AMPK AS A NOVEL TARGET FOR TREATMENT OF NEUROPATHIC AND POST-SURGICAL PAIN

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SIGNED: Dipti Vilas Tillu

DEDICATION

To my beloved parents

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CHAPTER I

INTRODUCTION

1.1. Pain

Pain is an unpleasant sensory experience to a noxious stimulus which everyone experiences in their daily life. It is an essential early warning sign to injury as well as a defense mechanism that usually elicits reflex withdrawal and thereby promotes survival and well-being of an individual from further injury (Stein, 2013). The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." Although it is essentially a sensation, pain has strong cognitive and emotional components. This characteristic makes it difficult to quantify or predict pain. It may vary in intensity (mild, moderate, or severe), quality (sharp, burning, or dull) and duration (transient, intermittent, or persistent). Nearly 50% of Americans seeking medical attention report pain as their primary symptom, thus making pain the single most frequent reason for physician consultation in the United States (Abbott and Fraser, 1998; Turk and Dworkin, 2004).

Physiological pain, in its simplest form, is initiated when a noxious stimulus to peripheral tissues such as skin is transduced and transmitted by specialized primary afferent neurons, the thinly myelinated Aδ-fibres and unmyelinated C-fibers, from the periphery to the superficial laminae of the dorsal horn of the spinal cord. In the spinal cord, neurotransmitters from the central terminals of Aδ- and C-fibers activate second order neurons at the first pain synapse which relay the nociceptive signal to higher brain centers

ultimately resulting in activation of specific central nervous system (CNS) areas leading to the perception of pain (Hansen and Malcangio, 2013).

1.2. Acute vs. Chronic pain

Pain is inherently protective in nature and therefore is usually transitory, lasting only until the noxious stimulus is removed or the underlying damage or pathology has healed, and known as acute pain. On some occasions changes of the pain pathway can lead to hypersensitivity such that pain becomes pathological and persists even after the initial noxious stimulus is removed or the pathology has completely healed. In such cases the pain becomes chronic and debilitating and serves no protective purpose (Markenson, 1996). Chronic pain can last for months or years after the initial injury has healed. What distinguishes acute transitory pain from the chronic persistent pain is not the duration of the pain but the inability of the body to restore its physiological functions to normal homoeostatic levels (Loeser and Melzack, 1999). Chronic pain is characterized by a heightened responsiveness to both noxious (hyperalgesia) and non-noxious stimuli (allodynia).

1.3. Chronic pain

Chronic pain is a major health problem affecting more than 1.5 billion people worldwide. In the United States about 100 million individuals are estimated to suffer from chronic pain (Institute of Medicine, of the National Academies 2011). Medical treatments for chronic pain in the US costs the country approximately 150 billion dollars in health care and lost work productivity each year (Tracey and Mantyh, 2007), American Productivity Audit). While the

management and treatment of acute pain is well developed and reasonably good, the needs of chronic pain patients are largely unmet. More than 50% of chronic pain patients experience little or no control over their pain (American Pain Foundation 2006), creating an enormous emotional and financial burden to sufferers, their families, and society (Tracey and Mantyh, 2007). Thus there is an urgent need for developing novel diagnostic and treatment approaches for chronic pain conditions.

Chronic pain patients comprise a heterogeneous group that includes patients with various pain conditions including inflammatory, neuropathic, cancer-induced, post-surgical, post-herpetic neuralgia, fibromyalgia etc. (Hansen and Malcangio, 2013). Based on pathophysiology i.e. the functional changes associated with or resulting from disease or injury, chronic pain can be classified as nociceptive, neuropathic and mixed or undetermined cause. Nociceptive pain is induced due to ongoing tissue injury e.g. post-surgical pain while neuropathic pain results from damage to the brain, spinal cord, or peripheral nerves. Chronic pain can also result due to mixed or undetermined causes e.g. fibromyalgia. The focus of this research work is mostly on the two types of chronic pain – neuropathic pain and the persistent post-surgical pain.

1.4. Neuropathic pain

1.4.1. Introduction

Neuropathic pain is a state of chronic pain resulting from peripheral or central nerve injury either due to acute events (e.g. amputation, spinal cord injury) or systemic disease (e.g. diabetes, viral infection and cancer) (Zhuo et al., 2011). Over 5 million Americans suffer from neuropathic pain (Berger et al., 2004)

making it a highly prevalent condition. International Association for the Study of Pain defines neuropathic pain as any pain caused by lesion or dysfunction of the somatosensory nervous system. Although neuropathic pain comprises of heterogeneous conditions which cannot be explained by a single etiology or specific lesions, the clinical manifestation of the pain is similar across the different neuropathic syndromes. These symptoms include paraesthesias i.e. tingling or skin crawling sensation, spontaneous i.e. not stimulus-induced ongoing pain, as well as electric shock-like sensations, burning and/or shooting pain. Many patients with neuropathic pain also experience evoked pain i.e., pain evoked by a stimulus which is characterized by allodynia (pain to non-noxious stimulus) and hyperalgesia (increased pain sensitivity of nociceptive stimulus). Another feature of neuropathic pain is summation, which is the progressive worsening of pain evoked by slow repetitive stimulation with mildly noxious stimuli e.g. pin pricks (Baron et al., 2010).

As mentioned previously, the origin of neuropathic pain can either be peripheral or central. Even so, peripheral neuropathic pain appears to have a greater prevalence than the central neuropathic pain. Peripheral neuropathic pain (PNP), defined as pain arising as a direct consequence of a lesion or disease affecting the peripheral somatosensory system, is associated with many conditions such as diabetic neuropathy, trigeminal neuralgia, and postherpetic neuralgia. PNP is estimated to affect up to 8% of the general US population and is responsible for at least 25% of the clinic visits related to pain (Bouhassira et al., 2008; Perez et al., 2013). PNP is also marked with a greater severity of pain than chronic pain of non-neuropathic origin and can coexist with other conditions depression, anxiety disorders, and impairment in sleep (Gilron et al., 2006; Smith

et al., 2007). Thus with the comorbidities and the significant impact on the health of individuals, PNP causes severe socio-economic burden through effect on the patient's family, the social and work environments, overall reduced productivity and major healthcare costs (Athanasakis et al., 2013; Navarro et al., 2011; Rodriguez et al., 2007). Hence it is important to understand the mechanisms driving neuropathic pain and develop novel therapeutic strategies.

1.4.2. Pathophysiology

Since the development of animal models of peripheral neuropathic pain, considerable advances have been made in understanding the mechanisms driving neuropathic pain. The neuro-imaging techniques such as Positron Emission Tomography (PET) and Functional Magnetic Resonance Imaging (FMRI) provide insights in blood flow and metabolic changes in the brain reflecting the local synaptic activity in defined brain regions which can be co-related to changes in pain threshold (Vranken, 2012). It is known that the mechanisms driving neuropathic pain can be both peripheral (ectopic nerve activity and peripheral sensitization) and central (central sensitization) (Baron et al., 2010; Ludwig and Baron, 2005). In the periphery, after any damage to a peripheral nerve immune cells release mediators including cytokines (IL-1β, IL-6, TNF-α), nerve growth factor (NGF) and protons which contribute to neuropathic pain by activating nociceptors. In addition degenerating nociceptive neurons release neuropetides like CGRP and Substance P which can further trigger vasodilatation and extravasation of inflammatory mediators from peripheral immune cells. As a result the primary afferent neurons, which are silent and unresponsive under normal physiological conditions, exhibit enhanced

sensitivity and start firing spontaneously. This increased spontaneous activity is evident in both, injured fibers as well as adjacent un-injured intact nerve fibers (Moalem and Tracey, 2006; Sommer and Kress, 2004). These changes in the ectopic activity parallel many molecular and cellular changes in the primary afferent neurons. There is an increase in mRNA and protein levels of voltage gated sodium channels and calcium channels and hyperpolarization-activated nucleotide-gated ion channels in the injured and the adjacent intact primary afferents fibers (Blackburn-Munro and Jensen, 2003; Cummins and Rush, 2007; Emery et al., 2011; Liu and Wood, 2011; Perret and Luo, 2009; Pexton et al., 2011; Wood et al., 2004). These changes can lower the threshold for action potential generation leading to spontaneous ectopic activity. Following nerve injury there can also be down regulation of voltage gated potassium channels and up-regulation of TRPV1 channels in nociceptive endings of injured C- fibers resulting in peripheral sensitization i.e. increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields (Baron et al., 2010; Ma et al., 2005). Additionally, sprouting of collateral fibers from intact adjacent sensory axons as well as neuroma formation due to sprouting of damaged nerve ending and involvement of the sympathetic nervous system can further increase the ectopic firing from primary afferent fibers (Amir and Devor, 1992; Vranken, 2012). Continuous ectopic activity in the primary nociceptive afferent neurons can lead to central sensitization. Ongoing discharges of peripheral afferent fibers that release excitatory amino acids (glutamate and aspartate), neurotrophins (BDNF) and neuropeptides (Substance P and CGRP) from the central terminals of nociceptive Aδ and C fibers within the dorsal horn of the spinal cord lead to postsynaptic changes of second-order

nociceptive neurons, such as phosphorylation of NMDA and AMPA receptors (Ultenius et al., 2006) or expression of voltage-gated sodium and calcium channels (Costigan et al., 2009; Hains et al., 2004; Larsson and Broman, 2011; Millan, 1999; Willis and Westlund, 1997). The activation of these ion channels along with activation of microglia, oligodendocytes, astrocytes and loss of spinal inhibitory control can induce neuronal hyperexcitability. This enables low-threshold mechanosensitive Aβ and Aδ afferent fibers to activate second-order nociceptive neurons such that otherwise non-noxious inputs, are perceived as being painful. This condition is called as allodynia. The response of these neurons to supra-threshold stimuli leads to hyperalgesia and there is a progressive increase in the magnitude of C-fiber evoked responses of dorsal horn neurons produced by repetitive activation of C-fibers referred to as the wind-up phenomenon (Woolf, 2011; Woolf et al., 1995). Additionally, supra-spinal structures such as peri acquaductal grey area (PAG), locus coerulus, rostroventromedial medulla (RVM) and several nuclei from the reticular formation, which give rise to the descending modulatory pathways, become dysfunctional after nerve lesions leading to disinhibition or facilitation of spinal cord dorsal horn neurons and to further central sensitization and maintenance of neuropathic pain (Baron et al., 2010; Cui et al., 1999; Millan, 2002).

1.4.3. Current Treatment Strategies

The heterogeneous nature of neuropathic pain mechanisms and the frequently coexisting psychological and emotional components pose a significant challenge in the treatment strategies of neuropathic pain. Several therapeutic classes of drugs are currently prescribed for treatment of PNP depending on the

etiology, symptoms and presence of other comorbidities. Tricyclic antidepressants are often the first choice of drugs selected to alleviate neuropathic pain (O'Connor and Dworkin, 2009; Swenson et al., 2006; Vranken, 2012). And though these drugs are very effective in reducing pain in several peripheral neuropathic pain disorders, treatment is outweighed by their side effects like sedation, urinary retention, dry mouth and weight gain. SSRIs such as duloxetine and venlafaxine and calcium channel α2-δ ligands such as gabapentin and pregabalin are emerging as first-line treatment for various painful polyneuropathies and diabetic neuropathy (Dworkin et al., 2010; Lunn et al., 2009; Moore et al., 2009; O'Connor and Dworkin, 2009; Vranken, 2012; Vranken et al., 2011). Though these drugs have a favorable safety profile and minimal drugs interactions, their effectiveness in managing neuropathic pain is disputed due to conflicting reports in the literature and inconclusive results in clinical trials (Vranken, 2012; Vranken et al., 2011). In addition, high concentrations of gabapentin and its longer half-life can result in renal failure (Bennett and Simpson, 2004). Topical administration of lidocaine has shown efficacy and good tolerability in patients with diverse peripheral neuropathic conditions such as post-herpetic neuralgia (Binder et al., 2009; Dworkin et al., 2010; Galer et al., 2002; Ritchie et al., 2010). Opioid agonists have demonstrated efficacy in a subset of patients with PNP but accompanying complications such as constipation, long term safety, possible association with development of immunologic impairment, opioid-induced hyperalgesia and risk of addiction have been a deterrent in their use as a treatment for neuropathic pain (Dworkin et al., 2010; Kalso et al., 2004; Manchikanti et al., 2012). Finally there are a number of medications that are generally used as third-line treatments for conditions like post-herpetic neuralgia

because of weak efficacy, discrepant results and/or safety concerns (Amr, 2010; Cohen et al., 2011; Dahan et al., 2011; Hocking and Cousins, 2003). These include NMDA receptor antagonists such as dextromethorphan, ketamine and amantadine and topical capsaicin (Derry and Moore, 2012; Wong and Gavva, 2009).

Despite of advances in the treatment strategies and availability of numerous treatment options available to alleviate neuropathic pain, there is no consensus on the most appropriate treatment (Vranken, 2012). Many patients are refractory to existing treatment options. Several randomized control trials that have examined pharmacotherapy for neuropathic pain have indicated that less than 50% of patients experience pain relief following pharmacotherapy and the relief is only partial. Existing treatment options only provide general pain relief values for specific etiologies, which might partially explain the failure to obtain complete pain relief in neuropathic pain conditions. Hence, increased understanding of pain-generating mechanisms and their translation into novel therapeutic targets is necessary for a better therapeutic outcome in patients with neuropathic pain (Baron, 2006).

1.5. Post- surgical pain

Incision associated with surgery causes acute pain and surgery has been identified as a potential major cause of chronic pain conditions (Aasvang et al., 2010; Kehlet et al., 2006; Perkins and Kehlet, 2000). Although pain is a predictable part of the postoperative experience, inadequate management of pain is common and can have profound implications to patient recovery. Unrelieved postoperative pain may result in clinical and psychological changes that

increases morbidity and mortality, costs and decrease in quality of life (Apfelbaum et al., 2003). Pain at the incisional site is the most common medical cause of delayed recovery and discharge after ambulatory surgery and a frequent cause of unplanned admission (Pavlin et al., 2002). Between 10 and 50% of patients who undergo surgery develop chronic pain following surgical procedures such as groin hernia repair, breast and thoracic surgery, leg amputation, or coronary artery bypass surgery (Kehlet et al., 2006). Up to a quarter of all chronic pain patients suffer from persistent pain because of a prior surgery (Crombie et al., 1998). This chronic pain can be debilitating in 2-10% of this population (Johansen et al., 2012; Kehlet et al., 2006) highlighting the importance of gaining a better understanding of chronic pain following surgery and developing novel therapeutics. Population-based studies suggest that both pre-surgery pain state and acute post-surgical pain are predictors of chronic pain following surgery (Hanley et al., 2007; Kehlet et al., 2006; Perkins and Kehlet, 2000). Surveys of hospital care of post-surgical pain demonstrate clear deficiencies in adherence to protocols and utilization of multimodal analgesia, however the occurrence rate of these shortcomings ~15% (Benhamou et al., 2008) are incongruent with the notion that this is the primary reason that surgery patients develop chronic pain (as high as 40-50% in some populations). Moreover, the exact mechanisms involved in the development of persistent pain following surgery are still not clear.

Currently opioids are the mainstay treatment for post-surgical pain patients. Though opioids can be efficacious in providing pain relief acutely, persistent use of opioids can induce delayed hypersensitivity due to reduction in their analgesic efficacy and development of tolerance. In a pre-clinical study

administration of fentanyl in rats in association with the surgical incision induced exaggerated post-operative pain (Laboureyras et al., 2009). Hence apart from their known potential for abuse liabilities, administration of opioids also poses serious side-effect of potential for exacerbation of chronic pain following surgery. Despite some success to control pain during and immediately after surgery with other class of drugs such as local anesthetic agents and cyclo-oxygenase (COX) inhibitors and improvements in post-surgical pain treatment strategies, the incidence of moderate to severe pain after surgery is still high in several patient populations (Apfelbaum et al., 2003; Pavlin et al., 2002). As a result, it is not clear whether chronic post-surgical pain is due to inadequate treatment of acute post-surgical pain or whether the currently available therapeutics used to treat acute post-surgical pain do not prevent the transition to chronic pain (Price and Dussor, 2013). Novel therapeutics that directly target molecular events responsible for driving incision-induced pain and its transition to chronic pain may relieve the burden that this problem presents for patients, clinicians and healthcare systems (Price and Dussor, 2013; Schug, 2012).

1.6. Chronic pain and protein translation

Though the exact mechanisms driving chronic pain are still poorly understood there is ample evidence implicating the key role of activity dependent local protein translation in sensory axons in the initiation and maintenance of pain hypersensitivity in a several preclinical pain models. Protein translation is comprised of three steps: initiation, elongation, and termination. The initiation of protein translation which is the rate-limiting step, is regulated by a subset of upstream kinases that are critical for neuronal plasticity: the

mechanistic target of rapamycin complex 1 (mTORC1) and extracellular regulated kinase (ERK), which signal to the translational machinery, eukaryotic initiation factors (eIF) eIF4E and eIF4G and the eIF4E binding protein (4EBP) (Sonenberg and Hinnebusch, 2009). There is already compelling evidence that mTOR is a key kinase controlling nociceptive plasticity(Geranton et al., 2009; Jimenez-Diaz et al., 2008; Liang et al., 2013; Melemedjian et al., 2011; Price et al., 2007; Shih et al., 2012). mTORC1 mediated ongoing local protein translation can not only maintain sensitivity of subset of nociceptors but can also contribute to local changes in gene expression in response to injury to promote nociceptive hypersensitivity (Bogen et al., 2012; Ferrari et al., 2013; Geranton et al., 2009; Jimenez-Diaz et al., 2008; Price et al., 2007; Tillu et al., 2012). Similarly, ERK which controls translation initiation via mitogen activated interacting kinases (MNK1 and MNK2) to the eukaryotic initiation factor (eIF) 4E has been shown to be important factor for regulating pain hypersensitivity (Adwanikar et al., 2004; Cruz et al., 2005a; Cruz et al., 2005b; Ji et al., 1999; Ji et al., 2002; Ji et al., 2009; Karim et al., 2001; Obara et al., 2012). There is an early and long-lasting upregulation of ERK in the primary afferent neurons (Galan et al., 2002; Karim et al., 2001; Kawasaki et al., 2004), DRGs (Averill et al., 2001; Cruz et al., 2005a; Cruz et al., 2005b; Dai et al., 2004; Dai et al., 2002; Schicho et al., 2005) and superficial laminae of spinal dorsal horn (Karim et al., 2001; Kawasaki et al., 2004) in wide range of animal models of chronic pain. Phosphorylated ERK can further exert its effects by controlling gene regulation and/or modulating membrane receptors and ionic channels to promoting pain hypersensitivity. The picture emerging from this body of work is that activity-dependent translation control, either controlled by mTORC1 or ERK/MNK/eIF4E, plays a key role in the

hypersensitivity developing in the nociceptive system and critical for neuronal plasticity following injury (Price and Geranton, 2009).

However pharmacological targeting of these pathways must be carefully controlled due to the extensive feedback kinase signaling networks activated by targeting individual kinases (Price and Dussor, 2013). While local, short-term inhibition of mTORC1 leads to blockade of pain hypersensitivity, it has been demonstrated that long-term inhibition of mTORC1 leads to feedback activation of ERK causing nociceptive hypersensitivity (Melemedjian et al., 2013). These feedback mechanisms in mTORC1 signaling have been indicated previously as well (Ghosh et al., 2006) and have almost certainly contributed to the failure of mTORC1 inhibitors in several cancer clinical trials (Carracedo et al., 2008). Therefore the effective strategy to prevent acute hypersensitivity and its chronification would be to target both these signaling pathways with a single factor that can negatively regulate activity-dependent translation and block feedback signaling mechanisms.

