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Potential Strategies for Improving Pain Management in the Opioid Patients: The Clinical Challenge

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Abstract: The rates of opioid prescription and use have continued to increase over the last few decades. In turn, a greater number of patients suffer from opioid tolerance. Treatment of acute pain is a clinical challenge for these patients. Acute pain can arise from common occurrences like surgical pain and pain resulting from the injury. P-glycoprotein (p-gp) is a transporter at the blood-brain barrier (BBB) associated with a decrease in the analgesic efficacy of morphine. Peripheral inflammatory pain (PIP) is a pain state known to cause a change in p-gp trafficking at the BBB. P-gp traffics from the nucleus to the luminal surface of endothelial cells making up the BBB. This surface where circulating blood interfaces with the endothelial cell is where p-gp will efflux morphine back into circulation. Osmotic minipumps were used as a long-term delivery method in this model of opioid tolerance in female rats. PIP induced p-gp trafficking away from nuclear stores showed a 2-fold increase when animals were exposed to opioids for 6 days. This observation presents a possible relationship between p-gp trafficking and the challenges of treating post-surgical pain in opioid tolerant patients. This could reveal potential strategies for improving pain management in these patients.

Keywords: peripheral inflammatory pain, p-glycoprotein, chronic opioid exposure

INTRODUCTION

Pain management is an important part of recovery for patients following surgery. Poor pain management can lead to slower recovery, an increased probability of readmission, increased cost of care and decreased patient satisfaction (1). Intravenous opioid analgesics, such as morphine, are currently the standard of care for post-surgical pain. Opioids are the most effective therapy for reducing reported pain in most patients. In a hospital setting, opioids are most commonly administered by nursing staff or through a patient-controlled analgesia (PCA) system (2). A problem arises when a patient with a previous history of chronic opioid use is treated for post-surgical pain. It has been reported that when these patients receive opioid analgesics following surgery, the treatment is less effective and some patients feel more pain (3). The tolerance associated with long-term use of opioids leads reduced efficacy. Opioid-induced hyperalgesia (OIH) is a related pathology in which patients become more sensitive to stimuli following long-term exposure to opioids (4). These two phenomena are examples of the clinical challenges associated with long-term opioid therapies. The ATP-binding cassette protein P-glycoprotein (p-gp) at the blood-brain barrier (BBB) is thought to play a role in decreased opioid efficacy. At the BBB, p-gp acts as an efflux protein transporting a variety of compounds back into circulating blood before these compounds can enter the brain. Rats chronically exposed to morphine, a substrate of p-gp, have a 4-fold decrease in morphine entering the CNS as well as a 2-fold increase in expression of the protein in sampled whole brain tissue (5). Pain has also been shown to be sufficient to decrease the antinociceptive efficacy of morphine (6). A combinatory effect of these two observations may explain a role of the BBB in the challenge of treating post-surgical pain in long-term opioid-treated patients.

The Problem of Pain

In his famous novel *1984*, George Orwell describes "Of pain you could wish only one thing: that it should stop. Nothing in the world was so bad as physical pain. In the face of pain there are no heroes."

Even with this negative association, pain serves as an invaluable tool for survival. Acute pain acts as a signal of noxious stimuli as well as reinforcing behaviors that avoid these stimuli. Pain also acts as a clue of internal injuries such as muscular damage or broken bones. Changes can occur in pain pathways resulting in an altered, chronic state. As a protective adaptation, this can alter behavior to protect the site of an injury allowing the injury to heal without further harm. In some cases, this chronic pain will persist at the site of an injury well past the time protective pain is beneficial to healing.

In the central nervous system, nociceptors project to differing laminae of the dorsal horn of the spinal cord depending on the type of nociceptive fiber. A variety of signaling molecules act at the

synapses between the central terminal of the nociceptors and the laminae of the spinal cord (8). Neurons within these laminae are responsible for transmitting the nociceptive signal through the spinal cord in a contralateral manner to the thalamus of the brain. From here, signals are sent to the somatosensory cortex and limbic system. While this process is short-lived for acute pain, persistent or chronic pain can arise when there is an anomaly in this system. The anomaly can be caused by over sensitization of nociceptors or because of spontaneous firing. Pharmacological modification of this pathway can be used as a strategy to reduce or eliminate pain.

Opioids

Opioids are a class of drugs with several useful effects including cough suppression and gastric slowing but are most commonly known and prescribed for analgesic effects. These drugs, as well as a few endogenous opioids, work at the class of receptors known as opioid receptors. There are three subtypes of opioid receptors: kappa, delta and, mu. Mu opioid receptors are believed to be the most important to the analgesic effects of opioids. All opioid receptors are inhibitory G protein-coupled receptors. The endogenous agonists for these receptors are dynorphins, enkephalins and endorphins, respectively (9). Opioid analgesics can be administered through suppository or intrathecally, but are most commonly administered intravenously or orally. More lipophilic opioids can also be administered transdermally. As described by Yaksh and Wallace in *Goodman and Gilman's: The Pharmacological Basis of Therapeutics,* oral opioids are subject to the first pass effect as well as poor absorption due to ion trapping and have a bioavailability of about 25% (10). Yaksh and Wallace continue to describe intravenous administration of opioids results in prompt action. The speed of action is affected by the lipophilicity of the compound which contributes to differences in the speed at which the compound can cross the BBB and enter the central nervous system (CNS). Morphine does not persist in tissue and is found in trace quantities 24 hours after the last administered dose. Metabolism of morphine relies on conjugation with glucuronic acid producing two metabolites, morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G). M6G has an analgesic effect. It is twice as potent as morphine, and is thought to make up a significant portion of morphine's analgesic effect in patients treated with long-term opioid therapy (11). The more prevalent metabolite, M3G, is known to have neuroexcitatory effects (12). M3G is also the primary form excreted from the body (10). While almost no unmodified morphine is excreted, morphine's metabolites are excreted through the kidneys.

