitors, and proinflammatory cytokine inhibitors (Song and Zhao, 2001; Shen *et al.*, 2011, 2014; Kang *et al.*, 2017; Mischel *et al.*, 2018).

Everything considered, mechanisms underlying opioid tolerance are very complex and involve many different pathways, therefore cannot be explained by one single cellular mechanism.

ii. Inflammation and tolerance

It was originally thought that tolerance to opioids is solely due to neuronal adaptation, however convincing data have contested this hypothesis, suggesting that glial activation plays an important role in opioid tolerance (Song and Zhao, 2001; Johnston *et al.*, 2004; Raghavendra *et al.*, 2004; Shavit *et al.*, 2005; Watkins *et al.*, 2005; Cui *et al.*, 2006; Liu *et al.*, 2006). Over the last decade, animal studies have reported that morphine induces inflammatory responses in the brain (Milligan and Watkins, 2009), spinal cord (Hutchinson *et al.*, 2008), and gastrointestinal tract (Meng *et al.*, 2013; Kang *et al.*, 2017). There is a growing evidence that chronic morphine analgesia may be decreased by inflammation (Johnston *et al.*, 2004; Shavit *et al.*, 2005; Hutchinson *et al.*, 2008) which could drive the development of antinociceptive tolerance. Proinflammatory

cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNFα) and chemokines (fractalkine/CX3CL1) are shown to mitigate the analgesic effects of morphine (Hutchinson *et al.*, 2008) and modulate antinociceptive tolerance (Johnston *et al.*, 2004) following intrathecal administration of morphine. Johnston *et al.* observed elevated spinal IL-1 & IL-6 proteins and TNF mRNA expressions after chronic, but not acute, intrathecal morphine injection. Upon administration of neutralizing antibodies to IL-6, TNFα, and fractalkine or IL-1 receptor antagonist, analgesia was restored, and tolerance was reversed (Johnston *et al.*, 2004; Hutchinson *et al.*, 2008). Furthermore, our laboratory has demonstrated that by depleting commensal bacteria in the gut with a broad-spectrum antibiotic not only prevented the pathological changes that occurs in the gut with chronic morphine exposure such as disruption of intestinal epithelial barrier, bacterial translocation, and inflammation, but also prevented the development of antinociceptive tolerance (Kang *et al.*, 2017) suggesting a possible involvement of inflammatory cytokines on the development of morphine antinociceptive tolerance.

The exact mechanism by which morphine produces inflammation remains a mystery, however, studies have suggested that it might either be through binding of classic opioid receptors or via Toll-like Receptors (TLR) signaling pathway. TLRs are single, membrane-spanning proteins that recognize a variety of pathogen-associated molecular patterns (PAMPs) conserved on microbes. Activation of these receptors stimulates an innate immune response, thus triggering downstream signaling and inducing the release of inflammatory substances such as cytokines and chemokines in humans and animals (Kawai and Akira, 2010; O'Neill *et al.*, 2013).

Several studies have implicated TLRs in morphine-induced inflammation, but whether or not morphine directly activates TLRs is debatable. On one hand, murine studies demonstrated that morphine indirectly activate TLR receptors through unknown mechanisms by first binding to mu-

opioid receptors that are expressed on the epithelial cells of the gut (Meng *et al.*, 2013) and then triggers the phosphorylation of myosin light chain (MLC) by MLC kinase resulting in reorganization of tight junction proteins and increase in intestinal permeability (Forsythe *et al.*, 2002; Moriez *et al.*, 2005; Meng *et al.*, 2013). The translocation of luminal bacteria thus causes an immune activation and colonic inflammation. On the other hand, morphine was shown to directly bind to TLR4 receptor (one of the 13 TLRs discovered which particularly recognizes and is activated by lipopolysaccharide (LPS), a component present on gram-negative bacteria cell wall). Morphine binds in a specific LPS-binding pocket of accessory protein, myeloid differentiation protein 2 (MD-2) (a co-receptor of TLR4) resulting in release of proinflammatory substances (Wang *et al.*, 2012). Thus, genetic knockout of TLR4/MD2 in mice suppressed neuroinflammation and restored morphine analgesia (Wang *et al.*, 2012).

All things considered, it is now well-documented that morphine can interact with glial and TRL signaling pathways to induce pro-inflammatory cytokine release from different systems in a host, which is shown to interfere with morphine antinociception and linked to the enhancement of morphine-induced tolerance, dependence, and reward associated with drug seeking behavior (Hutchinson *et al.*, 2008, 2010; Narita *et al.*, 2008; Milligan and Watkins, 2009; Watkins *et al.*, 2009; MR Hutchinson *et al.*, 2010). This dissertation has explored the role of gut inflammation on the development morphine antinociceptive tolerance in mice.

IV. Aims

The overall goal of this dissertation project was to investigate the effects of colonic inflammation on morphine-induced antinociceptive tolerance in mice. As inflammation was reported to mitigate morphine analgesia and modulate analgesic tolerance in the brain and the spinal cord, we sought to determine the effects of inflammation originating from the gastrointestinal tract, specifically in the colon on morphine antinociceptive tolerance. We hypothesized that colonic inflammation would enhance morphine antinociceptive tolerance.

The specific aims of this project were:

Aim 1: To determine if the antinociceptive tolerance to morphine correlated with the induction of pro-inflammatory cytokine in the colon

Aim 2: To demonstrate if the rate of antinociceptive tolerance development to morphine was enhanced by colonic inflammation

Aim 3: To test if peripheral opioid antagonists would prevent antinociceptive tolerance in the presence of colonic inflammation

CHAPTER 2

Materials and Methods

Animals

Male Swiss Webster mice weighing 25–35 g (Harlan Sprague Dawley, Inc. Fredrick, MD, USA) were housed five per cage in animal care facility under a 12-hour light/dark cycle with food and water available ad libitum. The experimental protocol was approved by the Institutional Animal Care and Use committee at Virginia Commonwealth University (VCU IACUC).

Chronic morphine treatment

For chronic morphine administration, a 25 mg, 50 mg (2x25), or 75 mg morphine or placebo pellet was implanted subcutaneously on the dorsum of individual mouse. Surgical implantation of pellets took place under anesthesia (2.5% isoflurane). Mouse hair was shaved from the base of the neck and the skin was thoroughly cleansed with 10% povidone iodine (General Medical Corp., Walnut, CA) and then rinsed with 70 % ethanol. A 1-cm horizontal incision was made at the cleansed area and the pellet was inserted in the space. The site was closed by stapling the skin with Clay Adams Brand, MikRon AutoClip 9-mm Wound Clips (Becton Dickinson, Franklin Lakes, NJ). The animals were allowed to recover in their home cages where they remained throughout the experiment until testing day.

Nociceptive response tests

Nociceptive response was tested in the warm water tail-immersion assay and hot plate assay set to 56 °C. For the warm water tail-immersion assay, mice were gently wrapped in a cloth and the distal 1/3 of the tail was submerged in the warm water bath. A baseline latency to tail-flick for all placebo pelleted or naïve mice was between 2-4 seconds. A maximum latency of 10 sec was used to prevent tissue damage. For the hot plate assay, mice were gently placed in a cylindrical tube on the hot plate to prevent escaping. The licking of the hind paw and jumping from the hot surface were used as the end point with a maximum latency of 30 sec. Morphine antinociception was tested 30 min after administration of a morphine challenge dose (10 mg/kg, s.c.). The tail-withdrawal data was quantified using the percentage of maximum possible effect (%MPE) equation: %MPE = [(challenge time − baseline time) / (10 − baseline time)] × 100. The hot plate data was calculated as %MPE = [(challenge time − baseline time) / (30 − baseline time)] × 100. Higher %MPE indicates less tolerance to morphine.

Experimental model of colitis

Colonic inflammation was induced by administering mice with 2,4,6-Trinitrobenzenesulfonic acid (TNBS) solution containing a 1:1 dilution mixture of 2.5% picrylsulfonic acid solution (5% w/v in H_2O)(Sigma-Aldrich, St. Louis, MO) and 50% ethanol. 100 μL of the mixture was administered to each animal intra-rectally. Control animals received 100 μL of a 1:1 dilution mixture of saline and 50% ethanol as vehicle. For chronic morphine experiments, morphine pellets (25 mg, 2x 25 mg, or 75 mg) or placebo pellets (NIDA) were implanted under anesthesia and then the TNBS solution was immediately administered rectally. The overall health of the animals was monitored each day and their weights were recorded daily.

Peripheral opioid receptor antagonist treatment

For the reversal of tolerance study, two peripheral opioid receptor antagonists were used, methylnaltrexone (MNTX) and 6β-N-heterocyclic substituted naltrexamine derivative (NAP). After the morphine pelleting and the TNBS administration, the mice were immediately injected with 25 mg/kg methylnaltrexone or 0.5 mg/kg NAP subcutaneously and they were injected daily until testing day. Mice treated with daily MNTX were pelleted with 75 mg morphine pellets and tested on day 3. Mice treated with daily NAP or VEH were pelleted with 25 mg, 50 mg (2x25), or 75 mg morphine or placebo pellets and tested on day 5, 4, or 3, respectively. For mice treated with methylnaltrexone, baseline tail-flick and hot plate latencies were recorded daily 20 min after drug administration. On day 3, the mice were challenged with 10 mg/kg morphine and tested in the tail-withdrawal and hot plate assays after 30 min. NAP and MNTX were dissolved in ddH_2O; therefore, the control mice were injected with ddH_2O as vehicle. The volume for the injections was 10 uL/g for body weights ranging from 25 – 35 grams.

Dose-response studies

Naïve Swiss Webster mice were injected with methylnaltrexone or NAP subcutaneously (s.c.) and tested in the warm water tail-immersion assay after 20 min. A morphine challenge dose (10 mg/kg, s.c.) was administered to both groups and latency was recorded after 30 min.

For NAP studies, a cumulative dosing schedule was used. On the day of testing, baseline tail-withdrawal latency was recorded from mice at 56 °C followed by subcutaneous challenge doses of morphine every 20 min. Mice treated with NAP were injected 0.5 mg/kg NAP 20 min prior to baseline tail-withdrawal latency recording followed by subcutaneous challenge doses of morphine every 20 min using the same paradigm.

Hematoxylin and eosin (H&E) staining

Colons samples were isolated from treatment groups and fecal pellets were gently flushed with 1 X PBS and then embedded in Optimal Cutting Temperature (OCT) Compound (Sakura Finetek, Torrance, CA). The embedded tissue was immediately flash frozen in liquid nitrogen and then stored at -80 °C. Colon tissues were cyrosectioned at 12-µm intervals. The cross-sections were fixed in 4% paraformaldehyde in PBS and stained using the standard hematoxylin and eosin (H&E, Sigma-Aldrich, St. Louis, MO) protocol.

Immunohistochemical staining

Colons samples were isolated from treatment groups and fecal pellets were gently flushed with 1 X PBS and then embedded in Optimal Cutting Temperature (OCT) Compound (Sakura Finetek, Torrance, CA). The embedded tissue was immediately flash frozen in liquid nitrogen and then stored at -80 °C. Colon tissues were cyrosectioned at 12-µm intervals. The cross-sections were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. After washing in PBS and permeabilization with Triton X-100 (0.1%) and blocking nonspecific binding sites with 5% goat serum, tissues were incubated with mouse anti-occludin at 2 ug/mL (Invitrogen, Carlsbad, CA) in PBS with 5% goat serum overnight at 4 °C. After washing in PBS, sections were incubated with goat anti-mouse IgG (H + L), Alexa Fluor 488 conjugated secondary antibody (Life Technologies, Carlsbad, CA) at 2 ug/mL for 2 h at room temperature. Sections were then washed and mounted under coverslips using ProLong Gold antifade reagent with DAPI (Life Technologies, Carlsbad, CA). Sections were imaged using a confocal microscope (Nikon).

RNA isolation and real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a Mini-Opticon real-time PCR system (Bio-Rad, Hercules, CA). 18 S ribosomal RNA was used as an internal control. Sample tissues were isolated, and total RNA was extracted using TRIzol reagent (ThermoFisher Scientific, Waltham, MA). The first-strand cDNA synthesis was amplified at 42 °C for 30 min and subsequent polymerization was performed in a single step using the SensiMix One-Step kit (Bio-Rad, Hercules, CA). The reaction mixture (20 uL) contained 200 nM forward primer, 200 nM reverse primer, 1 uL of 2X SensiMix One-Step buffer, 10 units RNase inhibitor, and 200 ng total RNA. The PCR protocol consisted of 40 cycles of denaturation (15 sec at 95 °C), annealing (30 sec at 58 °C), and extension (30 sec at 72 °C). Relative expression of the respective genes to 18 S expression was calculated using the ΔΔCt method and values were expressed as a fold change from the control groups. Primers used in this study are IL-1β, IL-6, IL-10, TNF-α and 18S rRNA.

List of primers

Table 1: Primers used for PCR

Target	**Sequence**
IL-1β	Forward: 5'-GTACAAGGAGAACCAAGCAA-3' Reverse: 5'-TGTTGAAGACAAACCGTTTT-3'
IL-6	Forward: 5'-CTAAAAGTCACTTTGAGATCTACTC-3' Reverse: 5'-TGTCCCAACATTCATATTGT-3'
IL-10	Forward: 5'-GGACTCCAGGACCTAGACAG-3' Reverse: 5'-GTTCACAGAGAAGCTCAGTG-3'
TNF-α	Forward: 5'-GTTGTACCTTGTCTACTCCC-3' Reverse: 5'-GTATATGGGCTCATACCAGG-3'

18 s	Forward: 5'-TCAAGAACGAAAGTCGGAGG-3' Reverse: 5'-GGACATCTAAGGGCATCAC-3'

Cytokine measure

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the level of IL-1β production. Colon tissues were homogenized in cell lysis buffer 2 (R&D Systems) and incubated on ice for 30 min, followed by centrifugation (10,000 rpm, 15 min at 4 °C). Supernatants were collected into clean tubes and stored at -80 °C until time of assay. Protein concentrations were measured using spectrophotometer (Bio-Rad, SmartSpec Plus) right before the assay and equal concentration of protein from each sample was added to the ELISA plate. IL-1β levels were measured using mouse IL-1beta/IL-1F2 Quantikine ELISA kit (R&D Systems) following the manufacturer recommendation.

FITC-dextran assay

For in vivo intestinal permeability studies, FITC-conjugated dextran (Sigma-Aldrich, St. Louis, MO) was administered by oral gavage (44 mg/100 g body weight of FITC-labeled dextran). After four hours, mice were euthanized using guillotine and a sample of their whole blood was collected into a heparinized blood collection tube. Collection process was carried out with lights off due to the light sensitivity of the FITC. Blood samples were centrifuged for 15 min at 3000 rpm at 15 °C and plasma was transferred into a new clean 1.5 mL tube. Samples were diluted at a 1:1 ratio with 1xPBS. 100 µl of diluted samples and standards (serial dilution of FITC-dextran: 0, 125, 250, 500, 1000, 2000, 4000, 6000, & 8000 ng/ml) were pipetted into a 96-well plate in duplicate. FITC concentration was quantified by emission spectrometry (Promega, Madison, WI) at 528 nm, using

an excitation wavelength of 485nm. Sample concentrations were measured against a standard curve of serially diluted FITC-dextran.

Statistical analysis

GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA) was used for statistical analyses. Data was analyzed using Student's unpaired two-tailed t-test, or ordinary two-way ANOVA with multiple comparison using a Tukey or Bonferroni *post-hoc* test or ordinary one-way ANOVA with comparison to a single control group using Dunnett *post-hoc* test, as indicated in the figure legends. Differences were considered significant if $p \leq 0.05$. For the dose-response studies, nonlinear regression analysis was performed and the best-fit line was generated. The results are expressed as mean value ± SEM. Power analysis was performed on %MPE data from each chapter using G*Power 3.1. *Post-hoc* power analysis was used to calculate the minimum sample size required to detect an effect. The power ranges from 0 to 1 and are presented as P $(1 - \beta)$; where β is the false negative rate. The higher the statistical power, the less probability it is to make a type II error, which is wrongly accepting the null hypothesis.

CHAPTER 3

Morphine-induced inflammatory mediators contribute to the development of antinociceptive tolerance

Over the last decade, a plethora of studies have reported that chronic morphine administration leads to the induction of inflammatory responses in the brain (Milligan and Watkins, 2009), spinal cord (Hutchinson *et al.*, 2008), and gastrointestinal tract (Meng *et al.*, 2013; Kang *et al.*, 2017) resulting in decreased efficacy of the drug, which would inherently drive antinociceptive tolerance. Previous studies from our laboratory have demonstrated that by decreasing the inflammatory responses from the gut that was potentially caused by chronic morphine exposure using a cocktail of antibiotics prevented the development of antinociceptive tolerance to morphine in mice (Kang *et al.*, 2017), suggesting a link between inflammation and tolerance. In the present study, we investigated the effects of morphine-induced inflammation on antinociceptive tolerance in mice. Our goal was to determine at what point during chronic morphine treatment the inflammatory cytokines gene such as interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), and tumor necrosis factor alpha (TNF-α) were expressed in both colon and ileum, and if the level of the inflammatory cytokines correlated to the development of tolerance.

The effects of Acute Morphine in Drug-Naïve Mice

To determine whether an acute morphine treatment induced an inflammatory response in the gastrointestinal (GI) tract, naïve mice were administered morphine at a dose of 10 mg/kg or

saline subcutaneously. After 20 minutes, whole colons were removed from mice and inflammatory cytokine mRNA expressions were examined by real-time polymerase chain reaction RT-PCR. Interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), and tumor necrosis factor alpha (TNF-α) were our chosen inflammatory cytokines because their expressions were frequently up-regulated during an inflammatory state and in inflammatory bowel diseases (Sanchez-Muñoz *et al.*, 2008; Strober and Fuss, 2011). The result showed no significant difference in the cytokine mRNA expressions from the colons of control and acute morphine-treated animals (figure 3.1).

Figure 3.1: Inflammatory cytokine mRNA expressions in mice colons after acute morphine exposure

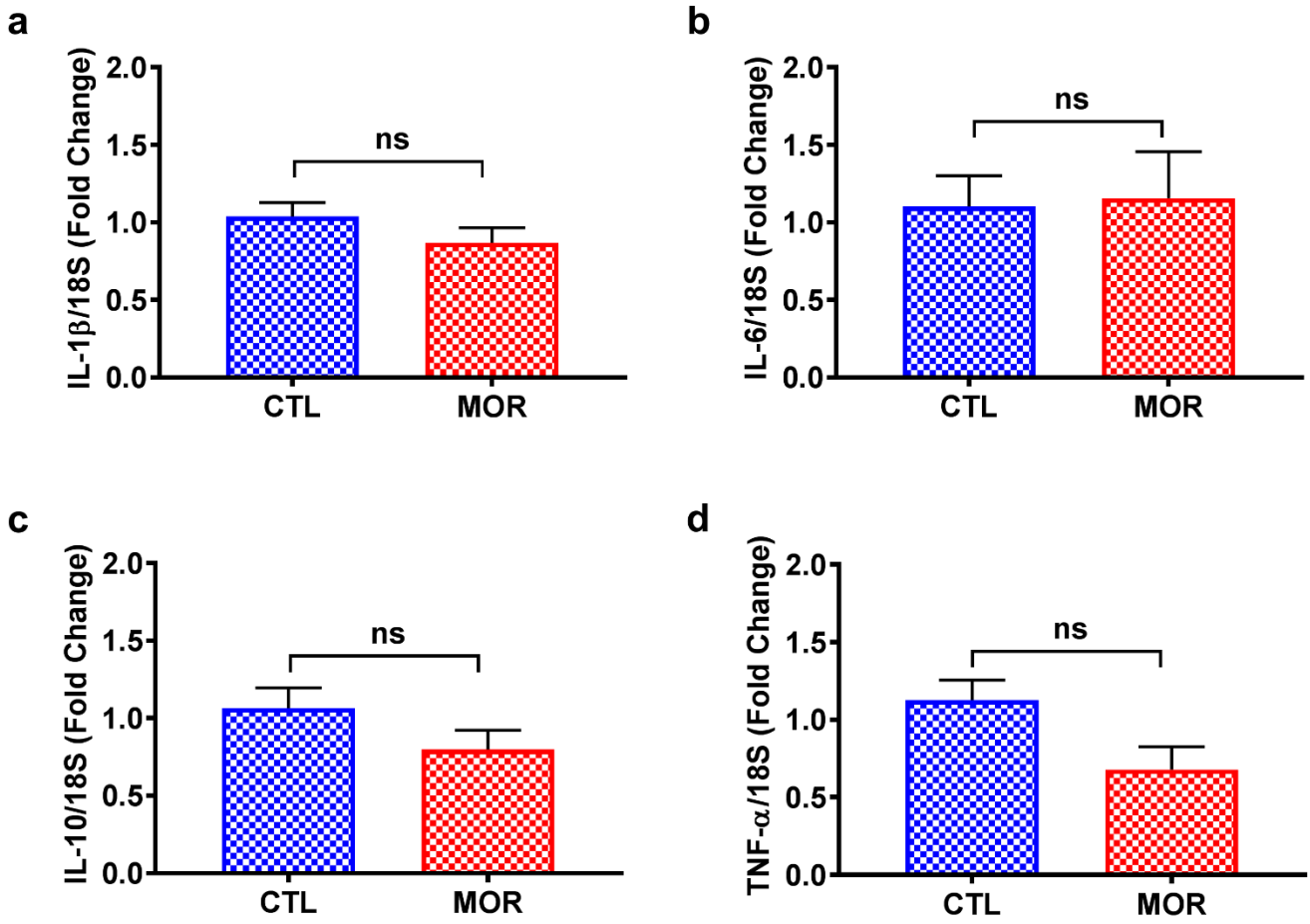

Figure 3.1: Inflammatory cytokine mRNA expressions in mice colons after acute morphine exposure. (**a** – **d**) Acute exposure of 10mg/kg morphine did not significantly increase IL-1β, IL-6, IL-10, and TNF-α inflammatory cytokine expressions in the colons of control and morphine-treated mice. N = 5/group, ns not significant by Student's unpaired t-test.