CHAPTER II

AMP-ACTIVATED PROTEIN KINASE – INTRODUCTION AND RATIONALE FOR TARGETING AMPK FOR PAIN

2.1. Introduction

In a cell, anabolic processes like protein synthesis are orchestrated by upstream kinases that signal to the translation machinery such as mTORC1 and ERK. (Sonenberg and Hinnebusch, 2009). Activity of these two kinases can be controlled either through individual targeting by selective inhibitors or through negative modulation of both pathways by one of the single endogenous signaling factors (Zoncu et al., 2011). AMP-activated protein kinase (AMPK) is one such factor that is crucial to the negative regulation of translation. AMPK, the energy sensor of the cell, is a heterotrimeric Ser/Thr protein kinase which plays a central role in regulation of energy homeostasis (Hardie and Carling, 1997; Hardie et al., 1998; Kemp et al., 1999). Any cellular stress causing depletion in cellular ATP and increase in AMP and ADP levels leads to activation of AMPK which in turn leads to elevation of the cellular AMP:ATP ratio. Once activated, AMPK inhibits ATP consuming anabolic processes such as protein translation, fatty acid and cholesterol synthesis as well as glycogen synthesis (Corton et al., 1995; Hardie, 2007b; Henin et al., 1995) and stimulates ATP generating catabolic processes such as fatty acid oxidation (Hardie, 2007a).

2.2. Role of AMPK in protein translation

Activation of AMPK initiates a diverse number of cellular processes controlled largely through the regulation of AMPK-mediated phosphorylation of

downstream targets (Price and Dussor, 2013). The main mode of inhibition of protein translation by AMPK is through inhibition of mammalian target of rapamycin (mTOR) and MAPK signaling pathways (Carling et al., 2012; Hardie, 2007b; Melemedjian et al., 2011). AMPK is responsible for negative regulation of mTOR through activation of tuberous sclerosis complex 2 (TSC2) as well as direct phosphorylation of mTOR (Carling et al., 2012; Corradetti et al., 2004a). In addition, AMPK also phosphorylates IRS1(Jakobsen et al., 2001) and AKT at negative regulatory sites (Carling et al., 2012) further suppressing mTORC1 activity. This results in a profound inhibition of mTOR and its downstream targets involved in translation control like eukaryotic elongation factor 4E binding protein (4EBP) and ribosomal S6 kinase (Corradetti et al., 2004b). Strong evidence also exists demonstrating that the activation of AMPK leads to inhibition of mitogen activated protein kinase (MAPK) signaling pathways including ERK (Carling et al., 2012; Melemedjian et al., 2011; Price and Dussor, 2013; Tillu et al., 2012). Although the mechanisms through which AMPK activation achieves inhibition of ERK activity are still not completely understood, this is likely linked to AMPK-mediated phosphorylation of IRS1 at Ser-794 (Tzatsos and Tsichlis, 2007a) and concomitant inhibition of small GTPase signaling molecules that link Trk signaling to MAPK activity (Price and Dussor, 2013).

2.3. Role of AMPK in cellular metabolism and other cellular processes

AMPK activation is not exclusively linked to negative regulation of protein synthesis. AMPK has been recognized as a key regulator of mammalian metabolic functions as well e.g. glucose metabolism, bile and fatty acid

metabolism. AMPK regulates fatty acid metabolism by stimulating lipolysis and fatty acid oxidation as well as inhibiting lipogenesis (Anthony et al., 2009; Foretz et al., 1998; Koh et al., 2007; Sullivan et al., 1994a; Woods et al., 2000; Yin et al., 2003). The kinase stimulates fatty acid oxidation and reduces cholesterol synthesis through phosphorylation and inactivation of acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA reductase respectively (Carling et al., 2012; Hardie et al., 2012; Viollet and Andreelli, 2011). AMPK can inhibit lipogenesis by negatively regulating fatty acid synthase (FAS) gene expression (Anthony et al., 2009; Foretz et al., 1998; Woods et al., 2000). AMPK is also known to improve glucose metabolism by increasing the rate of glucose uptake and glycolysis (Massie et al., 2011). Furthermore the kinase is involved in maintaining a balance between nutrient homoeostasis and excessive internal degradation by regulating cell autophagy through phosphorylation of ULK-1 a kinase required for initiation of autophagy (Carling et al., 2012; Egan et al., 2011). Hence it is clear that AMPK plays a central role in regulation of diverse aspects of cellular metabolism.

2.4. Structure and Regulation of AMPK

Since its initial identification as a protein kinase activated by AMP, significant advances have been made in understanding the structure of the kinase and its regulation (Bijland et al., 2013; Carling et al., 1987). Briefly, AMPK is heterotrimeric complex comprised of catalytic α and regulatory β and γ subunits (Carling et al., 2012). Multiple subunit isoforms of each subunit, two isoforms of α subunit, two isoforms of the β subunit and three isoforms of γ subunit, have been identified in mammals. The α subunit contains the

serine/threonine kinase domain and the β and γ subunits are involved in regulation of the kinase containing subunit. These isoforms exhibit differential tissue expression and may influence subcellular localization of the resultant AMPK complex (Bijland et al., 2013; Cheung et al., 2000; Salt et al., 1998).

AMPK is subject to regulation by allosteric mechanisms and post-translational modifications including phosphorylation, myristoylation and acetylation (Bijland et al., 2013; Carling et al., 2012; Hardie et al., 2012). The primary mode of regulation of the kinase is by reversible phosphorylation (Carling et al., 2012). Several phosphorylation sites have been identified in the α and β subunits but the key site for upstream regulation appears to localize to the α subunit at threonine residue 172 (Carling et al., 2012; Hawley et al., 1996). The level of Thr172 phosphorylation of the α subunit is determined by the relative activities of the upstream kinases responsible for Thr^{172} phosphorylation, CaMKKβ (Ca^{2+}/calmodulin-dependent protein kinase kinase β), the tumor suppressor kinase, LKB1 (liver kinase B1) as well as the protein phosphatases acting on AMPK (Hawley et al., 2003; Hawley et al., 2005; Woods et al., 2005; Woods et al., 2003). CaMKKβ when activated by calcium and calmodulin and conditions which increase intracellular calcium can increase phosphorylation of Thr^{172} (Carling et al., 2012; Stahmann et al., 2006; Tamas et al., 2006; Tokumitsu and Soderling, 1996). On the contrary, the mechanism by which the constitutively active LKB1 mediates changes in Thr^{172} phosphorylation is still not completely understood. Even the central endogenous regulators of AMPK, AMP and ADP promote phosphorylation of Thr^{172} to activate the kinase. While the precise mechanisms through which the phosphorylation by AMP occurs are still not completely understood, it is clear that AMP binding to the γ subunit of the

kinase protects the kinase from dephosphorylation through a diverse array of phosphatases including protein phosphatase (PP) 1, 2A and 2C (Carling et al., 2012; Price and Dussor, 2013; Sanders et al., 2007).

In addition to phosphorylation, AMPK can also be allosterically regulated by AMP or the AMP analogs such as ZMP (Carling et al., 1989; Cheung et al., 2000; Corton et al., 1995; Sullivan et al., 1994b). The degree of allosteric activation of the kinase depends on the nature of the γ isoform present in the AMPK complex. AMPKβ subunits are also myristoylated at the N-terminus, and AMPKα subunits are acetylated on lysine residues by p300 acetyltransferase but the physiological relevance of these modifications is still unclear (Bijland et al., 2013; Carling et al., 2012; Lin et al., 2012).

2.5. Pharmacological activation of AMPK in cells

Apart from being activated endogenously by the adenine nucleotides, AMP and ADP, AMPK can also be activated pharmacologically. The property of the γ subunit of AMPK in protecting the kinase from dephosphorylation also provides several avenues for activating AMPK (Hawley et al., 2010). Pharmacological activators of AMPK can be categorized as indirect or direct activators. The indirect AMPK activators include biguanides such as metformin and phenformin, thiazolidinediones (TZDs) such as rosiglitazone and plant-derived compounds such as resveratrol. The direct AMPK activators include compounds such as A-769662, ZMP and OSU-53. (Carling et al., 2012; Hawley et al., 2010; Lee et al., 2011). The mechanism of action for the indirect AMPK activators is activation of CaMKKβ through increasing levels of cellular AMP or ADP, or intracellular calcium levels. Biguandies like metformin, an indirect

AMPK activator, interferes with mitochondrial function leading to activation of LKB1 and subsequent AMPK phosphorylation (Shaw et al., 2005). Polyphenols like resveratrol may act via a similar mechanism (Baur et al., 2006; Dasgupta and Milbrandt, 2007). Although evidence exists suggesting that in neurons polyphenols may activate AMPK through CaMKKβ, (Dasgupta and Milbrandt, 2007). However neither biguanides nor polyphenols have proved to be particularly potent activators of AMPK (EC50s of at least 100 μM levels for short time courses) and these compounds also have other targets that may be important for their physiological effects. Direct activators such as A769662 or ZMP bind allosterically to AMPK potentially causing the γ subunit to change conformation in order to protect the α subunit from dephosphorylation (Price and Dussor, 2013). Specific subunit knockdown studies have demonstrated that the mechanism of action for A769662 at AMPK requires the presence of a specific γ subunit (Cool et al., 2006). OSU-53 is believed to possess a similar mechanism of action as A769662, although the exact mechanism has not been clearly characterized (Lee et al., 2011). Hence a strategy of activating AMPK directly via positive allosteric modulation, perhaps through engagement of the γ subunit appears to offer a potent and potentially selective mechanism for pharmacological manipulation of AMPK without the requirement for stimulation of upstream kinases (Carling et al., 2012; Price and Dussor, 2013).

2.6. Rationale for targeting AMPK for pain

The unique ability of AMPK to negatively regulate multiple signaling pathways that are linked to chronic pain makes this kinase a potential target to recruit an endogenous regulatory mechanism that may diminish nociceptor

sensitization (Obara et al., 2012; Price and Dussor, 2013). As mentioned previously, the activity-dependent translation control, either by mTORC1 or ERK/MNK/eIF4E, plays a key role in the sensitization developing in the nociceptive system following injury and AMPK activation inhibits both the mTORC1 and ERK/MNK/eIF4E pathways making AMPK an attractive candidate for treating chronic pain.

Interestingly, there is also an extensive cross-talk between these kinase signaling pathways. It has been demonstrated that disruption of mTORC1 signaling with rapamycin in sensory neurons causes suppression of IRS-1-mediated negative feedback, leading to AKT and ERK activation in sensory neurons causing nociceptive hypersensitivity and increased sensory neuron excitability (Melemedjian et al., 2013). Therefore it is important and advantageous to utilize a signaling factor that can negatively regulate activity-dependent translation as well as block feedback signaling mechanisms. AMPK phosphorylates IRS1 and AKT at negative regulatory sites blocking the feedback of ERK activation induced by rapamycin treatment reversing rapamycin induced nociceptive hypersensitivity (Carling et al., 2012; Jakobsen et al., 2001; Melemedjian et al., 2013; Tzatsos and Tsichlis, 2007b; Zakikhani et al., 2010).

Hence we propose that AMPK activators represent a unique class of drugs for the treatment of acute neuropathic and post-surgical pain and the prevention of transition to persistent pain state because these drugs will: A) inhibit signaling activity involved in pain sensitization without the side-effects that might be expected with direct kinase inhibitors of the individual pathways, B) decrease signaling evoked by nerve growth factor (NGF) and interleukin 6 (IL-6) in

nociceptors and C) directly inhibit the excitability of primary afferent nociceptors via inhibition of ERK-mediated phosphorylation of Nav1.7.

In summary, the following studies will elucidate the importance of local protein translation and translation control machinery in peripheral sensitization of nociceptors and establishment of persistent pain state and further provide evidence for activation of AMPK as a novel treatment avenue for acute and chronic pain states.

2.7. Aims of the Thesis

The overall aim of this thesis is to establish the importance of protein translation regulation pathways in initiating and maintaining chronic pain conditions such as neuropathic and post-surgical pain and to understand the role of AMPK activators in modulating translation regulation. The thesis also seeks to understand how AMPK activators can be utilized to inhibit development of an acute pain state and prevent the transition to chronic pain e.g post-surgical pain; and to investigate if AMPK activators can reverse a chronic pain state once established e.g neuropathic pain.

2.8. Organization of the Thesis

In Chapter 3, the role of dysregulated protein translational pathways in neuropathic pain and the importance of AMPK activators, in inhibiting the translation regulation signaling pathways and reversing the allodynia induced by nerve injury, is discussed. Chapter 4 focuses on the role of resveratrol, a potent and efficacious AMPK activator, in preventing development of acute allodynia in a model of post-surgical pain and preventing the transition to

chronic pain. The aim of Chapter 5 is establish AMPK activation as a bona-fide mechanism for the alleviation of post-surgical, and possibly other persistent pain states and to invent novel therapeutics and therapeutic strategies that employ this mechanism of action by screening multiple AMPK activators with different mechanisms of action, efficacies and route of administration.

CHAPTER III

TARGETING ADENOSINE MONOPHOSPHATE-ACTIVATED PROTEIN KINASE (AMPK) IN PRECLINICAL MODELS REVEALS A MECHANISM FOR THE TREATMENT OF NEUROPATHIC PAIN

3.1. Abstract

Neuropathic pain is a debilitating clinical condition with few efficacious treatments, warranting development of novel therapeutics. We hypothesized that dysregulated translation regulation pathways may underlie neuropathic pain. Peripheral nerve injury induced reorganization of translation machinery in the peripheral nervous system of rats and mice, including enhanced mTOR and ERK activity, increased phosphorylation of mTOR and ERK downstream targets, augmented eIF4F complex formation and enhanced nascent protein synthesis. The AMP activated protein kinase (AMPK) activators, metformin and A769662, inhibited translation regulation signaling pathways, eIF4F complex formation, nascent protein synthesis in injured nerves and sodium channel-dependent excitability of sensory neurons resulting in a resolution of neuropathic allodynia. Therefore, injury-induced dysregulation of translation control underlies pathology leading to neuropathic pain and reveals AMPK as a novel therapeutic target for the potential treatment of neuropathic pain.

3.2. Background

Neuropathic pain is a debilitating condition wherein a large cohort of patients fail to achieve even partial pain relief (Baron, 2006). Hence, novel treatment approaches targeting molecular mechanisms of pathology induced by

peripheral nerve injury (PNI) are needed. PNI leads to changes in sensory neuron phenotype and function resulting in hyperexcitability and ectopic activity in these neurons driving neuropathic pain (Campbell and Meyer, 2006). The important role of translation regulation in learning and memory has elucidated translation control as a critical factor for neuronal plasticity (Costa-Mattioli et al., 2009). Multiple lines of evidence suggest that translation regulation at the level of the primary afferent neuron is crucial for the establishment and maintenance of enhanced pain states (Geranton et al., 2009; Huang et al., 2008; Jimenez-Diaz et al., 2008; Melemedjian et al., 2010; Price and Geranton, 2009; Price et al., 2007). Several recent reports have suggested an important role for the mammalian target of rapamycin complex 1 (mTORC1) pathway in neuropathic pain (Geranton et al., 2009; Jimenez-Diaz et al., 2008); however, mechanistic links between mTORC1 and pathology induced by PNI are still lacking. Moreover, treatment strategies that target translation control have not been clearly identified as potential treatments for neuropathic pain.

Translation control is orchestrated by upstream kinases that signal to the translation machinery (Sonenberg and Hinnebusch, 2009). These kinases can be targeted individually by selective inhibitors or they can be negatively modulated by endogenous signaling factors that act on these pathways (Zoncu et al., 2011). A crucial kinase for negative regulation of translation is the ubiquitous, energy-sensing kinase AMP-activated protein kinase (AMPK). Activation of AMPK by depletion of cellular nutrients or through pharmacological intervention results in a dampening of signaling to the translation machinery (Zoncu et al., 2011) but the potential effects of AMPK activation on neuronal excitability, an important component of neuropathic pain conditions (Campbell and Meyer, 2006), is not

known. AMPK can be targeted pharmacologically via a number of investigational compounds (e.g. AICAR and A769662) and by the widely clinically available and relatively safe drug metformin.

Herein we have tested the hypothesis that AMPK may represent a novel and efficacious opportunity for the treatment of chronic neuropathic pain. We find that PNI is linked to reorganization of translation machinery in injured nerves and demonstrate that pharmacological activation of AMPK leads to normalization of aberrant gene expression at the level of translation, decreased sensory neuron excitability and the resolution of neuropathic allodynia in preclinical models. Importantly, these effects are achieved by metformin suggesting an immediately available novel avenue for the potential treatment of neuropathic pain in humans. Hence, AMPK represents a novel therapeutic target for the treatment of neuropathic pain disorders.

3.3. Materials and Methods

3.3.1. Surgery and behavioral testing

Male ICR mice (Harlan, 20-25 g) and male Sprague Dawley rats (Harlan, 250-300 g) were used. All animal procedures were approved by the Institutional Animal Care and Use Committee of The University of Arizona and were in accordance with International Association for the Study of Pain guidelines. Prior to surgery all animals were assessed for mechanical withdrawal thresholds (Chaplan et al., 1994). Spared nerve injury (SNI) was performed on the mice as described previously (Bourquin et al., 2006). Spinal nerve ligation (SNL) was done on rats by tight ligation of the L5 and L6 spinal nerves as described by Kim

and Chung (Kim and Chung, 1992). Sham control animals underwent the same surgery and handling as the experimental animals but without the SNL or SNI. All animals were allowed to recover for 14 days and all testing commenced day 14 day post-surgery (except as noted in the text when testing was done 7 weeks post SNI). Following nerve injury, only animals that developed paw withdrawal thresholds less than 1g for SNI and less than 4.7 g for SNL by day 14 post-surgery were used. Animals were placed in acrylic boxes with wire mesh floors and allowed to habituate for 1 hr. Pre-drug mechanical thresholds were recorded and the animals received intraperitoneal injections of vehicle, metformin (200mg/kg) (Combs et al., 2002) or A7969662 (30mg/kg) (Cool et al., 2006). Calibrated von Frey filaments (Stoelting, Wood Dale, IL) were used for mechanical stimulation of the plantar surface of the left hindpaw and withdrawal thresholds were calculated using the up-down method (Chaplan et al., 1994). For Western blotting, eIF4F complex formation and nascent protein synthesis studies, tissues were harvested 17 days post SNL or SNI unless drug treatments were initiated on day 14, in which case they were harvested on day 21.

3.3.2. Primary neuronal cultures

Mouse trigeminal ganglia (TG) were excised aseptically and placed in Hank's Buffered Salt Solution (HBSS, Invitrogen) on ice. The ganglia were dissociated enzymatically with collagenase A (1 mg /ml, 25 min, Roche) and collagenase D (1 mg/ml, Roche) with papain (30 U/ml, Roche) for 20 min at 37 °C. To eliminate debris 70 μm (BD) cell strainers were used. The dissociated cells were resuspended in DMEM/F12 (Invitrogen) containing 1X pen-strep

(Invitrogen), 1X GlutaMax, 3μg/ml 5-FDU (Sigma), 7μg/ml uridine (Sigma), 50ng/ml NGF (Millipore) and 10% fetal bovine serum (Hyclone). The cells were plated in 6-well plates (BD Falcon) and incubated at 37 °C in a humidified 95% air /5%CO2 incubator. On day 5 the cells were washed in DMEM/F12 media for 30 mins followed by treatment.