As previously mentioned, in addition to the pain relieving effects of opiates, opioid analgesics can elicit strong feelings of euphoria. Because of this and severe withdrawal symptoms, addiction and abuse are problems for many individuals including both those who began as therapeutic users and exclusively recreational users (24). Opioid addiction, also known as opioid use disorder, is a psychological condition defined as "compulsive, prolonged self-administration of opioid substances that are used for no legitimate medical purpose or, if another medical condition is present that requires opioid treatment, that are used in doses greatly in excess of the amount needed for that medical condition," (25). Both those using opioids recreationally for euphoric effects and those who begin using them for medical conditions are at risk of addiction. Addiction will take over the individual's life, and most of the affected individual's resources will be spent attempting to obtain more of the drug. Addiction and abuse have been a problem associated with opiates ever since man first discovered them.

An Epidemic

Today, the abuse of opioids has been described as an epidemic in the United States. The use of opioids affects all demographics of Americans and continues to become more common. On average, 3,900 individuals begin the non-medical use of prescription opioids, and 580 individuals begin heroin use every day (39). A study revealed emergency room visits caused by non-medical opioid use have doubled from 2004 to 2011, totalling a staggering 488,000 visits in 2011 alone (40). A study by Rudd *et al.* examining drug overdose deaths related to opioids, including both opioid pain relievers and heroin, demonstrated an increase in deaths of 200% between 2000 and 2014 (41). The study went on to demonstrate the increase in opioid-related deaths was much higher than the increase in overdose related deaths including all causes which were 137%. This trend is still continuing currently, with an increase of 14% of opioid-related deaths from 2013 to 2014 compared to a 6.5% increase in overall overdose-related deaths. The increase in deaths was significant for both sexes, people 25-44 and those 55 and older and in the Northeastern, Southern and Midwestern regions of the United States. Deaths related to natural and semi-synthetic opioids, heroin and synthetic opioids, excluding methadone, have all had significant increases. Synthetic opioids, excluding methadone, had the greatest increase in overdose related deaths with a 90% increase between 2013 and 2014. Methadone has not had an overall increase in overdose related

deaths between 2013 and 2014. Increasing opioid overdose related to opioids prescribed as pills meant for pain management is not surprising given that this is how a majority of modern recreational opioid users begin their experience with opioids (44). Monitoring of opioid consumption of patients that receive them by prescription may not be sufficient to prevent abuse. Misuse of prescription refills and "doctor shopping," a situation where an individual seeking opioids may go to several different doctors to receive multiple prescriptions for the drugs, are common problems associated with prescribed opioids (32). Prescription opioids can also be sold or shared by patients with a legitimate prescription. The sale of opioids through the internet is also a uniquely modern challenge for monitoring the consumption of opioid analgesics (44).

From 2010 to 2013, individuals who had used an opioid in the past month began to use only prescription opioids less and used a combination of opioids and heroin more, according to a self-administered survey of diagnosed opioid abusers (54). A possible contributing factor to increasing heroin use is the increasing availability of heroin in the United States. A federal report states that much of the heroin in the United States comes from Mexico and production of the drug in that country continues to rise (55). This report also states heroin is less expensive than prescription opioids on the streets. The estimated cost of a 10 mg dose of oxycodone is approximately $10 while it is estimated 50 mg of 50% pure heroin is around the same price. Heroin use may also be favorable because of the increased potency of the drug compared to morphine due to a larger amount being able to cross the BBB compared to morphine (56).

The Blood-Brain Barrier

The BBB is a barrier formed by the endothelial cells surrounding the lumen of the brain microvasculature. Adjacent endothelial cells attach to themselves and each other with tight junctions. These junctions, as described by Campbell, are made up of several transmembrane proteins and are responsible for a large part of the impermeability of the barrier (57). Several forms of the claudin protein are expressed at the BBB and seem to be very important for the formation of these tight junctions. Adherens junctions are another type of cell junction at the BBB and allow the endothelial cells to link to themselves. These junctions help set up cell polarity and are formed with cadherin proteins. Pericytes surround the endothelial cells. Pericytes belong to the vascular smooth muscle cell family and provide structural support for the BBB and play an important role in the establishment of the BBB (58). Both pericytes and the endothelial cells are found in the basement membrane which contains many proteins that play a direct role in the activity of endothelial cells. Disruption of this basement membrane in certain disease states is very closely related to disruption in the activity of the BBB (59). Astrocytes are important to the maintenance of the BBB and co-culture of endothelial cells with astrocytes improves BBB characteristics *in vitro* (58,60). *In vivo* studies in which loss of astrocytes at a particular location have confirmed astrocytes play an important role in the integrity of the BBB (59).

The ability of the BBB to act as a selectively permeable barrier is heavily reliant on transport proteins. Because the tight junctions of the BBB are mostly impermeable, transport proteins are essential for the movement of nutrients into and keeping potentially dangerous compounds out of the brain. Glucose, essential for brain function, requires a transporter to cross the barrier. The GLUT1 transporter is responsible for glucose transport and allows glucose to travel into the brain along its concentration gradient (61). Some transporters act to export compounds from the BBB, most notably the ATP-Binding Cassette (ABC) proteins (62). Of these, p-gp, also known as multiple drug resistant protein 1 (Mdr1), plays a major role in the mechanism by which these potentially dangerous compounds are removed (63,64). This protein is important for the management of certain disease states. In a study of Alzheimer disease, p-gp was shown to mediate the clearance of amyloid-β from the brain (65). P-gp is of particular interest because it has a wide range of substrates, and a poorly understood system of regulators. P-gp is regulated both by the level of expression and through post-translational regulation through trafficking. Breast cancer resistance protein (BCRP) is another important efflux protein at the BBB (66,67). BCRP may also have a higher importance related to xenobiotic regulation in the BBB than p-gp (68).