Chronic morphine administration induced inflammatory cytokine production

To determine when inflammatory cytokines were triggered in the gut as a result of chronic morphine treatment, a time course study was performed on mice pelleted with 75 mg morphine pellets (MP) or placebo pellets (PP) for 7 days. On alternating days, the nociceptive effects of morphine were assessed in the warm-water tail immersion assay and mice were euthanized afterward and their colon and ileum tissues were extracted for inflammatory cytokine mRNA expression examination by RT-PCR. Daily baseline tail-withdrawal latency indicated a progressive loss of morphine antinociception from day 1 to 7 (figure 3.2a). With a daily morphine challenge (10 mg/kg) after the baseline recordings, the development of morphine antinociceptive tolerance was not observed until day 5 after pelleting indicated by a lower percent maximum possible effect (%MPE) (figure 3.2b). Upon examination of mice colon tissues, IL-1β, IL-6, IL-10, and TNF-α mRNA expressions were up-regulated on day 1 after morphine pelleting in the colon; however, only IL-6 mRNA expression was significant on day 1 (figure 3.3b). By day 3, all four cytokines returned to basal levels and remained low, except for IL-1β whose mRNA expression was significantly elevated on day 5 and remained high by day 7 (figure 3.3a). The increased IL-1β mRNA expression on day 5 of chronic morphine treatment in the colon correlated with the development of antinociceptive tolerance. We next assessed IL-1β protein levels from MP and PP treated mice colon by ELISA on day 7 and found no significant difference in the IL-1β levels in both groups, however there was a trend toward an increased IL-1β levels in the MP group compared to the PP group (figure 3.4).

In the ileum, none of the inflammatory cytokine mRNA expressions (IL-1β, IL-6, IL-10, TNF-α) tested were significantly different in either morphine or placebo pelleted mice (figure 3.5). However, a similar trend was also observed on day 1 as all the cytokines were slightly up-

regulated, indicating an immune reaction to the morphine pellets in both colon (figure 3.3) and ileum (figure 3.5). The housekeeping gene 18S rRNA was used to normalize the mRNA expression data whose expression was not altered by chronic morphine treatment on any particular day (figure 3.6).

Figure 3.2: Tail withdrawal latency after chronic morphine exposure

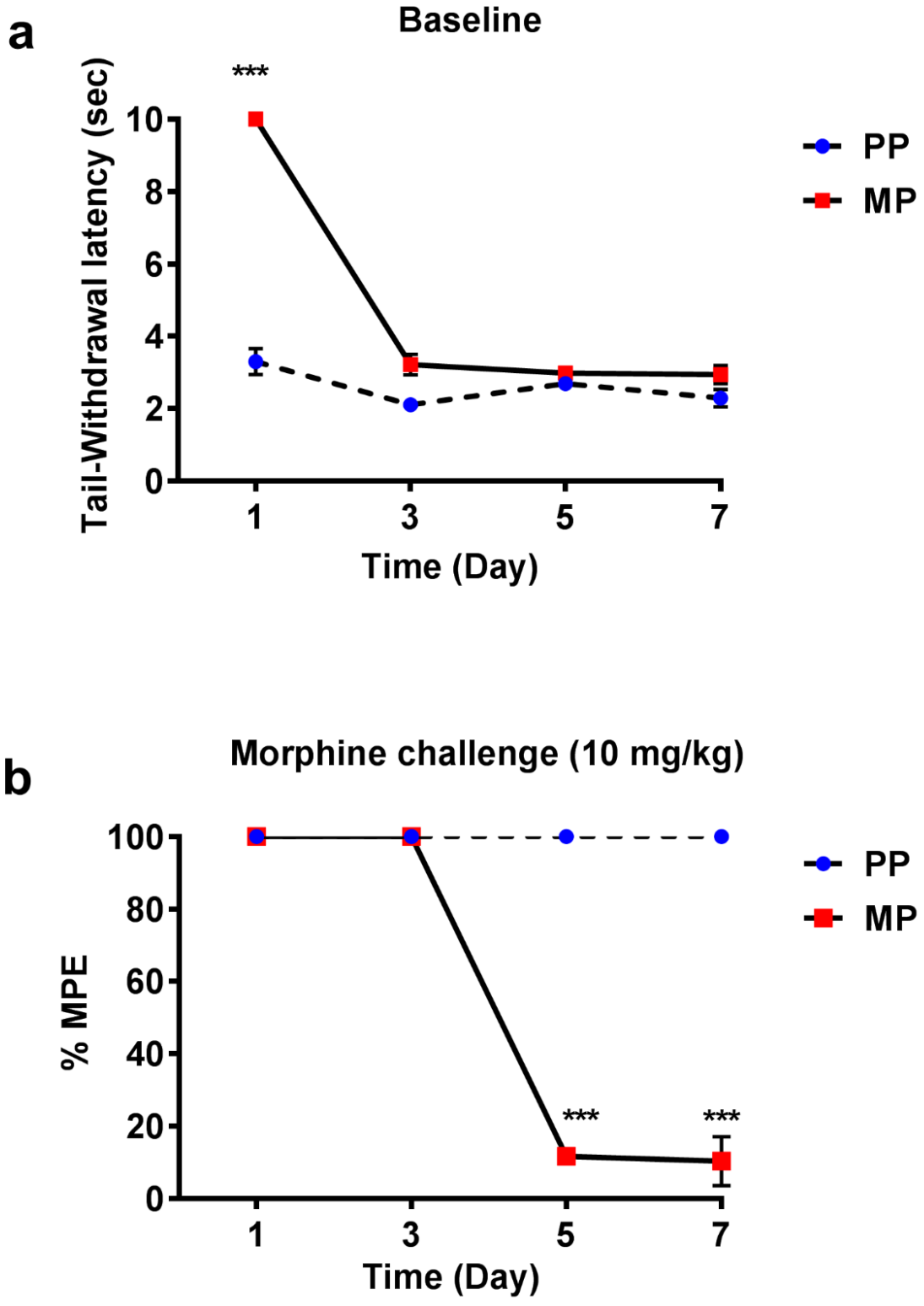

Figure 3.2: Tail withdrawal latency after chronic morphine exposure. (**a**) Daily baseline recording from morphine-pelleted mice showed a progressive decline in morphine's antinociceptive effects over the course of treatment. N = 5/group, ***p < 0.001 by two-way ANOVA with Bonferroni's post-hoc analysis. (**b**) Chronic morphine exposure (75mg) resulted in antinociceptive tolerance to morphine challenge (10 mg/kg) in the tail-immersion assay after 5 days of exposure. Results are expressed as the percentage of maximum possible effect. N = 5/day/group, P $(1 - \beta) = 0.99$, ***p < 0.001 by two-way ANOVA with Bonferroni post-hoc analysis.

Figure 3.3: Time course of inflammatory cytokine mRNA expression in mice colons after chronic morphine exposure

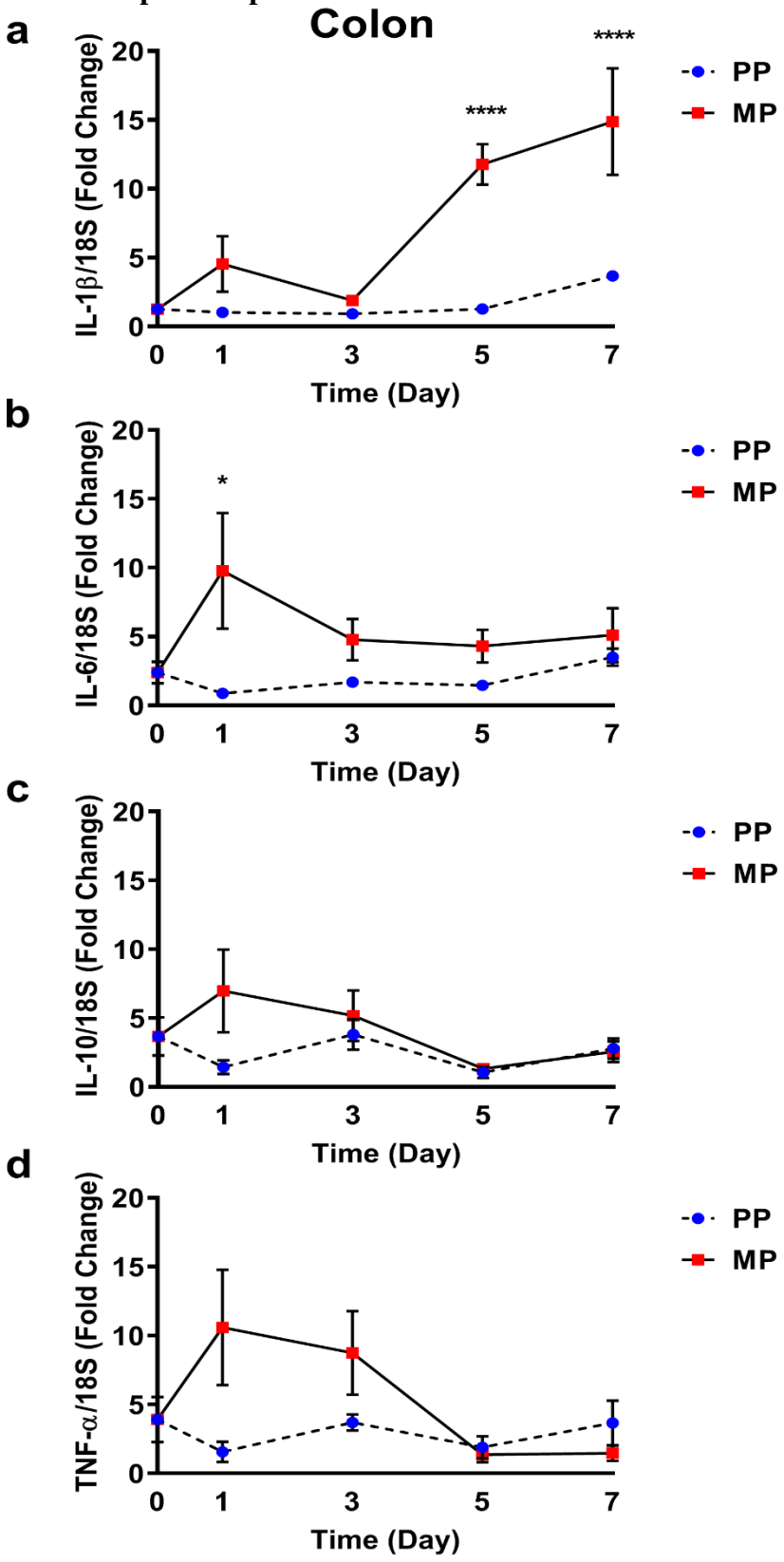

Figure 3.3: Time course of inflammatory cytokine mRNA expression in mice colons after chronic morphine exposure. (**a**) Chronic morphine (75mg) significantly increased colonic IL-1β mRNA expression at day 5 and 7 post pellet implantation. $N = 6$ for each sample day, ****$p < 0.0001$ by two-way ANOVA with Bonferroni's post-hoc analysis. (**b - d**) Chronic morphine (75mg) increased colonic IL-6, IL-10, and TNF-α mRNA expression at 1 day post pellet implantation. $N = 6$ for each sample day, *$p < 0.05$ by two-way ANOVA with Bonferroni post-hoc analysis.

Figure 3.4: IL-1β protein levels after 7 days of chronic morphine exposure in mice colon

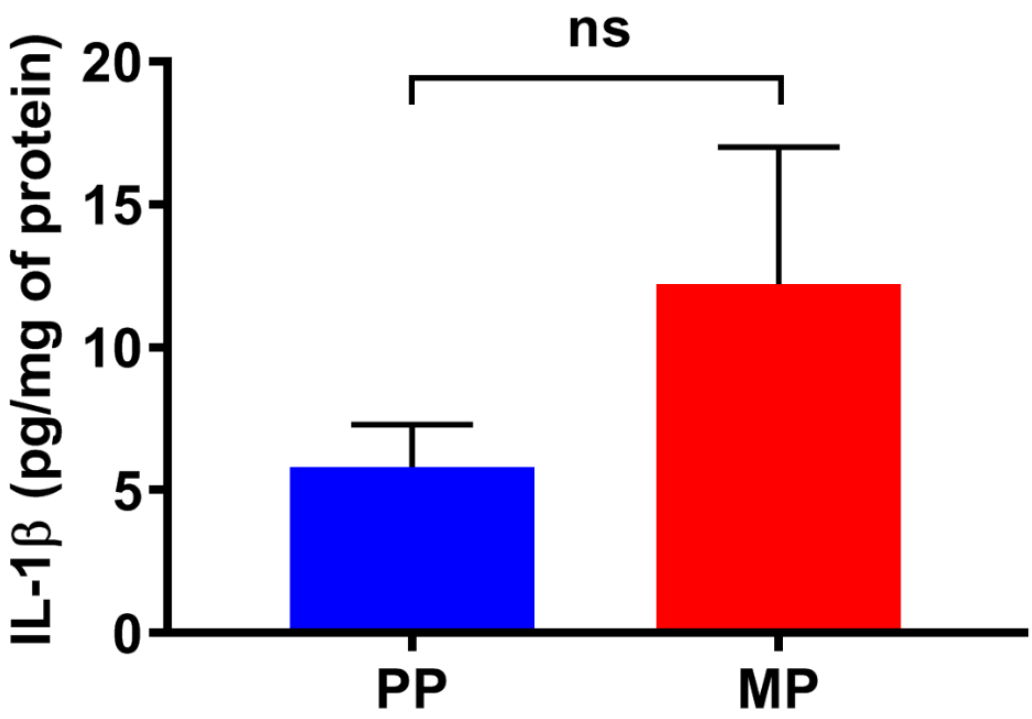

Figure 3.4: IL-1β protein levels after 7 days of chronic morphine exposure. IL-1β levels are higher in the morphine pelleted (75 mg) mice colons on day 7 than in the placebo pelleted group but there was no significant between the two groups. N = 5/group, p = 0.24, ns not significant by Student's unpaired t-test.

Figure 3.5: Time course of inflammatory cytokine mRNA expression in mice ileum after chronic morphine exposure

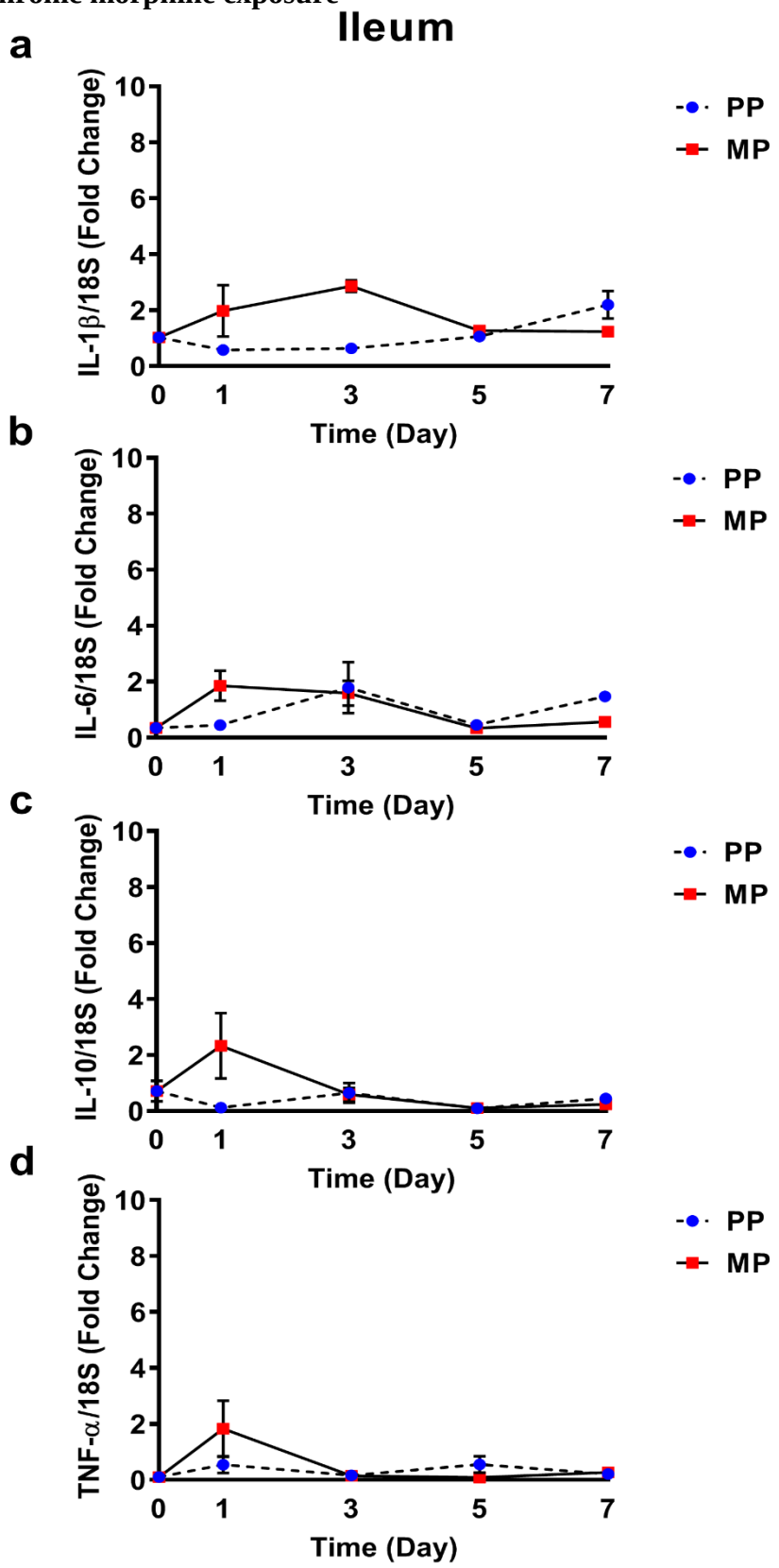

Figure 3.5: Time course of inflammatory cytokine mRNA expression in mice ileum after chronic morphine exposure. (**a - d**) Chronic morphine (75mg) did not induce any significant inflammatory responses in the ileum. N = 4 for each sample day per group, ns not significant by two-way ANOVA with Bonferroni post-hoc analysis.

Figure 3.6: Time course of housekeeping gene 18S rRNA in mice colons and ileum after chronic morphine exposure

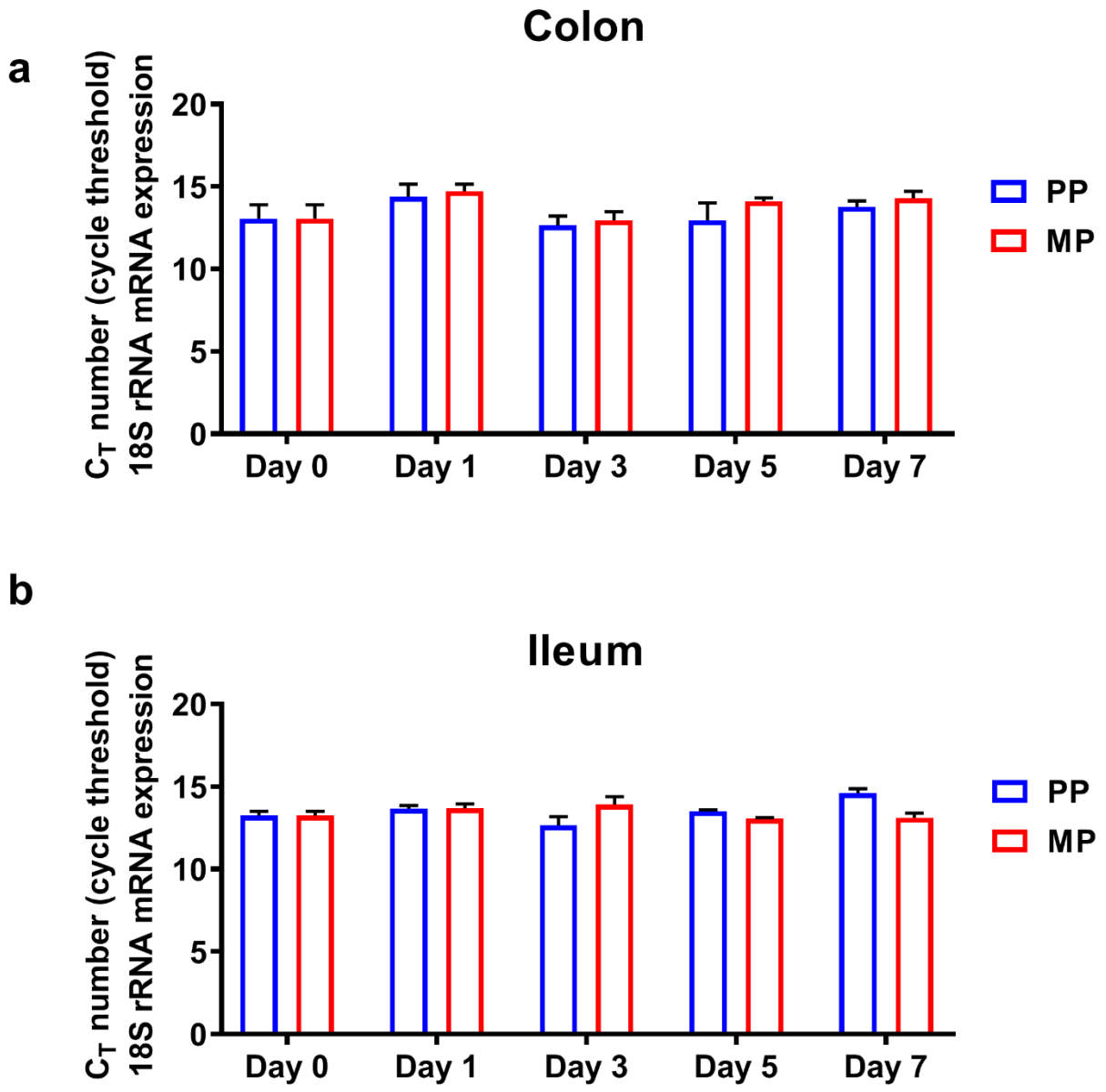

Figure 3.6: Time course of housekeeping gene 18S rRNA in mice colons and ileum after chronic morphine exposure. (**a - b**) 18S rRNA expression was not significantly altered by chronic morphine (75mg) in both colon and ileum samples from the PP mouse samples. N = 6/sample/day – colon; N = 4/sample/day – ileum; ns not significant by two-way ANOVA with Tukey post-hoc analysis.