3.3.3. Western blotting

Protein was extracted from the cells and tissue in lysis buffer (50 mM Tris HCl, 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA at pH 7.4) containing protease and phosphatase inhibitor mixtures (Sigma) with an ultrasonicator on ice, and cleared of cellular debris and nuclei by centrifugation at 14,000 RCF for 15 min at 4°C. Fifteen micrograms of protein per well were loaded and separated by standard 7.5% or 10% SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore) and then blocked with 5% dry milk for 3 h at room temperature. The blots were incubated with primary antibody overnight at 4°C and detected the following day with donkey anti-rabbit antibody conjugated to horseradish peroxidase (Jackson Immunoresearch). Signal was detected by ECL on chemiluminescent films. Each phosphoprotein was normalized to the expression of the corresponding total protein which in turn were normalized to GAPDH and βIII tubulin on the same membrane. Densitometric analyses were performed with Image J software (NIH).

3.3.4. Enzyme Linked Immunosorbent Assay (ELISA)

ELISAs for NGF (Promega) and IL-6 (Thermo) were performed on the sciatic nerves form rats with SNL according to the manufacturer's instruction.

The optical density was read by a plate reader (Multiskan Ascent, Themo) using a 450 nm filter. Ascent Software (Themo) was used for the data analysis and cubic spline curve fit was chosen for that purpose.

3.3.5. 5′ mRNA cap complex analysis

After the protein extraction, 50 μg protein was incubated with 7- methyl GTP Sepharose 4B beads (GE Healthcare) in the presence of 100μM GTP for 2 h at 4 °C. Unconjugated sepharose 4B beads were used for the negative controls. The beads were then pelleted and washed twice with lysis buffer. After a final centrifugation the pellet was suspended in 1X Laemmli Sample Buffer containing 5 % v/v β-mercaptoethanol and eIF4E, eIF4G eIF4A and 4EBP bound to the precipitated beads was analyzed by Western blotting.

3.3.6. Immunohistochemistry

Slide-mounted sections were fixed in ice-cold 3.7% paraformaldehyde in 1x PBS for 1 h and then washed 3 times for 5 min in PBS. Slides were transferred to a solution containing 0.1 M sodium citrate and 0.05% Tween 20 and then microwaved on high power for 3 min in a 900 W microwave oven for antigen retrieval. After a 30 min cooling period, slides were again transferred to 1x PBS, washed 3 times for 5 min, and then permeabilized in 1x PBS, containing 0.05% Triton X-100. Slides were then blocked for at least 1 h in 1x PBS, containing 10% normal goat serum, before the addition of anti-phospho-mTOR antibody overnight at 4°C. The anti-peripherin and N52 antibodies were applied together. Immunoreactivity was visualized after subsequent incubation with goat anti-rabbit and goat anti-mouse Alexa-Fluor antibody for 1 h at room temperature.

All immunohistochemistry (IHC) images are representative of samples taken from three animals. Confocal IHC micrographs were acquired on Ziess LSM 510 META NLO upright microscope using a 40X, 1.3 numerical aperture oil immersion objective.

3.3.7. Nascent protein synthesis in sciatic nerves

Ipsilateral and contralateral sciatic nerves from SNL rats or ipsilateral sciatic nerves from sham rats were excised at a length of 2 cm. The nerves were then cut to a length of 1 cm and incubated in DMEM/F12 supplemented with 50μM of Azidohomoalanine (AHA). After 2 hours of incubation at 37 °C in a humidified 95% air /5%CO2 incubator, protein was extracted from the nerves by ultrasonication in RIPA buffer. AHA incorporating proteins were labeled with biotin using Click-iT Biotin Protein Analysis Detection Kit (Invitrogen). The samples were analyzed using western blotting with the biotin labeled proteins detected by avidin-HRP chemiluminescence.

3.3.8. Patch clamp electrophysiology

Whole cell patch-clamp experiments were performed on isolated mouse TG using a MultiClamp 700B (Axon Instruments) patch-clamp amplifier and pClamp 10 acquisition software (Axon Instruments). Recordings were sampled at 5 kHz and filtered at 1 kHz (Digidata 1322A, Axon Instruments). Pipettes (OD: 1.5 mm, ID: 0.86 mm, Sutter Instrument) were pulled using a P-97 puller (Sutter Instrument) and heat polished to 2.5–4 MΩ resistance using a microforge (MF-83, Narishige). Pipette offsets were zeroed automatically before seal formation and liquid junction potentials were not corrected. Pipette capacitance neutralization

and bridge balance were adjusted automatically in current-clamp mode. All recordings were performed at room temperature. Data were analyzed using Clampfit 10 (Molecular Devices) and Origin 8 (OriginLab). Pipette solution contained (in mM) 140 KCl, 11 EGTA, 2 MgCl2, 10 NaCl, 10 HEPES, 1CaCl2 pH 7.3 (adjusted with N-methyl glucamine), and was 320 mosM. External solution contained (in mM) 135 NaCl, 2 CaCl2, 1 MgCl2, 5 KCl, 10 Glucose and 10 HEPES, pH 7.4 (adjusted with N-methyl glucamine), and was 320 mosM. The cells were treated with relevant drug for 1 hour before patch clamp studies were carried out. Ramp stimulus protocols for Nav1.7 were performed as described previously (Stamboulian et al., 2010) as these protocols have been shown to preferentially elicit function from Nav1.7 channels (Cummins et al., 1998). The viability of the neurons was verified by eliciting currents in response to a voltage step protocol before and after each current ramp protocol was carried out. Al recordings were made on mouse TG neurons from ~20 gram mice. Capacitance of all cells analyzed was between 20-30pF.

3.3.9. Drugs and primary antibodies

U-0126 and U0124 were from Tocris; metformin was from LKT Laboratories; mouse 2.5S NGF was from Millipore; The following rabbit polyclonal antibodies were obtained from Cell Signaling: p-ERK (Thr202/Tyr204, cat# 4377), total ERK, p-eIF4E (Ser209, cat# 9741), total eIF4E, p-mTOR (Ser2448, cat# 2971), total mTOR, p-4EBP(Thr37/46, cat # 9459), total 4EBP, p-eIF4G (Ser1108, cat# 2441), total eIF4G, p-AKT (Ser473, cat# 4058), total AKT, GAPDH and eIF4A. Mov10 was from Bethyl Labs and rck/p54 was from MBL international. A769662 was from LC Laboratories.

3.3.10. Statistical Analysis and Data Presentation

Data are shown as means and the standard error of the mean (± SEM) of eight independent cell culture wells, 6 tissue samples (for in vivo Western blotting, eIF4F complex formation and nascent protein synthesis) or 6 animals (for behavioral studies). Graph plotting and statistical analysis used Graphpad Prism Version 5.03 (Graph Pad Software, Inc. San Diego, CA, USA). Statistical evaluation was performed by one- or two-way analysis of variance (ANOVA), followed by appropriate post-hoc tests. The a priori level of significance at 95% confidence level was considered at $p < 0.05$.

3.4. Results

3.4.1. PNI induces a major reorganization of translational machinery in the peripheral nervous system

We utilized the rat spinal nerve ligation (SNL) and mouse spared nerve injury (SNI) models to assess biochemical changes in translation machinery occurring after PNI in the dorsal root ganglion (DRG) and sciatic nerve. SNL induced a significant increase in translation regulation signaling pathway components as well as translation machinery in the uninjured and injured DRG and in the sciatic nerve (Fig 3.1A). In the uninjured DRG (L4, Fig 3.1A) these changes included increased phosphorylation of mTOR, eukaryotic initiation factor (eIF) 4E binding protein (4EBP) and increased total 4EBP. Phosphorylation of eIF2α is negatively correlated with translation (Costa-Mattioli et al., 2005) and, consistent with enhanced translation in the L4 DRG, we observed decreased p-

eIF2α (Fig 3.1A). Proteins associated with RNA processing and binding (e.g. Moloney leukemia virus 10 (Mov10) (Banerjee et al., 2009)) were also increased (see Table 3.1 for quantification). Likewise, in the injured DRGs (L5/6) p-mTOR and p-4EPB were increased as well as total mTOR. Increases in total Mov10 and fragile X mental retardation protein (FMRP) were also observed (Fig 3.1A, see Table 3.2 for quantification).

SNL induces a degeneration of axons from the L5 and L6 DRGs resulting in pathology to uninjured sciatic axons originating from L4 DRG (Campbell and Meyer, 2006) that comingle in the nerve. In the sciatic nerve, PNI led to enhanced mTOR and extracellular signal-regulated kinase (ERK) pathway activity reflected by increased p-4EBP, p-ribosomal S6 protein (rS6p) and p-eIF4E, respectively. Upstream activation of mTOR in this setting is likely linked to enhanced AKT activity as increased p-AKT and p-mTOR at S2448 was observed (Nave et al., 1999). We have recently shown that nerve growth factor (NGF) and interleukin 6 (IL-6) signal to the translational machinery in dorsal root ganglion (DRG) and trigeminal ganglion (TG) neuronal axons via mTOR and ERK, respectively (Melemedjian et al., 2010). We found that NGF (ipsilateral 1532 ± 185.6 pg/ml vs. contralateral 924.5 ± 55.7 pg/ml, $p = 0.011$) and IL-6 (ipsilateral 1282 ± 100.8 pg/ml vs. contralateral 988.6 ± 35.2 pg/ml; $p = 0.021$) were increased by PNI in the sciatic nerve suggesting that NGF and IL-6 may contribute to local mTOR and ERK activation following injury. We further noted increases in translational machinery (total eIF4E, eIF4A, eIF2α) and RNA processing and binding proteins (FMRP, Mov10 and rck/p54; Fig 3.1A, see Table 3.1 for quantification). Hence, these results demonstrate a fundamental reorganization of translation signaling pathways and machinery in the sciatic nerve induced by PNI.

To verify that these findings are generalized to other models of PNI in other species, we utilized the mouse SNI model to confirm reorganization of biochemical components mediating translation regulation. In the injured sciatic nerve of the mouse SNI model we observed an upregulation of total 4EBP, eIF4E, eIF4A, Mov10 and rck/p54 and increased ERK activity (Fig 3.1B, see Table 3.4 for quantification). To demonstrate localization of enhanced mTOR signaling to DRG axons we used immunohistochemistry (IHC) for p-mTOR. Uninjured sciatic nerves from rats did not exhibit detectable levels of p-mTOR on serine 2448 (Fig 3.1C). However, following PNI robust p-mTOR IHC co-localized with markers of DRG neuron axons (peripherin + N52, Fig 3.1C). Collectively, these findings support the conclusion that PNI induces a fundamental reorganization of translation regulation signaling in the injured PNS and IHC findings with p-mTOR suggest that these changes occur in sensory neuron axons.

3.4.2. PNI enhances eIF4F complex formation and causes an increase in nascent protein synthesis in the sciatic nerve

To directly demonstrate stabilization of the eIF4F complex on the 5′-mRNA-cap we incubated proteins extracted from sciatic nerves ipsilateral and contralateral to SNL with sepharose beads conjugated to 7-methyl-GTP. Ipsilateral to the injury we observed 1200% increase in eIF4A association with the 7-methyl-GTP conjugated beads (Fig 3.2A and 3.2C). Conversely, only a modest increase in 4EBP was detected associated with 7-methyl-GTP (Fig 3.2A and 3.2B) despite a robust increase in total 4EBP induced by PNI (Fig 3.1A and 3.1B). The ratio of eIF4A to 4EBP (750% increase; Fig 3.2D) associated with 7-methyl-GTP strongly suggests a robust increase in cap-dependent translation in the sciatic

nerve induced by PNI. To directly ascertain altered nascent protein synthesis following PNI we incubated excised sciatic nerves ipsilateral and contralateral to SNL with a click-chemistry compatible methionine analogue L-azidohomoalanine (AHA) (Dieterich et al., 2007; Dieterich et al., 2006). AHA incorporation into nascently synthesized proteins was biotinylated using click-chemistry and detected by Western blotting. With 2 hr incubation, injured nerves incorporated 50% more AHA than either contralateral uninjured nerves or sciatic nerves taken from sham rats (Fig 3.2E and 3.2F). Taken together, these findings directly demonstrate that PNI-induced reorganization of translation regulation in the sciatic nerve results in enhanced eIF4F complex formation and increased protein synthesis.

3.4.3. AMPK activators reverse PNI induced allodynia

We hypothesized that activation of AMPK signaling may represent a novel mechanism for treatment of neuropathic pain. AMPK activation inhibits the mTOR pathway, is associated with decreased ERK activity (Zoncu et al., 2011) and inhibits insulin receptor substrate (IRS)-mediated feedback signaling (Tzatsos and Tsichlis, 2007). Metformin stimulates the AMPK pathway through multiple mechanisms (Ouyang et al., 2011; Shaw et al., 2005). Mice treated with metformin (200 mg/kg/day (Combs et al., 2002) for 7 days) starting 2 (Fig 3.3A) or 7 (Fig 3.3B) weeks post-SNI displayed a complete reversal of tactile allodynia. No changes in threshold were observed in sham mice (Fig 3.3A and 3.3B). Likewise, treatment with A769662 (30 mg/kg/day (Cool et al., 2006) for 7 days), a positive allosteric modulator of AMPK (Cool et al., 2006), led to a full reversal of tactile allodynia with no effect in shams (Fig 3.3C). Metformin (200 mg/kg/day for 7 days) also alleviated SNL-induced neuropathic allodynia without influencing

thresholds in sham rats (Fig 3.3D). These results establish AMPK activators as a potentially efficacious class of drugs for the treatment of PNI-induced neuropathic pain.

3.4.4. AMPK activators reverse PNI-induced biochemical changes

We asked if AMPK activators inhibit signaling pathways associated with translation regulation in sensory neurons in the presence of nerve growth factor (NGF). We used mouse TG neurons for these studies because we have previously shown that NGF signaling to ERK and mTOR in vitro is identical in TG and DRG neurons from this species (Melemedjian et al., 2010). Utilizing TG neurons greatly reduces the number of animals required for these studies. NGF promotes mTOR and ERK signaling pathways in these neurons (Melemedjian et al., 2010), contributes to neuropathic pain in preclinical models (Wild et al., 2007) and is associated with neuropathic pain in humans (Anand, 2004). Mouse TG neurons were treated with metformin (Fig 3.4A), A769662 (Fig 3.4B) or AICAR (Fig 3.4C) for 1 hour. A769662 and AICAR activated AMPK and suppressed activity in the mTOR and ERK pathways whereas metformin activated AMPK and selectively inhibited the mTOR pathway but did not lead to feedback signaling through IRS (e.g. no increase in ERK or AKT phosphorylation (Kinkade et al., 2008)). Moreover, A769662 and metformin inhibited eIF4F complex formation (Fig 3.5A and B) in primary cultures of TG neurons treated with NGF. To determine if metformin inhibited dysregulated translation in vivo, we excised sciatic nerves from SNL rats treated with vehicle or metformin (200 mg/kg/day for 7 days). A 50% increase in nascently synthesized proteins was observed in the sciatic nerve of SNL rats treated with vehicle, whereas metformin treatment restored

nascently synthesized protein levels to those observed in uninjured sciatic nerves (Fig 3.5C). Hence, AMPK activators suppress translation regulation pathways in sensory neurons and inhibit nascent protein synthesis in the sciatic nerve associated with PNI.

3.4.5. AMPK activators block hyperexcitability in sensory neurons

Based on the biochemical findings above, we hypothesized that AMPK activators might attenuate ramp current-evoked sensory neuron excitability. Small diameter (20-30 pF), mouse TG neurons cultured in the presence of NGF displayed increased excitability when stimulated with depolarizing ramp current injections (Fig 3.6A, 3.6B and 3.6C). Treatment with metformin restored these parameters to levels observed in vehicle-treated cultures (Fig 3.6A, 3.6B and 3.6C). Treatment with A769662 dose-dependently prolonged latency to first action potential (Fig 3.6C) and potently reduced the number of action potentials in response to ramp currents (Fig 3.6A). This effect persisted for at least 1hr after washout (Fig 3.6) demonstrating that the observed effects are not due to direct channel blockade. AMPK activators did not significantly influence resting membrane potential (vehicle = -60.52 ± 0.74; NGF + vehicle = -60.61 ± 0.70; NGF + metformin = -61.27 ± 1.22; NGF + A769662 (50 μM) = -58.3 ± 1.63; NGF + A769662 (200 μM) = -56.13 ± 2.37). As noted in biochemical experiments, A769662 suppressed ERK activation in sensory neurons (Fig 3.4B) whereas metformin only influenced the mTOR pathway (Fig 3.4A). This discrepancy in signaling, which likely reflects differential pharmacological upstream mechanisms of these compounds (Cool et al., 2006; Shaw et al., 2005), may provide insight into the enhanced efficacy of A769662 in reducing sensory neuron excitability in response

to ramp currents. Thus, AMPK activators not only suppress translation regulation signaling and eIF4F complex formation but also decrease the excitability of sensory neurons.

3.5. Discussion:

The present findings provide several novel insights into the pathology and potential treatment of neuropathic pain. We have demonstrated that PNI induces a major reorganization of translation regulation signaling, translation machinery and RNA-binding proteins in the injured PNS. These changes are directly linked to increased eIF4F complex formation and augmented nascent protein synthesis. We have identified AMPK activation as a novel avenue for the potential treatment of neuropathic pain in humans. Metformin and A769662 both alleviated neuropathic allodynia, inhibited translation regulation pathways associated with PNI and decreased sensory neuronal excitability. No clinical trials have assessed the efficacy of metformin for neuropathic pain. These studies provide a compelling preclinical rationale for the clinical assessment of metformin for neuropathic pain in humans and for the future development of more efficacious AMPK activators for the treatment of chronic pain disorders.

Translation control in the axonal compartment of neurons contributes to development of the peripheral and central nervous systems (Martin, 2004), is involved in axonal regeneration following injury (Willis et al., 2005; Zheng et al., 2001) and contributes to pain plasticity (Melemedjian et al., 2010; Price and Geranton, 2009). Likewise, the repertoire of mRNAs localized to the axonal compartment is developmentally regulated and shows plasticity upon injury

(Gumy et al., 2011; Wang et al., 2007; Willis et al., 2005; Willis et al., 2007; Zheng et al., 2001). Interestingly, a recent non-biased approach to mRNA profiling of the axonal compartment of DRG neurons revealed a developmental shift toward localization of mRNAs involved in immune regulation and nociception in the adult DRG axon (Gumy et al., 2011). In support of this mRNA profile, we have recently demonstrated that NGF and/or IL-6-induced allodynia is dependent on local, axonal translation from existing pools of axonally localized mRNAs (Melemedjian et al., 2010). The present findings enhance our understanding of plasticity of translation control after PNI. While changes in mRNA localization have been observed after pre-conditioning peripheral nerve lesions (Willis et al., 2005), we found that PNI induces profound changes in activity of kinases associated with translation control (e.g. mTOR and ERK), phosphorylation of their downstream targets and in overall levels of proteins involved in RNA processing and transport (e.g. Mov10, FMRP and rck/p54). This reorganization results in increased eIF4F complex formation and nascent protein synthesis in the injured sciatic nerve. Moreover, these changes are directly correlated to normalization of neuropathic allodynia as metformin reversed changes in protein synthesis in injured sciatic nerves and resolved PNI-induced allodynia.

