PERIPHERAL INFLAMMATORY PAIN AND P-GLYCOPROTEIN IN A MODEL OF CHRONIC OPIOID EXPOSURE

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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.

Chapter I- Background Information

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.

To understand pain, one must first examine the mechanisms by which pain originates, and what occurs in the nervous system following this signal. The physical component of pain, called nociception, is the process by which nociceptors, a group of nerve cells found in the peripheral nervous system, recognize intense thermal, mechanical or chemical stimuli (7). Nociceptors have a unique physiology; they have a cell body in specific areas known as ganglia. In the periphery, the cell bodies of nociceptors are located in the dorsal root ganglia. Trigeminal nerves of the face have cell bodies located in the trigeminal ganglion. Nociceptors have two axonal branches, a peripheral branch that innervates the target organ and a central axon that innervates the spinal cord (8). A key feature of

nociceptors is the ability to limit the initiation of a signal to noxious stimuli by requiring a relatively high activation signal. Nociceptors are divided into two groups of fibers. The Aδ-fibers and Aβ-fibers are thinly myelinated fibers responsible for transmitting "acute, well-localized, fast pain," specifying the location of the stimulus (7). The second type of fiber is the unmyelinated C-fiber which is responsible for poorly-localized "slow" pain often described as an ache. Both of these fiber types can be organized into subtypes that are more or less sensitive to thermal or mechanical stimulation.

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.

The analgesic effects of opioids are due to pharmacological action in the brain, in the spinal cord, and potentially in the periphery. In the brain, opioids are primarily believed to act at mu opioid receptors (MOR). In fact, mutations in the mu opioid receptor at

position 118 are sufficient to modify post-cesarean pain perceptions and the amount of morphine used by patients on a PCA system (13). Experiments involving microinjections at the medulla, substantia nigra, nucleus accumbens, and periaqueductal gray (PAG) resulted in the reduction of pain behaviors in animal models (14). The action in the PAG is thought to cause a disinhibition of the medulla at tonically active neurons (10). This disinhibition leads to the release of norepinephrine and serotonin to the spinal dorsal horn, attenuating dorsal horn excitability (14). This attenuation results in a reduction of nociceptive signaling through the spinal cord.

Opioids have an analgesic effect on the spinal cord by depressing the signal through small afferent nerve fibers in the dorsal horn (10). Intrathecal injection is sufficient to induce the analgesic effect of opioids in mammals. Systemic administration of low dose naloxone, a mu opioid receptor antagonist, is sufficient to reverse the analgesic effect of intrathecal injections of morphine, demonstrating the importance of the mu opioid receptor in opioid-mediated analgesia (10,15). The study by Chen and Pan demonstrated an injection of a selective mu opioid receptor antagonist into the lumbar spine was sufficient to stop the analgesic effect of systemically administered opioids at the rodent hind paw, without having an effect on the analgesic effect of morphine at the forepaw. This study demonstrated that the effects of systemically administered opioids are specific enough to be limited to certain regions of the spinal cord. Opioids act at the presynaptic terminal of the afferent nerves by limiting the release of neurotransmitters from the presynaptic terminal of the synapse between the nociceptor and the dorsal horn of the spinal cord (16). Opioids prevent the release of neurotransmitters at these synapses through inhibition of N-type calcium channels. Alternative splicing of these channels

plays a role in the effectiveness of opioids, suggesting a possible reason for a stronger effect on nociceptors (17). Observations that glutamate can block the effect of opioids demonstrate a role of postsynaptic neurons due to action at potassium channels, leading to hyperpolarization of the postsynaptic neuron, preventing the transmission of the action potential (10).

Although opioids are traditionally thought to be centrally acting compounds, there is evidence opioid analgesics have an effect in the peripheral nervous system. These experiments have largely focused on the effect of opioid analgesics on inflammatory models of pain in rodents. Mu opioid receptor agonists affect peripheral hyperalgesia associated with inflammatory pain by limiting prostaglandin E_2-induced hyperalgesia. (18,19). A recent study suggests this effect is not through action on mu opioid receptors, but possibly through an off-target effect (20). This result suggests a therapeutic role of opioids in the peripheral nervous system, but this effect may be through a non-traditional mechanism. Literature in this area is lacking but presents a potential avenue for new pain therapies.

One of the most challenging aspects of prolonged treatment with opioids is the progressive loss of function in as little as two weeks (21). This loss of function is referred to as opioid tolerance. Opioid tolerance is defined by Yaksh and Wallace in *Goodman and Gilman's: The Pharmacological Basis of Therapuetics* as the reduction of analgesic efficacy of a particular dose of an opioid as that dose is repeatedly given over time (10). This results in a right-hand shift of the dose-response curve of the drug and a reduction in the maximally achievable effect of lower doses. The ability of higher doses to surmount this loss of efficacy and switching between classes of opioids without a complete cross-

tolerance are the current clinical strategy to overcome tolerance. Tolerance is observable not only at the level of reduced analgesic and sedative effects but also at the level of the cell (22). There is a reduction of inhibition of adenyl cyclase. Detailed in by Yaksh and Wallace, different physiological responses to opioids develop tolerance at different rates (10). The constriction of the pupil (pupillary miosis) is an example of a response with little development of tolerance. Tolerance to the euphoric effects of opioids develops rapidly and at a rate higher than many other effects, presenting a danger to recreational users. Diminishing euphoria means users seeking this feeling are prone to ingesting a dose which can elicit a dangerous effect from a different response with a slower rate of tolerance. Analgesia, sedation, respiratory depression, and constipation are examples of responses to which tolerance will build at a slower, more moderate pace. Cross-tolerance between different opioids can occur, but this is not always the case, suggesting small but meaningful differences in the action of different types of opioid agonists. Tolerance is reversible and suspension of administration of the drug will, over time, return efficacy of a particular dose to the original, basal levels.

Chronic administration of opioids will also lead to the development of a state of dependence. Dependence presents as a state in which cessation of opioid use, or administration of an opioid receptor antagonist such as naloxone or naltrexone, will result in the precipitation of withdrawal syndrome symptoms. Because opioids are an inhibitory signal to the cell, cells will increase signaling to compensate and return to normal function. Removing the inhibitory signal will result in an overactivation of affected cellular pathways leading to a variety of symptoms caused by the overactivation of the somatomotor cortex and autonomic nervous system (10). The major physical symptoms

of withdrawal syndrome include diarrhea, vomiting, agitation, hyperalgesia, hyperthermia and hypertension. Feelings of depression, dysphoria and anxiety are also associated with withdrawal. Due to the fact these symptoms are highly aversive, prevention of withdrawal can act as a major motivator to continue use of the drug. This incentive to continue use can lead to overuse of, abuse of and addiction to opioids (23).

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.