The effects of morphine-induced inflammation on gut integrity

Morphological examinations of colon and ileum tissues after 5 days of morphine and placebo pelleting was determined by immunohistochemistry and hematoxylin and eosin (H&E) staining in cross sections. In the colon, tight junction protein occludin was redistributed in morphine treated mice; whereas, it was mainly localized at the epithelium in placebo treated mice (figure 3.7). Moreover, H&E staining of the colon showed a clear damage to the epithelial and mucosal layers after 5 days of morphine pelleting, indicated by arrows which was not noted in placebo pelleted mice (figure 3.8), allowing for bacterial translocation from the gut lumen across the gut wall. This would result in immune reaction, hence the increased IL-1β mRNA expression on day 5 (figure 3.3a). H&E staining of the ileum sections did not show any morphological changes as a result of morphine or placebo pelleting (figure 3.9). These findings indicate that chronic morphine-induced disruption of the epithelial integrity in the colon results in gut permeability (Kang *et al.*, 2017), translocation of bacteria, and a subsequent increase in IL-1β mRNA expression.

Figure 3.7: Effect of chronic morphine on the tight junction protein organization

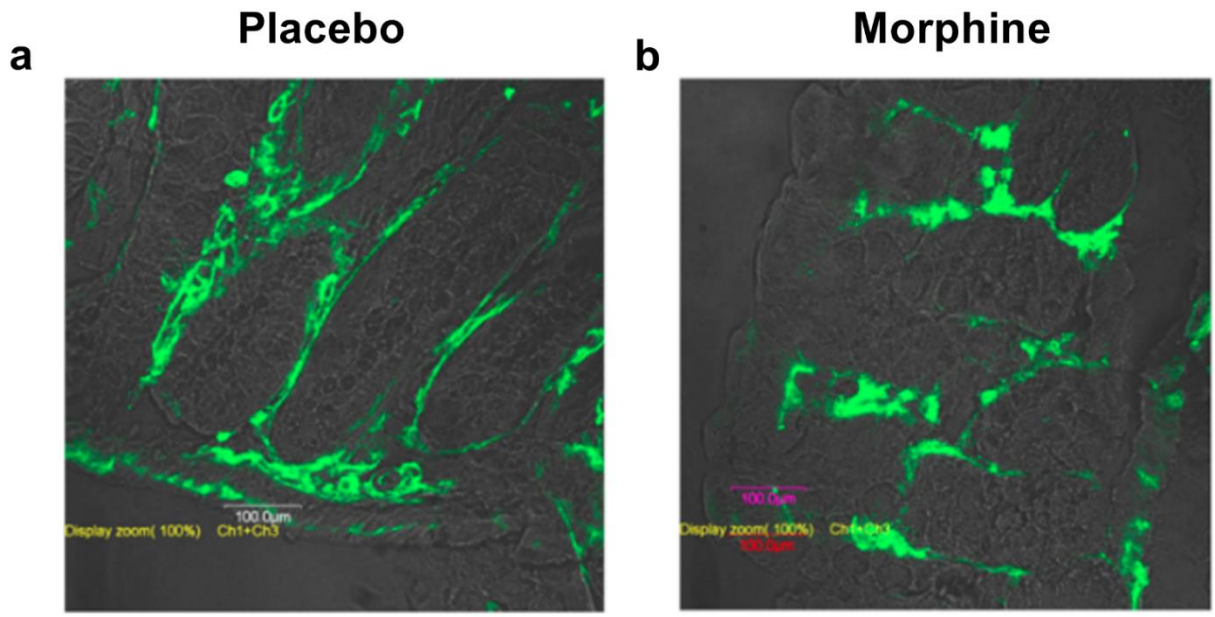

Figure 3.7: Effect of chronic morphine on the tight junction protein organization. (**a - b**) Representative immunohistochemical staining of colon sections from placebo-pelleted (PP) and morphine-pelleted (MP) mice at day 5 post pelleting. The tight junction protein occludin staining in green was superimposed on phase contrast image. Occludin distribution indicated an intact intestinal barrier in the PP mice; whereas, it was redistributed and disorganized in the MP mice. Scale bar: 100 µm.

Figure 3.8: Effect of chronic morphine on the epithelial barrier of the colon

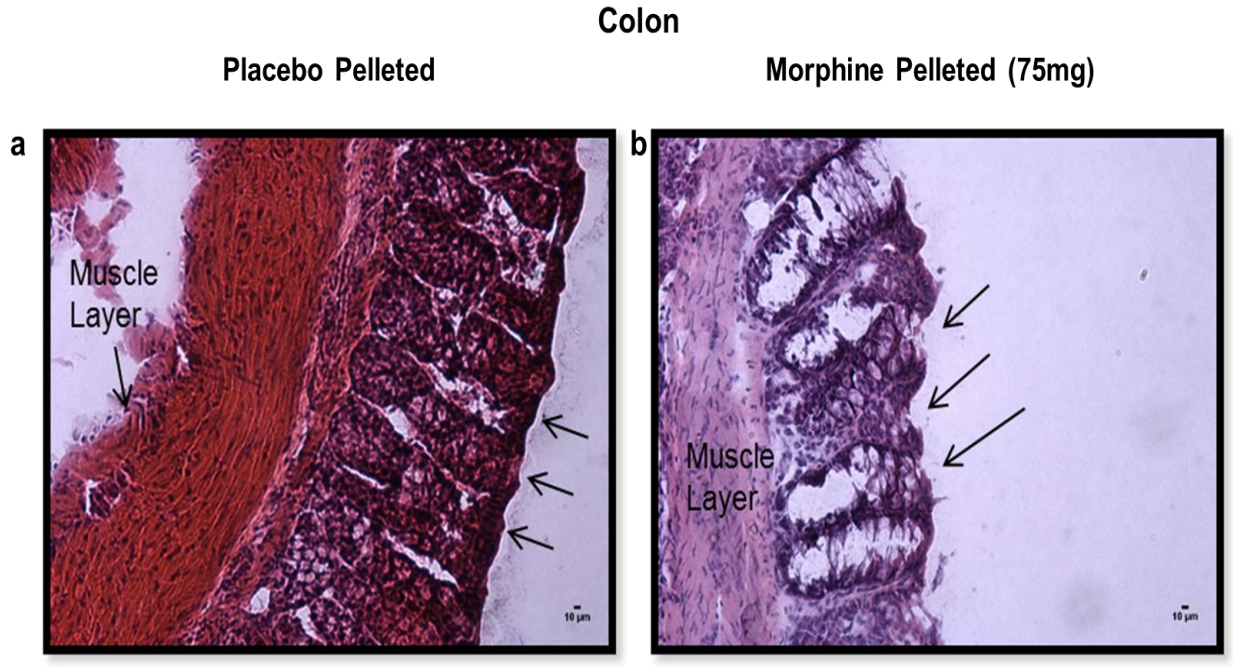

Figure 3.8: Effect of chronic morphine on the epithelial barrier of the colon. (**a - b**) H&E staining of the distal colon on day 5 of placebo and morphine pelleting demonstrated an intact epithelial barrier and mucosal organization in the placebo-pelleted mice; whereas, in the morphine-pelleted mice the epithelial barrier was disrupted and the mucosal layer was damaged. Arrow indicates epithelial barrier. Scale bar: 10 µm.

Figure 3.9: Effect of chronic morphine on the epithelial barrier of the ileum

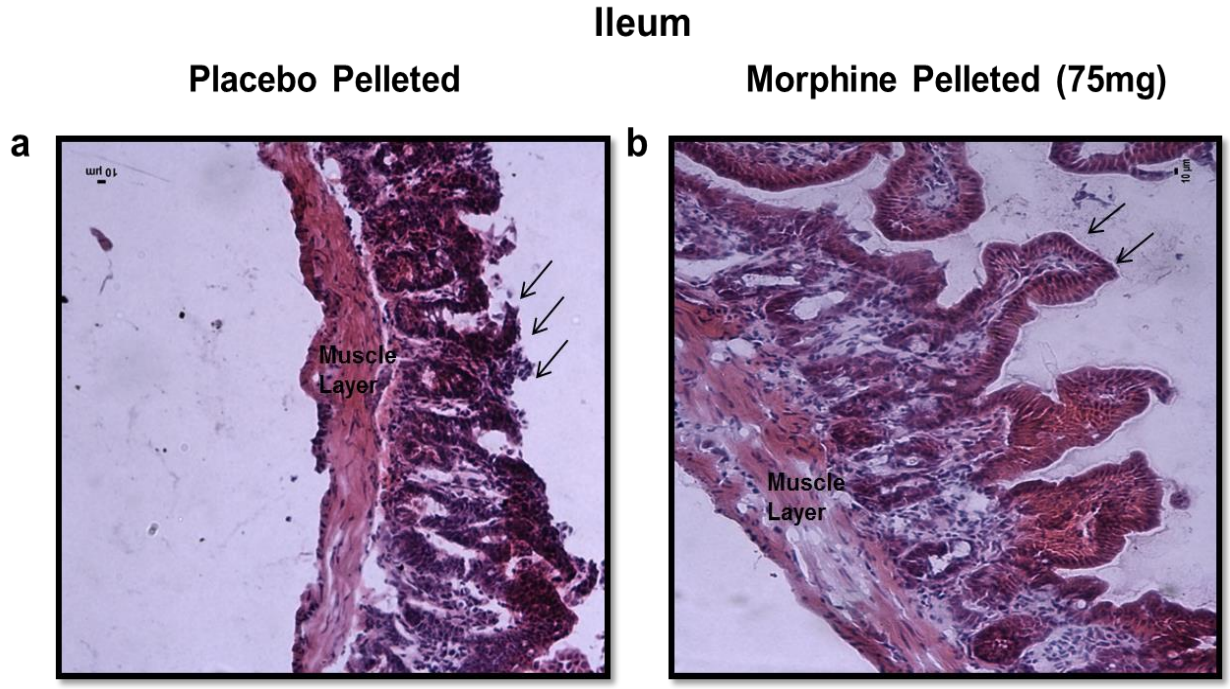

Figure 3.9: Effect of chronic morphine on the epithelial barrier of the ileum. (**a - b**) H&E staining of the ileum on day 5 of placebo and morphine pelleting demonstrated an intact epithelial barrier in both placebo-pelleted and morphine-pelleted mice. Arrow indicates epithelial barrier. Scale bar: 10 µm.

Summary

- The present study investigated the effects of morphine-induced inflammation on antinociceptive tolerance in mice and determined the time which chronic morphine treatment triggered inflammatory cytokines such as IL-1β, IL-6, IL-10, and TNF-α gene expressions and protein levels in the colon and ileum.
- Our findings demonstrated that acute morphine (10mg/kg) treatment did not result in inflammatory cytokine production.
- On the contrary, chronic morphine exposure significantly elevated IL-1β mRNA expressions on day 5 and 7 of morphine pelleting (75mg) in the colon (not the ileum) correlating with the development antinociceptive tolerance. The protein levels of IL-1β were also elevated on day 7 after morphine pelleting, however, they were not statistically significant from the placebo pelleted group.
- 5 days of morphine (75mg) pelleting also altered the integrity of the colon resulting in the breakdown of the epithelial barrier and the redistribution of tight junction protein occludin. These effects were not seen in the ileum.

Discussion

It is well established that chronic exposure of morphine results in differential rates of tolerance development in different systems in the body. The development of tolerance to the euphoric and analgesic effects of morphine occurs relatively faster than in the gastrointestinal tract and the respiratory center. Over the last decade, a plethora of studies have reported the effects of immune activation on morphine-induced analgesia and tolerance (Song and Zhao, 2001; Raghavendra *et al.*, 2002; Shen *et al.*, 2011). In the current study, we demonstrated the correlation of antinociceptive tolerance with inflammatory cytokines production in mice treated with chronic morphine.

Our findings indicated that 5 days of morphine pelleting (75 mg) resulted in the development of antinociceptive tolerance (figure 3.2). The use of morphine pellets to induce tolerance in mice has been a method of choice for many decades as the animals are constantly exposed to morphine (Way *et al.*, 1968) and the degree of tolerance development can occur in a relatively short amount of time (Way *et al.*, 1969). Although the pelleting method may not directly translate to the clinic, it has consistently produced tolerance and dependence in rodents in various nociceptive assays (Ross *et al.*, 2008). With 75 mg morphine pellets, the initial circulating levels of morphine was found to be 2 μg/ml in the blood and by 48 hours that level went down and stabilized at 0.6 μg/ml lasting for 5 days (Bryant *et al.*, 1988); whereas in humans, the circulating levels of opioids found in the blood of addicts who had overdosed was averaged to 0.8 ± 0.1 μg/ml (Ozaita *et al.*, 1998). The similarity in the blood concentration level of morphine in both rodents and humans makes our pelleting method an ideal tool to study tolerance in mice.

As morphine pelleted animals were significantly tolerant to the antinociceptive effects of morphine on day 5, the assessment of inflammatory cytokine Il-1β mRNA expression from colon

tissues was also significantly elevated indicating a link between tolerance development and inflammation. This finding is interesting as studies have found that attenuating inflammation has prevented morphine tolerance (Johnston *et al.*, 2004; Hutchinson *et al.*, 2008; Kang *et al.*, 2017). In one study, the development of antinociceptive tolerance to intrathecal morphine injections was inhibited by treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) ketorolac and ibuprofen (Powell *et al.*, 1999) and with a selective cyclooxygenase-2 (COX_2) inhibitor, NS-398 and with a non-selective cyclooxygenase (COX) inhibitor, indomethacin (Wong *et al.*, 2000). Whereas in another study, intrathecal injections of Il-1 receptor antagonist (IL-1ra) or inducing the expression of anti-inflammatory cytokine IL-10 by injecting adenovirus expressing interleukin-10 (ADIL-10) intrathecally not only attenuated morphine antinociceptive tolerance but also prevented the development of hyperalgesia and allodynia in rats (Johnston *et al.*, 2004). Therefore, our findings support the evidence that cytokines, chemokines, and glial activation contribute to the development of antinociceptive tolerance.

Of all the cytokines measured, only Il-1β was significantly expressed on the later days of morphine treatment in colon tissues. The other cytokines, IL-6, IL-10, and TNF-α, were proven to be the earliest genes expressed during the morphine-induced inflammation process. However, these effects were only seen in the colon and not in the ileum indicating a region-specific activation of the pro-inflammatory systems during systemic morphine treatment. Moreover, Il-1β protein levels were determined in morphine and placebo pelleted mouse colon tissues on day 7 by ELISA in order to confirm the translation of Il-1β gene into proteins. The protein levels of Il-1β failed to correspond to the mRNA expression, however there was a trend toward an elevated Il-1β levels in the morphine pelleted mice than the placebo mice. Perhaps, increasing the sample size would resolve the lack of statistical difference between the two groups. It is also possible that the level

of Il-1β produced fell below the sensitivity of the ELISA kit used; therefore, the low-grade inflammation produced by chronic morphine could not be detected. Alternatively, the detection window may have been missed when Il-1β levels were assessed on day 7 instead of day 5. Nonetheless, others have shown that chronic morphine induces Il-1β release in different organs in both animal models and *in vitro* models (Apte *et al.*, 1990; Peng *et al.*, 2000; Azuma and Ohura, 2002; Hutchinson *et al.*, 2007, 2008). Our findings are consistent with what others have found.

These results also show that by day 5 when the mice became tolerant and the Il-1β levels increased, the damage to the gut wall also became apparent. Five days of morphine pelleting demonstrated an injured mucosa, inflammatory infiltrates, and tight junction protein redistribution in the mice. These effects were observed in the colon alone and not in the ileum. In contrast, Meng et al. observed the same morphological changes within 24 hours after morphine pelleting in the small intestine but not in the colon of C57BL/6J mice (Meng *et al.*, 2013). The differential effects of morphine in the small intestine and the colon indicate a region-specific strain difference between Swiss Webster mice and C57BL/6J mice. Studies from our laboratory and others have reported C57BL/6J mice to have higher sensitivity to morphine in tolerance and dependence studies (Liu *et al.*, 2011; Fitting *et al.*, 2015)(unpublished data), which could explain earlier time point morphological changes observed in the small intestine in C57BL/6J mice.

Overall, the present results show that chronic morphine exposure via subcutaneous implantation is paralleled with an increased release of Il-1β within five days of treatment when antinociceptive tolerance developed. However, it is not known whether these are direct or indirect effects of the morphine pellets. It is noteworthy to further investigate if inflammation affects the rate of tolerance development to the antinociceptive effects of morphine, which the following chapters in this dissertation will address.

CHAPTER 4

Non-opioid-induced inflammation enhances the development of antinociceptive tolerance

This chapter is a modified version of an article submitted to the *Journal of Pharmacology and Experimental Therapeutics*

Komla, E, Stevens, DL, Zheng, Y, Zhang, Y, Dewey, WL, and Akbarali, HI. (2019). Experimental colitis enhances the rate of antinociceptive tolerance to morphine via peripheral opioid receptors. (*Under Review)*

Opioids are frequently used for the treatment of moderate to severe pain and non-cancer pain. Their therapeutic use is primarily limited due to the adverse effects with chronic use, particularly the development of tolerance and dependence. Tolerance develops to different degrees to the various pharmacological effects of opioids (Hayhurst and Durieux, 2016), with tolerance to respiratory depression occurring at a slower rate than analgesic and euphoric effects which may be one of the primary reasons for overdose related deaths.

Mechanisms by which tolerance occurs are complex and not well understood. We and others have previously reported that chronic morphine administration in mice alters the gut microbiome that affects the development of antinociceptive tolerance, dependence and reward pathways (Meng *et al.*, 2013; Kang *et al.*, 2017; Lee *et al.*, 2018). It has also been reported that

chronic morphine induces neuroinflammation in the brain and spinal cord via glial cell activation (Hutchinson *et al.*, 2008; Milligan and Watkins, 2009), and in the gastrointestinal tract via cytokines released from the enteric glia (Bhave *et al.*, 2017). Studies have suggested that inflammation may contribute to morphine tolerance (Song and Zhao, 2001; Raghavendra *et al.*, 2002; Watkins *et al.*, 2005), thus blocking inflammation with a glial-modulating agent propentofylline (Raghavendra *et al.*, 2004) or with neutralizing antibodies to IL-6, TNFα, or with IL-1 receptor antagonist (Johnston *et al.*, 2004; Hutchinson *et al.*, 2008), attenuated the development of antinociceptive tolerance at the spinal level in animal models. Additionally, depletion of gut bacteria with antibiotics also prevented the induction of antinociceptive tolerance as well as inflammation (Kang *et al.*, 2017).

Clinical supporting evidence from the Crohn's Therapy, Resource, Evaluation, and Assessment Tool (TREAT) registry comprising of over 6000 Crohn's disease patients showed that narcotic use carried a high risk for mortality and enhanced infection (Cross *et al.*, 2005; Lichtenstein *et al.*, 2006, 2012). Since chronic morphine also induces inflammation, the combination of inflammatory bowel disease (IBD) such as Crohn's disease and opioids likely enhances the severity of the disease. According to Targownik et al. IBD patients taking opioids are three times more likely to become heavy opioid users than their matched controls (Targownik *et al.*, 2014). It is possible that heavy opioid usage could be due to the development of tolerance to opioids. Furthermore, it is not known whether the increased inflammatory response from the combination of both IBD and opioids alters the rate at which tolerance to the opioid develops. This prompted us to investigate the rate of tolerance development to the antinociceptive effects of morphine in a model of experimental colitis. Notably, our results demonstrate that colonic inflammation enhances the rate of tolerance to morphine.