pioids and the Blood-Brain Barrier

Morphine is the international standard for opioid analgesics. As previously discussed, morphine is metabolized into M3G and M6G via glucuronidation, leading to blood concentrations of these metabolites several times higher than that of the parent compound. Morphine can be metabolized to M3G and M6G in the brain directly (87). Morphine and M3G, the metabolite with no analgesic activity, are strong substrates for p-gp, and thus have poor penetration into the brain (88). M6G, the metabolite with higher analgesic potency than the parent compound, has not been demonstrated to be a p-gp substrate, however still does not penetrate the BBB (89). Genetic

polymorphisms in ABCB1, the gene which encodes p-gp, in cancer patients have been shown to play a major role in intracellular concentrations of morphine and both metabolites (89). Inhibition of p-gp at the time of administration of morphine has also been shown to increase the observed analgesic effect, confirming p-gp inhibits the analgesic effect of morphine (90). It has been suggested that other members of the Mdr protein family may play roles in the transport of morphine's metabolites (91).

Research regarding the effect of chronic morphine exposure on the BBB is sparse and more information is needed to draw definitive conclusions. From what has been reported, the expression of several different genes in isolated microvessels of rats has been shown to change following chronic administration of morphine, including those in the Mdr family (92). Whole brain samples have been shown to have an increased expression of p-gp following chronic exposure to morphine. However, isolates of brain microvessels do not show this increase (92).

Opioids have been a part of human history since the earliest civilizations. Throughout history, opioids have been recognized for the ability to eliminate pain. Opioids achieve an analgesic effect through inhibitory action at mu opioid receptors in both the brain and spinal cord. While opioids have a positive effect on pain management, negative side effects include respiratory depression and gastric slowing. Chronic opioid use is associated with the development of tolerance and dependence. Chronic exposure to opioids has become increasing prevalent for both clinical and recreational users. Patients in this population have few options for acute pain management. At the BBB, p-gp is an efflux protein known to have increased trafficking to the luminal surface of the endothelial cells of the BBB during a pain event. Understanding the effect of chronic morphine on the signal required to initiate p-gp trafficking at the BBB in a pain state could lead to a novel therapeutic target for improving acute pain management in long-term opioid exposed patients.

MATERIALS AND METHODS

Reagents:

EDTA-free complete proteinase inhibitor was purchased from Roche (Sigma Aldrich, St. Louis, MO). Morphine was acquired from the National Institute of Drug Abuse (Bethesda, MD). Tris(2-carboxyethyl)phosphine hydrochloride, 20x sample reducing agent, 4x sample loading buffer and Precision Plus prestained molecular weight standards were purchased from Bio-Rad (Hercules, CA). Any other chemical was acquired through Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Animals and Treatments:

All animal protocols used in these studies were written in compliance with the written guidelines of the National Institutes of Health and approved by the University of Arizona Animal Care and Use Committee. Results were reported according to the ARRIVE guidelines. Female Sprague-Dawley rats (175-200 g) (Harlan Sprague-Dawley, Indianapolis, IN) were cared for using the standard conditions in the University of Arizona Animal Care Facility. All animals were allowed to acclimate for one week before being used in any experiment.

Induction of peripheral inflammatory pain:

A 0.1 mL injection of either λ-carrageenan (3% in 0.9% saline) or 0.9% saline was administered to the left hind paw of animals 3 hours before sacrifice.

Pump insertion surgery:

Alzet (Cupertino, CA) osmotic mini-pumps were filled to maximum capacity with morphine sulfate dissolved in 0.9% saline or 0.9% saline. Morphine concentration was appropriate to deliver 5 mg/kg/day to a rat weighing 200 g. Pumps were submerged in 0.9% saline and incubated overnight at 37°C. Rats were anesthesitized under 5.0% isoflurane in air and maintained at 2.5% isoflurane in air. A 1-inch square area at the bottom of the scapula was shaved, and an approximately 1.0 cm incision was made at this spot through the skin. A set of hemostats were used to create a cavity large enough to insert the mini-pump under the skin with the pumping end of the pump facing away from the incision. The incision was closed using two surgical staples. Staples remained in the animal until sacrifice. Minipumps were weighed empty, after filling, after priming, and after removal to monitor proper function. Remaining volume in the mini pump was determined at sacrifice to ensure proper function as well.

von Frey mechanical sensitivity:

Two people were present for all behavior studies. The up-down method described by Dixon *et al*. was used to establish mechanical allodynia (111). Briefly, the rats were placed into the chambers for at least 10 minutes to allow them to acclimate before any measurements were taken. Rats were treated with an acute dose (2.5 mg/kg) of morphine in 0.9% saline or 0.9% saline 3 hours after injection of 0.1 mL λ-carrageenan or saline into the left hind paw as previously described. Mechanical sensitivity was tested using von Frey

filaments in the assay described by Dixon et al. in the ipsilateral paw (111). Mechanical sensitivity was measured before surgery, before λ-carrageenan injection, before morphine injection, and 10, 20, 30, 45, 60, 90, 120, and 150 minutes after injection of morphine. Pre-surgery and pre-λ-carrageenan injection values were used to determine opioid-induced hyperalgesia. Animals that achieved a maximal threshold score following exposure were excluded from these calculations because the true change in threshold of these individuals could not be determined.

Hargraves' thermal sensitivity:

Two people were present for all behavior studies. Thermal sensitivity was tested using the method described by Hargraves *et al*. (112). Briefly, rats were placed into the chambers for at least 10 minutes to allow them to acclimate before any measurements were taken. The rats were treated with an acute dose (2.5 mg/kg) of morphine in 0.9% saline or 0.9% saline 3 hours after injection of 0.1 mL λ-carrageenan or saline into the left hind paw. The infrared emitter was placed under each foot and turned on. Time to paw withdrawal (seconds) was measured using a laboratory timer and was started and stopped by the person operating the infrared emitter. Thermal sensitivity was measured before surgery, before carrageenan injection, before morphine injection, and 10, 20, 30, 45, 60, 90, 120, and 150 minutes after injection of morphine.