Protein synthesis is an energy intensive process and, for this reason, an intricate system to control energy consumption has evolved in the form of the ubiquitous kinase, AMPK (Zoncu et al., 2011). Activated AMPK blocks protein synthesis by inhibiting components of the mTOR and ERK signaling pathways (Zoncu et al., 2011). Concurrent inhibition of multiple signaling pathways inherently prevents signaling crosstalk, which is commonly observed with the inhibition of a single kinase involved in these signaling cascades (e.g. inhibition

of mTORC1 with rapamycin (Kinkade et al., 2008)). Receptor tyrosine kinases are associated with IRS, which mediates activation of the mTOR and ERK pathways. Inhibition of mTORC1 with rapamycin removes negative feedback onto IRS, meditated by phosphorylation of IRS (S1101) by rS6K (Tremblay et al., 2007). Thus, inhibiting mTORC1 and, in turn, rS6K, with rapamycin releases disinhibition of IRS signaling resulting in activation of ERK and mTORC2/AKT pathways (Zoncu et al., 2011). ERK activation in the PNS is a well known mechanism for increasing the excitability of nociceptors (Ji et al., 2009). For these reasons we focused on AMPK as a therapeutic target for neuropathic pain as AMPK suppresses IRS signaling by phosphorylation on Serine 794 (Tzatsos and Tsichlis, 2007). Activation of AMPK with distinct pharmacological tools failed to promote ERK or AKT activation and, in the case of A769662 and AICAR, led to inhibition of these kinases. These findings highlight advantages of targeting AMPK for the treatment of neuropathic pain.

We have also demonstrated that activation of AMPK in mouse sensory neurons leads to decreased excitability. We used ramp current-evoked spiking to assess the influence of AMPK activation on sensory neuron excitability. Our findings are consistent with a potential modulation of the voltage-gated sodium channel Nav1.7 by AMPK activators. Human genetic studies clearly demonstrate an important role for Nav1.7 in inherited pain conditions and a growing body of evidence suggests an important role for Nav1.7 in acquired chronic pain states (Dib-Hajj et al., 2010). In humans, Nav1.7 expression is increased in painful neuromas (Black et al., 2008) and dental pulp (Beneng et al., 2010; Luo et al., 2008). Moreover, inhibition of Nav1.7 decreases sensory neuron excitability (Estacion et al., 2010; Schmalhofer et al., 2008). Genetic deletion of

Nav1.7 in mice leads to marked decreases in acute and inflammatory pain (Nassar et al., 2004). Finally, pharmacological inhibition of Nav1.7 with several distinct classes of inhibitors leads to a reduction in allodynia in preclinical neuropathic pain models (Hoyt et al., 2007a; Hoyt et al., 2007b; London et al., 2008; Tyagarajan et al., 2010). Hence, human clinical findings and pharmacological inhibition of Nav1.7 creates a compelling rationale for targeting Nav1.7 in neuropathic pain disorders. The present findings indicate that the AMPK signaling axis regulates sensory neuronal activity by decreasing action potential firing induced by ramp current injection and increasing the latency to the first action potential, both of which are consistent with modulation of Nav1.7 (Stamboulian et al., 2010). We hypothesize that this AMPK-mediated modulation of sensory neuron excitability may be linked to inhibition of ERK because ERK phosphorylates Nav1.7 altering channel gating properties toward a hyperexcitable state and leads to decreased neuronal hyperexcitability (Stamboulian et al., 2010). While further work will be needed to directly test the effect of AMPK activators on ERK-mediated Nav1.7 phosphorylation and Nav1.7 current kinetics, the present findings demonstrate a clear role for AMPK modulation in sensory neuronal excitability.

The results presented here are consistent with a peripheral action for AMPK activators in the alleviation of SNI- and SNL-induced allodynia; however, we cannot exclude a potential central mechanism of action. Several recent studies have demonstrated an important role for dorsal horn mTOR in preclinical pain models (Asante et al., 2009, 2010; Asiedu et al., 2011; Norsted Gregory et al., 2010; Price et al., 2007; Xu et al., 2011) and AMPK activators influence the mTOR pathway in central neurons (Potter et al., 2010; Ropelle et al.,

2008). Moreover, metformin crosses the blood brain barrier (Labuzek et al., 2010). We favor a peripheral mechanism of action for several reasons. AMPK activators had a clear effect on mTOR and, in some cases, ERK signaling, in cultured sensory neurons. These compounds also negatively influenced the excitability of these neurons, consistent with the alleviation of neuropathic pain. Moreover, in vivo treatment led to a reversal of PNI-induced enhanced nascent protein synthesis, consistent with a direct action of AMPK activators on the injured PNS. Finally, inhibition of translation regulation signaling (e.g. with mTORC1 inhibitors) in the CNS is thought to play a critical role in the initiation but not maintenance of plasticity (Kelleher et al., 2004). To this end, we have recently shown that mTOR inhibiton in the dorsal horn is incapable of reversing an established preclinical pain state (Asiedu et al., 2011) and, in the setting of neuropathic pain, other investigators have concluded that even centrally applied mTOR inhibitors act via inhibition of DRG neuron excitability (Geranton et al., 2009). Additional pharmacokinetic/pharmacodynamic studies will be required to resolve this question with certainty.

The present findings have important implications for AMPK-based drug discovery for the treatment of pain. Metformin activates AMPK via LKB1 stimulation (Shaw et al., 2005) and inhibition of AMP deaminase (Ouyang et al., 2011). AICAR results in 5-aminoimidazole-4-carboxamide ribonucleoside accumulation in cells mimicking AMP binding to AMPK (Corton et al., 1995). On the other hand, A769662 is a direct, positive allosteric modulator of AMPK (Cool et al., 2006) which requires the ◎1 subunit of the kinase heteromer (Scott et al., 2008). Unlike metformin and AICAR which activate AMPK by inducing the phosphorylation T172 on the alpha subunit, A769662 induced AMPK activation

does not require this post-translational modification (Goransson et al., 2007). While both metformin and A769662 led to a full reversal of neuropathic allodynia, A769662 was more potent in vivo and had a more profound effect on sensory neuron excitability with a near complete blockade of ramp current evoked spiking. Moreover, A769662 (and AICAR) led to robust ERK inhibition in sensory neurons in culture whereas metformin had no impact on ERK activity. While further experimentation will be needed to gain insight into the exact mechanisms through which different modes of AMPK activation achieve differential signaling endpoints, these results point to a potential pharmacological advantage for positive allosteric modulators of AMPK for the treatment of chronic pain. Furthermore, the efficacy of A769662 in mouse models of neuropathic pain suggests that targeting the ◎1-subunit of AMPK may be a viable drug development target for the pain pathway.

In conclusion, we have demonstrated a novel pathway for the potential treatment of neuropathic pain, AMPK activation. Pharmacological AMPK activation negatively regulates aberrant translation control after PNI, resolves neuropathic allodynia and decreases sensory neuron excitability. Due to the clinical availability and safety of metformin, these preclinical findings have the potential to lead to rapid translation into the clinic.

3.6. Figures

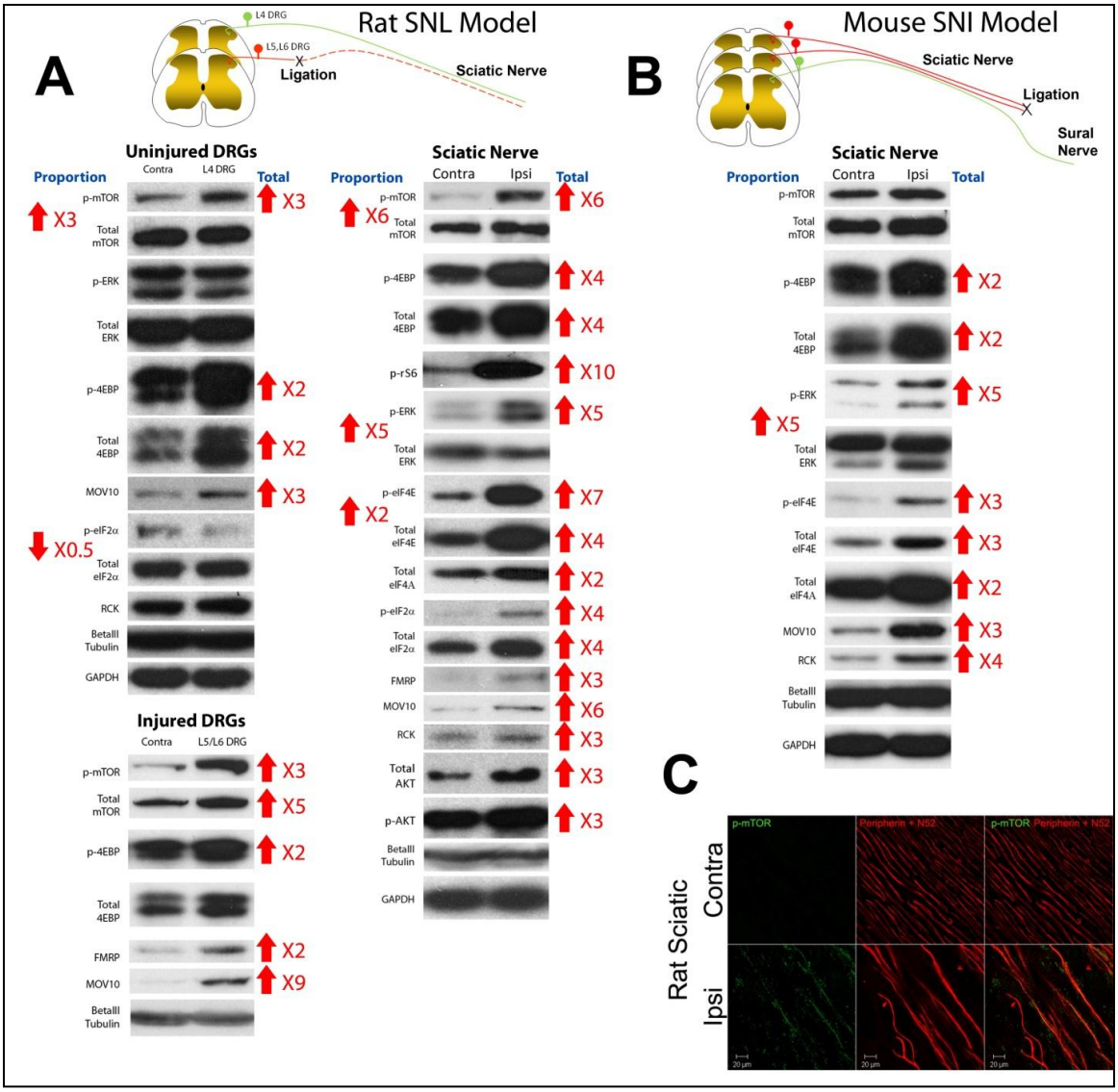

Figure 3.1: PNI induces a fundamental reorganization of translation signaling and machinery in the injured PNS. A) PNI in rats causes upregulation of translation machinery and signaling pathways associated with protein translation. These changes are observed in the injured sciatic nerve and in both injured (L5 and L6) and uninjured (L4) DRGs. **B)** PNI in mice increases proteins that enhance translation and associated signaling pathways. In both panels, up arrows indicate an increase while down arrows indicate a decrease; numbers indicate fold change in ipilateral vs. contralateral (right) and phospho-protein vs. total protein (left). **C)** Immunohistochemical colocalization of p-mTOR with sensory neuronal markers (peripherin + N52) show that activated mTOR localizes to axons of injured sciatic nerve from rats with SNL.

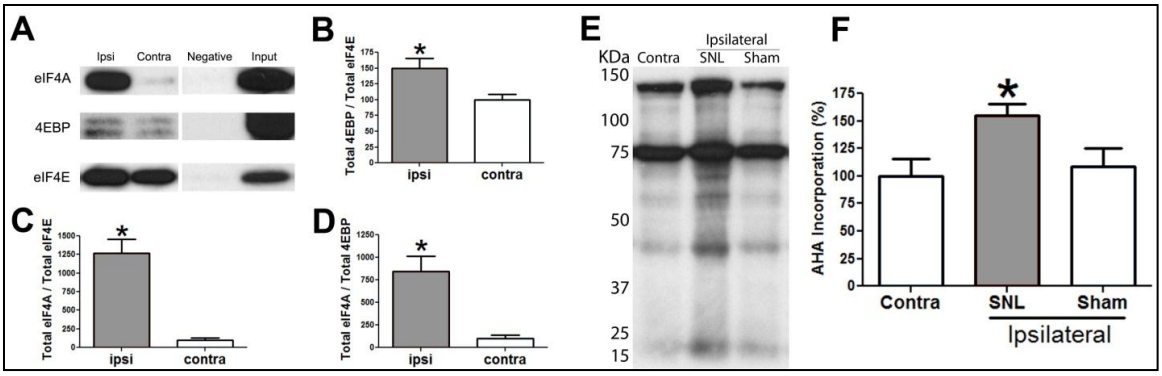

Figure 3.2: PNI enhances cap-dependent protein translation in the injured sciatic nerve. A) Western blot of eIF4A, 4EBP and eIF4E from sciatic nerve co-precipitated using 7-methyl-GTP conjugated beads. **B)** PNI induces only 50% increase in 4EBP (negative regulator of translation) association with cap-binding protein eIF4E, **C)** while injury induces a 1200% increase in the association of eIF4A (a component of eIF4F complex) with eIF4E. **D)** PNI induces a 750% increase in the ratio of eIF4A to 4EBP associated with eIF4E bound to 7-methyl-GTP conjugated beads. PNI increases nascent protein synthesis in injured sciatic nerve. **E)** Western blot of AHA incorporated into nascently synthesized proteins. **F)** PNI induces a 50% increase in the incorporation of AHA into nascently synthesized proteins. All samples taken 17 days post SNL from rats with n = 6 per condition. *$p<0.05$

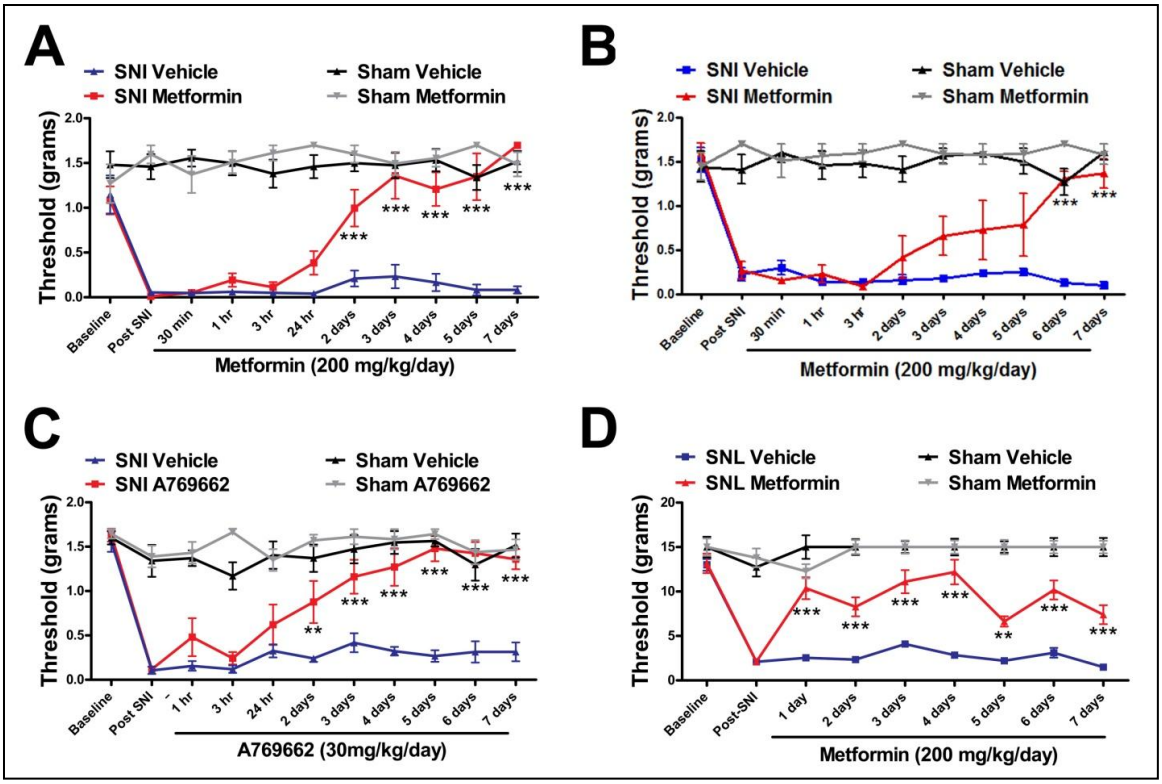

Figure 3.3: AMPK activators reduce PNI-induced neuropathic allodynia. Daily intraperitoneal injections of metformin in mice starting 2 **(A)** or 7 **(B)** weeks post PNI leads to a reversal of mechanical allodynia. **C)** A769662 daily treatment also reversed PNI-induced allodynia when started 2 weeks post PNI. **D)** Treatment of SNL rats (2 weeks post surgery) with metformin also significantly reduces neuropathic allodynia. N = 6 per group. ***p<0.01 and ***p<0.001*

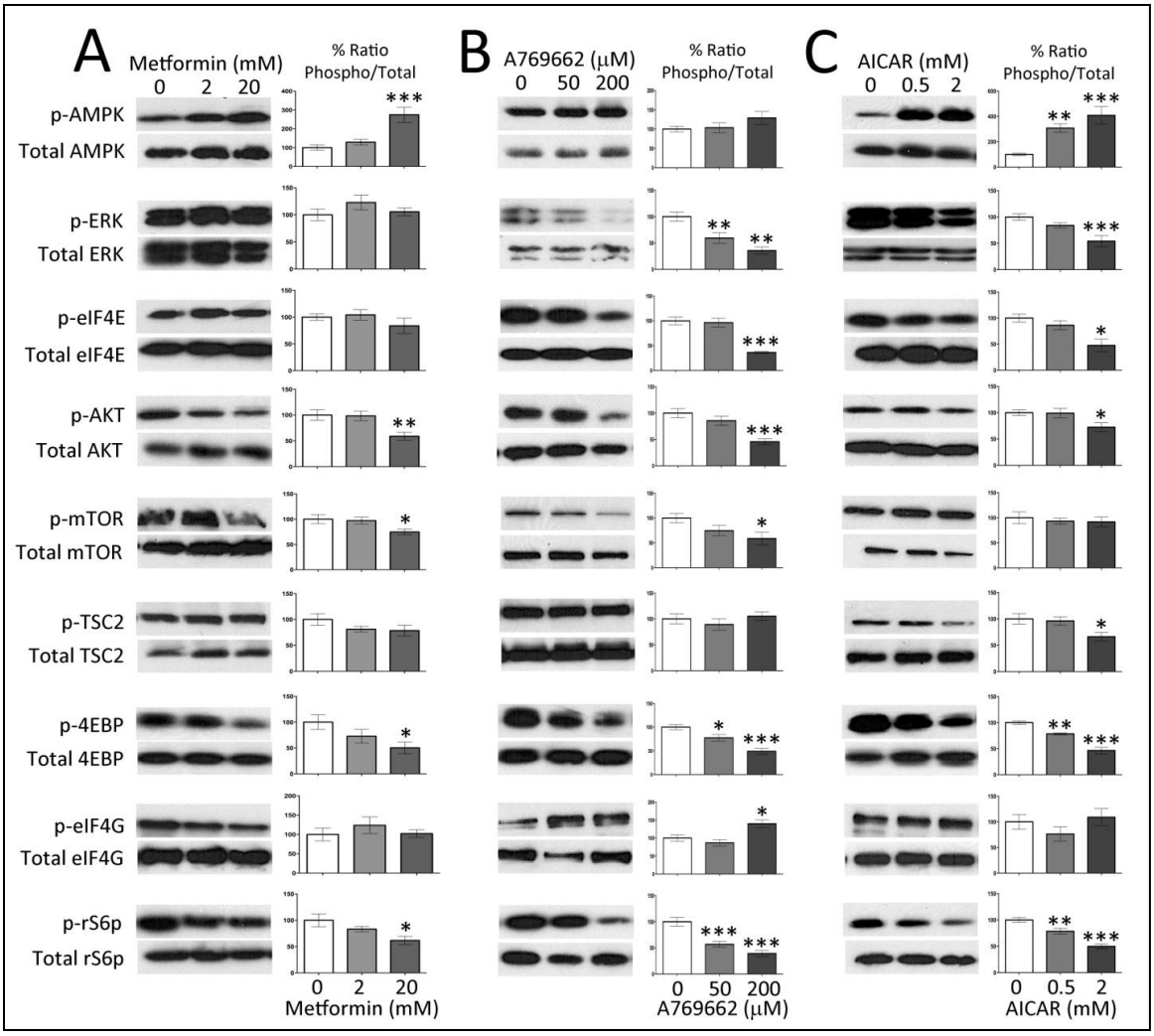

Figure 3.4: Treatment with AMPK activators suppresses translation regulation signaling. A) Treatment of mouse sensory neurons cultured in the presence of NGF (50 ng/ml) with metformin (2 and 20 mM) for 1 hour induces a dose-dependent increase in the phosphorylation of AMPK. Metformin treatment abrogates the phosphorylation of mTOR, 4EBP and rS6 in a dose-dependent manner. Metformin does not suppress the ERK-eIF4E pathway. **B)** AMPK allosteric activator A769662 suppresses translation regulation signaling. Treatment of mouse sensory neurons with A769662 (50 and 500 μM) results in a dose dependent suppression of phosphorylation of ERK, eIF4E, mTOR, 4EBP, AKT and rS6. **C)** Treatment of mouse sensory neurons with AICAR (0.5 and 2 mM) for 1 hour results in a dose dependent activation of AMPK. Moreover, AICAR dose-dependently suppresses the phosphorylation of ERK, eIF4E, AKT, TSC2, 4EBP and rS6. N = 6 per group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