A History of Opioids

The exact origins of the use of the opium poppy are not known. References to the use of a drug that can "lull all pain and anger and bring forgetfulness of every sorrow," believed to be opium, an active liquid extract from the opium poppy, can be found in writings even

predating this selection from Homer's The Odyssey. Opium was used for pain and when combined with hemlock, used as a means to execute the condemned in ancient Greece. The Greeks had such a strong association with opium, the gods associated with sleep (Hypnos), dreams (Morpheus), night (Nyx) and the god of death (Thanatos) were often depicted with wreaths of poppy blossoms (26). Opium's history is not limited to the ancient Greeks and has a history predating Greek civilization entirely.

The original use of opium is unknown but is thought to have been originally employed as a euphoriant in religious ceremonies. A review by Brownstein states knowledge of the process used to produce opium was likely limited to priests (27). The review goes on to say that the earliest written records of medicinal use of the opium poppy date back to the dawn of human civilization. The Sumerians, an early civilization originating in what is now southern Iraq, were the first people to record the production and use of opium. Clay tablets dated back to around 3000 BC written in Cuneiform script describe the process by which the opium poppy was cultivated. The tablets also describe how to extract the juice from the cultivated flowers and the process by which this juice is processed into opium. These tablets were written with enough detail to specify the juice is best collected in the morning, demonstrating the importance of opium to the people of the time. Cultivation of this plant remained popular for thousands of years, spanning many centuries and empires and eventually led to the distribution of opium throughout Eurasia. A country commonly associated with opium use is China. A review by Schiff reports Arabian traders brought opium and knowledge of the medicinal use of the drug to the country at some point between the 11^{th} and 13^{th} centuries AD (26). This review goes on to say, following a ban on smoking tobacco by Tsung Chen in 1644, smoking opium became a popular

replacement for many Chinese citizens. Opium sold in China originated from large growing operations in India distributed by the East India Company. Following the acquisition of the East India Company by the British government, large quantities of opium were sold to smaller companies who would smuggle the drug into China. These companies sold the opium through Canton. Following the replacement of the Viceroy of Canton in 1838, opium distribution was severely reduced. In 1839, millions of pounds of British and American opium were confiscated and destroyed by the Viceroy. This sparked the first opium war resulting in Britain being awarded control of the island of Hong Kong for over 150 years. By 1913, 25 percent of the Chinese population was addicted to opium. This epidemic prompted the British government to suspend the sale of opium, but this action came too late. Widespread use of opium would not stop in China until the years following World War II with the establishment of the People's Republic of China. This widespread addiction was only the first taste the modern world would have of the addictive and destructive potential of opioids.

In 1806, morphine was isolated from the opium poppy by Friedrich Sertüner (28). Morphine was named after Morpheus, the Greek god of sleep. The isolate could be produced in large quantities and became popular to use for minor surgical procedures and for the management of post-surgical and chronic pain. This discovery was not the solution for opiate addiction that many had hoped for, being just as addictive as opium and the widespread search for a nonaddictive replacement began. In 1898, heroin was first synthesized with the claim of being more potent than morphine and being free from an addictive nature like other opiates (29). Only one of these claims would prove to be true, and both heroin abuse and the search for a non-addictive opiate continue today (27).

The synthesis of methadone in 1946 led to the first potential treatment for opiate addiction (30). The symptoms of withdrawal syndrome associated with methadone use were markedly more manageable than those associated with traditional opiates. While these symptoms have a longer duration, the effects experienced are milder. This observation inspired a treatment plan in which patients would be switched from another opioid to methadone then administration would be tapered off entirely (27). These programs rely on very careful monitoring of drug intake combined with the addition of supportive therapies and lead to lowered mortality rates than in those who do not use this therapy (31). Those using this therapy are also able to maintain mostly normal lives, easing the transition out of addiction (27).

Chronic opioid therapy for non-cancer related chronic pain has been a standard use of these drugs throughout history. While this did fall out of favor though much of the 20th century due to the danger of addiction and other adverse effects, attitudes began to change in the 1980s (32). A letter written to the New England Journal of Medicine made a significant impact on attitudes towards the addictive nature of opioids in chronic pain patients (33). The letter explained that of the 11,882 examined patients who received at least one prescription of a narcotic, only four had well-documented addiction after leaving the hospital. The feeling of safety related to chronic opioid use was further reinforced by letters and scholarly reviews throughout the following decades. These studies often involved patients with a history of addiction presenting little to no instances of addiction (34–36). Of these studies, an article published in *Pain* was particularly notable. This study followed 38 patients who had received opioids for an extended period reporting misuse in only two patients (35). This gave the impression that if an opioid was

prescribed for pain, there was little danger of addiction. This shift in attitudes towards opioids as a complete solution for all types of pain management seemed to answer the increasing demand for pain management in clinical settings (32).

The relaxed attitudes surrounding opioids only began to be questioned after a decade long trend beginning in 2000 resulted in large changes of opioid use. Articles and reviews were published detailing the increase in opioid prescriptions across all types of clinical settings (37,38). Increasing trends in opioid use, as well as the increase in opioid prescription, began to raise public safety concerns.

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, totaling 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.

The rates of opioid dependence and overdose deaths associated with the current epidemic in the United States are not limited to one region. The northeastern United States is a region which has been particularly affected and has reacted by creating a significant network for treatment of opioid addiction (39,42). Massachusetts, a heavily impacted state, has increased the number of Recovery Support Centers and specialty Recovery High Schools (42). These and similar resources have helped to make New England the region with the highest overall treatment capacity in the country. The western states and many states in the southern half of the country, markedly Oklahoma, have high rates of overdose deaths and opioid dependence, these states do not have the treatment capacity necessary to address the opioid dependence problem. (39). This gap between those needing help and the capacity to help is likely due to multiple factors, but the cost of treatment is a logical example of a challenge that may impact some states more than others. Approximately 25 billion dollars was spent in 2007 on extra healthcare costs related to opioid abuse (43). Further research into affordable, effective treatments and government funding for these programs will be essential to changing these trends.

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). The familiarity of this route of administration of opioids combined with the widespread availability of these opioids makes for an unfortunate combination. A study of patients diagnosed with opioid abuse disorder showed that almost 80% of these patients had a prescription for opioids before the first diagnosis of opioid abuse (45). This study was also able to show that of the 20% that did not have a previous prescription, over half of them had a close family member who had a prescription before the first diagnosis of opioid abuse. This suggests that the availability of opioids from a family member can be a risk factor for abuse. Most individuals who overdose on prescription opioids either took more than the dose they were prescribed or took pills that were prescribed to someone else (40). 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).

In the mid-2000s, at least some of the opioids being used on the streets came from online pharmacies. These were websites one could visit and order prescription drugs to be sent to the consumer through the mail. As many as 89% of these pharmacies did not require a prescription to order controlled substances in 2005, and of those that did, a majority of them accepted a fax of the prescription (44). The faxed documents did not have safety measures put in place to ensure these documents were not duplicated or forged. Since

2008, several laws have been passed to reduce the illicit trade of opioids through these online pharmacies (44,46). The rise of internet prevalence and internet anonymity has also led to a new online avenue for the distribution of opioids. The dark web is a system of encrypted websites which can only be accessed using The Onion Router (Tor) browser. This is a browser designed to allow the user to browse the internet in complete anonymity. This anonymity has opened up the door for illicit sale of prescription opioids. A study of drug sale by Aldridge and Decary-Hetu on an "open" online "cryptomarket" showed an estimated monthly revenue of $284,972 from the sale of opioids, many of which were for orders ranging from $100.01-$500.00 (47). Because the dark web is not regulated and the Tor browser allows for complete anonymity, the internet will continue to be a challenge for those trying to monitor the illicit sale of opioids.