Pathological characteristics of TNBS-induced colitis

Colonic inflammation was induced in mice by 2.5% of 2,4,6-trinitro-benzene sulfonic acid (TNBS) administered intra-rectally. Histological examination of the colon by hematoxylin and eosin (H&E) staining and post-mortem necropsy images three days after initial TNBS treatment showed an inflamed colon, neutrophil infiltration, and damaged mucosal and crypt architecture compared to vehicle treated group (fig 4.1a). The extent of inflammation was assessed by examining inflammatory cytokine interleukin-1beta (IL-1β) mRNA expression and protein levels from colon tissues using real-time polymerase chain reaction (RT-PCR) and Enzyme-linked immunosorbent assay (ELISA), respectively. IL-1β was used as a marker of inflammation and its expression was normalized with housekeeping gene 18S rRNA. Figure 4.1b shows a significant higher IL-1β mRNA expression in TNBS treated animals compared to the vehicle treated mice. The housekeeping gene 18S rRNA was not altered by TNBS or VEH treatment (fig 4.1c). IL-1β protein levels were similarly higher in TNBS treated mice (fig 4.1d), consistent with the mRNA expression. Other inflammatory cytokine mRNA expressions such as interleukin-6 (IL-6), interleukin-10 (IL-10), and tumor necrosis factor alpha (TNF-α) were also measured. Their expressions were significantly increased in TNBS mice colons after 3 days (fig 4.2). In addition, mice were monitored daily for their overall health and their body weights were recorded. Mice treated with TNBS exhibited a significant weight loss (15%) after the initial TNBS exposure (fig 4.1e).

Figure 4.1: Evaluation of TNBS-induced colitis

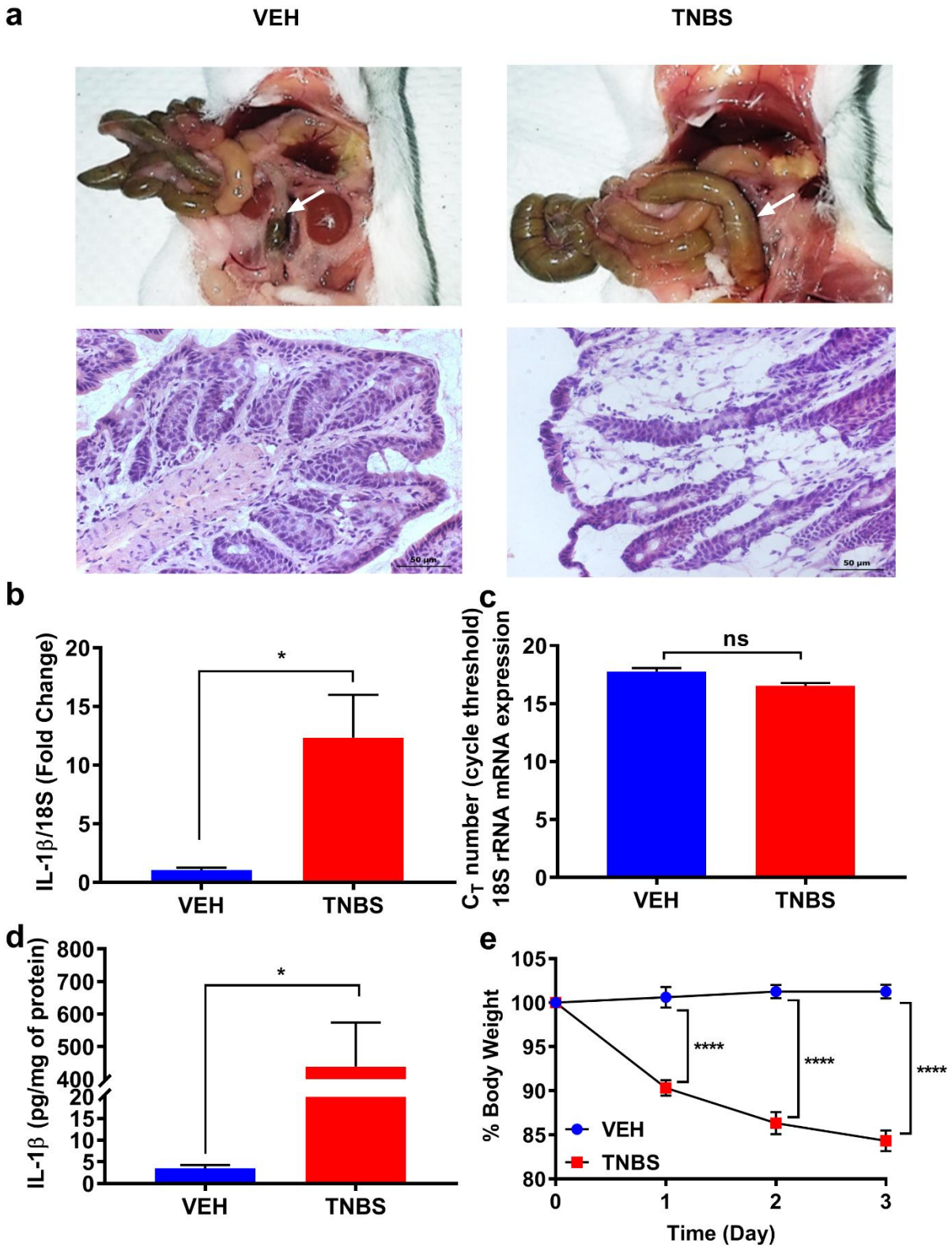

Figure 4.1: Evaluation of TNBS-induced colitis (**a**) Mice treated with TNBS show a clear inflammation of colon indicated by arrows compared to vehicle treated mice. H&E staining of colon sections demonstrates mucosal damage in TNBS treated mice. (**b**) TNBS treatment significantly increases mRNA expression of IL-1β from colon tissues on day 3. N = 6/group, *p = 0.0114 by unpaired t-test. (**c)** qPCR data are normalized to housekeeping gene 18S rRNA, whose expression is not altered during an inflamed state. (**d**) The level of IL-1β produced in TNBS treated mice is significantly increased in whole colon tissues on day 3 correlating with the mRNA expression data. N = 5/group, *p = 0.0120 by unpaired t-test. (**e**) Percent weight loss of TNBS treated mice is significantly higher than VEH group. N = 5/group, ****p < 0.0001 by two-way ANOVA with Tukey post-hoc analysis.

Figure 4.2: Inflammatory cytokine mRNA expressions in TNBS-treated mice colons

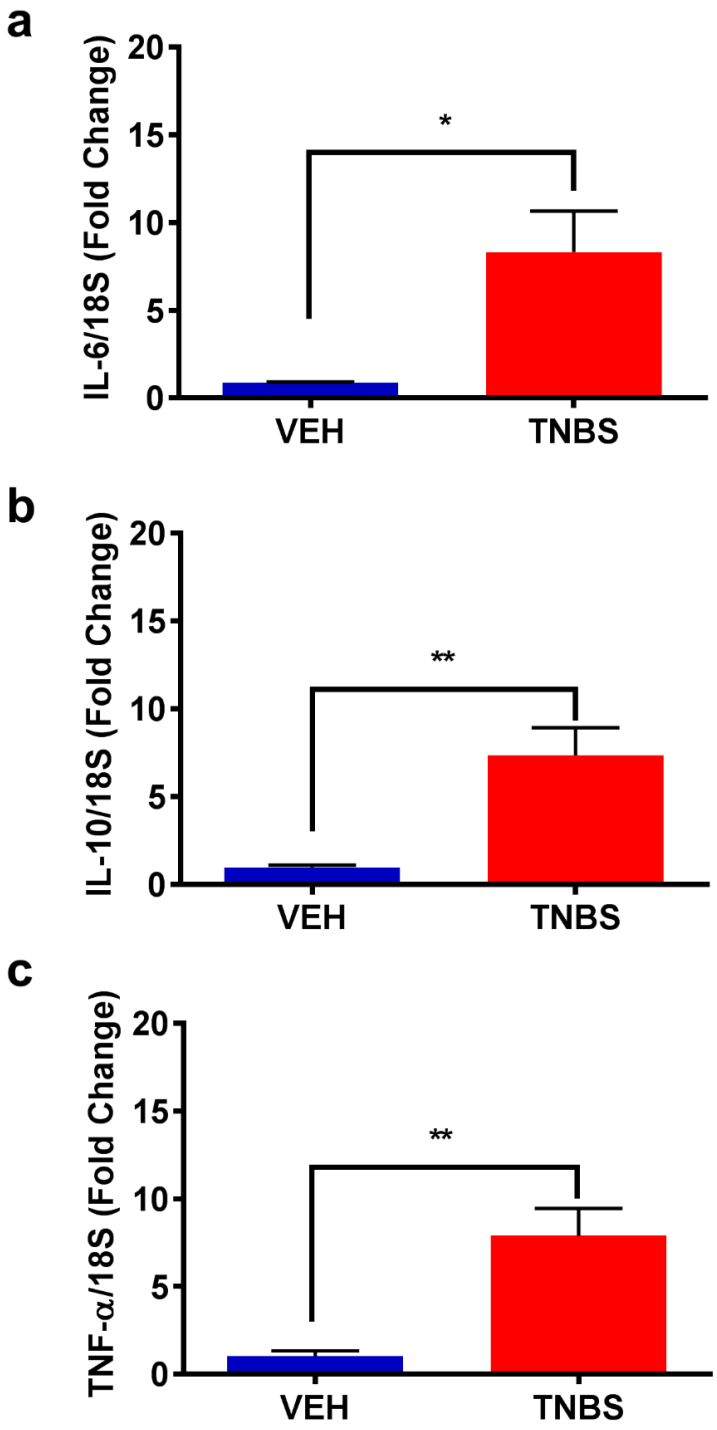

Figure 4.2: Inflammatory cytokine mRNA expressions in TNBS-treated mice colons. (**a** – **c**) TNBS treatment significantly increased mRNA expressions of IL-6, IL-10, and TNF-α in colon tissues on day 3. N = 6/group, *p = 0.01, **p = 0.002 by unpaired t-test.

Colonic inflammation altered the rate of antinociceptive tolerance

A cumulative dose-response to morphine was carried out in TNBS and VEH treated mice on day 3 following TNBS administration. The dose-response to morphine was shifted slightly to the left in the TNBS treated mice compared to the non-inflamed mice ((VEH ED_{50} = 4.7 mg/kg (4.03 - 5.47, 95% C.L.); TNBS ED_{50} = 3.14 mg/kg (2.58 - 3.82, 95% C.L.)) (fig 2) indicative of greater potency of the drug in the inflamed mice due to the lack of overlapping between the 95% confidence limits of the ED_{50} values. Although the two curves were not significantly different, the potency ratio (PR) of morphine in VEH treated mice was 1.39 (1.07 – 1.82, 95% C.L.); whereas in TNBS treated animals was 0.72 (0.55 – 0.93, 95% C.L.).

Figure 4.3: Dose-Response curves to the antinociceptive effect of morphine during TNBS inflammation

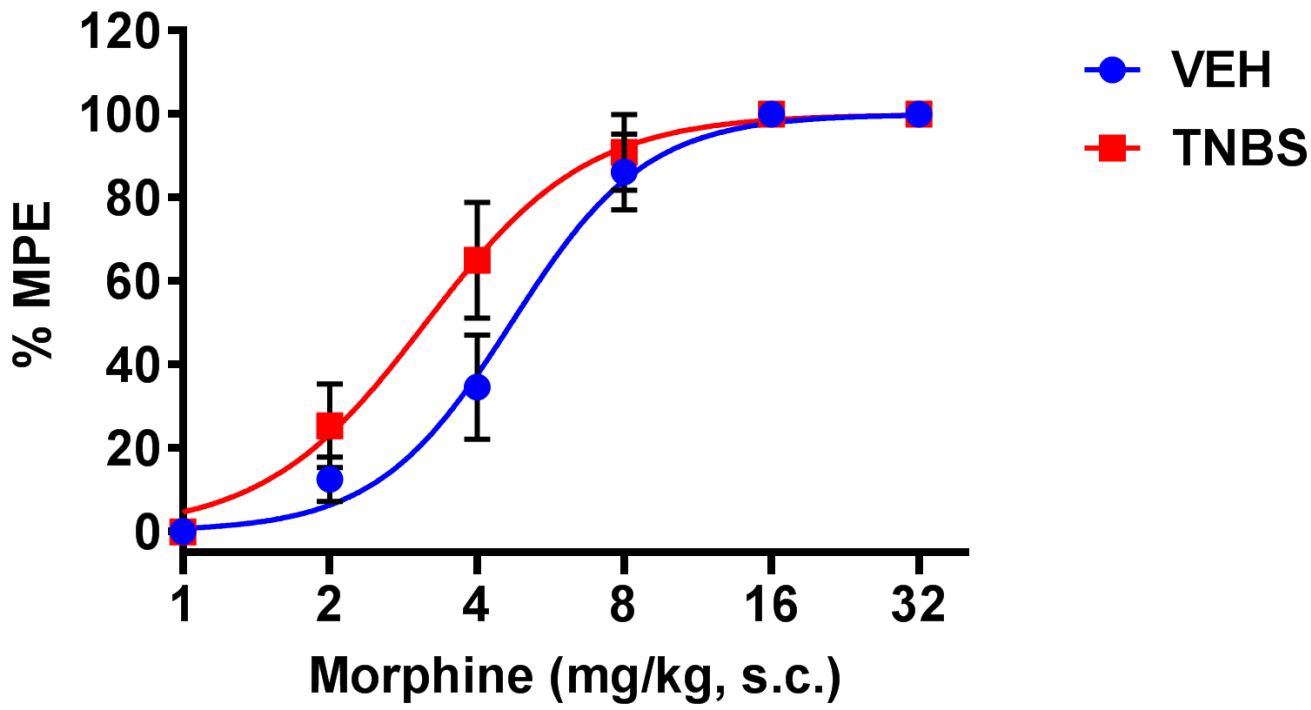

Figure 4.3: Dose-Response curves to the antinociceptive effect of morphine during TNBS inflammation. A cumulative dose-response to morphine (1, 2, 4, 8, 16, & 32 mg/kg) tested in TNBS and VEH treated mice on day 3 shows increased morphine potency in TNBS mice compared to VEH mice. N = 9/group, VEH ED_{50} = 4.7 mg/kg (4.03 - 5.47, 95% C.L.), PR = 1.39 (1.07 – 1.82, 95% C.L.); TNBS ED_{50} = 3.14 mg/kg (2.58 - 3.82, 95% C.L.), PR = 0.72 (0.55 – 0.93, 95% C.L.). No significant difference between the two curves p = 0.21 by two-way ANOVA with Tukey post-hoc analysis.

To test if the extent of tolerance developed after chronic morphine is altered by colonic inflammation, TNBS treated mice were pelleted with either 25 mg, 50 mg (2x25 mg), or 75 mg of morphine pellet (MP) or placebo pellet (PP) on the day of TNBS administration. TNBS treatment in combination with morphine pelleting resulted in rapid decline in body weight (fig 4.4). There was a 10 – 15% decrease in body weight with TNBS that was slightly enhanced in the presence of morphine, however the weight loss did not exceed 18%.

Figure 4.4: Percent body weight loss in morphine and TNBS-treated mice

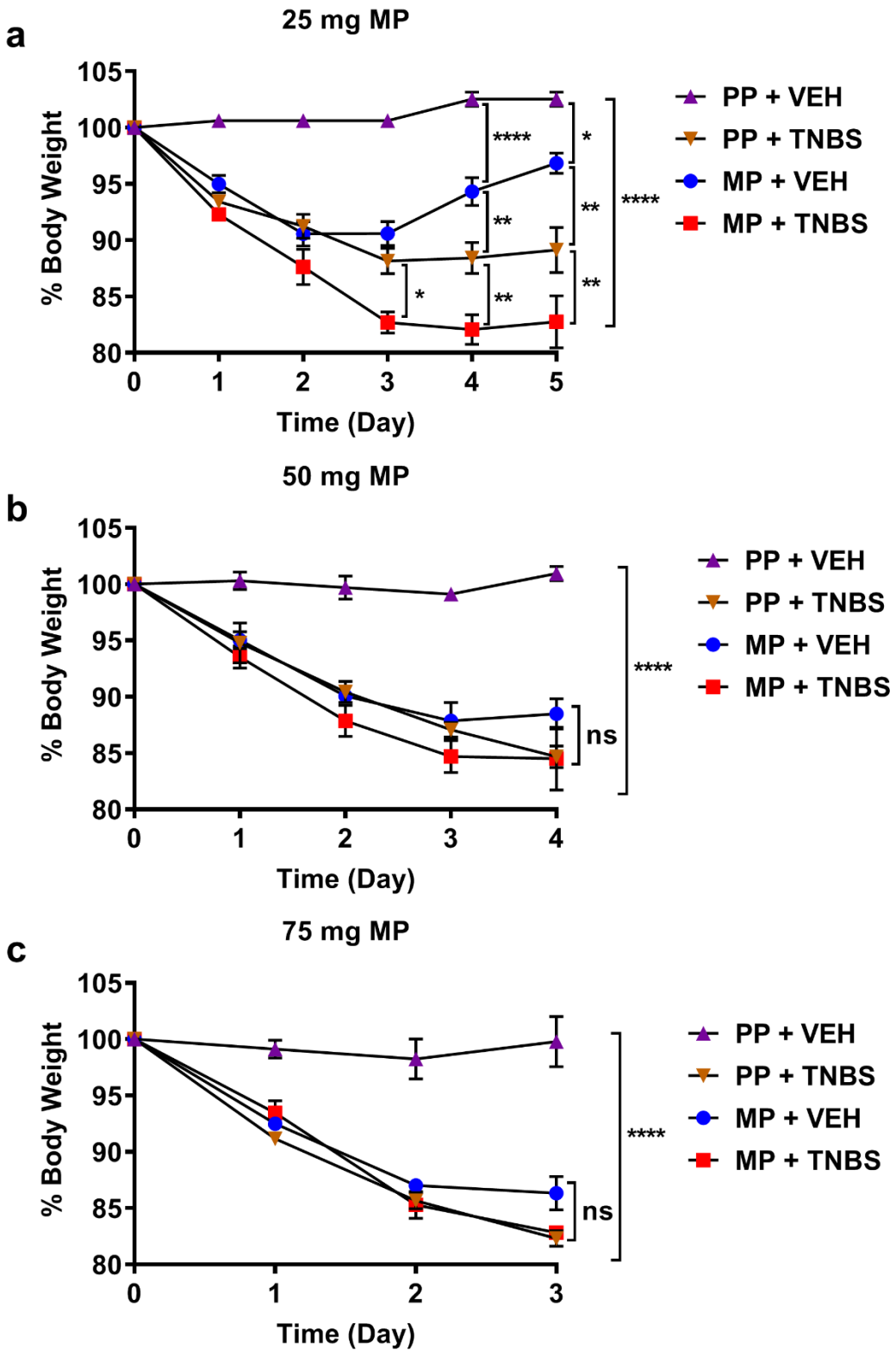

Figure 4.4: Percent body weight loss in morphine and TNBS-treated mice (**a - c**) Body weight gradually decreased over the course of morphine alone, TNBS alone, or the combination of TNBS and morphine treatment. N = 5/group, ns not significant, *p = 0.0311, **p < 0.01, ****p < 0.0001 by two-way ANOVA with Tukey post-hoc analysis.

The rate of tolerance to morphine was determined each day by measuring the latency to tail withdrawal in the warm water tail-immersion assay. As shown in figure 4.5a, b and c (left panels), as expected, the baseline latency in MP treated groups was higher on day 1 than PP treated groups. Over time, the baseline latency of MP treated mice progressively declined to the level of the PP treated mice in each of the groups, indicating the loss of morphine's effect. In preliminary studies, mice treated with TNBS and 75 mg morphine pellets had increased mortality; therefore, the subsequent experiments in which mice were treated with both TNBS + MP to determine tolerance were limited to 5 days (25 mg MP), 4 days (50 mg MP) and 3 days (75 mg MP). Tolerance was determined daily in each cohort of mice by a challenge dose (10 mg/kg) and the %MPE was determined (right panels). When the placebo groups were challenged with morphine (10 mg/kg), they all responded with a maximal antinociception at 100 %MPE. In the morphine pelleted groups, significant tolerance was observed to a morphine challenge on day 5 in the 25 mg MP + TNBS mice (19.3 ± 4 %MPE). Interestingly, at this dose of morphine pellet, tolerance did not develop in MP group alone up to 5 days. In the 50 mg MP group, tolerance was enhanced in the MP + TNBS mice (18.9 ± 13 % MPE) compared to MP mice alone by day 4 and similarly in the 75 mg MP group, tolerance in the MP + TNBS group was 20.9 ± 13.5 %MPE by day 3. Collectively, these findings indicate that antinociceptive tolerance to chronic morphine exposure in the presence of colonic inflammation are enhanced and occurred in a dose and time dependent manner.