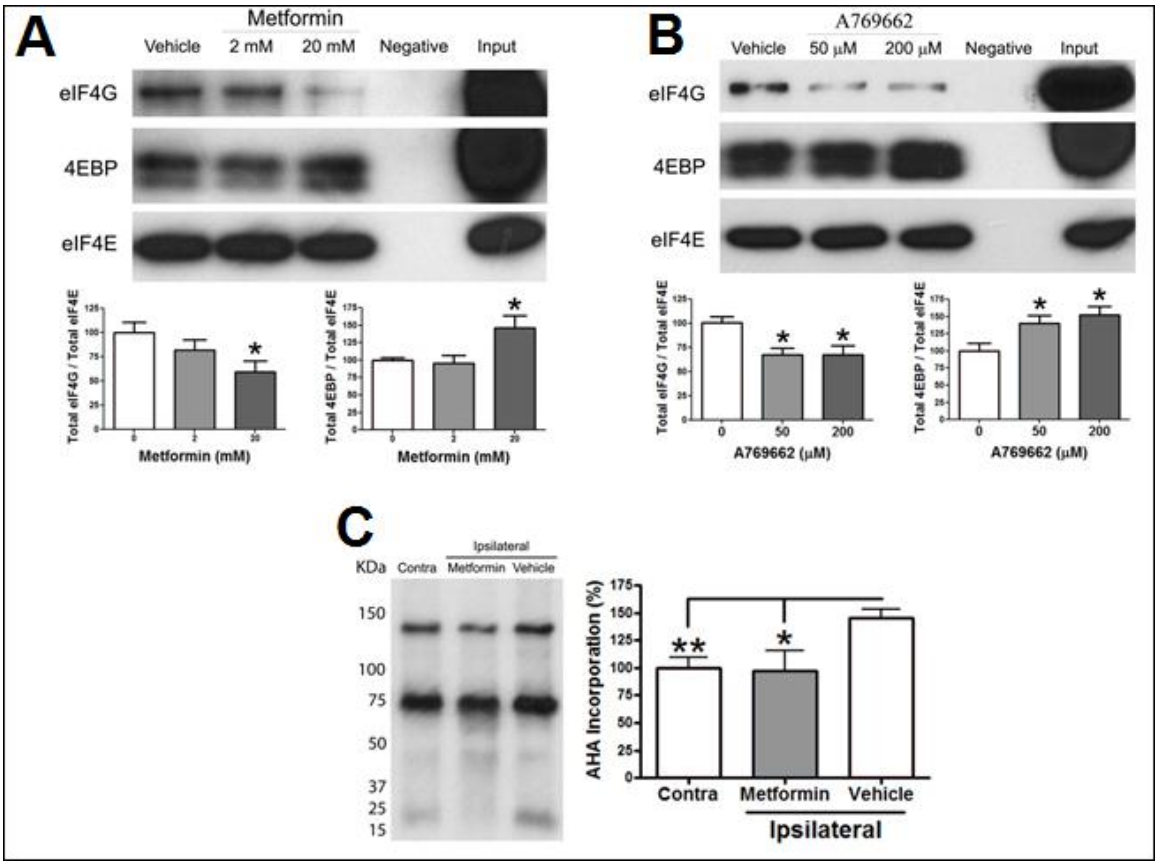

Figure 3.5: AMPK activators suppress translation in sensory neurons and the injured PNS. Treatment of mouse sensory neurons cultured in the presence of NGF (50 ng/ml) with **A)** Metformin (2 and 20 mM) or **B)** A769662 (50 and 500 μM) results in decreased binding of eIF4G and increased 4EBP binding to the m7GTP-cojugated sepharose beads in a dose-dependent manner consistent with a decrease in eIF4F complex formation. **C)** Metformin treatment of rats with SNL restores nascent protein synthesis in injured nerves to levels observed in uninjured nerves. N = 6 per group. **p<0.05, ** p<0.01.*

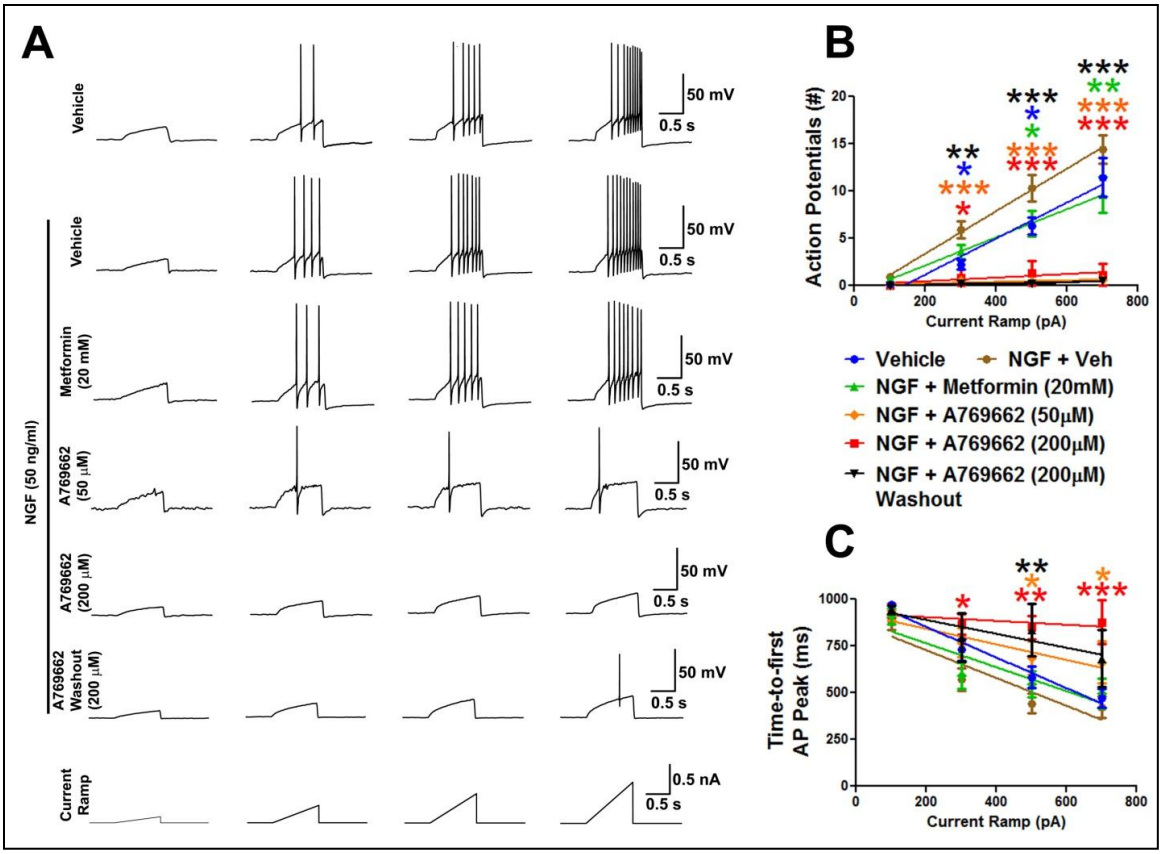

Figure 3.6: AMPK activators suppress hyperexcitability of sensory neurons. A) Patch clamp analysis of mouse primary sensory neurons cultured in the presence of NGF (50 ng/ml, n = 13) demonstrate an increase in the **B**) number of ramp current-evoked action potentials vs. vehicle (n = 14) and **C)** reduced latency to first action potential in response to ramp currents. Metformin (1hr, n = 14) and A769662 (1hr, n = 12) reverse these parameters. A769662 effect persists after washout (n = 8). Colored stars denote significant effects compared to the NGF + Vehicle group. **p <0.05, **p<0.01 and ***p<0.001*

3.7. Tables

Table 3.1: Quantification of western blots on ipsilateral vs contralateral L4 (uninjured) DRGs from SNL rats 17 days post-surgery

RAT SNL					
Uninjured L4 DRG vs. Contralateral					
	Contralateral	Contralateral	Ipsilateral	Ipsilateral	
Protein / Standardization	Mean %	SEM	Mean %	SEM	P-value
p-mTOR / total mTOR	100	17.9	402.8	63.6	0.001
p-mTOR / beta III tubulin	100	19.0	425.7	92.0	0.012
total mTOR / beta III tubulin	100	12.1	86.0	34.4	NS
p-4EBP / total 4EBP	100	11.6	100.1	9.3	NS
p-4EBP / beta III tubulin	100	15.8	190.3	22.2	0.003
total 4EBP / beta III tubulin	100	17.8	182.6	14.8	0.002
p-ERK / total ERK	100	11.0	121.0	17.1	NS
p-ERK / beta III tubulin	100	19.8	111.3	13.2	NS
total ERK / beta III tubulin	100	12.3	97.2	4.0	NS
p-eIF4alpha / total eIF4alpha	100	13.5	60.1	8.0	0.026
p-eIF2alpha / beta III tubulin	100	19.7	61.8	7.2	NS
total eIF2alpha / beta III tubulin	100	13.6	106.9	6.3	NS
mov10 / beta III tubulin	100	20.8	220.9	37.0	0.008
rck / beta III tubulin	100	12.5	123.5	14.1	NS
eIF4A / beta III tubulin	100	15.3	102.5	14.7	NS
p-AKT / total AKT	100	10.9	112.8	14.7	NS
p-AKT / beta III tubulin	100	6.0	107.8	12.2	NS
total AKT / beta III tubulin	100	10.8	92.5	3.9	NS
p-mTOR / GAPDH	100	14.1	387.2	72.4	0.006
p-4EBP / GAPDH	100	13.3	162.6	12.0	0.002
total 4EBP / GAPDH	100	11.8	163.1	9.6	0.000
mov10 / GAPDH	100	19.4	195.6	34.6	0.025

Table 3.2: Quantification of western blots on ipsilateral vs. contralateral L5 and L6 (injured) DRGs from SNL rats 17 days post-surgery.

RAT SNL					
Injured L5/L6 DRG vs. Contralateral					
	Contralateral	Contralateral	Ipsilateral	Ipsilateral	
Protein / Standardization	Mean %	SEM	Mean %	SEM	P-value
p-mTOR / total mTOR	100.0	22.2	71.6	16.2	NS
p-mTOR / beta III tubulin	100.0	27.5	321.0	59.1	0.003
total mTOR / beta III tubulin	100.0	23.6	579.4	178.3	0.016
p-4EBP / total 4EBP	100.0	12.7	98.4	7.3	NS
p-4EBP / beta III tubulin	100.0	33.1	222.1	46.2	0.049
total 4EBP / beta III tubulin	100.0	42.2	179.8	18.7	NS
FMRP / beta III tubulin	100.0	29.3	199.0	36.6	0.047
mov10 / beta III tubulin	100.0	36.4	875.0	304.2	0.035

Table 3.3: Quantification of western blots on ipsilateral vs. contralateral sciatic nerves from SNL rats 17 days post-surgery.

RAT SNL					
Sciatic Nerve Ipsilateral vs. Contralateral					
	Contralateral	Contralateral	Ipsilateral	Ipsilateral	
Protein / Standardization	Mean %	SEM	Mean %	SEM	P-value
p-mTOR / total mTOR	100	27.6	581.8	112.0	0.002
p-4EBP / total 4EBP	100	8.9	117.0	13.6	NS
p-4EBP / total 4EBP	100	12.6	408.0	104.2	0.008
p-rS6 protein / beta III-tubulin	100	22.6	1005.5	300.0	0.007
p-AKT / total AKT	100	17.3	101.6	22.0	NS
p-AKT / beta III-tubulin	100	14.4	276.4	67.5	0.034
total AKT / beta III-tubulin	100	11.2	261.6	36.9	0.003
p-ERK / total ERK	100	8.8	510.3	153.4	0.023
p-eIF4E / total eIF4E	100	14.8	164.0	17.6	0.024
p-eIF4E / beta III-tubulin	100	25.1	700.8	168.5	0.008
total eIF4E / beta III-tubulin	100	16.8	415.4	62.7	0.001
eIF4A / beta III-tubulin	100	14.6	241.0	31.0	0.001
p-eIF2alpha / total eIF2alpha	100	8.9	110.7	21.4	NS
p-eIF2alpha / beta III-tubulin	100	15.3	506.4	163.4	0.024
total eIF2alpha / beta III-tubulin	100	19.5	575.7	107.9	0.001
FMRP / beta III-tubulin	100	14.2	257.8	59.0	0.024
mov10 / beta III-tubulin	100	28.5	636.3	161.2	0.008
rck / beta III-tubulin	100	22.9	483.4	141.4	0.015
p-4EBP / GAPDH	100	25.4	224.6	18.6	0.004
total 4EBP / GAPDH	100	26.1	257.4	32.5	0.006
p-eIF4E / GAPDH	100	15.9	458.0	54.4	0.000
eIF4E / GAPDH	100	10.6	270.2	12.4	0.000
p-eIF2alpha / GAPDH	100	10.3	297.4	74.9	0.018
total eIF2alpha / GAPDH	100	4.7	244.3	15.7	0.000
rck / GAPDH	100	20.5	280.0	62.2	0.013
p-AKT / GAPDH	100	8.4	182.8	26.1	0.017
total AKT / GAPDH	100	14.1	177.2	18.6	0.011
eIF4A / GAPDH	100	9.3	249.6	33.0	0.001
fbxw7 / GAPDH	100	25.4	420.6	134.1	0.037

Table 3.4: Quantification of western blots on ipsilateral vs. contralateral sciatic nerves from SNI mice 17 days post-surgery.

MOUSE SNI					
Sciatic Nerve Ipsilateral vs. Contralateral					
	Contralateral	Contralateral	Ipsilateral	Ipsilateral	
Protein / Standardization	Mean %	SEM	Mean %	SEM	P-value
p-mTOR / total mTOR	100	7.6	128.0	19.6	NS
p-mTOR / beta III tubulin	100	13.4	211.4	68.9	NS
total mTOR / beta III tubulin	100	14.0	155.7	42.1	NS
p-4EBP / total 4EBP	100	5.8	94.0	5.7	NS
p-4EBP / beta III tubulin	100	15.0	150.6	17.6	0.049
total 4EBP / beta III tubulin	100	14.6	169.6	23.0	0.025
p-ERK / total ERK	100	13.6	511.9	187.6	0.049
p-ERK / beta III tubulin	100	13.3	500.4	109.5	0.003
total ERK / beta III tubulin	100	8.3	111.1	8.8	NS
p-eIF4E / total eIF4E	100	25.5	104.9	13.1	NS
p-eIF4E / beta III tubulin	100	22.7	301.0	41.3	0.001
total eIF4E / beta III tubulin	100	13.2	283.0	42.5	0.001
eIF4A / beta III tubulin	100	16.8	185.9	28.0	0.022
mov 10 / beta III tubulin	100	31.6	256.9	41.0	0.011
rck / beta III tubulin	100	26.3	482.7	155.0	0.032

CHAPTER IV

RESVERATROL ENGAGES AMPK TO INHIBIT ERK AND mTOR SIGNALING IN SENSORY NEURONS AND INHIBIT INCISION-INDUCED ACUTE AND CHRONIC PAIN

4.1. Abstract

Despite advances in our understanding of basic mechanisms driving post-surgical pain, treating incision-induced pain remains a major clinical challenge. Moreover, surgery has been implicated as a major cause of chronic pain conditions. Hence, more efficacious treatments are needed to inhibit incision-induced pain and prevent the transition to chronic pain following surgery. We reasoned that activators of AMP-activated protein kinase (AMPK) may represent a novel treatment avenue for the local treatment of incision-induced pain because AMPK activators inhibit ERK and mTOR signaling, two important pathways involved in the sensitization of peripheral nociceptors. To test this hypothesis we used a potent and efficacious activator of AMPK, resveratrol. Our results demonstrate that resveratrol profoundly inhibits ERK and mTOR signaling in sensory neurons in a time- and dose-dependent fashion and that these effects are linked to AMPK activation and independent of sirtuin activity. Interleukin-6 (IL-6) is thought to play an important role in incision-induced pain and resveratrol potently inhibited IL-6-mediated signaling to ERK in sensory neurons and blocked IL-6-mediated allodynia in vivo through a local mechanism of action. Using a model of incision-induced allodynia in mice, we further demonstrate that local injection of resveratrol around the surgical wound strongly attenuates incision-induced allodynia. Intraplantar IL-6 injection and plantar incision

induces persistent nociceptive sensitization to PGE2 injection into the affected paw after the resolution of allodynia to the initial stimulus. We further show that resveratrol treatment at the time of IL-6 injection or plantar incision completely blocks the development of persistent nociceptive sensitization consistent with the blockade of a transition to a chronic pain state by resveratrol treatment. These results highlight the importance of signaling to translation control in peripheral sensitization of nociceptors and provide further evidence for activation of AMPK as a novel treatment avenue for acute and chronic pain states.

4.2. Background:

Incision associated with surgery causes acute pain and surgery has been identified as a potential major cause of chronic pain conditions (Aasvang et al., 2010; Kehlet et al., 2006; Perkins and Kehlet, 2000). Between 10-50% of patients develop chronic pain following surgical procedures such as groin hernia repair, breast and thoracic surgery, leg amputation, or coronary artery bypass surgery (Kehlet et al., 2006). Despite improvements in post-surgical pain treatment strategies, the incidence of moderate to severe pain after surgery is still high in several patient populations (Apfelbaum et al., 2003; Pavlin et al., 2002). Moreover, the exact mechanisms involved in the development of persistent pain following surgery have not been elucidated. Interleukin 6 (IL-6), a pro-inflammatory cytokine, is a significant mediator of nociceptive plasticity in pre-clinical pain models and is implicated in several human pain conditions. Serum IL-6 levels increase significantly in patients immediately after surgery (Holzheimer and Steinmetz, 2000; Notarnicola et al., 2011; Pandazi et al., 2010)

and circulating IL-6 levels are proportional to the extent of tissue injury during an operation, rather than being proportional to the duration of the surgical procedure itself (Lin et al., 2000). Furthermore, IL-6 levels have been shown to be elevated in skin around incision sites (Bryan et al., 2005; Sato and Ohshima, 2000) and it has been implicated in preclinical incision-induced pain models (Clark et al., 2007; Liang et al., 2008; Sahbaie et al., 2009). Although these reports are suggestive of involvement of IL-6 in post-surgical pain, the precise mechanisms by which IL-6 drives post-surgical pain are poorly understood. However, IL-6 has been implicated as an important player in many preclinical pain models and elegant genetic studies have demonstrated that IL-6's pain promoting qualities are mediated by IL-6 receptors expressed by nociceptors (Andratsch et al., 2009; Quarta et al., 2011).