"Doctor shopping" does not make up a large proportion of the opioids prescriptions filled in the United States. However, this activity is concerning because opioids obtained in this manner could be diverted to illicit users. A study evaluating "doctor shopping" in the United States found only about 0.7% of those who receive opioid prescriptions are identified as probable doctor shoppers (48). This study reported these individuals average 32 prescriptions apiece from 10 different prescribers and make up 1.9% of the overall number of prescriptions and 4% of the total weight of prescribed opioids. These individuals are seeking a large number of prescriptions for large doses, a behavior not necessary for patients receiving opioids for legitimate medical uses. While studies definitively showing this activity is primarily used for acquiring non-medical use are not available, the number of these "doctor shoppers" more than doubled from 1999 to 2007 in California (49). The increase in the prevalence of these "doctor shoppers" over a time

coinciding with an overall increase in opioid use and demand indicates a connection to this behavior and opioid addiction and dependence. Efforts to improve coordination between medical practitioners and the prescription of opioids are already underway to help combat this problem (48). Further studies to determine risk factors for this type of opioid seeker could be very helpful for reducing this behavior.

One study found that about one-third of the chronic opioid patients in the study received early refills with no significant difference between the three hospitals included in the study (50). An early refill will, in general, mean one of two things, the patient has used more than was prescribed, or the patient has given their prescribed pills to someone else. While this is concerning, the former may be a more reasonable explanation given that opioids lose analgesic effectiveness over time, and a patient may resort to taking more pills to manage pain. This idea is reinforced by a study reporting much lower rates of early refills in an overall population of all patients prescribed opioids (51). Research concerning the rates at which prescription refills are sold is lacking and may represent important information useful for controlling the flow of opioids to the streets.

Physicians prescribing opioids must be examined as a factor contributing to the opioid epidemic. There are many factors involved in the physician's role in this problem, but some are of particular significance. Another factor is patient satisfaction and how these opinions have come to affect the way physicians practice. While most physicians will refuse to prescribe opioids to patients suspected of abusing them, there are times the physician may feel pressured to prescribe them anyway. This can be due to political reasons, such as patient satisfaction surveys, because of the availability of completely subjective patient reviews online. A feeling that suffering is something technology should

be able to prevent has contributed to this problem for physicians. A doctor who chooses not to prescribe opioids to a certain patient may be perceived as an undesirable physician and could face professional repercussions for using prudent judgment (52).

The rise in opioid abuse is not limited to prescription opioid pain pills. Heroin use has increased in the United States over the last decade. Heroin use, as well as the use of other illicit drugs, is monitored with a published survey conducted by the Center for Behavioral Health Statistics and Quality (CBHSQ) and published in the National Survey on Drug Use and Health. The number of people who had used heroin in the last 30 days and in the last year has increased over the last decade with a significant increase in individuals between the ages of 18 and 25 (53). This is likely due to the increase in popularity of opioid pain pills. A review of surveys interviewing heroin users who used opioid pain pills before the first time the individual used heroin range from 40% to 86% but was enough to suggest a relationship (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).

The BBB serves as a physical, through tight junction proteins, and biochemical, through transport proteins, barrier helping to maintain the ionic homeostatic environment of the central nervous system (CNS). The BBB is key to maintaining proper function of neurons. Evolutionary studies have shown that this type of barrier was essential for the development and function of increasing complex brains in vertebrates (69,70). Because the brain is the most crucial organ in the body, the final important role of the BBB is to

help protect the CNS from compounds that exist in the bloodstream. Unfortunately, this is not limited to dangerous compounds and serves as a challenge for pharmacological agents as well (68). Opioids are a particularly notable class of drugs affected by the BBB because of the control the BBB has on the access of opioids to the CNS (56).

The discovery of the BBB dates back to 1885 when German immunologist Paul Ehrlich noticed peripherally administered dyes would stain all of the organs of an animal except the brain (71,72). While this was originally attributed to differences in binding affinities, further pharmacological research suggested that this effect was not limited to dyes, but other compounds could be administered peripherally and not found in the brain. A student of Ehrlich named Edwin Goldmann coined the term "blood-brain barrier" following further experimentation of his own where he discovered intrathecal administration of dye resulted in staining of the brain and not the peripheral tissue (73,74). This observation showed that this barrier prevented both transport out of the brain and transport into the brain. Tore Broman was one of the first to argue that the vascular endothelial cells were responsible for the action of the barrier, not astrocytes or pericytes (75). Broman also showed hypertonic solutions injected intrathecally could open the barrier and that the integrity of the barrier was compromised in some disease states such as stroke. It was not until the introduction of electron microscopy in the 1960s that endothelial cells were shown to make up the barrier (76). These studies also led to visual proof of tight junctions between the endothelial cells making up the barrier (77). A study by William H. Oldendorf using radiolabeled modifiers showed that different amino acids, amines, and sugars were able to be taken up into the brain at different concentrations (78). This observation suggested that there was a level of control at the

BBB and that differences in characteristics of compounds affected the ability of these compounds to cross the barrier. Freeze fracture analysis showed that the tight junctions of the BBB could block molecules as small as 10-15 Å and the transendothelial electrical resistance was found to be very high, further reducing the permeability of the barrier (68).

Overcoming the BBB is essential to systemically administered drugs meant to enter into the brain. The challenges and basic techniques to overcome these challenges were described by Pardridge (79). Briefly, only lipid-soluble small molecules under 400-600 Da can pass through the barrier freely. This leaves an enormous catalog of compounds that are unable to cross the BBB in pharmacologically meaningful doses. Because the BBB has numerous proteins that transport compounds out of the brain, systemic administration has continued to be a challenge. Certain chemical strategies have been adopted to increase drug delivery into the brain, but these are primarily limited to reducing the size and increasing the lipophilicity of the compound. Limiting hydrogen bonding as much as possible is important to creating a compound that will be able to surmount the BBB.

Strategies meant to improve delivery by means other than modification of the original compound have also been adopted to overcome the problem of drug delivery to the CNS presented by the BBB. Invasive techniques, as discussed by Tam et al., include BBB disruption and direct injection into the CNS (80). Briefly, techniques for disruption of a specific region of the BBB have included osmotic shock, using focused ultrasound, or electromagnetic energy to cause barrier disruptions. These disruptions create a leak in the barrier sufficient for passive transport of drugs into the brain, but are not specific and could also transport undesirable compounds. Direct delivery into the CNS can be

accomplished with a transcranial injection or by implantation of a drug loaded wafer. These techniques require exact placement and rely on diffusion, limiting the range of delivery of the drug. Recent efforts to overcome the BBB have included the introduction of loaded nanoparticles as a method of delivery. A study in which nanoparticles were loaded with the analgesic dalargin, which normally has no effect when injected intravenously, showed the nanoparticles were able to elicit an analgesic effect in rats (81). Cell-penetrating peptides have also been suggested as means to cross the BBB. These are sequences of amino acids that are capable of traversing mammalian plasma membranes and the BBB and can act as a ferry by which molecules can be transported into the brain (82).