Paw Edema:

Paw edema was measured 3 hours after λ-carrageenan (or saline) injection, 30 minutes after morphine injection, and 150 minutes after morphine injection. A Ugo-Basile (Varese, Italy) plethysmometer was used to determine the paw volume (mL) of both the ipsilateral and contralateral hind paws. Rats were restrained, and the contralateral paw was measured first followed by the ipsilateral paw. Data are expressed as the difference between these two measurements.

Microvessel isolation:

Cerebral microvessels were isolated as previously described (84). Briefly, rats were anesthetized, decapitated, and the brains removed. Brains were minced and homogenized using a Potter-Elvjehm homogenizer. Samples were layered over 30% Ficol and centrifuged (20 min at 5800 x g at 4°C) to remove the majority of the lipids. The vessels found in the pellet were resuspended in buffer and filtered using a series of nylon mesh filters. These limited the remaining vessels to pieces which were between 300 μm and 40 μm. Samples were frozen at -20°C or used for a subsequent biochemical analysis.

Nuclear/cytosolic protein analysis:

Animals of like treatment were pooled to create samples consisting of 3 independent rats coming from different cages. Nuclei were isolated using the instructions provided with the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit (ThermoFisher Scientific, Rockford, IL). This includes Cytoplasmic Extraction Reagent I (CER I), Cytoplasmic Extraction Reagent II (CER II) and Nuclear Extraction Reagent (NER). Briefly, microvessels isolated using the previously described technique were suspended in an appropriate volume of CER I. These were vortexed vigorously for 15 seconds and left to incubate on ice for 10 minutes. An appropriate volume of CER II was then added to the sample. This sample was then vortexed for 5 seconds and left to incubate on ice for 1 minute. Vortexing again for 5 seconds, the sample was then centrifuged at 16,000 x g for 5 minutes. The supernatant represented the cytoplasmic extract and was saved. The pellet was suspended in an appropriate volume of NER. The sample was vortexed for 15 seconds every 10 minutes for 40 minutes as the sample incubated on ice. The sample was then centrifuged for 10 minutes at 16,000 x g. The supernatant contained the nuclear extract.

Western Blot and Quantification:

Equal concentrations of nuclear and cytosolic protein were separated via SDS-PAGE gel electrophoresis loaded onto Criterion TGX 4-20% gels (Bio-Rad). Proteins were detected and quantified using antibodies to MDR1 (sc8313) and nucleoporin p62 (sc25523) from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Dallas, TX). An HRP-linked anti-rabbit secondary (GE Healthcare, Piscataway, NJ) was used for the detection of these antibodies. Proteins were measured by chemiluminescence using the Clarity bioluminescence kit (Bio-Rad) and imaged on a ChemiDoc System (Bio-Rad). Bands were quantitated by importing the image into Image Lab (Bio-Rad) and exported them from the program. The bands were quantified following removal of background signal using the algorithms in FIJI (113). These images were cropped and the contrast and brightness adjusted for the entire cropped portion before constructing the figure.

Statistics:

Difference between means was tested using the Student's t-test using the algorithms in Microsoft Excel (Microsoft, Redmond, WA).

RESULTS

Osmotic minipumps can be used to establish tolerance to morphine

The long-term goal in this line of experimentation is to establish a mechanism for post-translational regulation of P-gp and to apply this mechanism to more effective pain therapies by improving opioid delivery to the brain. In previous experiments, peripheral inflammatory pain (PIP) has been shown to increase PIP mediated p-gp trafficking at the (86). Experimentation by Zong and Pollack has shown that the chronic administration of p-gp substrates is sufficient to induce changes in whole brain p-gp (114).

Tests using the up-down method of von Frey mechanical sensitivity demonstrated that morphine exposure from osmotic minipumps induced opioid-induced hyperalgesia (OIH) (Fig 1) (111). OIH was identified by a marked increase in mechanical sensitivity between thresholds determined before the pump insertion surgery and after six days of morphine exposure. Animals that received a morphine pump had a 50% reduction in mechanical thresholds after morphine exposure ($p<0.001$). Post-exposure thresholds for animals treated with morphine were significantly lower than the post-exposure values for animals that were treated with the saline control ($p<0.001$). Animals not exposed to morphine showed no significant decrease in mechanical thresholds following exposure. There was no significant difference in pre-surgery mechanical sensitivity between the two groups.

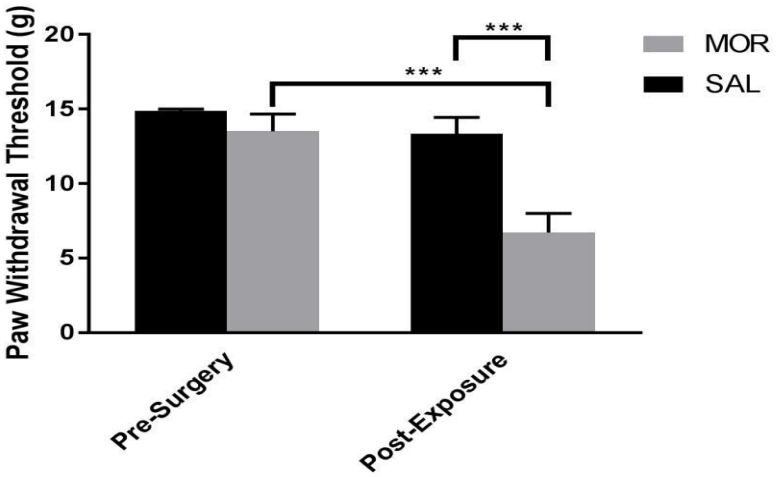

Figure 1. Six day exposure to morphine from osmotic mini-pump causes opioid-induced hyperalgesia in female rats. Mechanical allodynia was measured in female Sprague-Dawley rats before receiving surgery to insert an osmotic mini-pump and after six days exposure to the pump. Animals received morphine (5 mg/kg/day) in 0.9% saline (MOR) or 0.9% saline (SAL) for six days before testing again. Values are mean + SEM (n=9) Lines indicate compared values. *** denotes significantly different ($p<0.001$).