Figure 4.5: TNBS-induced colitis enhanced the rate of tolerance development in a dose- and time-dependent manner

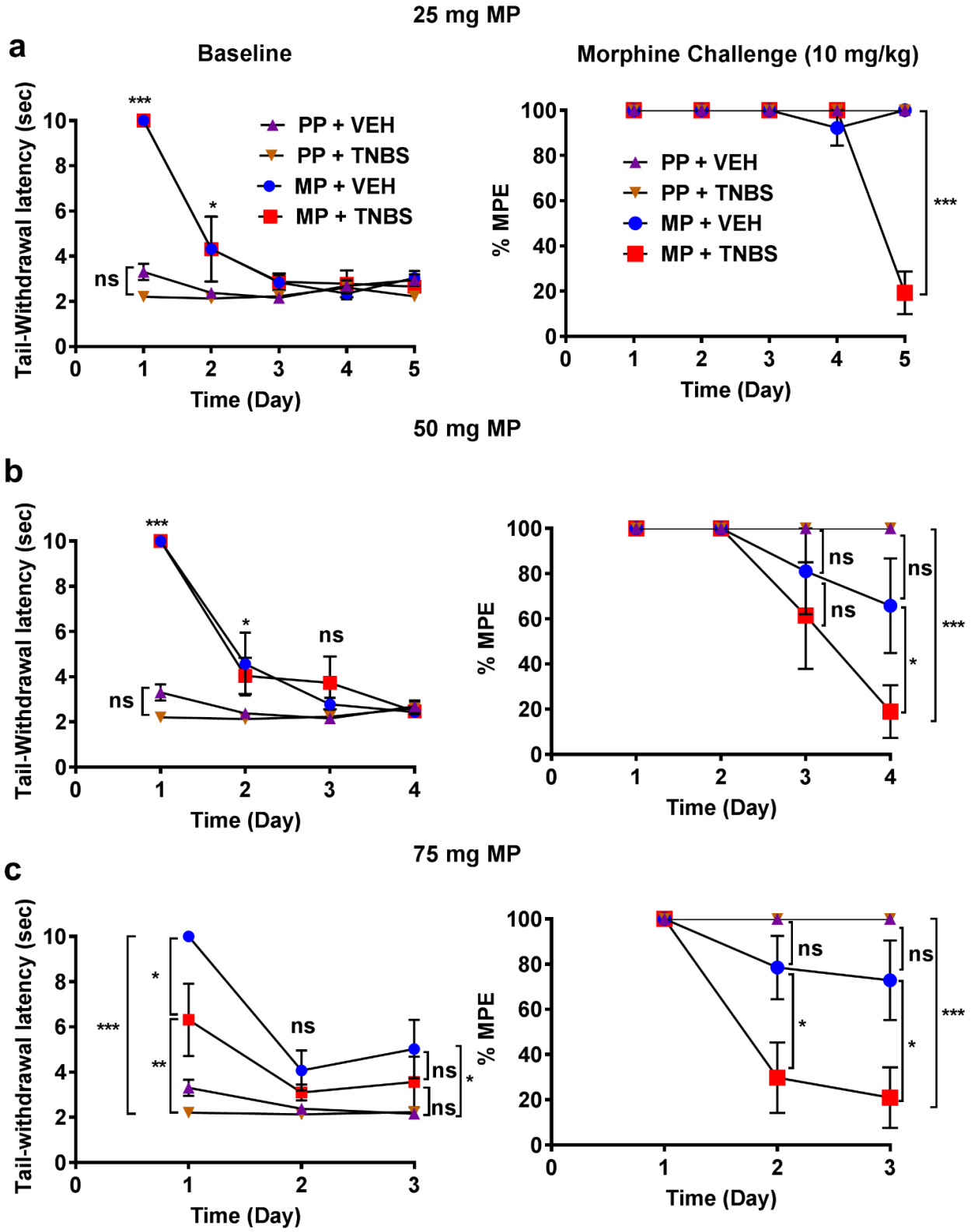

Figure 4.5: TNBS-induced colitis enhanced the rate of tolerance development in a dose- and time-dependent manner. (**a - c**) Daily baseline recording (left panel) shows a progressive loss of morphine response in 25 mg, 50 mg, and 75 mg MP groups over the course of 5, 4, and 3 days, respectively. Daily 10 mg/kg morphine challenge (right panel) restored the loss of morphine response in all groups except in 25 mg MP + TNBS group on day 5, 50 mg MP + TNBS group on day 4, and 75 mg MP + TNBS group on day 3, indicating the development of tolerance in the inflamed mice. (**a**) (right panel) MP + TNBS (N = 5 – 7/day), MP + VEH (N = 5/day), PP + TNBS (N = 5/day), PP + VEH (N = 5/day), P (1 – β) = 1, ***p < 0.001 by two-way ANOVA with Tukey post-hoc analysis. (**b**) (right panel) MP + TNBS (N = 5 – 8/day), MP + VEH (N = 5 – 7/day), PP + TNBS (N = 5/day), PP + VEH (N = 5/day), P (1 – β) = 0.99, ns not significant, *p < 0.05, ***p < 0.001 by two-way ANOVA with Tukey post-hoc analysis. (**c**) (right panel) MP + TNBS (N = 7 – 8/day), MP + VEH (N = 7/day), PP + TNBS (N = 5 – 8/day), PP + VEH (N = 5 – 7/day), P (1 – β) = 1, ns not significant, *p < 0.05, ***p < 0.001 by two-way ANOVA with Tukey post-hoc analysis.

We next determined the time course of IL-1β expression in the colon from each of these groups. The IL-1β mRNA expression in the 25 mg MP + VEH and PP + VEH groups was not significantly altered over 5 days (fig 4.6a). However, IL-1β mRNA expression was significantly increased in TNBS treated groups. Morphine treatment did not enhance the expression of IL-1β over the levels induced by TNBS in any of the groups. These results indicate that the level of inflammation produced by TNBS alone masks any potential increase in the inflammation induced by morphine over this time frame.

Figure 4.6: TNBS treatment induced the gene expression of pro-inflammatory cytokine IL-1β

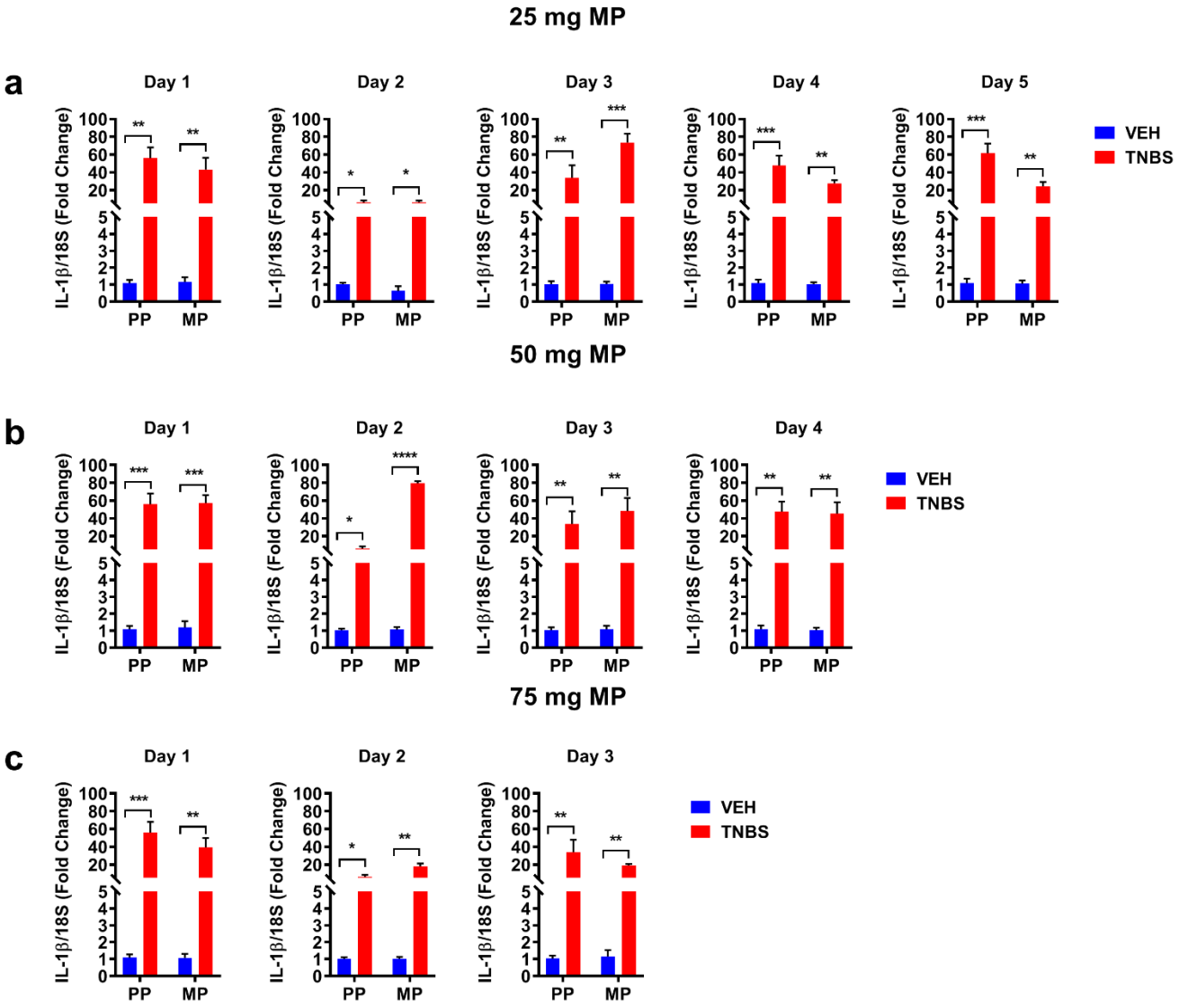

Figure 4.6: TNBS treatment induced the gene expression of pro-inflammatory cytokine IL-1β. (**a - c**) Increased IL-1β mRNA expression observed in TNBS-treated mice in the presence of 25 mg MP, 50 mg MP, and 75 mg MP. IL-1β expressions are not significantly different between MP + TNBS and PP + TNBS groups, and between MP + VEH and PP + VEH groups. N = 5/group *p < 0.05, **p < 0.01, ***p < 0.001. ****p < 0.0001 by two-way ANOVA with Tukey post-hoc analysis.

Summary

- Three days of TNBS treatment significantly increased IL-1β mRNA expressions and protein levels in the colon.
- TNBS treatment significantly decreased mice body weight.
- Mice treated with TNBS for three days had an increased morphine potency indicated by the leftward shift in the cumulative dose-response curve.
- Morphine pelleted mice exhibited a progressive decrease in analgesia over time, but were not tolerant as they responded to the morphine (10 mg/kg) challenge.
- At high morphine concentration (75 mg), analgesia was mitigated in the presence of TNBS on day 1 post pelleting.
- TNBS treated mice with morphine pellets showed an enhanced development of antinociceptive tolerance in a dose and time dependent manner.

Discussion

In the present study, we found that colonic inflammation enhances the rate and extent of antinociceptive tolerance to morphine. These findings may have major clinical implications for inflammatory bowel diseases (IBD) patients using narcotic opioids. There is growing evidence that opioids may be a risk factor for IBD patients as these drugs increase the severity of the disease, the risk of infection and mortality, as well as the risk of becoming heavy opioid users (Cross *et al.*, 2005; Hanson *et al.*, 2009; Long *et al.*, 2012; Targownik *et al.*, 2014). Clinical data have shown that prior opioid use before diagnosis with IBD predisposes patients to heavy opioid use later in life and about 5% of IBD patients using opioids become heavy opioid users (Targownik *et al.*, 2014). The enhanced rate of tolerance development to morphine in the presence of colonic inflammation may be an important factor in predisposing for increased opioid use, thus setting up a vicious cycle.

While inflammation-mediated tolerance to morphine has been reported in experimental arthritis model with complete Freund's adjuvant (CFA) (Li *et al.*, 1999; Fernández-Dueñas *et al.*, 2007), to our knowledge, the present study is the first demonstration of the differential rate of morphine tolerance in experimental colitis model. The TNBS-induced inflammation is suggested to model Crohn's disease in terms of both clinical and histopathological finding (Strober *et al.*, 1998; Antoniou *et al.*, 2016). In the experimental colitis model, inflamed mice were more sensitive to acute morphine (fig 4.3). Previous studies by Fernandez-Duenas *et al.* also found increased sensitivity to acute morphine in the presence of CFA-induced inflammation(Fernández-Dueñas *et al.*, 2007). The increase morphine potency in inflamed mice indicated by the leftward shift in the dose response curve could be due to the increase in μ-opioid receptor (MOR) expression during inflammation (Philippe *et al.*, 2006), which aligns with previous studies in rodents demonstrating

the association of peripheral inflammation with increased MOR axonal transport (Hassan *et al.*, 1993; Jeanjean *et al.*, 1995; Mousa *et al.*, 2001). The peripheral axonal transport of MOR during an inflammatory state is thought to be mediated by cytokine production and nerve growth factor (Stein and Lang, 2009) resulting in enhanced antinociceptive efficacy of morphine.

While others have found that morphine-induced proinflammatory mediators opposed acute and chronic morphine analgesia (Johnston *et al.*, 2004; Shavit *et al.*, 2005; Hutchinson *et al.*, 2008), we did not observe any significant changes in IL-1β expressions between placebo mice colons and morphine pelleted colons at the morphine doses (25 mg, 50 mg, and 75 mg) used on any particular day (fig 4.6). IL-1β expression was however elevated on day 5 as demonstrated in fig 3.3a in 75 mg MP colons, paralleling with the development of tolerance. In agreement with Hutchinson *et al*., only TNBS mice pelleted with a higher dose of morphine (75 mg) exhibited a decreased morphine analgesia at day 1 post morphine pelleting (fig 4.5 c). This is believed to be influenced by a progressive spinal glial activation in response to chronic morphine exposure (Song and Zhao, 2001; Watkins *et al*., 2005).

The development of tolerance to morphine was tested by providing a challenge dose of 10 mg/kg morphine in chronic morphine treated mice. Interestingly, while inflammation alone did not induce tolerance to morphine, tolerance occurred with the lowest dose of morphine in the presence of colonic inflammation (fig 4.5a). These findings suggest that inflammation sensitizes the process of tolerance development. A possible mechanism may involve the release of endogenous opioid peptides, specifically beta endorphins under inflammatory conditions that may in combination with the presence of morphine lead to rapid desensitization of the mu-opioid receptors resulting in greater tolerance (Stein *et al.*, 1990a; Stein *et al.*, 1990b; Cabot *et al.*, 1997). Another possibility for the enhanced tolerance could be due to gut-derived mediators that may

render tolerance to morphine in primary afferent neurons emanating from the colon. We recently reported that colonic supernatants from morphine treated mice rendered tolerance in the isolated dorsal root ganglion (DRG) neurons (Mischel *et al.*, 2018).

The data from this chapter support the notion that chronic inflammation facilitates the development of morphine antinociceptive tolerance. Our findings complement a vast literature on pro-inflammatory cytokines altering morphine analgesia and tolerance, thus increasing the dose of morphine in the presence of chronic inflammation results in a faster rate of tolerance development. Further studies are warranted to explore whether the enhanced morphine tolerance described in this chapter could be prevented by using peripheral opioid antagonists.

CHAPTER 5

Morphine antinociceptive tolerance is prevented by a peripheral opioid receptor antagonist

Morphine is an excellent analgesic that comes with many detrimental side effects including analgesic tolerance. Tolerance to the analgesic effects of morphine are originally thought to be centrally-mediated; however, over the last two decades, peripheral opioid receptors have been implicated to contribute to the development of tolerance (Stein *et al.*, 1989; Junien and Wettstein, 1992; Kolesnikov *et al.*, 1996; Kolesnikov and Pasternak, 1999; Corder *et al.*, 2017). Although the peripheral mechanisms of morphine are still not fully understood, most studies have used models of inflammatory pain and hyperalgesia to examine those mechanisms such as Freund's complete adjuvant (CFA), carrageenan, prostaglandin, acetic acid, or formalin to inflame subcutaneous tissues, viscera or joints (Joris *et al.*, 1987; Mays *et al.*, 1987; Raja *et al.*, 1992; Stein, 1993). These models represent the binding of opioids to its peripheral receptors that are expressed on primary afferent neurons (Fields *et al.*, 1980; Schäfer *et al.*, 1995). For example, in the CFA model of inflammation, chronic morphine exposure produced tolerance to the anti-hyperalgesic effects of acute morphine administration in mice (Hernández *et al.*, 2009). Furthermore, mechanical hyperalgesia was reversed by systemic administration of the peripheral opioid receptor antagonist naloxone-methiodide during paw inflammation (Fernández-Dueñas *et al.*, 2007), suggesting the development of tolerance to the peripheral effects of morphine during inflammation.

There are currently a few peripheral opioid antagonists that are FDA approved. They are called peripheral-acting mu-opioid receptor antagonists (PAMORAs) and were developed for the

purpose of diminishing the unwanted GI side effect of opioids such as constipation. Clinically approved PAMORAs for the treatment of opioid induced constipation (OIC) and post-operative ileus include but are not limited to Methylnaltrexone (MNTX) and alvimopan. These drugs are peripherally restrictive; therefore, do not cross the blood brain barrier (BBB) to interfere with opioid-induced analgesia or cause withdrawal symptoms. Given that the peripheral MOR antagonists are devoid of agonist activity at the MOR and they effectively treat OIC, it has led to the concept that peripheral MOR antagonists may also be useful in preventing the development of antinociceptive tolerance to morphine. In a recent report, Corder *et al*. have demonstrated the reversal of morphine antinociceptive tolerance and opioid-induced hyperalgesia in mice using methylnaltrexone bromide (Corder *et al.*, 2017). Therefore, in the following studies we tested the reversal of the enhanced tolerance developed in morphine pelleted mice treated with TNBS using peripheral opioid receptor antagonists.

Methylnaltrexone, a clinically approved peripheral opioid receptor antagonist failed to prevent the development of antinociceptive tolerance

The aforementioned data from chapter 4 demonstrates that there are peripheral and central components at play as the inflamed gut (peripheral) transduces signal via the primary afferent neurons (peripheral) to the dorsal horn of the spinal cord, which then relays that signal to the brain (central) resulting in enhanced tolerance development. This prompted us to determine if blocking the peripheral component would prevent the enhanced development of tolerance in the MP + TNBS groups. We therefore tested methylnaltrexone (MNTX). We first demonstrated injection in the tail-immersion assay the effect of MNTX on morphine-induced antinociception by subcutaneously injecting naïve mice with various doses (0.5 mg/kg – 25 mg/kg) of MNTX 20 min prior to morphine (10 mg/kg). In figure 5.1 the acute dose-response relationship showed a

decreased antinociceptive effect of morphine in the presence of MNTX at all the doses used, indicated by the low %MPE. To determine if MNTX would prevent morphine tolerance in our inflammatory model, TNBS or vehicle treated mice were pelleted with 75 mg morphine pellets and were subcutaneously injected with 25 mg/kg MNTX daily. MNTX treated animals had a very low daily tail-flick latency compared to morphine alone groups, even at day 1 post morphine pelleting (figure 5.2a). When the mice were challenged on day 3 with 10 mg/kg morphine, their %MPE remained low (figure 5.2b). This finding indicated that MNTX was crossing the blood brain barrier and antagonizing morphine-induced antinociception. To confirm this finding, we then sought to demonstrate similar results in a different nociceptive assay using a hot plate assay involving higher brain functions. Similar to the tail-immersion data, MNTX treated mice had a relatively low daily hot plate latency (figure 5.3a) and when the mice were challenged with 10 mg/kg morphine on day 3, their %MPE was significantly reduced compared to morphine alone groups (figure 5.3b). This finding confirms the notion that methylnaltrexone demethylates rapidly in rodents into naltrexone (Kotake *et al.*, 1989; Chandrasekaran *et al.*, 2010), hence the lack of morphine antinociceptive effects in our assays. In addition, to determine the effect of MNTX on TNBS-induced colonic inflammation, IL-1β and TNF-α mRNA expressions were measured from mice colon on day 3 post testing. MP + TNBS mice treated with MNTX had a significantly lower IL-1β mRNA expression compared to the other groups (figure 5.4a). TNF-α mRNA expression was lowered in the presence of MNTX, however it was not statistically significant (figure 5.4b). This finding indicates that MNTX may have some anti-inflammatory properties.

Figure 5.1: The dose-response of MNTX

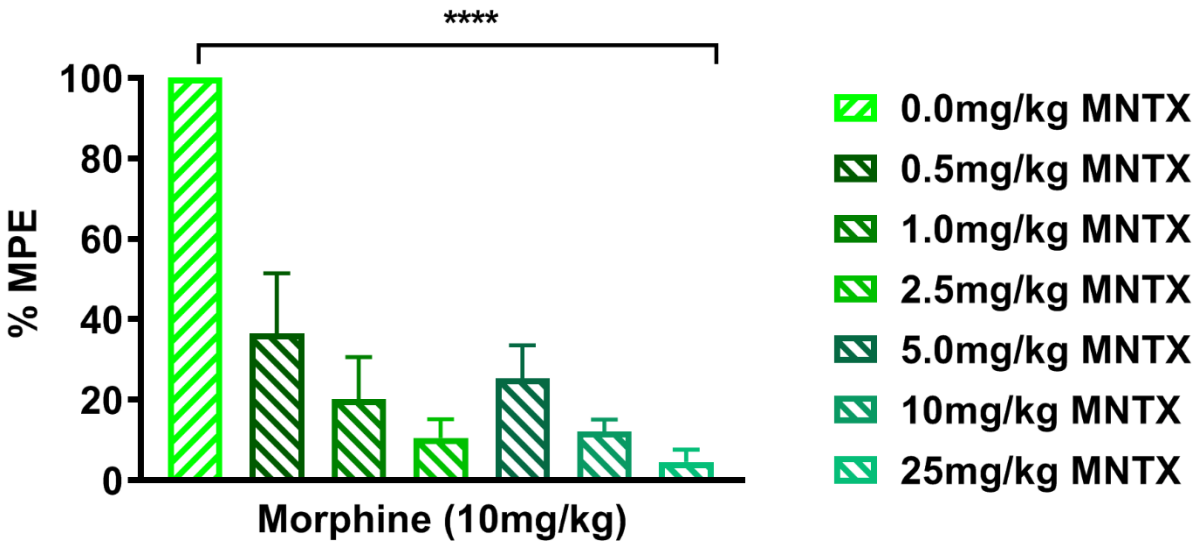

Figure 5.1: The dose-response of MNTX. 0.5 mg/kg to 25 mg/kg MNTX dose-dependently attenuated morphine's antinociceptive effects in the tail-immersion assay after an acute morphine challenge dose (10 mg/kg). N = 5 – 6/group, P (1 – β) = 0.99, ****p < 0.0001 by one-way ANOVA with Dunnett post-hoc analysis; compared to control (0.0 mg/kg).