Peripheral inflammatory pain (PIP) induced by an injection of λ-carrageenan is sufficient to cause alterations in integrity and p-gp function at the BBB (6,83–85). PIP is capable of altering the permeability of the membrane and allowing for the increased introduction of xenobiotics into the brain by creating a leakiness in the tight junctions at the BBB (83). PIP in the hind paw of a rat is sufficient to reduce the analgesic effect of morphine in the tail of a rat, suggesting a systemic effect on opioid efficacy (6). Interestingly, PIP has been demonstrated to be sufficient to trigger a change in trafficking of p-gp from nuclear stores to the luminal membrane of endothelial cells as well as an increase in ATPase activity (84,86). These observations highlight a relationship between PIP and post-translational modification of active p-gp expression at the BBB. Many opioid analgesics are substrates of p-gp, and if a pain state is sufficient to increase the activity of p-gp, this presents a clear challenge for ensuring opioid efficacy.

Opioids 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 (5,92).

Heroin has a potency 2-fold greater than morphine and crosses the BBB more readily than morphine (93). Although heroin is similar in structure to morphine, this drug is more

lipophilic than morphine leading to an increased potency. Heroin is metabolized into 6-monoacetylmoprhine (6-MAM) and subsequently to morphine in the blood (94). As stated before, morphine is a substrate of p-gp and inhibition of this protein increases transport into the brain. In a study by Seleman *et al.* in which the effect of a p-gp inhibitor co-administered with heroin, 6-MAM, and morphine, only morphine transport was increased (56). This study showed the transport of heroin and 6-MAM were unaffected by p-gp inhibition, suggesting that this may also play a role in the higher potency of heroin over morphine. 6-MAM has been shown to have an even greater affinity for mu opioid receptors than morphine and a greater analgesic effect (94). 6-MAM has a short half-life in humans and is rapidly metabolized into morphine. Although heroin 6-MAM can enter the BBB, p-gp still plays a role in the effect of heroin on the CNS because of the rapid metabolism to morphine, which is a p-gp substrate (95).

Oxycodone is a potent opioid often prescribed to manage moderate to severe pain. When co-administered with the p-gp inhibitor valspodar, transport of oxycodone into the brain was not shown to be affected (96). Oxycodone has a lower affinity for mu opioid receptors than morphine, but similar doses have been shown to be effective in the management of post-surgical pain (97). This prompted experimentation examining the relative transport of oxycodone into the brain compared with morphine. Oxycodone was shown to transport into the mouse brain in concentrations six times higher than morphine (98). The relationship between the BBB and oxycodone is unique because oxycodone can be found at concentrations three times higher in the brain than in the blood (99). This suggests oxycodone may have a transport mechanism that provides an influx into the brain, as opposed to the efflux mechanism associated with most opioids.

Methadone is a synthetic opioid that is used in the treatment of especially chronic pain and for opioid dependence (31,100). Methadone is thought to have lesser side effects than many other opioids, so the chronic administration is often considered more manageable than other opioids (31). Methadone is administered as a racemic mixture of both the R- and S-enantiomers of the drug (101). Methadone is metabolized into the pharmacologically inactive compound 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) (102). A study by Wang *et al.* showed both the R- and S-enantiomers of methadone are substrates of p-gp, limiting the delivery of the clinically used racemic methadone across the BBB (101). This study made a comparison of concentrations of methadone found in multiple tissues throughout the body of wild-type and ABCB1a (the gene encoding p-gp in mice) knockout animals showed significantly higher concentrations of both enantiomers of methadone in only the brain.

Conclusion

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.

Chapter II- Experimental Data

INTRODUCTION

The term “opioid epidemic” is used to describe a recent increase in the prevalence of opioid use in America. The number of individuals using opioids and the number of opioid prescriptions written and filled is at an all-time high with more than 240 million prescriptions written in 2014 (39). Long-term therapeutic and recreational use of illicit and prescription opioids have become increasingly common (54). Long-term opioid use, even in a therapeutic setting, will lead to the development of opioid tolerance (103). Opioid tolerance is a state in which a patient requires increasing doses of opioids to achieve the same analgesic effect. More long-term opioid use also led to more patients suffering from addiction and dependence disorders; this population exceeds the addiction treatment capacity of most states in the United States (39). Because opioid tolerant and dependent populations are numerous and growing, addressing the problem of acute pain management for these patients is a clinical challenge. Improper pain management is costly to both the patients and the institutions treating them. Problems adequately addressing pain lead to increased recovery times and more time committed by physicians to manage pain (21). Currently, Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and local anesthetics are the primary therapeutics used to manage acute pain in these patients (104). Treatment strategies involving NMDA receptor antagonists as a fast acting therapy for pain management in opioid tolerant patients have been attempted (105). Ketamine is the therapy that has been used clinically, but the interaction between ketamine and opioids is complex and not fully understood which must be considered (105–108). Surgical patients can benefit from complex plans consisting of pre-operative opioid dose

tapering with an increase in opioid dose after the procedure (104). This strategy is only an option when the surgery is planned and cannot be used in an emergency, giving this strategy limited clinical application. Strategies improving opioid efficacy for tolerant patients would eliminate the need for complicated strategies. The blood-brain barrier (BBB) offers a site where opioid efficacy at the central nervous system can be improved.

The BBB is both a physical and biochemical barrier primarily caused by the characteristics of the endothelial cells that line the lumen of brain capillaries. These cells control the delivery of compounds between the brain and circulating blood with high specificity. The prototypical clinically used opioid, morphine, is a known substrate of p-glycoprotein (p-gp). Increased p-gp at the BBB is associated with decreased morphine efficacy (5,6). The biochemical barrier created by a series of efflux transporters, the most notable being p-gp, is responsible for many of the therapeutic challenges associated with the BBB. P-gp is an efflux protein that utilizes ATP hydrolysis to transport substrates against their concentration gradient (62). This action allows p-gp to transport an impressive array of blood-borne substrates ranging from xenobiotics to therapeutic drugs back into circulation (109). A model of peripheral inflammatory pain (PIP) induced by an injection of λ-carrageenan into a rat's hind paw is sufficient to reduce the analgesic efficacy of morphine (6). This effect can be reversed by administration of a nerve block, demonstrating a clear relationship between pain and reduction of the analgesic effect of morphine (85). The relationship between pain and p-gp was further demonstrated by observations of a trafficking event of p-gp from nuclear reservoirs to the luminal surface of these brain endothelial cells (86). This trafficking moves p-gp to a location where the protein can be activated and increase the overall export of these substrates.