Recently we demonstrated that IL-6 causes induction of nascent protein synthesis in primary afferent neurons and their axons which can contribute to increased nociceptive sensitivity (Melemedjian et al., 2010). We have also shown that AMP-activated protein kinase (AMPK) activators reverse mechanical allodynia in neuropathic pain models and that these compounds negatively regulate protein synthesis in sensory afferents (Melemedjian et al., 2011b). AMPK, the energy sensor of the cell, is a heterotrimeric Ser/Thr protein kinase activated by alterations in cellular AMP: ATP ratio. Once activated, AMPK inhibits ATP consuming anabolic processes such as protein translation (Hardie, 2007). AMPK activation achieves these effects largely through inhibition of mammalian target of rapamycin (mTOR) signaling (Hardie, 2007) but AMPK activation has also been linked to inhibition of mitogen activated protein kinase (MAPK) signaling (Kim et al., 2001; Melemedjian et al., 2011b). We hypothesized

that activation of AMPK signaling pathway may represent a novel pharmacological mechanism for the treatment of post-surgical pain.

To test this hypothesis, we have utilized resveratrol, a natural polyphenol found in red grapes and wine, which has previously been shown to increase AMPK activity in neurons (Dasgupta and Milbrandt, 2007b). Although several studies originally described resveratrol as an activator of sirtuin enzymes, which are NAD-dependent deacetylases (Howitz et al., 2003; Jarolim et al., 2004; Viswanathan et al., 2005; Wood et al., 2004) these results have been challenged based on lack of specificity in screening assays (Beher et al., 2009; Pacholec et al., 2010). Moreover, several recent in vivo studies strongly suggest that resveratrol effects are independent of sirtuins. On the other hand, resveratrol is a highly potent and efficacious activator of AMPK (Baur et al., 2006; Dasgupta and Milbrandt, 2007a; Zang et al., 2006) and its metabolic effects are dependent on ◎ subunit AMPK expression suggesting that AMPK is the major, if not the primary, target for resveratrol in vivo (Um et al., 2010)

Herein, we demonstrate that resveratrol activates AMPK and suppresses translation regulation pathways in sensory neurons in a dose-dependent, time-dependent and reversible manner. We also show that resveratrol inhibits both acute and persistent sensitization in an IL-6-induced hyperalgesic priming model as well as in a in a model of postsurgical pain. These findings suggest that resveratrol may have utility in the treatment of post-surgical pain and further implicate AMPK as a novel target for the development of analgesics.

4.3. Materials and Methods

4.3.1. Experimental animals

Male ICR mice (Harlan, 20-25 g) were used for the study. All animal procedures were approved by the Institutional Animal Care and Use Committee of The University of Arizona and were in accordance with International Association for the Study of Pain guidelines.

4.3.2. Behavior testing

For the testing, animals were placed in acrylic boxes with wire mesh floors and allowed to habituate for approximately 1 hr on all testing days. Paw withdrawal thresholds were measured using calibrated von Frey filaments (Stoelting, Wood Dale, IL) by stimulating the plantar aspect of left hind paw using the up-down method (Chaplan et al., 1994).

4.3.3. IL-6 priming and behavior testing

A mouse model for 'hyperalgesic priming' originally developed by Levine and colleagues [for review see (Reichling and Levine, 2009)] and adapted for mice (Asiedu et al., 2011b) was used for the study. Baseline mechanical thresholds of the left hind paw were measured prior to IL-6 injection. For acute sensitization experiments, IL-6 (0.1ng) was injected into the plantar surface of the left hind paw in a volume of 25µl (diluted in saline). Resveratrol (0.1, 1, or 10µg) or vehicle was co-injected with IL-6 and paw withdrawal thresholds were measured at 1 hr, 3 hr, 24 hr, 48h and 72h post injection. For persistent sensitization experiments animals received an injection of PGE_2 (100ng) in the plantar surface of left hind paw in a volume of 25µl 4 days following initial intraplantar injection. Following PGE_2 injection, paw withdrawal thresholds were again measured at 1 hr, 3 hr and 24 hr following the PGE_2 injection.

4.3.4. Plantar incision and behavioral testing

Prior to surgery all animals were assessed for paw withdrawal thresholds. A mouse model of incisional pain was used for this study (Banik et al., 2006). A 5mm longitudinal incision was made with a number 11 blade through skin, fascia and muscle of the plantar aspect of the hindpaw in isoflurane-anesthetized rats. Sham controls underwent the same procedure but without the incision. The skin was apposed with 2 sutures of 5 mm silk. Animals received intraplantar injection of resveratrol or vehicle around the incision at times indicated after incision. Animals were allowed to recover for 24 hours and then paw withdrawal thresholds were measured at 24 hr, 48h, 72h, D5, D7, D9, D11, and D13 post-surgery. For persistent sensitization experiments, the animals received an intraplantar injection of PGE_2 (100ng/25μl) 14 days following incision or sham procedures. The paw withdrawal thresholds were again measured at 1 hr, 3 hr and 24 hr following the PGE_2 injection.

4.3.5. Primary neuronal cultures

Mouse trigeminal ganglia (TG) were excised aseptically and placed in Hank's Buffered Salt Solution (HBSS, Invitrogen) on ice. The ganglia were dissociated enzymatically with collagenase A (1 mg /ml, 25 min, Roche) and collagenase D (1 mg/ml, Roche) with papain (30 U/ml, Roche) for 20 min at 37 °C. To eliminate debris 70 μm (BD) cell strainers were used. The dissociated cells were resuspended in DMEM/F12 (Invitrogen) containing 1X pen-strep (Invitrogen), 1X GlutaMax, 3μg/ml 5-FDU (Sigma), 7μg/ml uridine (Sigma), 50ng/ml NGF (Millipore) and 10% fetal bovine serum (Hyclone). The cells were

plated in 6-well plates (BD Falcon) and incubated at 37 °C in a humidified 95% air /5%CO2 incubator. Cultures were maintained in resuspension media until time of treatment. For experiments where resveratrol treatments were done alone, cultures were maintained in the continuous presence of nerve growth factor (NGF) at a concentration of 50 ng/ml. NGF was excluded for IL-6 experiments. On day 5 the cells were washed in DMEM/F12 media for 30 mins and subsequently were treated as described in results.

4.3.6. Western blotting

Protein was extracted from cells in lysis buffer (50 mM Tris HCl, 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA at pH 7.4) containing protease and phosphatase inhibitor mixtures (Sigma) with an ultrasonicator on ice, and cleared of cellular debris and nuclei by centrifugation at 14,000 RCF for 15 min at 4°C. 15 μg of protein per well were loaded and separated by standard 7.5% or 10% SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore) and then blocked with 5% dry milk for 3 h at room temperature. The blots were incubated with primary antibody overnight at 4°C and detected the following day with donkey anti-rabbit antibody conjugated to horseradish peroxidase (Jackson Immunoresearch). Signal was detected by ECL on chemiluminescent films. Each phosphoprotein was normalized to the expression of the corresponding total protein on the same membrane. Densitometric analyses were performed using Image J software (NIH).

4.3.7. 5′ mRNA cap complex analysis

After the protein extraction, 50 μg protein was incubated with 7- methyl GTP Sepharose 4B beads (GE Healthcare) in the presence of 100μM GTP for 2 h at 4 °C. Unconjugated sepharose 4B beads were used for the negative controls. The beads were then pelleted and washed twice with lysis buffer. After a final centrifugation the pellet was suspended in 1X Laemmli Sample Buffer containing 5 % v/v β-mercaptoethanol and eIF4E, eIF4G, eIF4A and 4EBP bound to the precipitated beads was analyzed by western blotting.

4.3.8. Drugs and primary antibodies

Resveratrol was from Cayman Chemical; mouse 2.5S NGF was from Millipore; The following rabbit polyclonal antibodies were obtained from Cell Signaling: p-ERK (Thr202/Tyr204, cat# 4377), total ERK, p-eIF4E (Ser209, cat# 9741), total eIF4E, p-mTOR (Ser2448, cat# 2971), total mTOR, p-4EBP(Thr37/46, cat # 9459), total 4EBP, p-eIF4G (Ser1108, cat# 2441), total eIF4G, p-AKT (Ser473, cat# 4058), total AKT, GAPDH and eIF4A.

4.3.9. Statistical Analysis and Data Presentation

Data are shown as means and the standard error of the mean (± SEM) of eight independent cell culture wells, 6 tissue samples (for in vivo Western blotting, eIF4F complex formation and nascent protein synthesis) or 6 animals (for behavioral studies). Graph plotting and statistical analysis used Graphpad Prism Version 5.03 (Graph Pad Software, Inc. San Diego, CA, USA). Statistical evaluation was performed by one- or two-way analysis of variance (ANOVA),

followed by appropriate post-hoc tests. The a priori level of significance at 95% confidence level was considered at $p < 0.05$.

4.4. Results

4.4.1. Resveratrol suppresses signaling to translation machinery in sensory neurons

While resveratrol has been shown to stimulate AMPK and inhibit mTOR signaling in cell-lines and some neural tissues, its effect on sensory neurons is unknown. Hence, we first asked whether resveratrol treatment influenced AMPK activity or signaling pathways involved in regulating cap-dependent protein translation in cultured trigeminal ganglion (TG) neurons from mice grown in the presence of NGF (50ng/ml) for 5 days. TG cultures were treated with vehicle or increasing doses (10, 30 or 100μM) of resveratrol (Fig 4.1) for 1 hr. Resveratrol activated AMPK in a dose dependent manner (Fig 4.1A) and suppressed activity in signaling pathways that promote cap-dependent translation. Specifically, these changes included significantly decreased phosphorylation of extracellular signal regulated kinase (ERK, Fig 4.1B) and its downstream target involved in translation control eukaryotic initiation factor (eIF) 4E (Fig 4.1C). Resveratrol also decreased protein kinase B (AKT Fig 4.1D), mTOR (Fig 4.1E) and tuberin sclerosis protein 2 (TSC2, Fig 4.1F) indicating negative regulation of the mTOR pathway in TG neurons. Consistent with this notion, eiF4E binding protein (4EBP, Fig 4.1G) and ribosomal S6 protein (rS6p, Fig 4.1I), which are downstream mTOR targets, demonstrated decreased phosphorylation upon resveratrol treatment. Finally, resveratrol increased eIF4G

phosphorylation (Fig 4.1H), an effect inconsistent with known mechanisms of eIF4G regulation but an effect that we have consistently observed with other AMPK activators (e.g. A769662) (Melemedjian et al., 2011b). Thus, in cultured TG neurons, resveratrol activates AMPK and suppresses signaling via the ERK and mTOR pathways to translation machinery suggesting a dose-dependent inhibition of cap-dependent translation by resveratrol in sensory neurons.

Having established a dose-dependent effect of resveratrol on TG neurons in culture, we next asked whether these effects were time dependent. Resveratrol, at a maximally effective dose (100μM) was applied to TG neurons for 10, 30 or 100 min and activity in signaling pathways was assessed by Western blot (Fig 4.2). Resveratrol activated AMPK maximally at 10 and 30 min treatment (Fig 4.1A). Similarly, resveratrol suppressed activity in the ERK (Fig 4.2B and 4.2C) and mTOR pathways (Fig 4.1D-I) over the time course of resveratrol exposure.

Because resveratrol led to a profound inhibition of ERK and mTOR signaling pathways, we next asked whether this effect is reversible. TG cultures were treated with resveratrol for 1 hr followed by 1 or 2 hr washout periods. Resveratrol led to a reversible activation of AMPK (Fig 4.3A) and a reversible inhibition of both ERK (Fig 4.3B and 4.3C) and mTOR signaling (Fig 4.3D-H). Hence, resveratrol dose- and time-dependently activates AMPK and inhibits ERK and mTOR signaling in a reversible fashion in sensory neurons.

Finally, we asked whether resveratrol treatment leads to an inhibiton of cap-dependent translation in TG neurons. Cap-dependent translation requires eIF4F complex formation on the 5'cap structure of mRNAs (Sonenberg and Hinnebusch, 2009) and this can be assayed with a cap pull-down assay that

assesses eIF4G and 4EBP binding to eIF4E (Beretta et al., 1996). The eIF4F complex is composed of eIF4E bound to eIF4A and eIF4G whereas 4EBP binding to eIF4E is indicative of inhibition of cap-dependent translation because 4EBP represses eIF4A and 4G binding to eIF4E (Sonenberg and Hinnebusch, 2009). Resveratrol treatment for 1 hr led to a profound increase in 4EBP binding to eIF4E and a parallel decrease in eIF4G binding (Fig 4.4A-C). Hence, resveratrol dose-dependently inhibits eIF4F complex formation in sensory neurons consistent with inhibition of cap-dependent translation.

4.4.2. Resveratrol-mediated inhibition of ERK and mTOR does not require SIRT1

While the above results strongly suggest that resveratrol acts via activation of AMPK, there have been conflicting reports suggesting that resveratrol produces its effect by activation of SIRT1, a NAD-dependent deacetylase (Howitz et al., 2003; Jarolim et al., 2004; Viswanathan et al., 2005; Wood et al., 2004). To assess whether Sirt1 may play a role in this process we utilized a Sirt1 inhibitor to ask whether it would block the effects of resveratrol on ERK and mTOR signaling. Trigeminal primary neuronal cultures were pre-treated for 1 hr and then co-treated with nicotinamide (10 mM), which inhibits SIRT1, in presence of resveratrol (100 μM) for 1 hr (Table 4.1). Nicotinamide coincubation had no effect on resveratrol-mediated activation of AMPK or inhibition of ERK or mTOR signaling. Likewise, if resveratrol activates SIRT1, SIRT1 activators should be able to recapitulate effects produced by resveratrol. Hence, to further rule out a role for Sirt1, TG cultures were treated with vehicle or a SIRT1 activator, CAY10602 (Nayagam et al., 2006) (20 and 60μM) for 1 hr.

Treatment with CAY10602 did not change AMPK, mTOR or ERK levels (Table 4.2). These results rule out a role for Sirt1 in resveratrol mediated regulation of AMPK, ERK and mTOR signaling and support the conclusion that resveratrol engages AMPK signaling to inhibit ERK and mTOR in primary sensory neurons.

4.4.3. Resveratrol blocks IL-6-induced signaling and IL-6-mediated allodynia

Multiple lines of evidence indicate that IL-6 is an important mediator of nociceptive plasticity in postsurgical pain. IL-6 levels have been shown to be elevated systemically (Holzheimer and Steinmetz, 2000; Pandazi et al., 2010) as well as locally around the incision (Bryan et al., 2005; Sato and Ohshima, 2000) and IL-6 has been linked to post-surgical pain in preclinical models (Clark et al., 2007; Liang et al., 2008; Sahbaie et al., 2009). We have recently demonstrated that IL-6 signals to the translational machinery in primary afferents, leading to enhanced cap-dependent translation by activating the ERK/eIF4E (Melemedjian et al., 2010). While the results above demonstrate that resveratrol decreases ERK and mTOR signaling in TG neurons, it is not known whether resveratrol is capable of blocking signaling via ERK or mTOR engaged by extracellular signals. Hence, we asked whether resveratrol blocks IL-6-induced changes ERK/eIF4E signaling in primary afferent neurons. Pretreatment of the TG cultures with resveratrol (100 μM, 15 min) and subsequent co-treatment with IL-6 (50ng/ml, 15 min) completely blocked IL-6 mediated phosphorylation of ERK and eIF4E in TG cultures (Fig 4.5). These findings indicate that resveratrol blocks IL-6-induced signaling in sensory neurons.

We have likewise demonstrated that IL-6 injection into the hindpaw of mice leads to allodynia that is dependent on translation regulation via the

ERK/eIF4E pathway (Melemedjian et al., 2010). Because resveratrol inhibits IL-6-mediated ERK/eIF4E signaling in sensory neurons we hypothesized that resveratrol would inhibit IL-6-mediated allodynia in vivo. Intraplantar injection of IL-6 (0.1ng) produces acute mechanical allodynia which lasts for ~3d, with complete resolution by day 4 (Fig 6A). Co-injection with resveratrol (0.1μg or 1μg or 10μg) dose-dependently blocked IL-6-induced allodynia (Fig 4.6A and 4.6B). There was no statistically significant difference between resveratrol and vehicle treated groups. Hence, resveratrol blocks both IL-6-mediated signaling via the ERK/eIF4E pathway and IL-6-induced allodynia. These findings suggest that resveratrol may be an efficacious compound for use in pain conditions linked to IL-6 signaling, such as post-incisional pain.

4.4.4. Resveratrol inhibits allodynia in a mouse model of post-surgical pain

The above results predict that resveratrol should be effective in blocking allodynia in a model of post-surgical pain. We utilized a mouse model of incisional pain to assess if resveratrol can prevent development of allodynia following the plantar incision. Animals received a plantar incision on the left hindpaw. Resveratrol (1μg or 10μg) or vehicle was injected into the paw around the incision either immediately following incision and 24 hrs post surgery or 1 and 3 days following incision. Mice with plantar incision that received vehicle displayed mechanical allodynia lasting for at least 9 days. In contrast, animals that received resveratrol at the time of incision and again 1 day later showed blunted allodynia and this effect was dose-dependent (Fig 4.7A and 4.7B). Moreover, administering resveratrol 1 and 3 days following incision significantly inhibited mechanical allodynia induced by incision (Fig 4.7C). No changes in

threshold were observed in sham animals receiving resveratrol. These results assert that resveratrol can be a potentially efficacious treatment for post-surgical pain.

4.4.5. Resveratrol blocks persistent sensitization induced by IL-6 injection and plantar incision

Persistent pain is a common feature experienced by many patients undergoing surgical procedures (Kehlet et al., 2006). Therefore, we assessed if resveratrol is effective in blocking persistent sensitization induced by IL-6 injection and plantar incision. Persistent sensitization can be revealed in both the models with a second intraplantar injection of inflammatory mediator, PGE_2 (100 ng), after the resolution of initial allodynia (Asiedu et al., 2011b). For the IL-6 induced persistent sensitization, co-treatment of resveratrol with IL-6 on Day 1 abolished the IL-6 induced persistent sensitization following PGE_2 injection on day 6 (Fig 4.8A). Similarly, in the incision model, treatment with resveratrol at the time of incision and 1 day later or 1 and 3 days following incision both abolished persistent sensitization precipitated by PGE_2 injection 14 days after incision. Hence, resveratrol not only inhibits allodynia induced by IL-6 or plantar incision but it also blocks the development of persistent nociceptive sensitization.