Figure 5.2: The effect of MNTX on morphine antinociceptive tolerance in the warm water tail-immersion assay

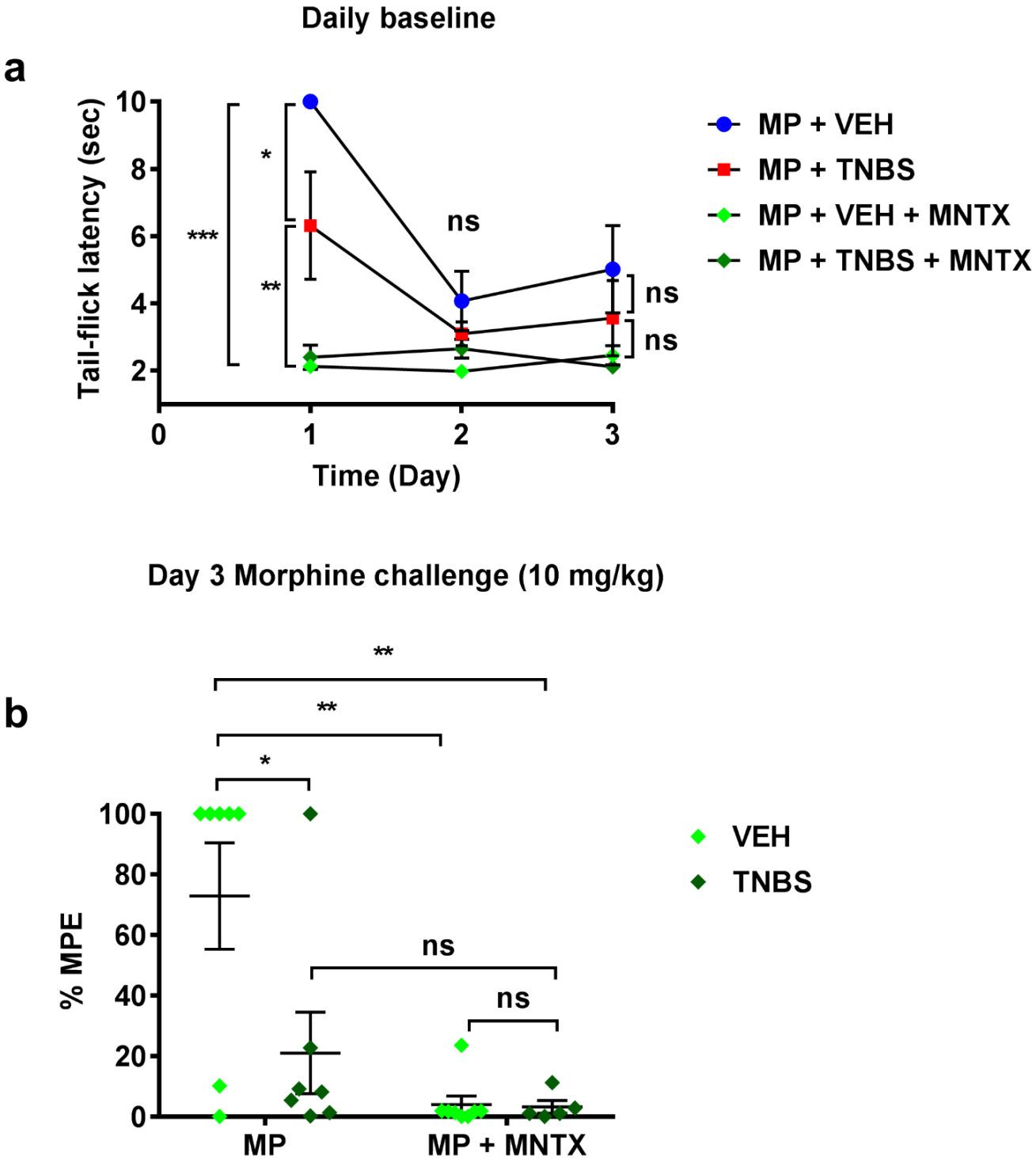

Figure 5.2: The effect of MNTX on morphine antinociceptive tolerance in the warm water tail-immersion assay. (**a**) Daily baseline recording from mice pre-treated with 25 mg/kg MNTX showed a very low tail-flick latency compared to the non-treated mice. N= 5-8/group, *P = 0.0169, **P = 0.0074, ***P < 0.001, n.s. not significant, two-way ANOVA with Tukey's post-hoc analysis. (**b**) Mice pre-treated with 25 mg/kg MNTX failed to respond to the 10 mg/kg morphine challenge indicated by the low %MPE. N= 5 – 8/group, P (1 – β) = 0.99, MP + VEH vs. MP + TNBS *p = 0.019, MP + VEH vs. MP + MNTX + VEH **P = 0.0011, MP + VEH vs. MP + MNTX + TNBS **P = 0.0035, n.s. not significant, two-way ANOVA with Tukey post-hoc analysis.

Figure 5.3: The effect of MNTX on morphine antinociceptive tolerance in the hot plate assay

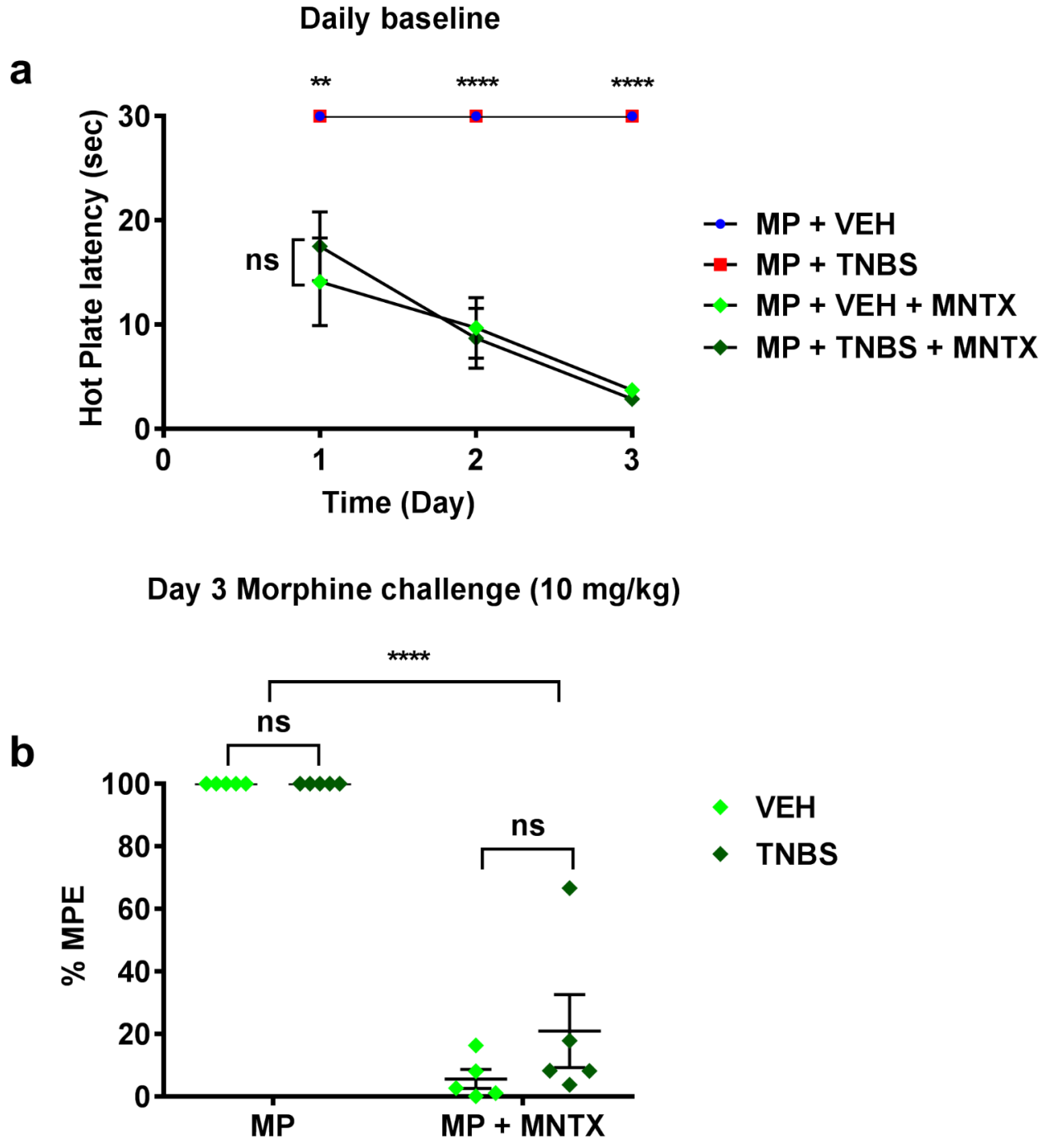

Figure 5.3: The effect of MNTX on morphine antinociceptive tolerance in the hot plate assay. (**a**) Daily baseline recording from mice pre-treated with 25 mg/kg MNTX showed a progressive decline in hot plate latency over the course of three days compared to the non-treated mice. N= 5/group, **P = 0.0023, ****P < 0.0001, n.s. not significant, two-way ANOVA with Tukey's post-hoc analysis. (**b**) Mice pre-treated with 25 mg/kg MNTX did not display antinociception to the 10 mg/kg morphine challenge indicated by the low %MPE. N= 5/group, P (1 – β) = 0.98, ****P < 0.0001, n.s. not significant, two-way ANOVA with Tukey post-hoc analysis.

Figure 5.4: MNTX decreased TNBS-induced IL-1β and TNF-α mRNA expressions

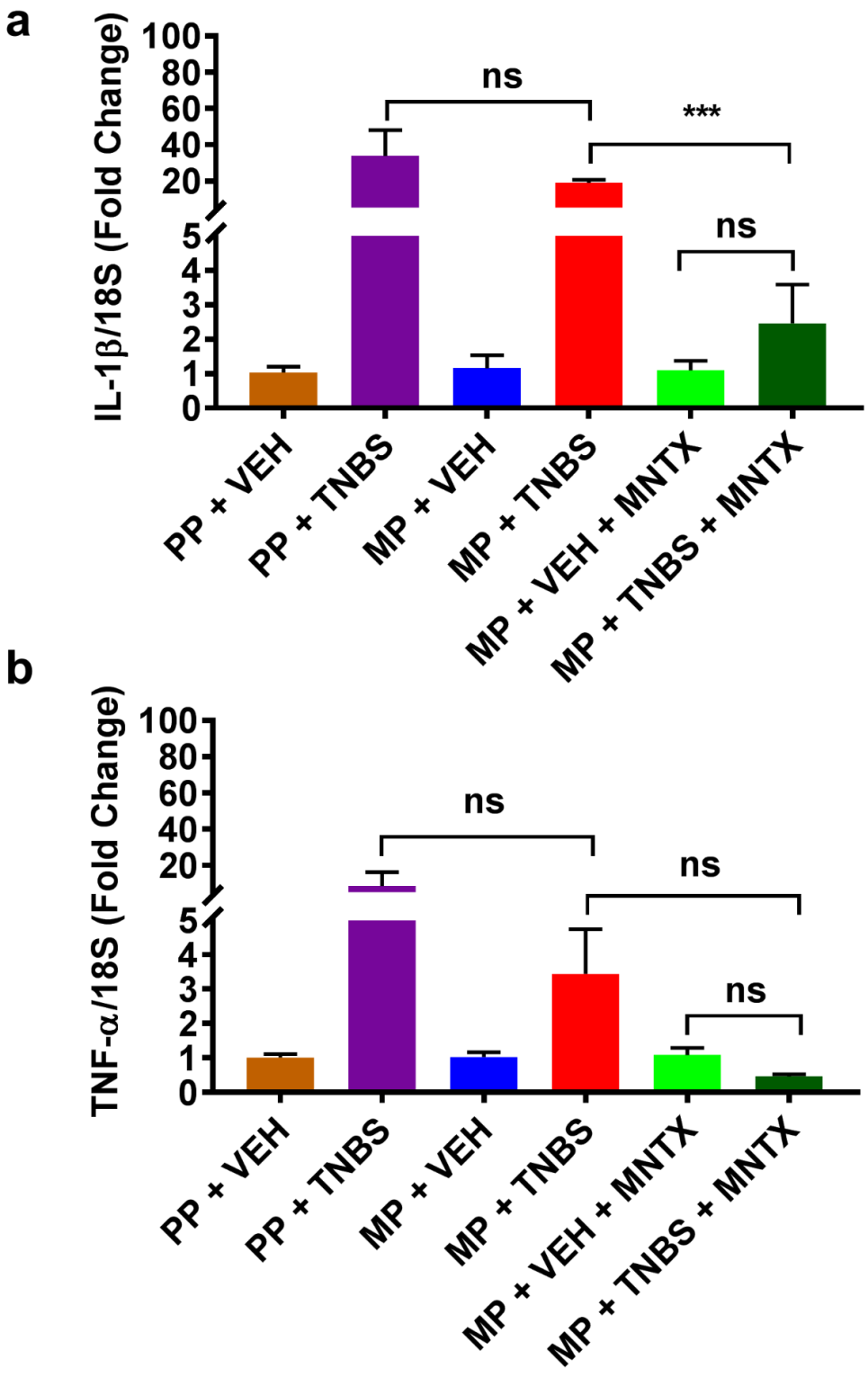

Figure 5.4: MNTX decreased TNBS-induced IL-1β and TNF-α mRNA expressions. (**a** – **b**) Three days of daily injection of 25 mg/kg MNTX decreased IL-1β and TNF-α mRNA expressions in the colons of TNBS treated animals. N= 3 – 4/group, ***P = 0.0002, n.s. not significant, by Student's unpaired t-test.

Peripheral opioid receptor antagonist, NAP prevented the development of antinociceptive tolerance

Due to the rapid demethylating nature of MNTX in mice, it was not best suited for our studies. We therefore moved to a different peripheral opioid receptor antagonist, 6β-N-heterocyclic substituted naltrexamine derivative (NAP), a highly selective μ-opioid receptor (MOR) antagonist that is impermeable to the blood-brain barrier. Published pharmacological and pharmacokinetic studies have suggested that NAP undergoes less metabolism (Mitra *et al.*, 2011; Zhang *et al.*, 2011) and there was no significant CNS effects observed up to 50 mg/kg (Yuan *et al.*, 2012); therefore, NAP may be a promising candidate for our studies.

NAP was administered daily at a dose of 0.5 mg/kg s.c. To confirm the peripheral selectivity of NAP, we initially evaluated the effect of NAP on morphine induced antinociception. NAP was administered at doses ranging from 0.1 mg/kg – 5.0 mg/kg 30 min prior to testing the analgesic effects of morphine in the warm water tail-immersion assay. Figure 5.5 shows that NAP did not affect morphine mediated antinociception at lower doses but only significantly reduced it at 5 mg/kg. This is consistent with our previous report of NAP as peripherally restricted μ-opioid receptor antagonist. At lower doses it substantially reverses morphine-induced inhibition of GI transit but not centrally-mediated antinociception (Li *et al.*, 2009; Zhang *et al.*, 2011; Yuan *et al.*, 2012).

Figure 5.5: The dose-response of NAP

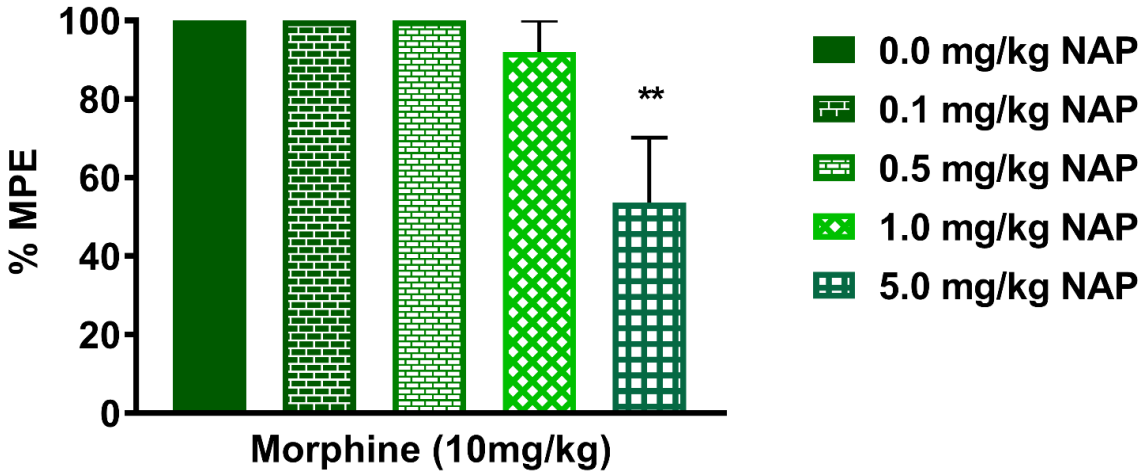

Figure 5.5: The dose-response of NAP. 0.1 mg/kg to 1 mg/kg NAP did not attenuate morphine's antinociceptive effects in the tail-immersion assay after an acute morphine challenge dose (10 mg/kg). At a dose of 5 mg/kg NAP, morphine's effect was diminished by 50%. N = 5/group, $F_{4, 20}$ = 5.984, P (1 – β) = 0.96, **p = 0.0027 by one-way ANOVA with Dunnett post-hoc analysis; compared to control (0.0 mg/kg).

As shown in figure 5.6, NAP prevented the enhanced tolerance developed in TNBS treated groups in each of the morphine pelleted cohort. Tolerance was determined by a challenge dose of morphine (10 mg/kg) in the tail-immersion assay at day 5 in the 25 mg MP group, at day 4 in the 50 mg MP group, and at day 3 in the 75 mg MP group. The substantial tolerance developed in the TNBS + morphine pelleted mice was prevented by NAP treatment in all three morphine pelleted groups. Since full tolerance to morphine alone did not develop in the time frame in the non-inflamed group, we further tested if NAP would also prevent tolerance to morphine alone in the absence of inflammation at a time when significant tolerance develops. Tolerance develops over 5 – 7 days with 75 mg morphine pellets (Figure 5.7a). As shown in Figure 5.7b, NAP reduced the extent of tolerance to morphine when tolerance was tested on day 7. This finding suggests a peripheral component to morphine antinociceptive tolerance.

Figure 5.6: Peripheral opioid receptor antagonist, NAP prevented the enhanced development of morphine antinociceptive tolerance in TNBS-treated mice

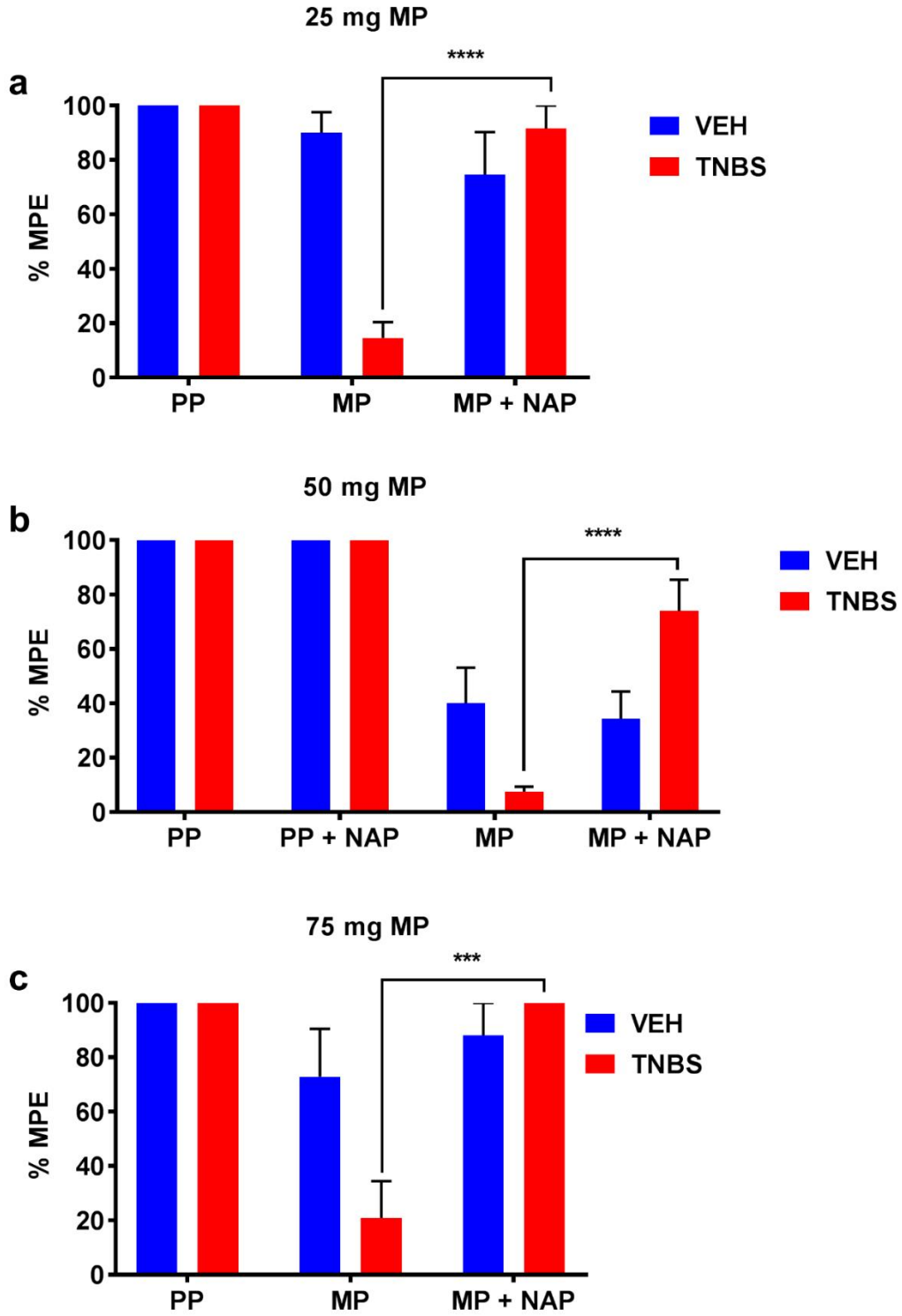

Figure 5.6: Peripheral opioid receptor antagonist, NAP prevented the enhanced development of morphine antinociceptive tolerance in TNBS-treated mice. (**a - c**) Daily subcutaneous injection of 0.5 mg/kg NAP significantly attenuated the development of antinociceptive tolerance to morphine challenge (10 mg/kg) in 25 mg, 50 mg, and 75 mg MP + TNBS + NAP mice compared to 25 mg, 50 mg, and 75 mg MP + TNBS mice on day 5, 4, and 3, respectively. (**a**) MP + TNBS (N = 10), MP + TNBS + NAP (N = 5), MP + VEH (N = 12), MP + VEH + NAP (N = 5), PP + TNBS (N = 5), PP + VEH (N = 5), P (1 – β) = 0.99, ****p < 0.0001 by two-way ANOVA with Tukey post-hoc analysis. (**b**) MP + TNBS (N = 10), MP + TNBS + NAP (N = 10), MP + VEH (N = 10), MP + VEH + NAP (N = 10), PP + TNBS (N = 6), PP + TNBS + NAP (N = 5), PP + VEH (N = 6), PP + VEH + NAP (N = 7), P (1 – β) = 0.99, ****p < 0.0001 by two-way ANOVA with Tukey post-hoc analysis. (**c**) MP + TNBS (N = 7), MP + TNBS + NAP (N = 5), MP + VEH (N = 7), MP + VEH + NAP (N = 5), PP + TNBS (N = 8), PP + VEH (N = 5), P (1 – β) = 0.99, ***p = 0.0004 by two-way ANOVA with Tukey post-hoc analysis.