Opioid tolerance is associated with an increase in overall brain p-gp expression. However, these changes are region specific and do not occur in the endothelial cells in the brain microvessels (92,110). Changes in efflux would thus be due to p-gp trafficking, not because of a change in overall p-gp expression. Trafficking of p-gp is related to a reduction of morphine efficacy and this trafficking is heavily modulated by acute PIP events. We hypothesized a relationship between the clinical challenges of acute pain management in chronic opioid users and this pain-mediated trafficking. This opened the line of investigation into a possible relationship between p-gp trafficking and long-term exposure to morphine. This study was undertaken to establish this relationship as a potential future target for more effective pain therapies in long-term opioid-treated populations. We established a model of long-term opioid exposure leading to tolerance in female rats using osmotic mini-pumps. This model was used to assess changes in p-gp trafficking between chronic morphine-exposed and non-exposed rats during a PIP challenge. Morphine exposure was sufficient to cause a 2-fold increase in p-gp trafficking away from the nucleus when animals were exposed to PIP.

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 BBB (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). This led to the investigation of the impact long-term opioid exposure may have on p-gp trafficking. Most long-term opioid experiments in rats available in the literature use male rats. To perform these experiments, there was a need to establish a model for chronic opioid exposure in female rats. Osmotic Mini-Pumps were used to deliver 5mg/kg/day of morphine at a consistent dose.

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.

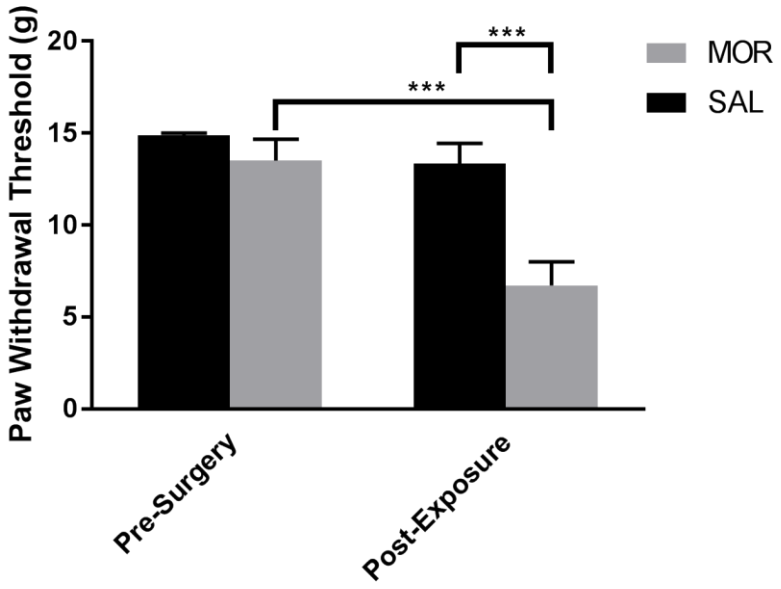

Figure 1. Six day exposure to morphine from osmotic mini-pump causes opioid-induced hyperalgesia in female rats. Mechanical allodynia was measured in female Sprague-Dawley rats before receiving surgery to insert an osmotic mini-pump and after six days exposure to the pump. Animals received morphine (5 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).

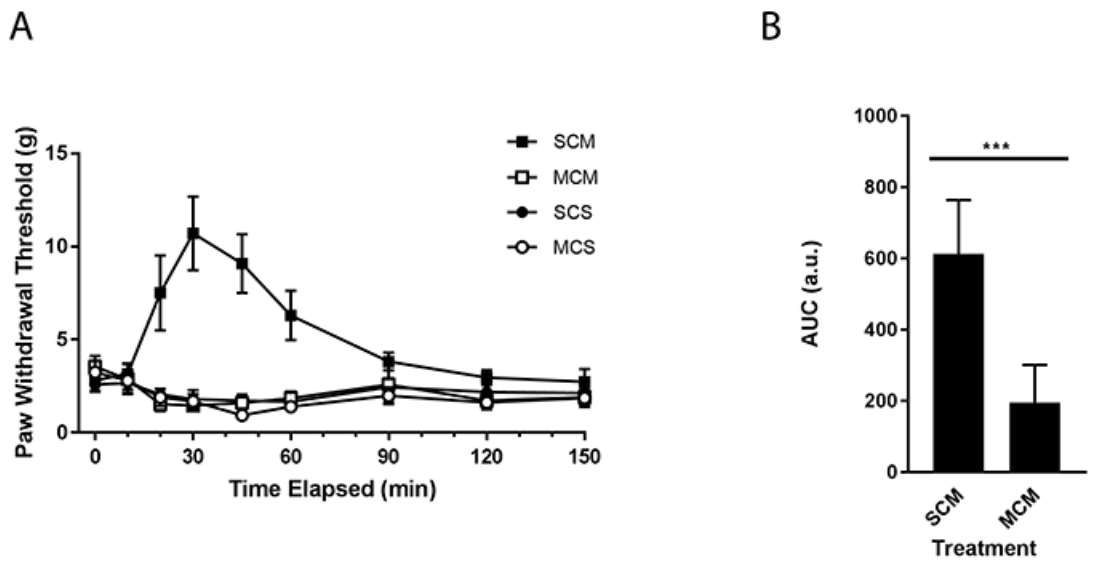

Figure 2.Prolonged morphine exposure eliminates the anti-nociceptive effect of acute morphine administration on mechanical sensitivity. (A) Mechanical paw withdrawal threshold was determined by the von Frey mechanical sensitivity test in rats exposed to 5 mg/kg/day of morphine or saline for 6 days, then 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)/ λ-carrageenan hind paw injection (0.1 mL)/ Saline intraperitoneal injection (1mL/kg);SCM: Saline Osmotic mini-pump (24μL/day)/ λ-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)/ λ-carrageenan hind paw injection (0.1 mL)/ Saline intraperitoneal injection (1mL/kg);MCM: Morphine Osmotic mini-pump (24μL/day) (5mg/kg/day)/ λ-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 ($p<0.0001$)