Osmotic minipumps can be used to establish tolerance to morphine (mechanical allodynia)

The von Frey up-down assay was used with the previously established model of PIP induced by λ-carrageenan injection in the rat hind paw to investigate morphine tolerance (111). In morphine tolerance, the reduction of mechanical allodynia by an acute dose of morphine is reduced or eliminated entirely following chronic exposure, such as that from the mini-pumps. Intraperitoneal injection of 2.5 mg/kg morphine was used for anti-nociception in these experiments.

By measuring the paw withdrawal threshold in the inflamed hind paw over a time course of 2.5 hours, rats pre-exposed to morphine then given the acute dose were indistinguishable from those that received no acute morphine at all (Fig 2A). Mechanical allodynia was determined to be reduced by an acute dose of morphine in animals with no pre-exposure to morphine. Animals that did not receive an acute dose of morphine did not have a change in sensitivity in the ipsilateral paw. The anti-nociceptive effect was seen from 20 to 60 minutes in these animals and the area under the curve for this period was quantified for both groups that received an acute dose of morphine (Fig 2B). A comparison of the determined area under the curve for these groups showed a significantly larger area for the morphine naïve animals ($p<0.0001$). Animals with no injection of λ-carrageenan tended to have a lower mechanical threshold when exposed to morphine for six days (Fig 3).

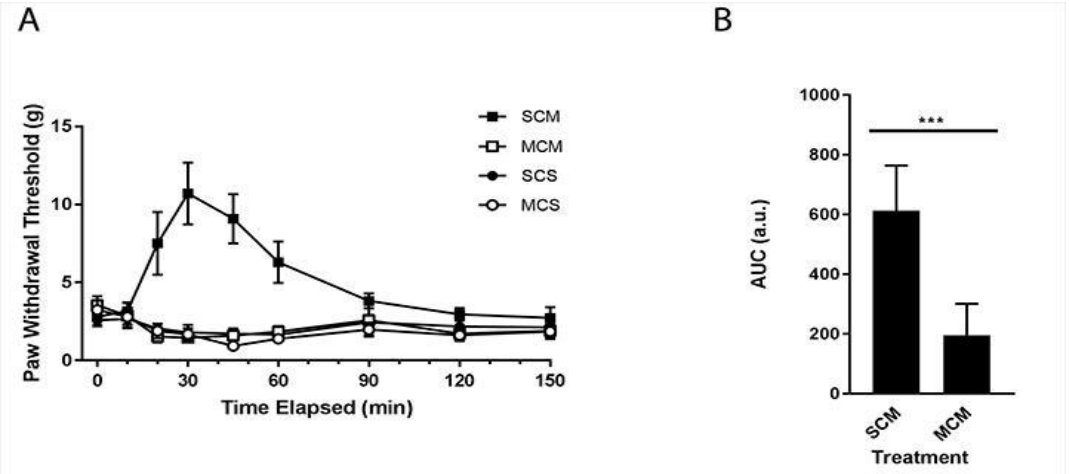

Figure 2.Prolonged morphine exposure eliminates the anti-nociceptive effect of acute morphine administration on mechanical sensitivity. (A) Mechanical paw withdrawal threshold was determined by the von Frey mechanical sensitivity test in rats exposed to 5 mg/kg/day of morphine or saline for 6 days, then treated with an acute dose (2.5 mg/kg) of morphine or saline 3 hours after injection of λ-carrageenan into the left hind paw. The symbols mean: SCS: Saline Osmotic mini-pump (24µL/day)/ □-carrageenan hind paw injection (0.1 mL)/ Saline intraperitoneal injection (1mL/kg);SCM: Saline Osmotic mini-pump (24µL/day)/ □-carrageenan hind paw injection (0.1 mL)/ Morphine intraperitoneal injection (1mL/kg) (2.5mg/kg);MCS: Morphine Osmotic mini-pump (24µL/day) (5mg/kg/day)/ □-carrageenan hind paw injection (0.1 mL)/ Saline intraperitoneal injection (1mL/kg);MCM: Morphine Osmotic mini-pump (24µL/day) (5mg/kg/day)/ □-carrageenan hind paw injection (0.1 mL)/ Morphine intraperitoneal injection (1mL/kg) (2.5mg/kg). Values are the mean +/- SEM (n=8). **(B)** The area under the curve for the animals treated with SCM and MCM during the peak observed morphine effect (between 20 minutes and 60 minutes). Values are the mean + SEM (n=8). *** denotes significantly different ($p<0.0001$)