Figure 5.7: Peripheral opioid receptor antagonist, NAP prevented the development of morphine-induced antinociceptive tolerance

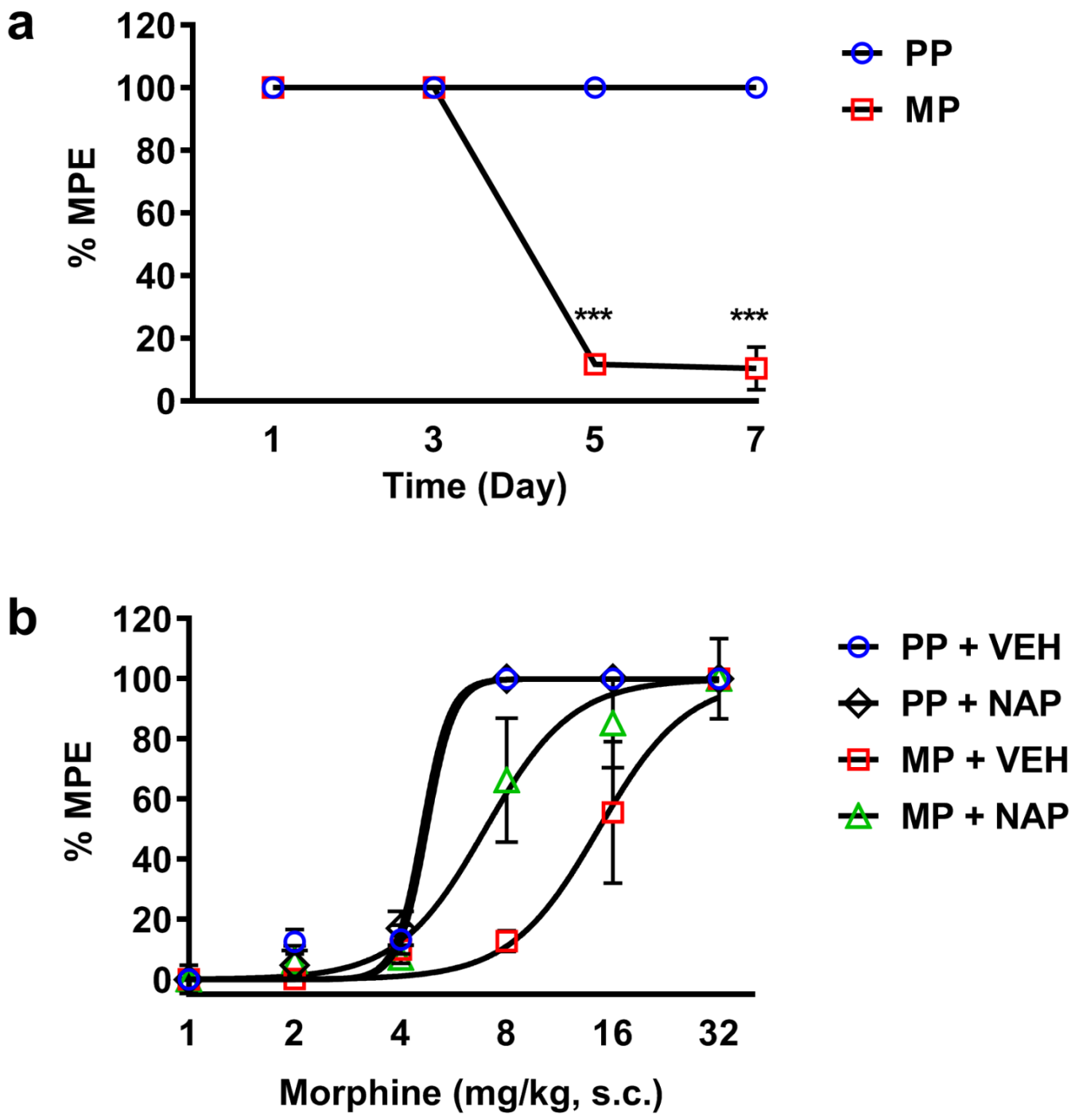

Figure 5.7: Peripheral opioid receptor antagonist, NAP prevented the development of morphine-induced antinociceptive tolerance. (**a**) Chronic morphine pelleting (75mg) resulted in antinociceptive tolerance to morphine challenge (10 mg/kg) in the tail-immersion assay after 5 days of exposure. N = 5/day/group, P (1 – β) = 0.99, ***p < 0.001 by two-way ANOVA with Bonferroni post-hoc analysis. (**b**) Daily NAP injection prevented the development of antinociceptive tolerance in morphine pelleted mice on day 7 indicated by the left-ward shift in the cumulative morphine dose-response curve (1, 2, 4, 8, 16, & 32 mg/kg). N = 5/group, PP + VEH ED_{50} = 4.74 mg/kg; PP + NAP ED_{50} = 4.61 mg/kg; MP + VEH ED_{50} = 14.57 mg/kg (11.13 – 18.4, 95% C.L.); MP + NAP ED_{50} = 6.95 mg/kg (5.54 – 8.92, 95% C.L.); P (1 – β) = 0.887, with non-linear regression analysis (best-fit lines).

In order to test if NAP reduced colonic inflammation, IL-1β mRNA expression was measured from the colons of 50 mg MP + TNBS mice that were treated with NAP (0.5 mg/kg) after 4 days and IL-1β protein levels were measured from the colons of 75 mg MP + TNBS mice that were treated with NAP (0.5 mg/kg) after 3 days. The mRNA expression and protein levels of IL-1β was not reduced by daily NAP injection in the inflamed animals (fig 5.8). Histological cross-sections of colons and gut permeability were examined from 50 mg MP groups on day 4. H&E staining of colon sections demonstrated a damaged mucosal and crypt architecture in both MP + TNBS + NAP and MP + TNBS groups (fig 5.9). Similarly, gut permeability which was determined by FITC-dextran concentration in blood plasma was increased in TNBS treated mice and in mice treated with morphine alone. However, NAP treatment did not reduce gut permeability (fig 5.10).

Figure 5.8: NAP did not decrease TNBS-induced IL-1β mRNA expression and protein levels

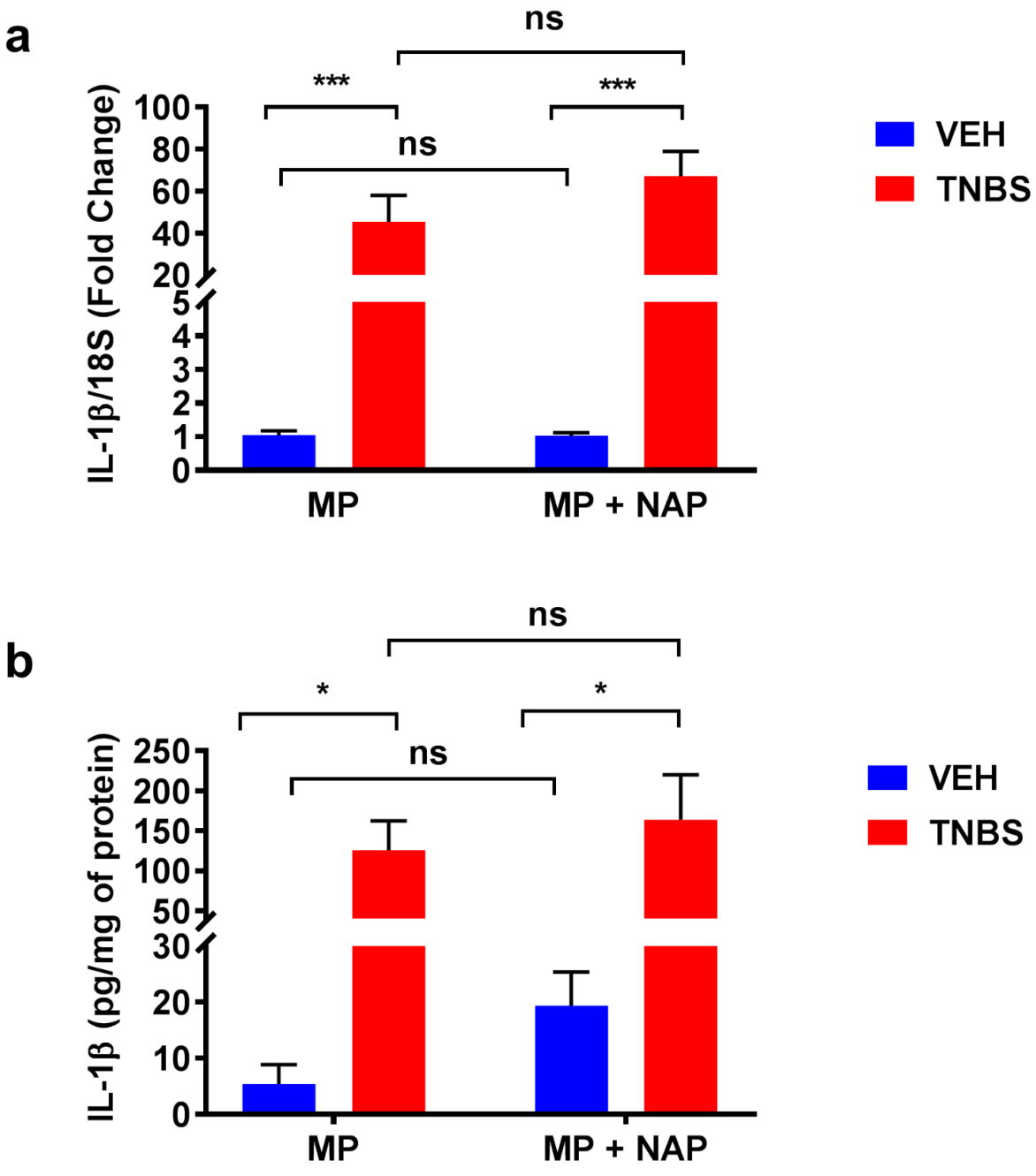

Figure 5.8: NAP did not decrease TNBS-induced IL-1β mRNA expression and protein levels. (**a**) IL-1β mRNA expression was not statistically different between 50 mg MP + TNBS + NAP and 50 mg MP + TNBS groups on day 4. N = 5/group, ns not significant, ***p < 0.001 by two-way ANOVA with Tukey post-hoc analysis. (**b**) Three days of daily NAP treatment did not reduce IL-1β protein levels in the colons of 75 mg MP + TNBS mice. N = 5/group, ns not significant, *p < 0.05 by two-way ANOVA with Tukey post-hoc analysis.

Figure 5.9: Morphological changes in the colons of mice treated with 50 mg MP

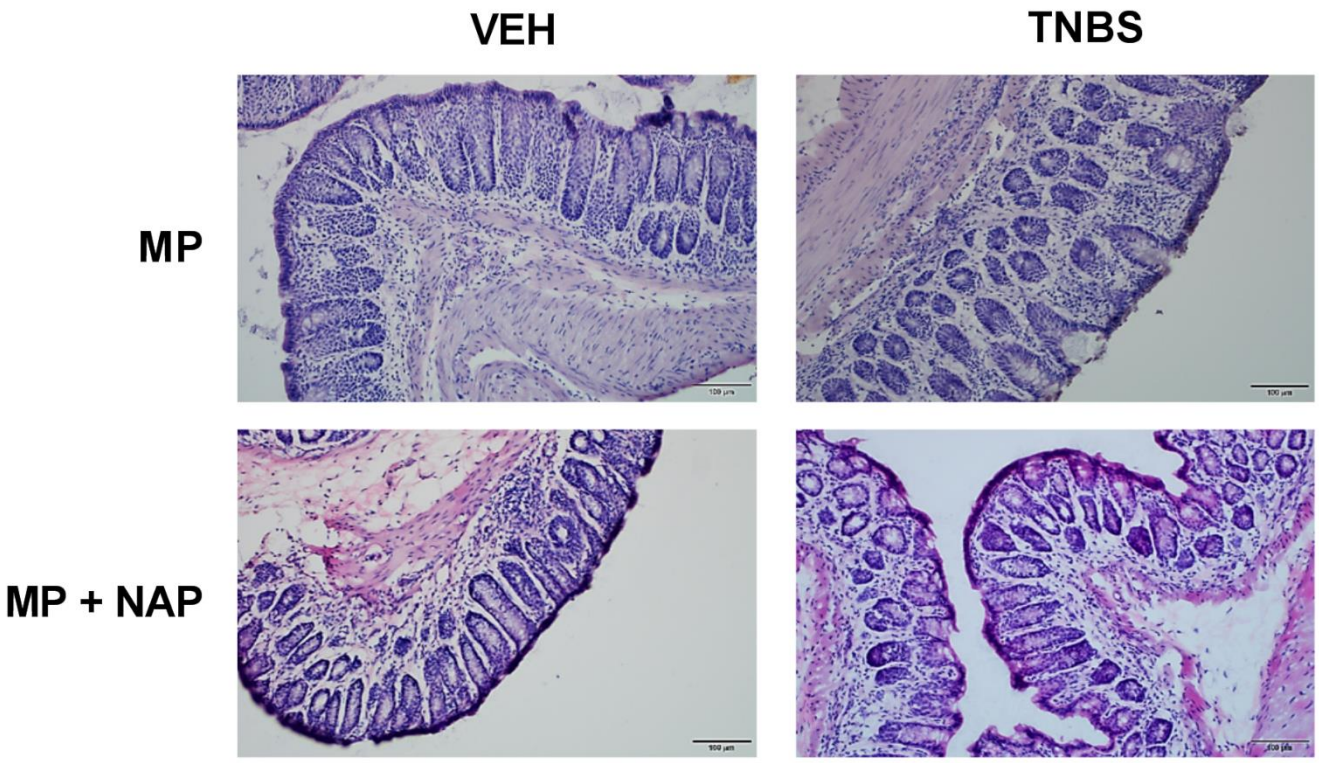

Figure 5.9: Morphological changes in the colons of mice treated with 50 mg MP. H&E staining of the distal colon from 50 mg MP groups showed an equally damaged mucosal layer and neutrophil infiltrations in the presence or absence of NAP and TNBS on day 4. Scale bar:100 μm.

Figure 5.10: Permeability of the colons of mice treated with 50 mg MP

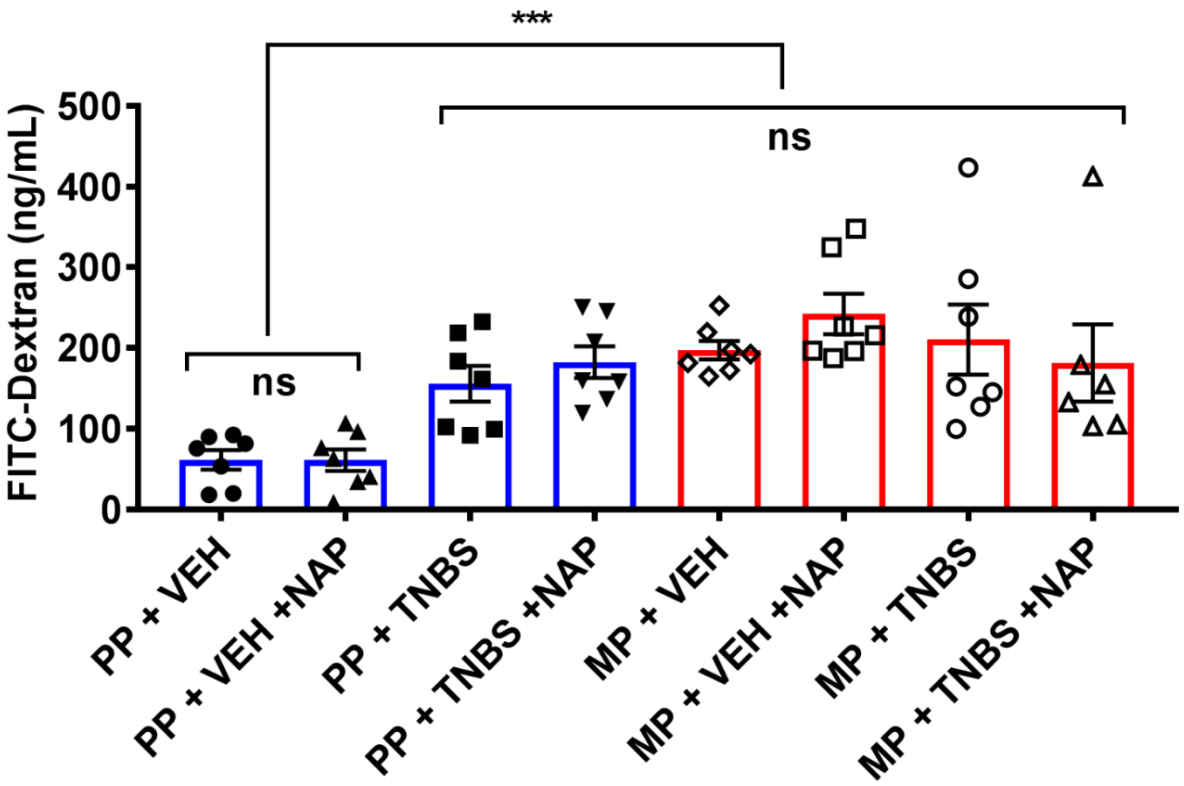

Figure 5.10: Permeability of the colons of mice treated with 50 mg MP. Fluorometric quantification of FITC from blood serum showed a significant fluorescence concentration in all groups except for PP+VEH and PP+VEH+NAP groups. Daily NAP injection did not block membrane permeability in 50 mg MP + TNBS + NAP mice. N = 6 - 7, ns not significant, ***p < 0.001by two-way ANOVA with Tukey post-hoc analysis.