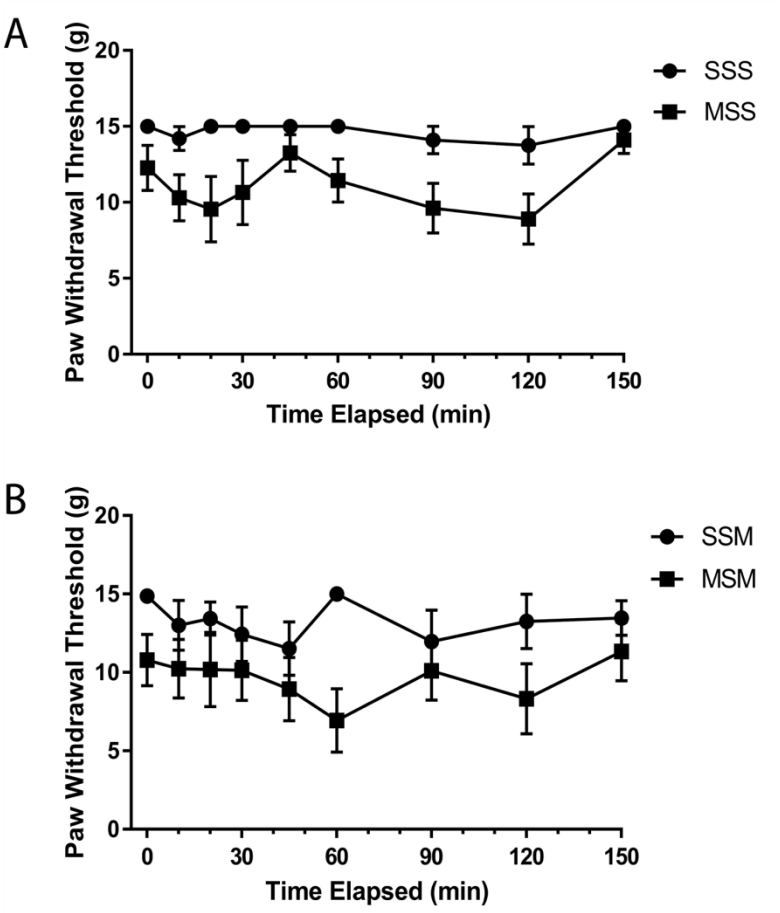

Figure 3. Chronic morphine administration tends to reduces mechanical sensitivity thresholds determined by the von Frey test in the ipsilateral paw. The symbols mean: SSS: Saline Osmotic mini-pump (24µL/day)/ saline hind paw injection (0.1 mL)/ Saline intraperitoneal injection (1mL/kg); SSM: Saline Osmotic 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.

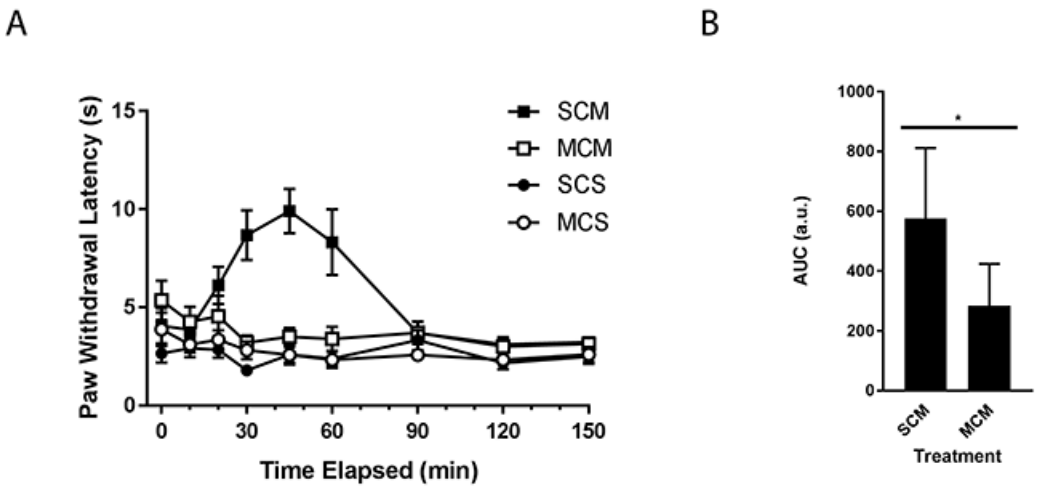

Figure 4. Prolonged morphine exposure eliminates the anti-nociceptive effect of acute morphine administration on thermal sensitivity. (A) Thermal paw withdrawal threshold was determined by the Hargraves thermal sensitivity test in the ipsilateral paw of rats exposed to 5 mg/kg/day of morphine or saline 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)/ λ-carrageenan hind paw injection (0.1 mL)/ Saline intraperitoneal injection (1mL/kg);SCM: Saline Osmotic mini-pump (24μL/day)/ λ-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)/ λ-carrageenan hind paw injection (0.1 mL)/ Saline intraperitoneal injection (1mL/kg);MCM: Morphine Osmotic mini-pump (24μL/day) (5mg/kg/day)/ λ-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).

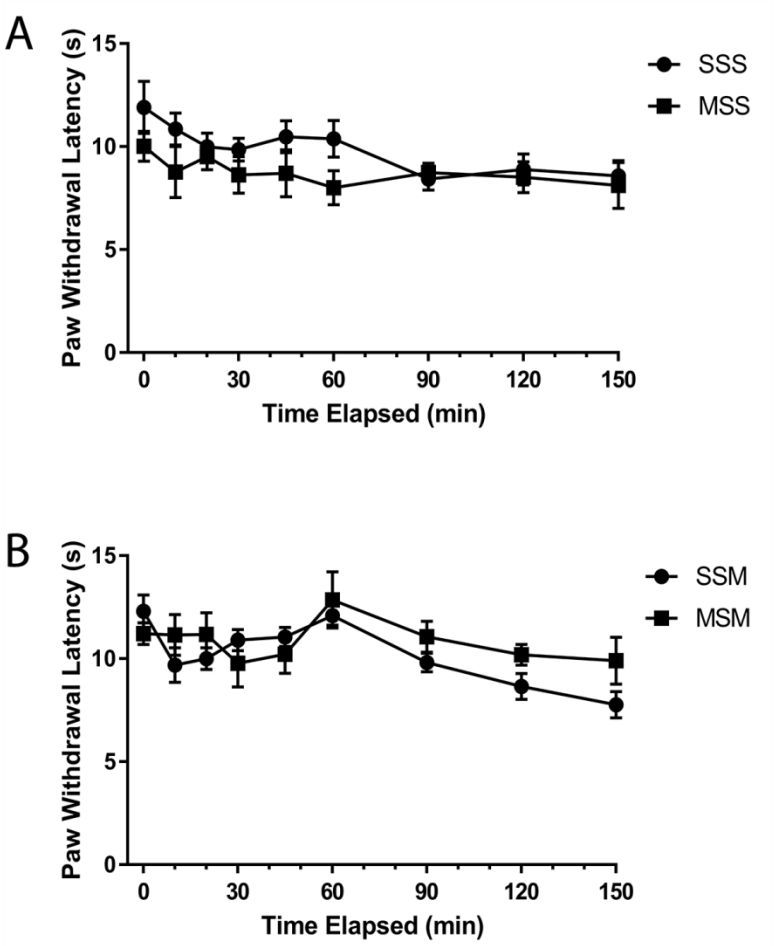

Figure 5. Chronic morphine administration had no effect on thermal paw withdrawal measurements using the Hargreaves test on the ipsilateral paw. The symbols mean: SSS: Saline osmotic mini-pump (24µL/day)/ saline hind paw injection (0.1 mL)/ Saline intraperitoneal injection (1mL/kg); SSM: Saline Osmotic 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 the mean +/- SEM (n=9).

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 6B). 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.