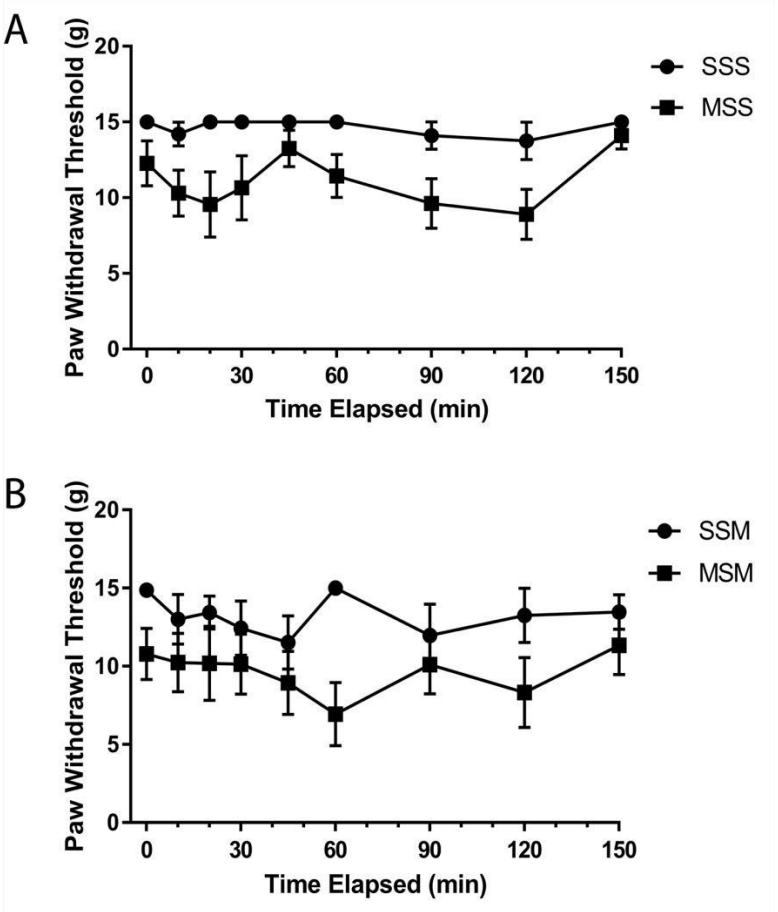

Figure 3. Chronic morphine administration tends to reduces mechanical sensitivity thresholds determined by the von Frey test in the ipsilateral paw. The symbols mean:

SSS: Saline Osmotic mini-pump (24µL/day)/ saline hind paw injection (0.1 mL)/ Saline intraperitoneal injection (1mL/kg); SSM: Saline Osmotic mini-pump (24µL/day)/ saline hind paw injection (0.1 mL)/ Morphine intraperitoneal injection (1mL/kg)

(2.5mg/kg);MSS: Morphine Osmotic mini-pump (24µL/day) (5mg/kg/day)/ saline hind

paw injection (0.1 mL)/ Saline intraperitoneal injection (1mL/kg);MSM: Morphine Osmotic mini-pump (24µL/day) (5mg/kg/day)/ saline hind paw injection (0.1 mL)/ Morphine intraperitoneal injection (1mL/kg) (2.5mg/kg). Values are mean +/- SEM (n=8).

Osmotic minipumps can be used to establish tolerance to morphine (thermal sensitivity)

The inability of an acute dose of morphine to reduce thermal sensitivity in animals previously exposed to morphine is another marker of acquired morphine tolerance. Using the same time course as the von Frey experiment, the Hargreaves method for determining thermal sensitivity was implemented to measure sensitivity (112). Paw withdrawal latency (s) was used as the measure for each point. Rats that had been pre-exposed to morphine had a greatly reduced anti-nociceptive response to morphine (Fig 4A). Morphine naïve animals had a distinct period in which thermal sensitivity was reduced. Animals that did not receive an acute dose of morphine did not have a change in sensitivity in the ipsilateral paw. For this experiment, an anti-nociceptive effect was seen from 30 to 60 minutes in the animals with no pre-exposure to morphine. The area under the curve was larger for the animals with no pre-exposure to morphine ($p<0.01$) (Fig 4B). Animals with no injection of λ-carrageenan showed no change over the time course (Fig 5). Measurements in the contralateral paw showed no changes regardless of treatment.

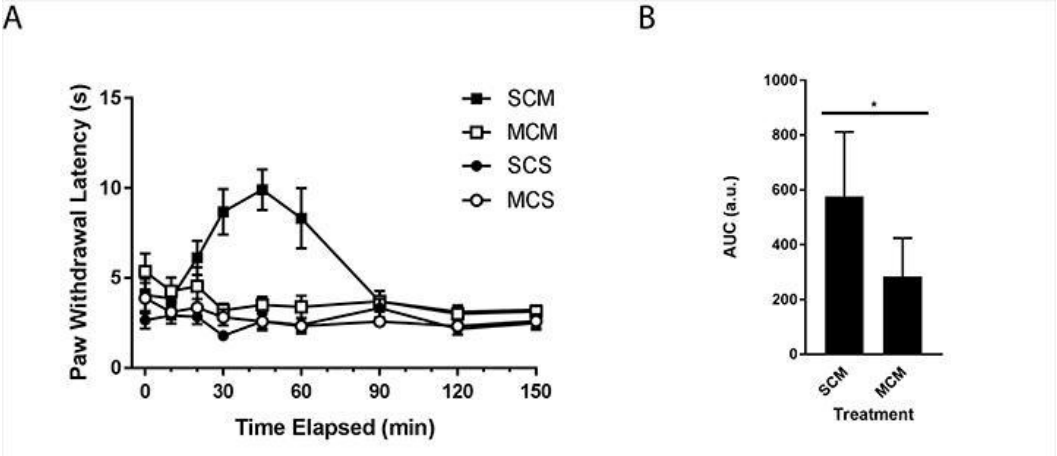

Figure 4. Prolonged morphine exposure eliminates the anti-nociceptive effect of acute morphine administration on thermal sensitivity. (A) Thermal paw withdrawal threshold was determined by the Hargraves thermal sensitivity test in the ipsilateral paw of rats exposed to 5 mg/kg/day of morphine or saline for 6 days, then treated with an acute dose (2.5 mg/kg) of morphine or saline 3 hours after injection of λ-carrageenan into the left hind paw (n=9). The symbols mean: SCS: Saline Osmotic mini-pump (24µL/day)/ □-carrageenan hind paw injection (0.1 mL)/ Saline intraperitoneal injection (1mL/kg);SCM: Saline Osmotic mini-pump (24µL/day)/ □-carrageenan hind paw injection (0.1 mL)/ Morphine intraperitoneal injection (1mL/kg) (2.5mg/kg);MCS: Morphine Osmotic mini-pump (24µL/day) (5mg/kg/day)/ □-carrageenan hind paw injection (0.1 mL)/ Saline intraperitoneal injection (1mL/kg);MCM: Morphine Osmotic mini-pump (24µL/day) (5mg/kg/day)/ □-carrageenan hind paw injection (0.1 mL)/ Morphine intraperitoneal injection (1mL/kg) (2.5mg/kg). Values are the mean +/- SEM (n=9). **(B)** The area under the curve for the animals treated with SCM and MCM during the peak observed morphine effect (between 30 minutes and 60 minutes).Values are mean + SEM (n=9). * denotes significantly different ($p=0.007$).