Figure 5.11: Proposed schematic of the prevention of antinociceptive tolerance by NAP

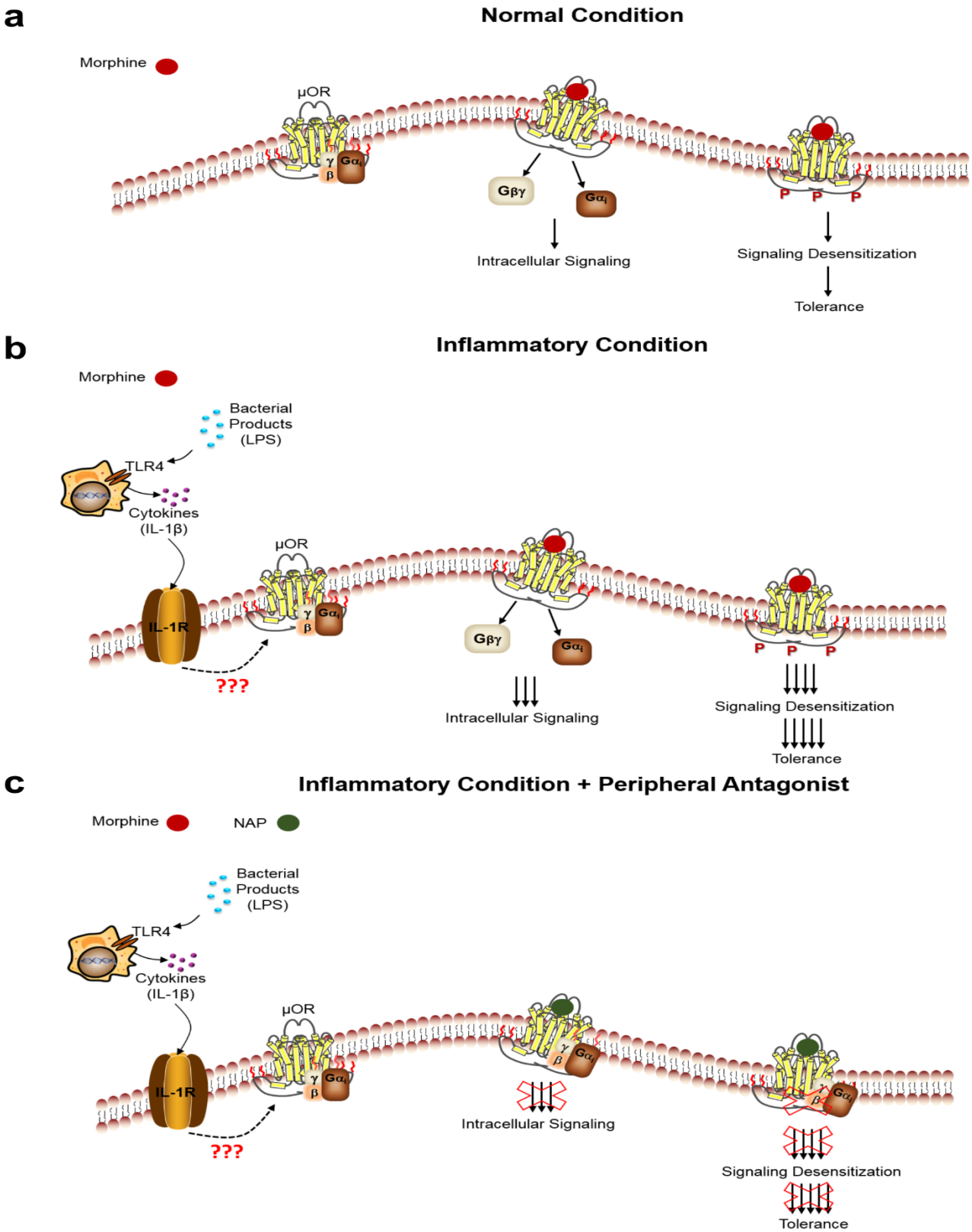

Figure 5.11: Proposed schematic of the prevention of antinociceptive tolerance by NAP. (**a**) Under a normal condition, when morphine is used, it binds to MOR, activates the receptor, and induces downstream intracellular signaling. Continual activation of the receptor by morphine results in signaling desensitization leading to morphine-induced tolerance. (**b**) During an inflammatory condition, cytokines released from immune cells may interact with the MOR directly or indirectly resulting in a potent morphine response upon binding, leading to an enhanced rate of tolerance development. (**c**) In the presence of a peripheral antagonist NAP, the enhanced morphine tolerance developed is attenuated.

Summary

- Two peripheral opioid receptor antagonists were used in this chapter: methylnaltrexone (MNTX) and 6β-N-heterocyclic substituted naltrexamine derivative (NAP).
- MNTX blocked morphine-induced antinociception in the tail-flick and hot plate assays.
- Daily MNTX injection failed to prevent the enhanced morphine antinociceptive tolerance developed in the presence of TNBS in the tail-flick and hot plate assays.
- MNTX may have an anti-inflammatory property as it lowered the IL-1β and TNF-α mRNA expressions after three days of treatment.
- NAP did not affect morphine-induced antinociception in the tail-flick assay.
- Daily NAP injection significantly prevented the enhanced morphine antinociceptive tolerance developed in the presence of TNBS at all three MP doses, 25 mg, 50 mg, and 75 mg.
- Daily NAP injection also prevented the development of antinociceptive tolerance in the 75 mg MP mice at day 7.
- NAP did not change the IL-1β mRNA expression and protein levels in the TNBS treated mice.
- NAP did not protect the integrity of the gut indicated by the lack of changes in the H&E staining and gut permeability assay in the presence or absence of TNBS.

Discussion

There is a growing appreciation for the peripheral effects of opioids and their importance. In the present study, our findings indicate that peripheral opioid receptors mediate morphine antinociceptive tolerance. Blockade of the peripheral receptors with its antagonist, NAP, but not methylnaltrexone, attenuated the development of morphine antinociceptive tolerance in the presence and absence of TNBS-induced colonic inflammation without interfering with morphine antinociception.

We have previously reported that NAP, a naltrexamine derivative is peripherally selective μ-opioid receptor antagonist (Li *et al.*, 2009; Yuan *et al.*, 2012). Unlike methylnaltrexone, which can be demethylated and have central effects in rodents (Kotake *et al.*, 1989; Chandrasekaran *et al.*, 2010) (fig 5.1), NAP did not block acute effects of morphine on antinociception (fig 5.5), but did reverse morphine-induced inhibition of gastrointestinal motility (Yuan *et al.*, 2012). At a dose that did not affect central mediated antinociception, NAP prevented the antinociceptive tolerance irrespective of the presence of inflammation suggesting that inhibition of the μ-opioid receptors in the peripheral sites was sufficient to prevent tolerance development. NAP has high binding affinity for MOR with more than 700 fold and 150 fold selectivity over delta and kappa opioid receptors, respectively, (Li *et al.*, 2009; Yuan *et al.*, 2012) . A peripheral component for the tolerance to opioids has also been reported recently by Corder et al, wherein methylnaltrexone bromide, a clinically available peripheral MOR antagonist prevented the development of tolerance and opioid-induced hyperalgesia without affecting morphine's antinociceptive effects (Corder *et al.*, 2017).

Given that the peripheral contribution is necessary for the development of tolerance, IBD patients may need to take a peripheral opioid receptor antagonist to prevent the development of analgesic tolerance. Methylnaltrexone and alvimopan are peripheral opioid receptor antagonists

currently used in the clinic for treatment of opioid-induced constipation and post-operative ileus, respectively. Up to now, there are no clinical studies that have directly assessed the use of peripheral opioid receptor antagonists in IBD patients to prevent analgesic tolerance, however there are clinical studies that have investigated the use of low-dose naltrexone (LDN) in IBD patients to decrease colonic inflammation and prolong remission in these patients (Younger *et al.*, 2014; Raknes and Småbrekke, 2017; Lie *et al.*, 2018; Raknes *et al.*, 2018). Intriguingly, studies have found that the biological effects of lipopolysaccharide (LPS), a component of gram-negative bacteria that contributes to the inflammatory pathway in IBD by activating Toll like Receptor 4 (TLR4) signaling could be blocked by both naltrexone and naloxone (Sziebert *et al.*, 1983) as the inactive isomers (+)-naltrexone and (+)-naloxone have shown to interact with TLR4, thus reducing inflammation (Watkins *et al.*, 2014; Wang *et al.*, 2016). Given that methylnaltrexone is rapidly converted to naltrexone in mice, it is possible that the naltrexone is interacting with TLR4, hence the decrease in both IL-1β and TNF-α mRNA expressions in the colons of MP + TNBS mice (fig 5.4).

In conclusion, the rate of morphine tolerance has a dose and time dependency that is greatly enhanced in the presence of inflammation. The enhanced rate of tolerance is not a direct effect of inflammation *per se*, but rather a receptor-mediated effect. Cytokines released during the inflammatory state may directly or indirectly modulate the sensitivity of the receptors, thus altering intracellular signaling and affecting antinociceptive tolerance. In the presence of NAP, this effect was blocked (figure 5.11). Our findings suggest a potential therapeutic avenue for peripheral opioid receptor antagonists to attenuate the development of opioid tolerance, without affecting analgesia.

CHAPTER 6

General discussion

The use of opioids for the treatment of pain has a very long history dating back to 3500 BC. The search for opioid analgesics that produces relief from moderate to severe pain has grown exponentially and today the treatment of pain remains a significant public health concern as two-thirds of patients have difficulty achieving pain relief from the currently available pharmacotherapies (Sindrup and Jensen, 1999) due to development of analgesic tolerance, among all other opioid side effects. The development of analgesic tolerance is especially problematic because as the dose of opioid increases to achieve analgesic efficacy, the more undesirable side effects it creates such as respiratory depression, dependence, addiction, hypernociception, constipation, and even death leading to a vicious cycle. Mechanisms underlying opioid tolerance are likely complex. Though it was originally thought to be solely based on neuronal adaptations (Williams *et al.*, 2001; Dumas and Pollack, 2008) including: desensitization, decoupling, internalization and/or downregulation of opioid receptors; upregulation of NMDA receptor function; downregulation of glutamate transporters; and production of nitric oxide; for the last couple of decades, immune activation/inflammation has been implicated to modulate morphine analgesia and tolerance. The objective of this dissertation was to determine the role of gut inflammation on the development of morphine antinociceptive tolerance. Our findings suggest that 1) morphine antinociceptive tolerance is paralleled with inflammatory cytokine IL-1β gene expression in the colon 2) the rate of morphine antinociceptive tolerance is enhanced in the presence of non-opioid-induced colonic inflammation 3) morphine antinociceptive tolerance can

be prevented with a peripheral opioid receptor antagonist. The studies described here shed light on the behavioral and cellular aspect of chronic morphine exposure in the presence of inflammation as well as the involvement of peripheral opioid receptors during morphine tolerance.

The first series of experiments demonstrated that inflammatory cytokine production correlated with the development of morphine antinociceptive tolerance. A great body of evidence supports that the morphine administration causes Il-1 release (Johnston *et al.*, 2004). Il-1 release is implicated with decreased morphine analgesia and tolerance (Johnston *et al.*, 2004; Hutchinson *et al.*, 2007, 2008). Notably, our studies have used Il-1β as a marker of inflammation and its expression and levels were elevated in our assays. As to how Il-1β production is triggered during chronic morphine treatment is likely due to glial activation (Watkins *et al.*, 2005, 2009; Hutchinson *et al.*, 2007) and gut dysbiosis (Meng *et al.*, 2013; Banerjee *et al.*, 2016; Kang *et al.*, 2017; Wang *et al.*, 2018). One study proposed that morphine activates nitric oxide production in p38 MAPK-dependent manner from glial cells (Cui *et al.*, 2006; Liu *et al.*, 2006) resulting in downstream up-regulation of proinflammatory cytokines (Raghavendra *et al.*, 2002; Johnston *et al.*, 2004). Thus, the increased glial production of nitric oxide (Ledeboer *et al.*, 2007) and proinflammatory cytokines (Milligan *et al.*, 2005) results in sensitization of the nociceptors and dampening of morphine analgesia. Another study from our laboratory demonstrated an enhanced purinergic activity in enteric glia after 5 days of morphine pelleting in primary glia cultures from the colon longitudinal muscle/myenteric plexus (LMMP) (Bhave *et al.*, 2017). Bhave *et. al* showed that the purinergic activity observed was due to ATP release from the enteric glia triggered by LPS. ATP is an essential activator of inflammatory responses (Cauwels *et al.*, 2014). During inflammatory conditions, ATP levels are increased as a result of active or passive release from damaged cells (Lazarowski *et al.*, 2003). Activation of purinergic receptors by ATP on immune cells during

morphine treatment would result in induction of inflammatory mediators and drive morphine tolerance. Interestingly, treatment with the connexin43 inhibitor, carbenoxolone (CBX) attenuated morphine tolerance in tail-immersion and hot plate assays (Gonek, M. unpublished) and abrogated the glia-mediated inflammation (Bhave *et al.*, 2017).

The gut microbiome has been a topic of interest as of late due to its involvement in multiple disease processes such as IBD, anxiety and depression, and obesity. There is accruing evidence that morphine treatment is associated with changes in the composition of gut microbiota (Meng *et al.*, 2013; Acharya *et al.*, 2017; Kang *et al.*, 2017; Lee *et al.*, 2018). Under normal conditions, the indigenous microorganisms of the gut exist in a symbiotic relationship with the host, providing metabolic benefits, immune homeostasis, immune responses and protection against pathogen colonization (Pickard *et al.*, 2017). Chronic morphine administration has shown to cause gut bacterial imbalance in man and mice (Acharya *et al.*, 2017; Kang *et al.*, 2017; Wang *et al.*, 2018). Perturbation of the gut microbial community is associated with loss of epithelial tight junction function and intestinal permeability, resulting in bacterial translocation across the gut wall and promoting secondary inflammation in the gut (Meng *et al.*, 2013; Kang *et al.*, 2017). Manipulation of the gut microbiome with antibiotics was shown to abrogate morphine-induced inflammation, restored intestinal barrier integrity, and prevented the development of morphine tolerance (Kang *et al.*, 2017). The findings from the gut microbiome and immune activation literature suggest a key role for microbial dysbiosis and glial activation during chronic morphine treatment. All these findings suggest that blockade of the inflammatory signaling is essential to morphine tolerance prevention. However, our findings indicates that it may not be entirely the case. We have found that morphine tolerance could be prevented by simply blocking the μ-receptors with a peripherally-restricted antagonist irrespective of the inflammatory signaling.

Our findings suggest that morphine antinociceptive tolerance is enhanced in the presence of inflammation in a dose and time dependent manner. The mechanism by which inflammation enhances tolerance remains elusive. The behavioral tolerance observed in our studies indicates MOR desensitization due to the lack of response to the morphine challenge which is likely driven by receptor phosphorylation and/or β-arr2 binding. This process has shown to be enhanced during inflammation at the whole animal level. Cellularly, one would assume the release of pro-inflammatory mediators from the primary afferent nerve endings would lead to intracellular changes in the signal transduction pathways in DRG neurons. This notion is supported by recent findings in our laboratory where naïve DRG neurons were incubated in colonic supernatant media from a morphine pelleted mouse colon showed tolerance upon acute morphine challenge (3 uM) in whole-cell current-clamp experiments (Mischel *et al.*, 2018). The idea was that the colon from a morphine pelleted mouse leached inflammatory mediators into the media, and by incubating the naïve DRGs in that media resulted in changes in MOR kinetics producing cellular tolerance. The enhanced behavioral tolerance observed at the whole animal level paralleled with the cellular tolerance in the presence of the inflammatory 'soup' confirming the involvement of inflammatory cytokines on the development of morphine antinociceptive tolerance.

The findings in chapter 5 suggest that blockade of MORs is sufficient to prevent morphine antinociceptive tolerance. Due to the demethylating nature of methylnaltrexone in mice, NAP which lacks those effects was used as a proof of concept to demonstrate that blocking the peripheral opioid receptors would attenuate morphine tolerance. The primary route for methylnaltrexone metabolism in humans is via sulfation at the phenolic group and carbonyl reduction (Chandrasekaran *et al.*, 2010), hence its lack of CNS effects in humans. Due to the fact that NAP is not clinically available and has no effect on immune cells, it is important to investigate other

peripheral opioid receptor antagonists that possess anti-inflammatory properties. There are currently no human studies on using peripheral opioid antagonists to prevent the development of analgesic tolerance but based on our findings and others (Corder *et al.*, 2017), they may be potential therapeutic candidates for patients while taking opioids. The problem that patients often experience taking MNTX for opioid-induced constipation is severe diarrhea and bloody stool, thus MNTX may not be the ideal candidate after all. Therefore, it is important to investigate other clinically available peripheral opioid antagonists such as alvimopan and naloxegol to test for the prevention of antinociceptive tolerance in mice. However, chronic use of alvimopan is not recommended as it could lead to myocardial infarction in humans (Becker and Blum, 2009).

Although, our findings indicate that blocking peripheral opioid receptors is sufficient to prevent tolerance without perturbing central analgesia or reducing proinflammatory mediators, it is imperative that systemic inflammation be blocked in order to maintain a healthy homoeostatic balance. Targeting opioid-induced immune activation is pivotal to the maintenance of analgesia. Studies have found these tools effective in attenuating inflammation and preventing morphine tolerance in animal models: glial modulators, neutralizing antibodies/antagonists to IL-1, IL-6, and TNF-α, inactive naltrexone/naloxone isomers, TLR2/4 knockout/knockdown, NSAIDs/selective cyclooxygenase (COX)-2 inhibitors (Wong *et al.*, 2000; Watkins *et al.*, 2005; Hutchinson *et al.*, 2010; Wang *et al.*, 2016). Furthermore, our studies mainly focused on morphine, the prototypical opioid. However, it is important to broaden our studies to include other clinically relevant opioids such as oxycodone, hydrocodone, hydromorphone, fentanyl, meperidine, and methadone. To date, studies have documented methadone, oxycodone, and 4,5-epoxymorphinan to be glial activators (Hutchinson *et al.*, 2007); whereas, etorphine was found to lack glial activation (Narita *et al.*, 2006).

Future studies

We found that the rate of morphine tolerance was enhanced in the presence of inflammation. The next step would be to determine the mechanisms that are driving tolerance and look into what happens to the MOR during inflammation. It is well established that β-arr2 association with the MOR desensitizes the receptor by preventing further downstream signaling. Future studies warrant to investigate if the enhanced tolerance development in the presence of inflammation is β-arr2-mediated. Using immunohistochemistry and qPCR techniques, we can determine the expression of β-arr2 in DRG and enteric neurons. Furthermore, it is worth replicating some of the TNBS + MP experiments using β-arr2 knockout and wild type mice. It is also important to look at receptor expressions after chronic morphine and TNBS treatment. We assume that the receptors are desensitized after constant activation by morphine but we do not know if they are internalized and then slowly re-sensitized back to the cell membrane. This can be accomplished using qPCR to determine the expression of MOR in DRG neurons and enteric neurons. Isolated DRG neurons and enteric neurons from the myenteric plexus could also be stained with antibodies specific to MOR. In line with this, it is also important to examine the turnover rate of the MOR from enteric, PAG, and DRG neurons by immunoprecipitation using western blots. Moreover, the activation and inactivation kinetics of the MOR from the enteric and DRG neurons during TNBS inflammation in response to morphine could be determined using the classical receptor binding assays or a more recent innovation such as the Fluorescence Resonance Energy Transfer (FRET)-based assays which were developed to accurately display drug-receptor interaction in real time.

Additionally, it is worth investigating the cellular tolerance in enteric, PAG, and DRG neurons from MP + TNBS groups to determine if the behavioral tolerance observed at the whole

animal level would correspond to the cellular tolerance and if that tolerance could also be prevented with both MNTX and NAP using patch clamping techniques to measure the excitability of the cells. Furthermore, other studies that suggested that morphine elicited pro-inflammatory cytokines in the spinal cord was after chronic intrathecal injection of morphine (Song and Zhao, 2001; Watkins *et al.*, 2005; Hutchinson *et al.*, 2008). It would be interesting to see that morphine pelleting with or without TNBS treatment prompted inflammatory responses in the spinal cord and the DRG neurons.

Limitations

According to the findings from this dissertation, inflammation drives antinociceptive tolerance development. One limitation of our study was that we did not measure systemic inflammation or LPS from the blood or other organs. Given that TNBS treatment damages the gut epithelial barrier promoting immune reactions, one would assume bacterial translocation from the gut lumen to the extraperitoneal sites such as the circulatory system. In fact, some studies have demonstrated that morphine administration to intensive care unit (ICU) patients results in sepsis due to the breakdown of the gut epithelial barrier (Banerjee *et al.*, 2013). Our laboratory has also found bacterial colonization in the liver, the spleen, the blood, and the mesenteric lymph nodes after chronic morphine treatment (Kang *et al.*, 2017). The presence of bacterial products such as LPS in the circulatory system would result in systemic inflammation which would further enhance tolerance.

Another limitation of our study was that we were not able to test the TNBS mice treated with different doses of morphine at the same time point. As mentioned in chapter 4, TNBS mice treated with 25 mg, 50 mg, and 75 mg MP were tested on day 5, 4, and 3, respectively due to a rapid weight loss compared to the placebo-vehicle treated mice. Extending the studies to day 7 for all the groups could have shown us if mice pelleted with 50 mg MP alone ever became fully tolerant. By day 4 these mice were 35% tolerant. On the other hand, mice pelleted with 25 mg MP were not tolerant up to day 5. Extending the study to day 7 could have shown us if they ever showed any sign of tolerance by day 7. In line with this, we were not able to measure the disease activity index (DAI) from the mice in order to evaluate the extent of the colonic inflammation. DAI encompasses weight loss, quality of stool, and presence of blood in stool. The morphine

pellets rendered the TNBS mice constipated; therefore, we couldn't investigate if there was blood in the stool. The extent of inflammation was based only on weight loss and cytokine measures.

Moreover, another limitation of our study was that we did not measure other inflammatory cytokines in addition to IL-1β. Our study mainly focused on IL-1β because it was the only cytokine that was significantly up-regulated in the colon after chronic morphine treatment. The use of the BioPlex system in the future would enable us to measure multiple cytokines from our samples.

Concluding remarks

The work presented in this dissertation supports the notion that pro-inflammatory mediators influence the development of antinociceptive tolerance to chronic morphine exposure. We found that as the dose of morphine increased in the presence of inflammation, the more tolerant the mice became to the antinociceptive effects of morphine. We also found that treatment with a peripheral opioid receptor antagonist prevented morphine antinociceptive tolerance. Our findings add to the body of literature that by increasing opioid intake during an inflammatory state resulted in decreased analgesia and enhanced analgesic tolerance. Patients with inflammatory bowel diseases, inflammatory joint diseases, and sickle cell anemia are especially at risk as chronic opioid use would worsen their disease state and prompt a heavy opioid use. Taking opioids in conjunction with a clinically available peripheral opioid receptor antagonist could circumvent the analgesic tolerance without attenuating centrally-mediated analgesia. But further clinical studies ought to investigate how well our findings translate to the clinic. Clinical findings using a peripheral antagonist could very well change how pain is managed using opioid analgesics. This could lead to further development of new pharmaceutical formulations of central agonistic activity and peripheral antagonistic activity.

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Vita

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