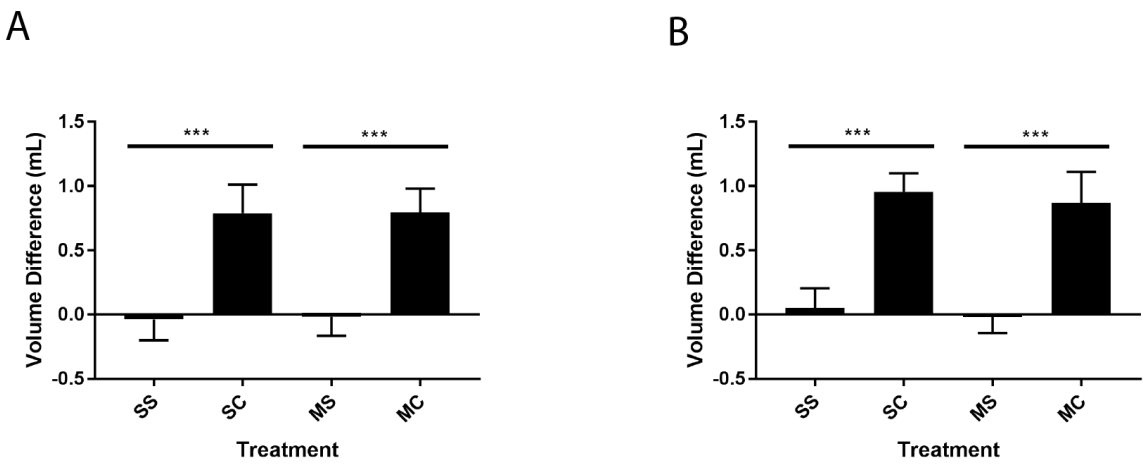

Figure 6. Chronic morphine exposure has no effect on hind paw edema following λ-carrageenan exposure paw edema measurements in the animals tested for mechanical allodynia (A) or thermal sensitivity (B). The symbols mean: SS: Saline mini-osmotic pump/ Saline hind paw injection (100µL); SC: Saline mini-osmotic pump/ λ-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/ λ-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 7C).

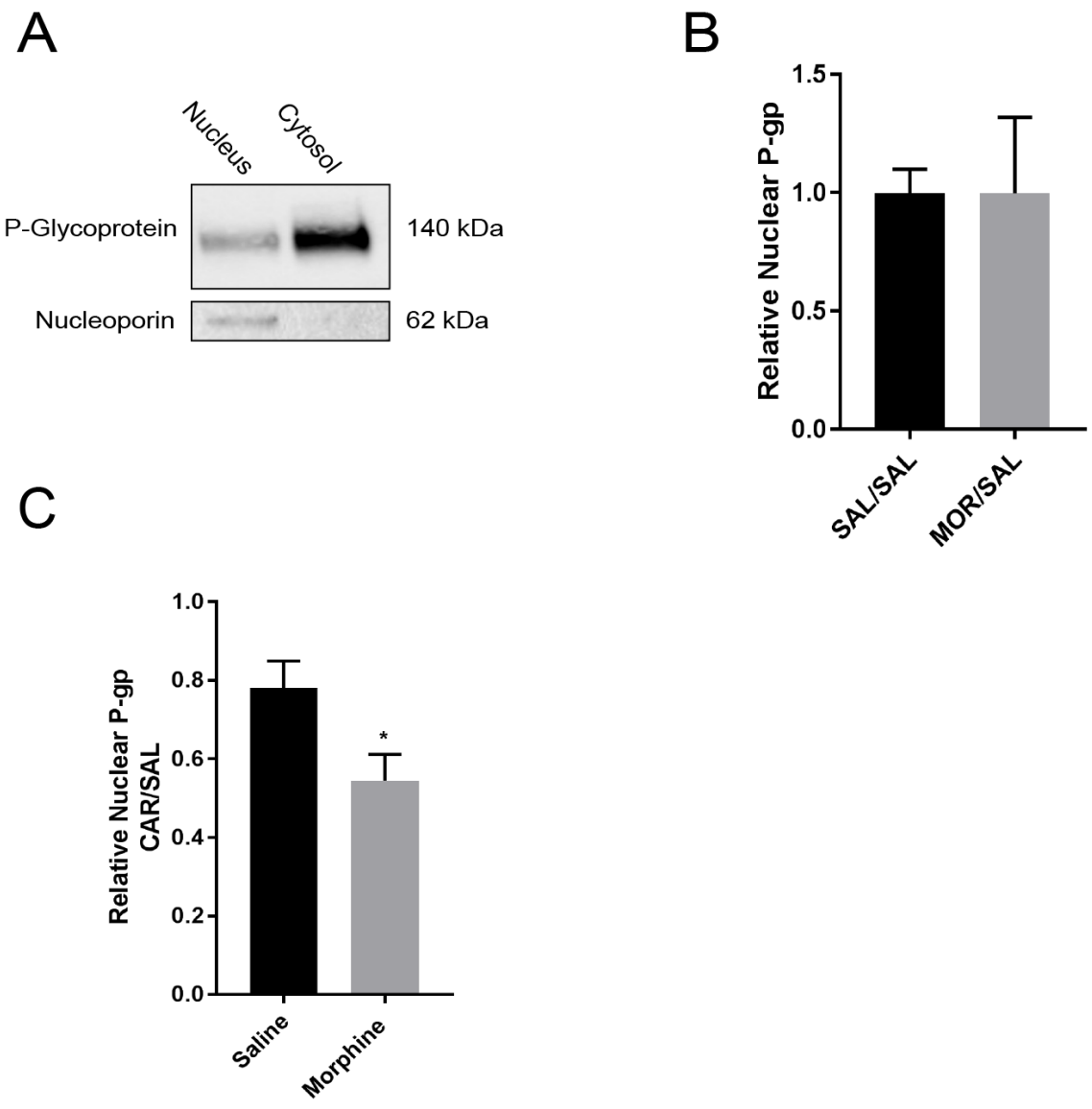

Figure 7. Chronic morphine exposure increases trafficking of p-glycoprotein away from the nucleus after peripheral inflammatory pain. (A) Representative immunoblot indicating the p-gp and nucleoporin in the nuclear and cyctosolic isolates. **(B)** P-gp expresion normalized to nucleoporin in the nuclear 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.

The observed effective window for an acute dose of morphine for reduction of mechanical allodynia was from 20 to 60 minutes in the animals who had not been pre-exposed to opioids with osmotic minipumps. Animals that had been pre-exposed to morphine and received an acute dose of morphine were indistinguishable from those that had received an acute injection of saline. The elimination of the antinociceptive effect of morphine at this dose is a clear demonstration of morphine tolerance.

This experiment shows that the nuclear trafficking of p-gp induced by PIP is increased in the presence of chronic opioids. There is a nearly 2-fold increase in the p-gp trafficking away from the nucleus in the morphine-treated animals relative to those who received a saline pump. There is robust literature evidence that there is an increase in p-gp expression in the brain of both rats and mice with long-term exposure to opioids (5,92,114). These changes are mostly isolated to the cortex, hippocampus and large vessels of the brain and the available literature on this subject shows there is no change in expression of p-gp in isolated microvessels (92,110). This is an important distinction for investigations of trafficking within these endothelial cells. A consistent level of expression of p-gp in these cells means that comparisons of the nuclear protein concentration between animals of different treatment groups could be made in a valid way. This assumption is further strengthened by this experiment because there is no difference between p-gp concentrations in the nuclei of BBB endothelial cells isolated

from animals without PIP. Of note is the observation that chronic exposure to morphine was not sufficient to induce p-gp trafficking. Another factor must be present to induce the trafficking. In this case, p-gp expression at the nucleus remained the same when animals were exposed to morphine without PIP. More investigation into the possible mechanism causing the increased response is required. Possible causes include a decrease in threshold required to induce the trafficking or an increase in the magnitude of the trafficking response due to the extended presence of a substrate.