Chronic morphine exposure does not affect paw edema in a λ-carrageenan induced model of peripheral inflammatory pain

While previous studies have shown that the pain component of the PIP is the important component for the effect on the BBB, the inflammation and edema may play an important role in the magnitude of the pain. To be sure long-term

morphine exposure did not affect the swelling in the paw, the volume of both feet was compared as a way to determine paw edema. At 3 hours post injection of λ-carrageenan, the injected paw was shown to be increased by 0.79 +/- 0.06 and 0.80 +/- 0.05 mL relative to the other hind paw in the saline exposed and morphine-exposed animals used for the von Frey experiment, respectively (Fig 6A). At 3 hours post injection of λ-carrageenan, the injected paw was shown to be increased by 0.96 +/- 0.03 and 0.87 +/- 0.06 mL relative to the other hind paw in the saline exposed and morphine-exposed animals used for the Hargreaves experiment, respectively (Fig 5B). Animals that received a paw injection of saline did not have a significant difference in paw volume, regardless of treatment demonstrating that chronic morphine exposure has no effect on paw edema.

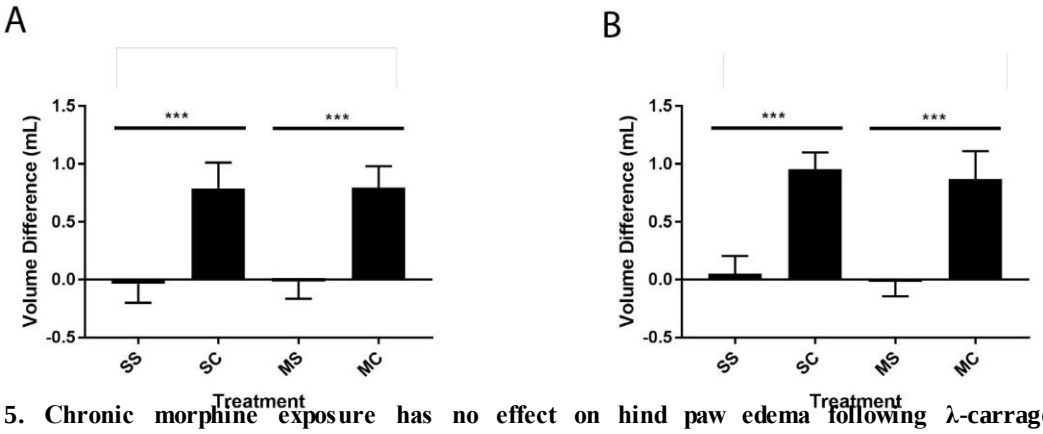

Figure 5. Chronic morphine exposure has no effect on hind paw edema following λ-carrageenan exposure paw edema measurements in the animals tested for mechanical allodynia (A) or thermal sensitivity (B). The symbols mean: SS: Saline mini-osmotic pump/ Saline hind paw injection (100µL); SC: Saline mini-osmotic pump/ □-carrageenan hind paw injection (100µL); MS: Morphine(5mg/kg/day) mini-osmotic pump/ Saline hind paw injection (100µL); MC: Morphine(5mg/kg/day) mini-osmotic pump/ □-carrageenan hind paw injection (100µL). Values are the mean + SEM (A n=16, B n=18). *** denotes significantly different ($p<0.0001$)

PIP mediated trafficking of P-gp leaving the nucleus is increased by long term opioid exposure

PIP is sufficient to induce trafficking of p-gp away from nuclear reservoirs (86). Using a nuclear protein isolation assay, protein from the cytoplasm and nuclear membrane of endothelial cells from isolated microvessels was isolated. Nucleoporin acted as a control for the purity of the nuclear fractions (Fig 7A). A six-day morphine exposure did not change the nuclear p-gp (Fig 7B). Animals exposed to morphine showed a 46% decrease in nuclear p-gp when given a PIP stimulus (Fig 7C). Animals with a saline pump showed a 24% reduction in nuclear p-gp when exposed to PIP (Fig 6C).