4.5. Discussion

The present findings make a compelling case for the use of resveratrol as a local treatment for both incision induced pain and prevention of chronic pain induced by incision. They show that resveratrol potently and efficaciously inhibits ERK and mTOR signaling in sensory neurons. The mTOR (Geranton et

al., 2009; Jimenez-Diaz et al., 2008; Obara et al., 2011; Price et al., 2007) and ERK pathways (Ji et al., 2009) have been linked to pathology in multiple preclinical pain models and our previous findings strongly implicate these pathways in induction of mechanical allodynia by IL-6 and NGF (Asiedu et al., 2011a; Melemedjian et al., 2010) and the development and maintenance of nerve-injury induced allodynia (Melemedjian et al., 2011b). The pharmacological action of resveratrol observed in our experiments is likely linked to activation of AMPK. We have previously implicated AMPK activation in alleviation of neuropathic pain (Melemedjian et al., 2011b), hence, the findings described herein expand the potential clinical usefulness of AMPK activators into the area of post-surgical pain. We conclude that diverse pharmacological mechanisms for activation of AMPK may have utility as novel analgesics for a variety of pain conditions.

Anabolic processes, such as protein synthesis, are orchestrated by upstream kinases that signal to the translation machinery (Sonenberg and Hinnebusch, 2009) such as mTOR and ERK. These kinases can be targeted individually by selective inhibitors or they can be negatively modulated by endogenous signaling factors that act on these pathways (Zoncu et al., 2011). A crucial kinase for negative regulation of translation is the ubiquitous, energy-sensing kinase AMPK. Activation of AMPK by depletion of cellular nutrients or through pharmacological intervention results in a dampening of signaling to the translation machinery (Zoncu et al., 2011). This is the natural cellular response to energy deprivation wherein high AMP levels signal to AMPK thereby shutting down anabolic processes when nutrient levels are low. AMPK is not solely regulated by cellular homeostatic mechanisms as it can also be targeted pharmacologically via a number of investigational compounds (e.g. AICAR and

A769662 (Cool et al., 2006)), natural products (resveratrol (Dasgupta and Milbrandt, 2007a; Zang et al., 2006)) and by the widely clinically available and safe drug metformin (Ouyang et al., 2011; Shaw et al., 2005). AMPK negatively regulates mTOR via activation of mTOR's negative regulator, TSC2 (Corradetti et al., 2004). This results in a profound inhibition of mTOR and its downstream targets involved in translation control (e.g. 4EBP and ribosomal S6 kinase and rS6p (Corradetti et al., 2004)). Activation of AMPK also negatively regulates ERK activity induced by growth factors and cytokines (Kim et al., 2001). This likely occurs via phosphorylation of the insulin receptor substrate 1 (IRS1) protein at Serine 794 (Tzatsos and Tsichlis, 2007). IRS-1 is a critical component of the signaling module of all tyrosine kinase receptors (Trks) and is linked to GP130 (the IL-6 signal transduction receptor) signaling (Argetsinger et al., 1995). This interaction may explain the inhibitory effect of resveratrol on IL-6-mediated ERK/eIF4E signaling observed here. Hence, engaging AMPK with potent activators of this pathway (e.g. resveratrol) represents a unique opportunity to achieve inhibition of pain-related signaling because it harnesses the cell's natural mechanism for dampening signaling in two pathways strongly implicated in pain amplification in the periphery, ERK (Ji et al., 2009) and mTOR (Geranton et al., 2009; Jimenez-Diaz et al., 2008; Melemedjian et al., 2011b; Obara et al., 2011; Price et al., 2007).

Accordingly, we hypothesized that AMPK activators may represent a novel tool for the treatment of post-surgical pain. We chose to focus on resveratrol for these experiments because resveratrol is a potent and efficacious activator of AMPK (Dasgupta and Milbrandt, 2007a). Our findings clearly demonstrate that local application of resveratrol to the site of incision reduces

mechanical allodynia, and, importantly, prevents the transition to a chronic pain-like state as measured by PGE_2 precipitated persistent nociceptive sensitization. These findings are consistent with previous experiments where we have shown that inhibition of translation regulation signaling during the initiation of allodynia induced by IL-6 or IL-6 and NGF prevents the development of persistent nociceptive sensitization (Asiedu et al., 2011a). Moreover, we have recently shown that AMPK activators reduce peripheral nerve injury-induced allodynia and decrease excitability of sensory neurons in vitro (Melemedjian et al., 2011a). These findings collectively create a compelling case for the further exploration and development of AMPK activators for the treatment of post-surgical pain.

While the pharmacological action of resveratrol has been an area of controversy, most evidence now points to AMPK as the major target of resveratrol. As described above, much attention was originally paid to resveratrol as an activator of sirtuins, in particular sirt1. However, subsequent studies have questioned these original results and recent studies in transgenic animals point to AMPK as a requisite component of resveratrol signaling (Um et al., 2010). Resveratrol, stimulates AMPK in a liver kinase B1 (LKB1) –dependent fashion, similar to the upstream activation of AMPK by metformin (Dasgupta and Milbrandt, 2007a; Shin et al., 2009). We found that resveratrol stimulates AMPK in TG neurons in a dose- and time-dependent fashion and that this AMPK activation is correlated with decreased ERK and mTOR signaling, events that we have previously shown are stimulated by other AMPK activators in TG neurons (Melemedjian et al., 2011b). Inhibition or activation of sirt1 failed to inhibit or recapitulate the effects of resveratrol, respectively, likely ruling out an

effect of sirtuins in our experiments. Several other mechanisms of action have been ascribed to resveratrol including inhibition of inducible cyclooxygenase (Subbaramaiah et al., 1998) and inhibition of cyclin-dependent kinase 5 (Utreras et al., 2011). It is unlikely that these mechanisms contribute to the inhibition of ERK and mTOR signaling that we have observed in TG neurons in vivo; however, we cannot exclude the potential contribution of these effects of resveratrol to our behavioral results. Finally, resveratrol has been shown to possess voltage gated-sodium channel inhibition properties (Kim et al., 2005). This effect has a slow onset (minutes of drug application is needed), inconsistent with direct channel blockade, and may be due to AMPK activation. In support of this conclusion, we have shown that other AMPK activators induce a profound decrease in sensory neuron excitability via a suppression of ramp-current evoked spiking (Melemedjian et al., 2011b).

Several previous reports have demonstrated an anti-allodynic or anti-hyperaglesic effect of resveratrol in preclinical pain models including the formalin model (Torres-Lopez et al., 2002), complete Freund's adjuvant-induced inflammation (Gentilli et al., 2001) and nucleus pulposus-induced allodynia (Wuertz et al., 2011). While, again, several mechanisms of action have been ascribed to resveratrol in these assays, our findings provide novel evidence linking resveratrol's anti-allodynic effects in the periphery to ERK and mTOR inhibition via activation of AMPK. Because resveratrol has poor bioavailability that can be sensitive to physiological factors when given systemically (Larrosa et al., 2011), we focused on its local effects in incision-induced pain. Resveratrol is a natural product that can be made in different preparations for human use (it is currently sold as a dietary supplement). Based on our present results, we

propose that preparations of resveratrol for local use in post-surgical pain situation may be clinically useful in a similar fashion (but with obvious different mechanisms of action) to highly purified capsaicin surgical wound infusions (Aasvang et al., 2008). Such preparations may afford inhibition of nociceptor sensitization and protect against a transition to chronic pain induced by surgery.

4.6. Figures

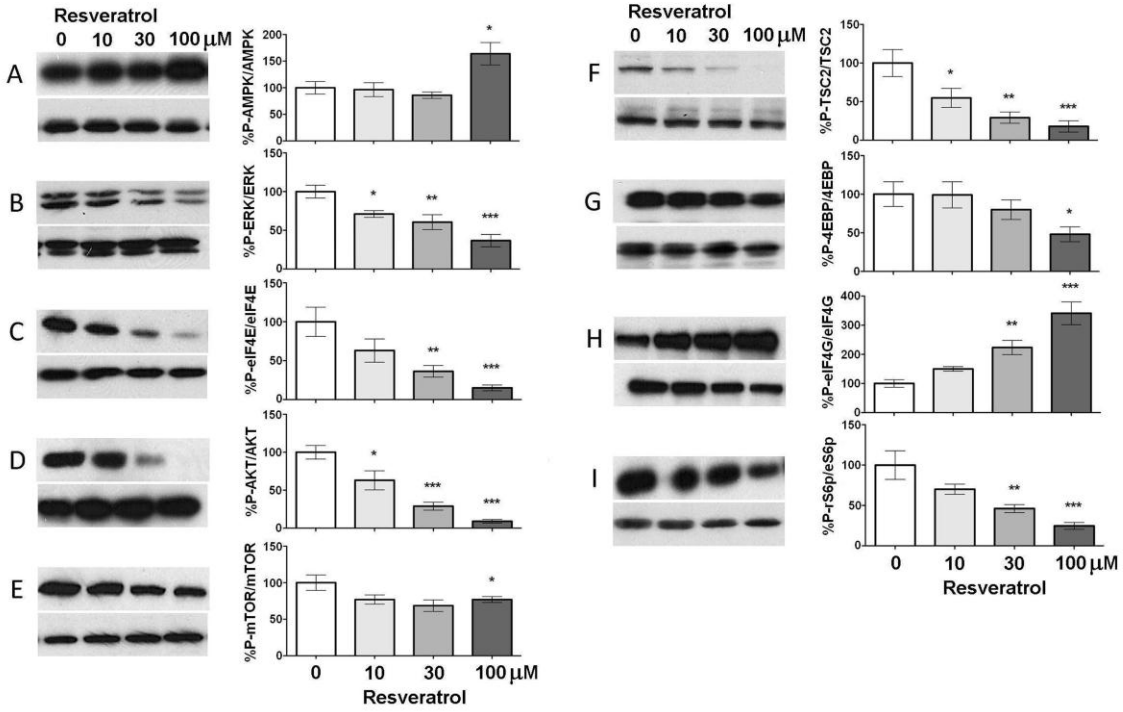

Figure 4.1: Resveratrol suppresses ERK/mTOR signaling in sensory neurons in a dose-dependent manner. Treatment of TG neurons with resveratrol (10, 30, and 100µM) for 1h induces a dose-dependent increase in phosphorylation of AMPK (A). Resveratrol treatment significantly decreases the phosphorylation of ERK (B), eIF4E (C), AKT (D), mTOR (E), TSC2 (F) 4EBP (G) and rS6p (I) but increases eIF4G phosphorylation (H).

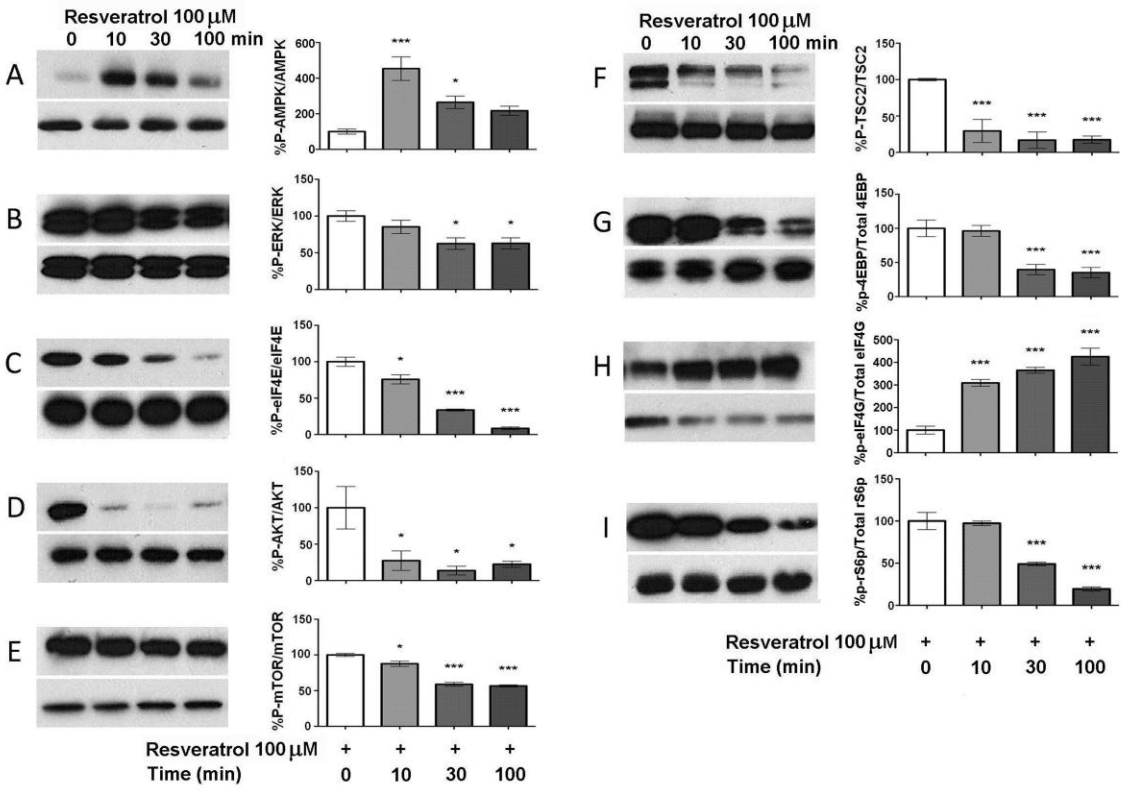

Figure 4.2: Suppression of ERK/mTOR signaling by resveratrol is time dependent. TG neurons were treated with 100μM resveratrol for 0, 10, 30, and 100 min. Resveratrol induces an increase in phosphorylation of AMPK (A) maximally with 10 and 30 min treatment. Resveratrol decreases the phosphorylation of ERK (B), eIF4E (C), AKT (D), mTOR (E), TSC2 (F) 4EBP (G) and rS6 (I) and this effect is time-dependent. Resveratrol increased eIF4G phosphorylation (H).

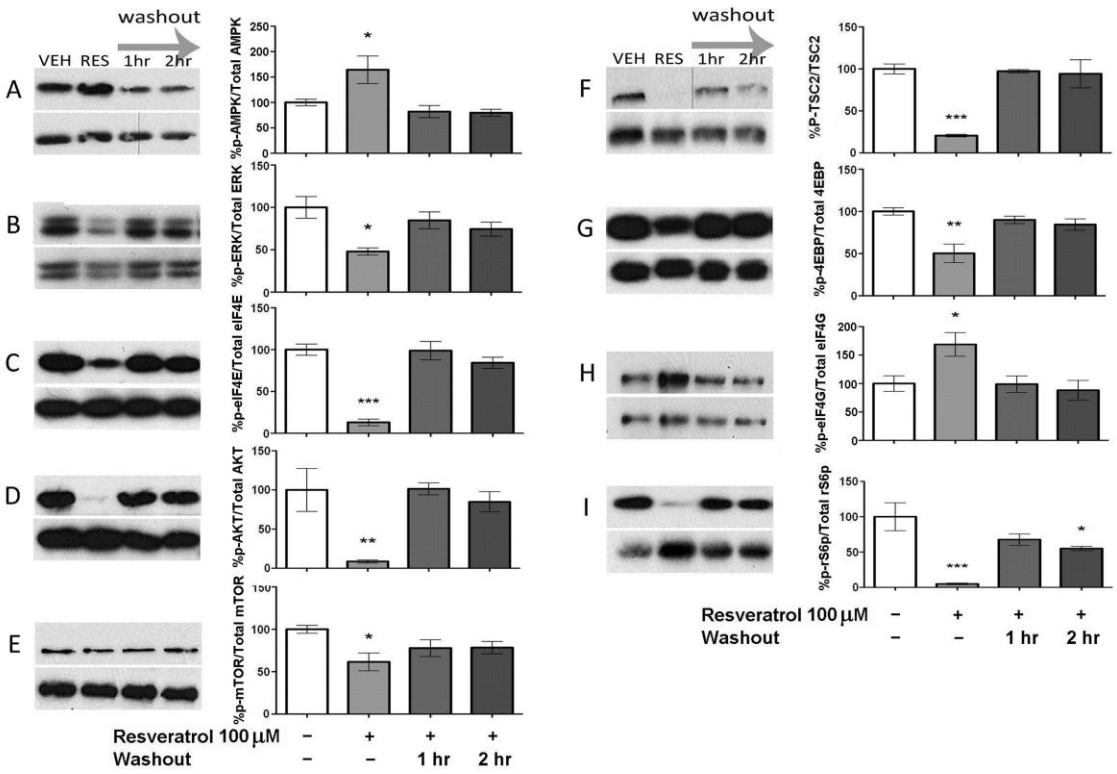

Figure 4.3: Suppression of ERK/mTOR signaling by resveratrol is reversible. TG neurons were treated with 100µM resveratrol for 1 hr followed by 1 or 2 hr washout periods. Effects of reseveratrol were reversible in all cases upon 1 hr washout.

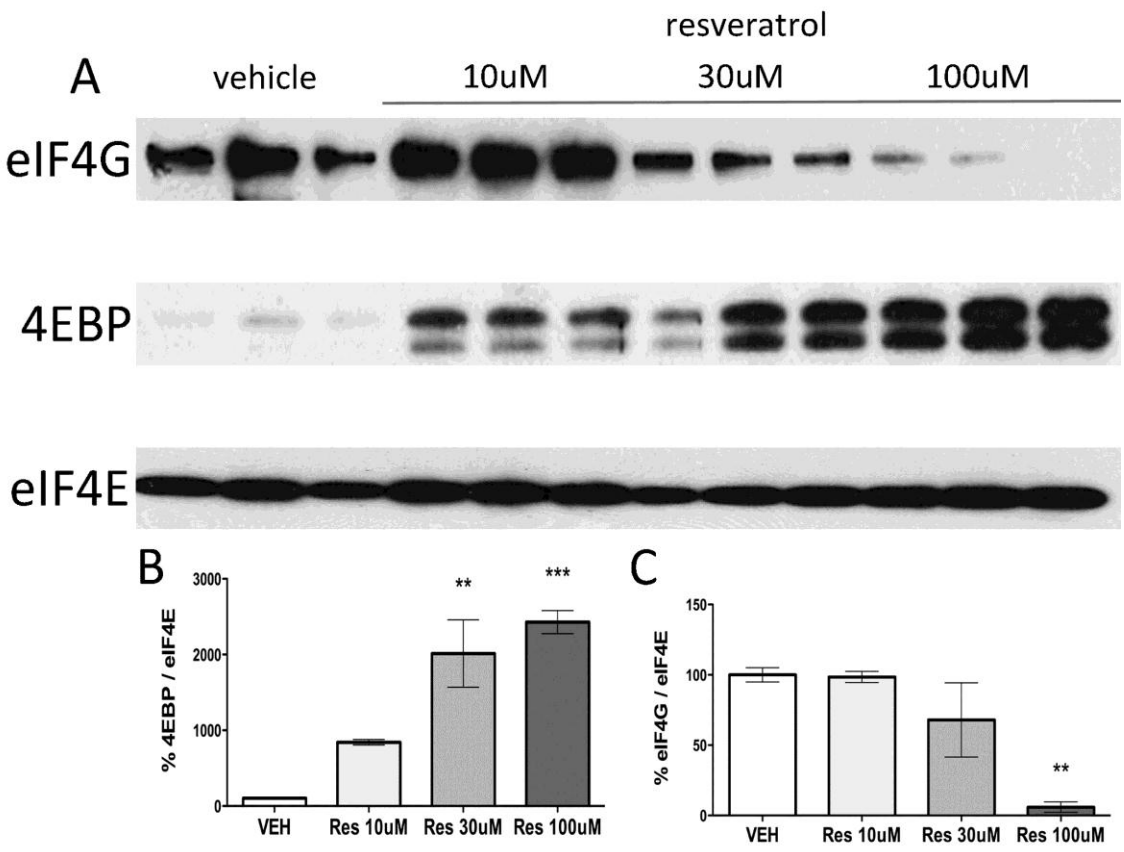

Figure 4.4: Resveratrol suppresses eIF4F complex formation in sensory neurons. TG neurons were treated with resveratrol (10, 30 and 100μM) for 1 hr. A) Western blot for eIF4G, 4EBP and eIF4E from trigeminal neurons co-precipitated using 7-methyl-GTP comjugated beads. B) Resveratrol induces a significant increase in 4EBP (negative regulator of translation) association with the cap-binding protein eIF4E in a dose-dependent manner. C) Resveratrol induces a significant decrease in eIF4G association with the cap-binding protein eIF4E (a component of eIF4F complex) in a dose-dependent manner.