The increase in PIP mediated p-gp trafficking caused by long-term morphine exposure can be expected to further lower morphine efficacy in long-term opioid-treated patients experiencing acute pain. PIP decreased opioid efficacy and caused trafficking of p-gp from the nucleus to the luminal surface of BBB endothelial cells in non-opioid tolerant animals (6,86). Because morphine is a p-gp substrate, these observations suggest a relationship between pain induced p-gp trafficking and the decrease in morphine efficacy in animals in pain. An increase in p-gp trafficking caused by long-term exposure to morphine would likely further decrease the efficacy of opioid analgesics. Sources of acute pain which require the use of opioid pain relievers in clinical patients include injury and post-surgical pain. Pain management is an essential component to recovery from surgery or an injury. Improper or insufficient pain management is related to both increased recovery times and time spent managing pain (104). With opioid use and the number of opioid prescriptions at all-time highs, there is an ever growing need for strategies to improve pain management in opioid tolerant patient populations (39,40). The observation that chronic exposure to morphine increases PIP induced p-gp trafficking

may lead to an increased quality in the treatment of patients with long-term opioid treatments if the trafficking can be mediated.

If trafficking could be attenuated, this would open up a novel avenue for treatment of acute pain in opioid tolerant patients. Reduction in p-gp trafficking would help increase the maximal concentrations of drugs into the brain. A potential benefit of reducing p-gp trafficking instead of using an antagonist is trafficking would spare the basal levels of p-gp. Preserving the basal level of p-gp ensures the protective nature of this protein is intact while the detrimental effect p-gp has on drug delivery is attenuated. During acute pain, if the trafficking does not occur, more opioids will be able to enter the brain, reducing the need for a higher dose. This means that the peripheral side effects, not affected by a less permeable BBB, will be less of a problem and pain can be more effectively managed.

Changes to p-gp trafficking induced by long-term exposure to morphine, a p-gp substrate, may have ramifications for a variety of therapies because p-gp has a large number of substrates including drugs of many different classes (118). Chronic administration of Rifampicin, an antibiotic that is also a p-gp substrate, has been shown to increase whole brain p-gp expression in a way similar to morphine (114). This suggests the mechanism may be triggered by the presence of a substrate, not due to a unique effect of a given compound. Characterization of the mechanism underlying long-term exposure to a p-gp substrate and increased p-gp trafficking induced by pain would benefit many patients.

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.

Chapter III- 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.

Studies have shown that other p-gp substrates, such as the antibiotic rifampicin, can elicit a similar increase to overall brain p-gp levels as those caused by chronic morphine

exposure (114). This suggests that any therapy involving the long-term administration of a p-gp substrate may also cause an increase in the trafficking response. Because so many compounds are substrates of p-gp, trafficking of this protein is a clinically significant problem. Investigation into the mechanism underlying p-gp trafficking could be used to the advantage of clinicians. Decreasing trafficking would allow a greater concentration of morphine to enter the CNS during pain.

A pain model mimicking an injury or surgical wound more closely would help to determine if any aspect p-gp trafficking is unique to PIP. Repeating this study with male rats would help determine if there is a sex difference in this effect. Chronic pain patients are a population who require long-term opioid therapies, determining if chronic pain has an impact on pain mediated p-gp trafficking could be helpful for applying this observation to these patients.

The model of pain utilized in this study utilizes an injection of the biological agent λ-carrageenan to induce PIP. PIP causes an increase in sensitivity at the site of injection and blockade of the nerve signal from the site of injection reverses the effect on the BBB (85). This study does not eliminate the possibility that the inflammatory component of PIP could play a role in the observed modification of the BBB. Experiments by Campos *et al.* show that the modification at the BBB is not due to an inflammatory response, but the pressure caused by the inflammation may impact the pain signal. The paw incision model of post-surgical pain is a procedure in which a 1 centimeter long incision is made down the length of the plantar surface hind paw of a rat and then resealed (119). This model allows for a reliable increase in mechanical sensitivity and could thus be used as a source of pain for BBB change investigations.

Repeating this study with male rats would determine the effect of sex on PIP mediated p-gp trafficking. Literature evidence supports estrogen mediating the analgesic effects of morphine and oxycodone (120,121). A study by Bodnar and Kest showed analgesia caused by mu opioid receptor agonists, like morphine, is generally more effective in male rats (117). This study also demonstrates that morphine tolerance, the primary concern with long-term opioid use, has a faster onset and stronger effect in male rats compared to female rats. This suggests that an effect caused by chronic morphine administration, such as the increased trafficking away from the nucleus during an acute pain stimulus observed in this study, may be increased as well. Opioid-induced hyperalgesia (OIH) is a paradoxical state in which long-term administration of opioid analgesics makes patients more sensitive to nociceptive stimuli. The study by Bodnar and Kest demonstrates that OIH causes a greater increase in sensitivity in female rats (117). Morphine without painful stimuli did not change basal levels of nuclear p-gp in this experiment; morphine only intensified the trafficking response. OIH could be involved in the increased trafficking of p-gp during PIP. If a relationship exists between OIH and this increase in trafficking, the relationship would suggest that female rats could have an increased trafficking event compared to males. Characterization of sex differences in PIP mediated p-gp trafficking would be important to investigating the mechanism underlying this observation. Repeating the study with male rats would also serve to put this research into a context in which it could be compared to many other studies already found in the literature which have predominantly used male rats.

A group of patients who is often given long-term opioid therapy is the group of patients being treated for chronic, non-cancer related pain. Chronic pain states like these are

pathological in nature and have many unique characteristics. While the use of opioids for chronic pain therapy is controversial, chronic opioid therapy has become an increasingly common treatment for chronic non-cancer pain (122). Many types of chronic pain can affect patients for extended periods of time, so the use of opioids to manage this pain results in patients receiving opioids for an undetermined amount of time and essentially sets them up for on-going use. (123). Because of this long-term treatment, injury or surgery requiring pain management will always be in the context of chronic opioid use for these patients. Performing experiments on animals using a model for chronic non-cancer pain would allow us to determine if p-gp trafficking could play a role in pain management in these patients. Because pain is a key component of the model used in this study, characterization of the effect chronic pain has on pain mediated p-gp trafficking at the BBB would be critical. There is a distinct lack of information concerning the effect of chronic pain states and changes to the BBB. Research concerning chronic pain and the BBB focuses on chronic models of inflammatory pain, echoing the results of previous studies using the acute PIP model used in this study (124). 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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