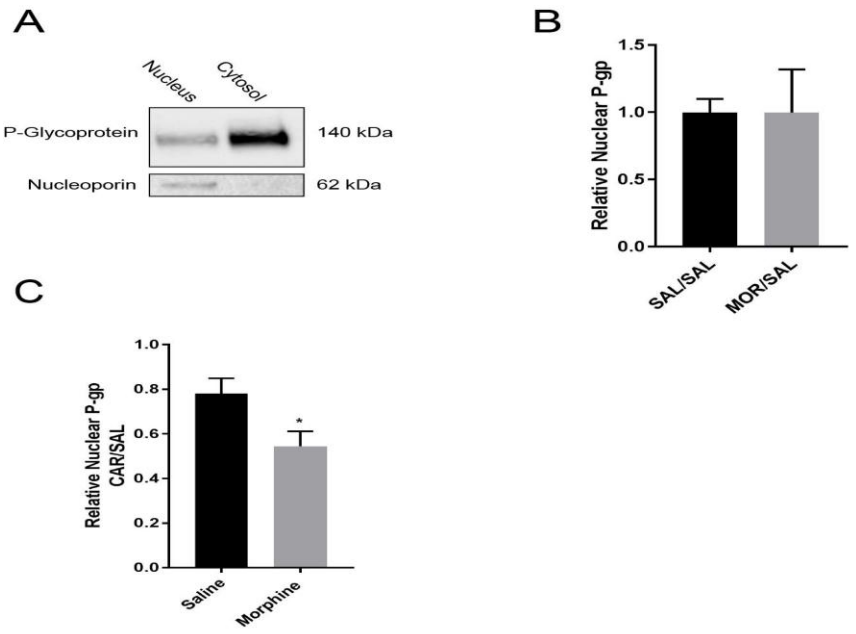

Figure 6. Chronic morphine exposure increases trafficking of p-glycoprotein away from the nucleus after peripheral inflammatory pain. (A) Representative immunoblot indicating the p-gp and nucleoporin in the nuclear and cyctosolic isolates. **(B)** P-gp expresion normalized to nucleoporin in the nuclear fractions. Values are the mean + SEM (n=3 pools of 3 rats each) **(C)** Ratio of nuclear p-gp normalized to nulcoporin in CAR/SAL injected animals as a measure of p-gp trafficking. Values are the mean + SEM (n=3). * denotes significantly differnent from control (saline) ($p<0.05$). The symbols mean: SAL/SAL represents animals with an osmotic mini-pump filled with 0.9% saline and a 0.9% saline hind paw injection.MOR/SAL represents animals with an osmotic mini-pump filled with morphine (5 mg/kg./day) in 0.9% saline and a 0.9% saline hind paw injection.

DISCUSSION

Long-term opioid exposure is sufficient to induce a 2-fold increase in peripheral inflammatory pain (PIP) mediated trafficking of p-glycoprotein (p-gp) away from the nucleus in rat brain endothelial cells. This presents a possible role of p-gp in the clinical challenges associated with decreased opioid efficacy in long-term opioid patients in need of acute pain management (2,3). Utilizing the previously established model of PIP via injection of λ-carrageenan and an observation that PIP induces p-gp trafficking from nuclear reservoirs to the plasma membrane (86), we tested whether there could be a relationship between p-gp trafficking and clinical challenges of acute pain management in long-term opioid patients.

This experimental approach required the establishment of a model of long-term opioid exposure and opioid tolerance in female rats. The subdermal osmotic mini-pump method of delivery allowed us to deliver a consistent dose of opioids (115). These behavior data suggest this delivery method was sufficient to induce tolerance to the mechanical allodynia and thermal sensitivity mediating effects of morphine after an acute pain stimulus. These data also showed an increase in baseline mechanical allodynia but not thermal sensitivity. The up-down method of analyzing von Frey filament data has been shown to be an effective way to detect mechanical allodynia in rodents when analyzed with the Dixon statistic (111,116). A 50% reduction in mechanical pain threshold compared to both pre-surgery values and animals who received a control pump demonstrates that using this method; morphine exposure via these osmotic pumps caused opioid-induced hyperalgesia (OIH) to occur in female rats. OIH to mechanical stimuli is an observed phenomenon that is particularly associated with the effects of long-term opioid exposure in female rats, making a particularly good marker for tolerance in these experiments (117). Baseline allodynia was observed 3 hours post injection of λ-carrageenan in the treated hind paw to the same extent in all animals, regardless of which pre-treatment the animal received.

P-gp trafficking from the nuclear membrane to the luminal membrane of endothelial cells making up the BBB presents a major problem for delivery of opioids into the CNS in the presence of acute pain. This trafficking effect is amplified by the persistent presence of a p-gp substrate such as morphine. A clinically relevant example of this is the challenges associated with post-surgical pain management in patients being treated long-term with opioids. Pain management in these patients is particularly difficult and causes increased recovery times and reduced patient satisfaction. The mechanism by which this trafficking occurs is currently unknown, but the characterization of this mechanism is a promising therapeutic target.

Conclusions and Future Directions

This study demonstrated that long-term exposure to morphine causes an increase in the p-glycoprotein (p-gp) trafficking response induced by peripheral inflammatory pain (PIP) at the blood-brain barrier (BBB). Pain induced trafficking of p-gp decreases the efficacy of morphine (6). Pain decreasing the efficacy of the analgesic morphine is a clear clinical problem. P-gp trafficking at the BBB impacts delivery to the central nervous system (CNS), but not to other peripheral targets. Peripheral mu opioid receptors mediate many of the side effects associated with opioids such as respiratory depression and gastric slowing leading to constipation. In a clinical setting, morphine is administered via self-administration systems called Patient Controlled Analgesia systems (2). Because this is controlled by the patient in an attempt to manage pain, the patient will continue to increase the dose of the analgesic, but because of a decreased delivery to the CNS caused by p-gp mediated trafficking at the BBB, peripheral side effects may become more severe. This study shows this could be more severe for patients with a history of long-term opioid use. This observation suggests the increased PIP-mediated trafficking of p-gp may be a potential factor in the challenge of managing acute pain in opioid tolerant patients. Characterizing this mechanism could lead to more effective post-surgical pain management in these patients. Altering p-gp trafficking would allow opioid tolerant patients to manage post-surgical pain more effectively. Improved pain management means improved recovery from injury and less time taken from the physician to manage pain..

Surgery is an increasingly common solution to attempt to correct chronic pain, seeing application in hernia pain, groin pain and neck pain (125–127). Patients already receiving chronic opioid therapy for chronic pain that desire to seek a surgical approach to managing pain are a group of particular interest. Investigations into the role of p-gp and the BBB could lead to more effective strategies for managing post-surgical pain in this growing population of chronic pain patients receiving long-term opioid therapy. The pain associated with the recovery from the mini pump insertion surgery performed on these rats makes this model a potential means of investigating this problem. Because there was no control for the chronic post-surgical pain included in this study, further investigation of the effect of chronic pain is needed.

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