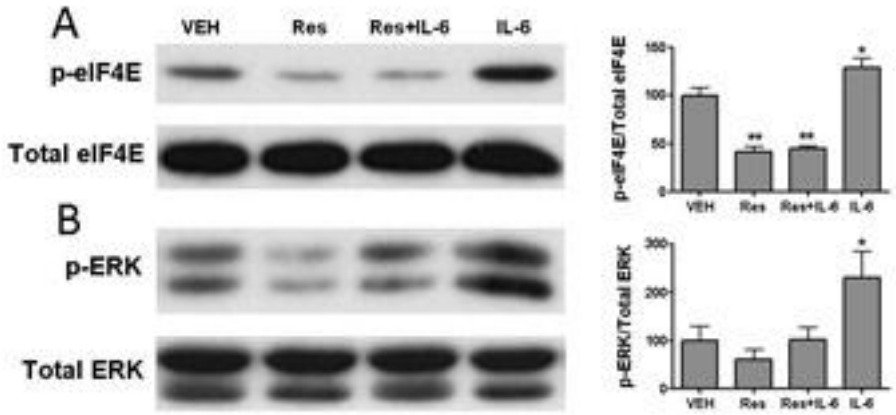

Figure 4.5: Resveratrol blocks IL-6 induced signaling in sensory neurons. TG neuron cultures were pre-treated with resveratrol (100 μM, 15 min) followed by subsequent co-treatment with IL-6 (50ng/ml, 15 min). Western blot for eIF4E (A) and ERK (B) from TG neurons treated with IL6 and/or resveratrol. Resveratrol blocked IL-6 mediated phosphorylation of eIF4E and ERK in TG cultures.

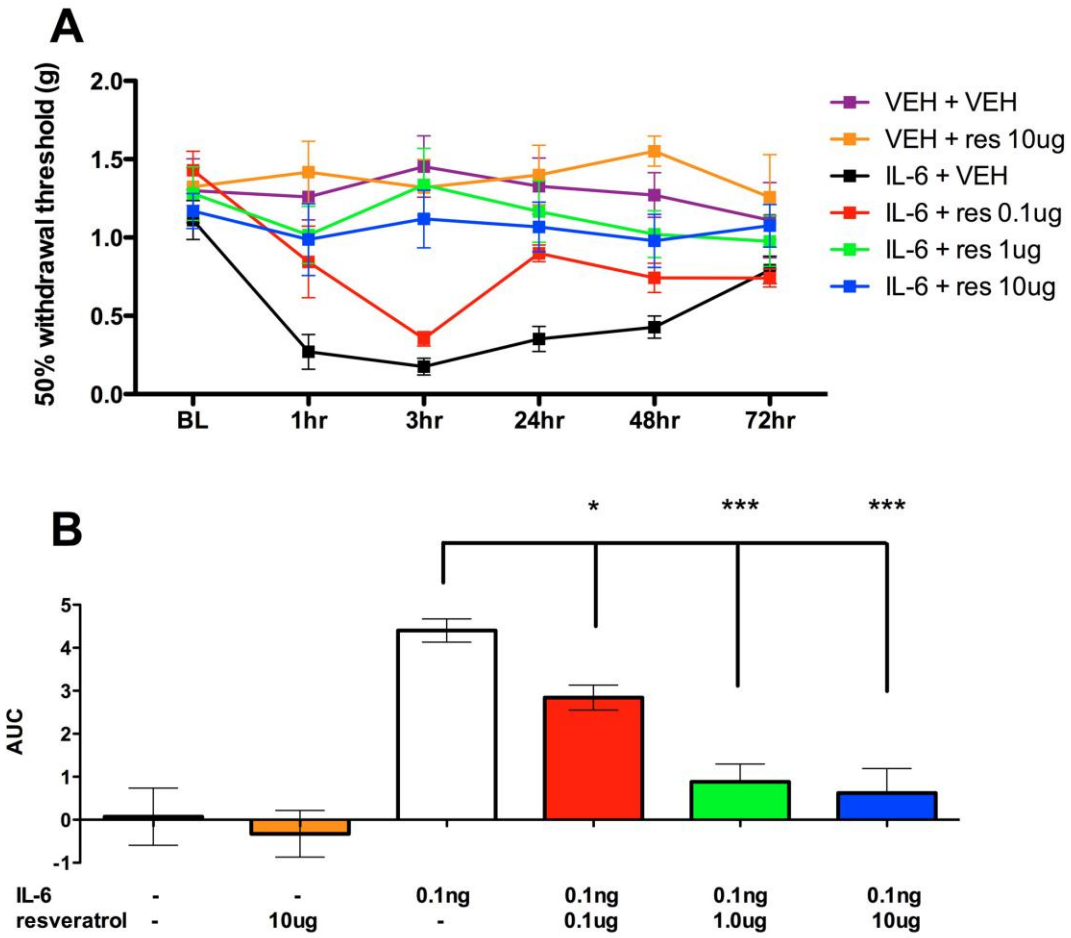

Figure 4.6: Local resveratrol blocks IL-6 induced acute allodynia in a dose dependent manner. A) Intraplantar injection of IL-6 (0.1ng) and co-treatment with resveratrol (0.1, 1 and 10μg) blocks IL-6 induced allodynia. B) Area under the curve (AUC) analysis shows that resveratrol reduces IL-6 induced allodynia in a dose-dependent manner.

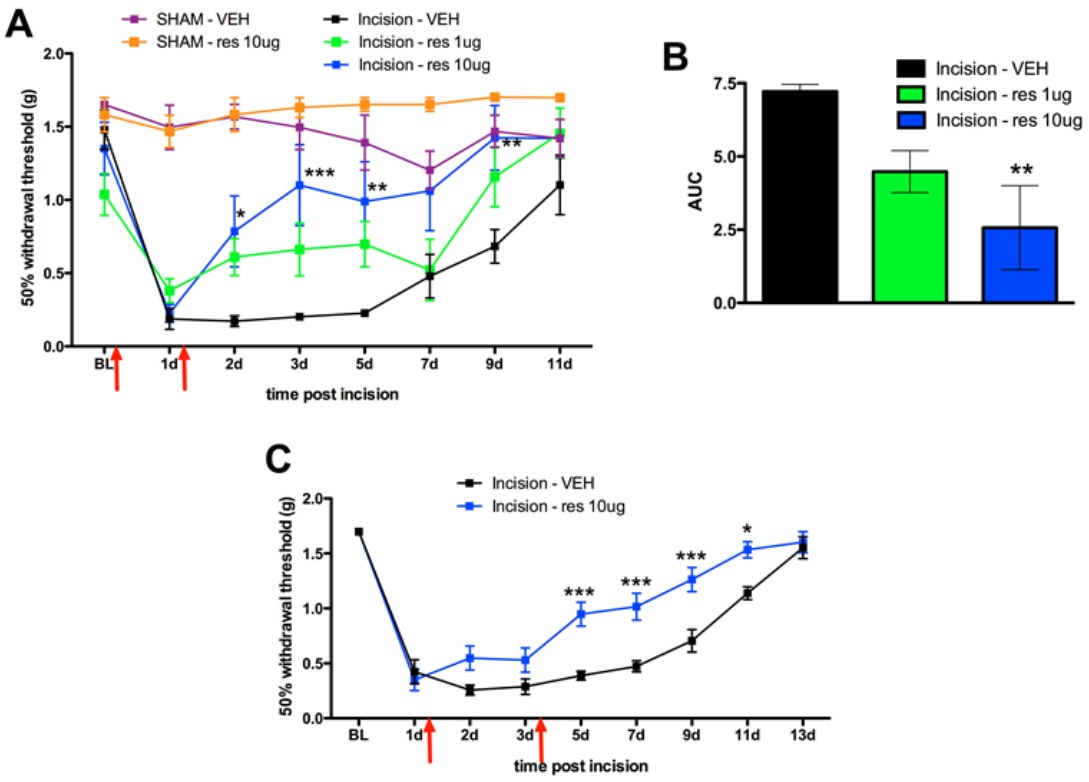

Figure 4.7: Local resveratrol blocks plantar incision-induced allodynia in a dose-dependent manner. Animals received a plantar incision on the left hindpaw. Resveratrol (1µg or 10µg) or vehicle was injected into the paw around the incision either immediately following incision and 1 day post incision (A, B) or 1 and 3 days following incision (C). A) Resveratrol injection (1µg or 10µg) immediately following incision and 1 day post incision significantly blocked plantar incision induced allodynia in a dose-dependent manner. B) Area under the curve (AUC) analysis showing dose-dependent effects in A. C) Resveratrol injection (10 µg) on day 1 and 3 following incision significantly blocks plantar incision induced allodynia. Red arrows show times of resveratrol injection.

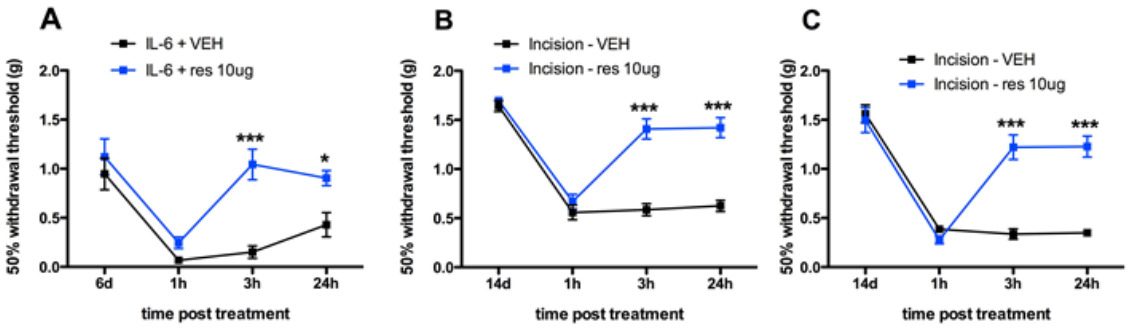

Figure 4.8: Resveratrol blocks IL-6- and plantar incision-induced persistent sensitization. A) Intraplantar injection of IL-6 (0.1ng) with resveratrol (10μg) co-treatment on Day 1 abolished the IL-6 induced persistent sensitization precipitated by PGE_2 injection on day 6. B) Intraplantar injection of resveratrol (10μg) at the time of incision and 1 day post incision abolished plantar incision-induced persistent sensitization precipitated by PGE_2 injection on day 14 after incision. C) Intraplantar injection of resveratrol (10μg) on day 1 and 3 post incision abolished plantar incision induced persistent sensitization precipitated by PGE_2 injection on day 14 after incision.

4.7. Tables

Table 4.1: Sirt1 inhibition does not reverse resveratrol-induced effects on TG neurons. TGneuronal cultures were pre-treated with nicotinamide or vehicle for 1 hr and then co-treated with nicotinamide (10 mM), a sirt1 inhibitor, in the presence of resveratrol for 1 hr. The phosphorylated levels of AMPK, ERK, eIF4E, AKT, mTOR, 4EBP and rS6p were unchanged by nicotinamide.

Antibody	Vehicle	nicotinamide 10mM + resveratrol 100μM	resveratrol 100 μM
p-AMPK/AMPK	100 ± 7.3	199.8 ± 28.1	222.4 ± 40.0
p-ERK/ERK	100 ± 11.8	41.8 ± 8.5 **	47.2 ± 8.8 **
p-eIF4E/eIF4E	100 ± 5.4	27.6 ± 4.7 ***	30.2 ± 6.2 ***
p-AKT/AKT	100 ± 6.2	11.6 ± 3.1 ***	12.5 ± 2.4 ***
p-mTOR/mTOR	100 ± 4.4	70.6 ± 4.4 *	62.9 ± 6.4 **
p-TSC2/TSC2	100 ± 8.3	26.6 ± 4.2 ***	36.5 ± 5.2 ***
p-4EBP/4EBP	100 ± 2.3	38.5 ± 3.0 ***	37.9 ± 7.2 ***
p-rS6p/rS6p	100 ± 7.0	33.9 ± 5.9 ***	36.5 ± 6.8 ***

Table 4.2: Sirt1 activators fail to recapitulate resveratrol induced effects on TG neurons. TG neuronal cultures were treated with the sirt1 activator, CAY10602 (20 and 60μM) for 1hr. The phosphorylated levels of AMPK, ERK, eIF4E, AKT, mTOR, 4EBP and rS6p were not changed by CAY10602.

Antibody	Vehicle	CAY10602 20μM	CAY10602 60μM
p-AMPK/AMPK	100 ± 13.6	95.0 ± 14.4	107.3 ± 16.6
p-ERK/ERK	100 ± 6.50	117.2 ± 5.91	126.4 ± 3.32 *
p-eIF4E/eIF4E	100 ± 13.0	105.9 ± 17.1	102.0 ± 17.0
p-AKT/AKT	100 ± 11.8	105.8 ± 5.0	107.2 ± 7.5
p-mTOR/mTOR	100 ± 8.8	103.8 ± 17.0	125.7 ± 18.5
p-TSC2/TSC2	100 ± 3.3	97.8 ± 6.8	115.1 ± 5.2
p-4EBP/4EBP	100 ± 8.3	126.2 ± 11.1	122.9 ± 8.5
p-eIF4G/eIF4G	100 ± 14.1	76.2 ± 13.8	109.0 ± 16.7

CHAPTER V

AMPK ACTIVATION IS SUFFICIENT TO INHIBIT INCISION-INDUCED CHRONIC PAIN

5.1. Abstract

Surgery is a major cause of persistent pain suggesting that treatments that directly target the molecular pathology promoting post-surgical pain, particularly those that contribute to the progression to chronic pain are needed. It has been previously demonstrated that dysregulated protein translation regulation pathways, in particular ERK/eIF4E and mTOR signaling pathways underlie persistent pain states and that AMPK activators can profoundly inhibit ERK and mTOR signaling in sensory neurons. We have further demonstrated that local injection of resveratrol, a potent AMPK activator, into the hindpaw following plantar incision dose-relatedly reverses incision-mediated mechanical hypersensitivity as well as hyperalgesic priming induced by incision. The aim of these studies is to establish AMPK activation as a bona-fide mechanism for the alleviation of acute and chronic post-surgical pain states and to invent novel therapeutics and therapeutic strategies that employ this mechanism of action for future use in humans. In order to achieve this multiple AMPK activators, including resveratrol, metformin, and A-769662, which possess different mechanisms of AMPK activation, were utilized to demonstrate a shared endpoint – inhibition of incision-induced mechanical hypersensitivity and hyperalgesic priming. Though local injection of resveratrol injection efficaciously blocked incision-induced allodynia, there is a need of better route of

administration which is convenient and causes minimal discomfort for clinical translability. To address this issue a novel cream formulation was developed with resveratrol that can be applied topically. Lyophilized resveratrol was serially dissolved in polyethylene glycol 400 (PEG 400) and solid PEG ointment to achieve a final concentration of 2 mg/ml. In the plantar incision model in mice, topical application of resveratrol attenuates incision-induced mechanical hypersensitivity as well as the development of hyperalgesic priming precipitated by hind paw injection of PGE2 following resolution of incision-induced mechanical hypersensitivity. Metformin, which is clinically available and widely prescribed drug, stimulates upstream LKB1 activity to activate AMPK whereas A-769662 is positive allosteric modulator that directly activates AMPK. Using the Brennan incision model in mice, we demonstrated that systemic metformin injection dose-dependently and efficaciously attenuates incision-induced mechanical hypersensitivity as well as the development of hyperalgesic priming precipitated by hindpaw injection of PGE2 following resolution of incision-induced mechanical hypersensitivity. Interestingly systemic A-769662 was not effective in blocking incision-induced acute mechanical hypersensitivity; however it significantly blocked hyperalgesic priming induced by injection of PGE2. This effect was paralleled by lower doses of metformin, which had no acute effect yet blocked hyperalgesic priming. Finally, co-treatment with systemic metformin and local resveratrol at individually sub-efficacious doses at the time of incision blocked acute hypersensitivity and hyperalgesic priming suggesting potential super-additive effects of combined AMPK activator use. None of these treatment approaches adversely affected wound healing. These results provide further evidence for activation of AMPK as a novel treatment

avenue for acute and chronic pain states induced by surgery. These preclinical findings afford the opportunity for immediate clinical testing due to the clinical availability of metformin.

5.2. Introduction

Post-surgical pain has been identified as a potential major cause for chronic pain. Between 10 and 50% of patients who undergo surgery develop chronic pain following surgical procedures such as groin hernia repair, breast and thoracic surgery, leg amputation, or coronary artery bypass surgery (Kehlet et al., 2006) and up to a quarter of all chronic pain patients suffer from persistent pain because of a prior surgery (Crombie et al., 1998). This chronic pain can be debilitating in 2-10% of this population (Johansen et al., 2012; Kehlet et al., 2006). Despite the fact that analgesics for the treatment of acute post-surgical pain are widely available, surgery remains a major cause of persistent pain suggesting that treatments that directly target the molecular pathology of post-surgical pain, particularly those that prevent the transition to chronic post-surgical pain, are needed. Recent advances in our understanding of the mechanisms of post-surgical pain have led to the elucidation of signaling pathways and mediators that play an important role in driving post-surgical pain. However, treatment approaches that target these pathways or mediators are not currently clinically utilized.

The development of a preclinical model for post-surgical pain by Dr. Tim Brennan and his group has greatly enhanced our understanding of how endogenous mediators released as a result of incision lead to activation and sensitization of nociceptors (Brennan et al., 1996). Two of the major endogenous

mediators released following incision are NGF and IL-6. In animal models of post-surgical pain, there is an increase in IL-6 and NGF levels in the serum and skin around the incision (Banik et al., 2005; Bryan et al., 2005; Matsuda et al., 1998; Sato and Ohshima, 2000). NGF and IL6 can lead to engagement of the mTORC1 and ERK pathways, respectively, in nociceptors and their axons (Melemedjian et al., 2010). Engagement of these pathways can lead to the development of acute mechanical allodynia following NGF or IL-6 injection and hyperalgesic priming to subsequent noxious stimuli following recovery from the initial NGF and/or IL-6 injection (Asiedu et al., 2011; Melemedjian et al., 2010). We have recently demonstrated that both these pathways can be negatively regulated by an endogenous signaling factor, adenosine monophosphate protein kinase (AMPK) (Melemedjian et al., 2011). AMPK is a ubiquitous energy-sensing kinase which can be activated physiologically by increase in intracellular AMP/ATP ratio which occurs during energy deprivation or cell starvation. AMPK can be activated pharmacologically as well by a number of clinically available drugs e.g. metformin or natural products such as resveratrol. AMPK can also be activated by a number of investigational compounds e.g. AICAR or A769662. We demonstrated that the AMP activated protein kinase (AMPK) activators, metformin and A769662, inhibited translation regulation signaling pathways and nascent protein synthesis in injured nerves neurons resulting in a resolution of neuropathic allodynia induced by peripheral nerve injury (Melemedjian et al., 2011). In addition, we also demonstrated that resveratrol, a potent and efficacious activator of AMPK, profoundly inhibits ERK and mTOR signaling in sensory neurons in a time- and concentration-dependent fashion and local injection of resveratrol around the surgery site attenuates the surgery

induced acute mechanical hypersensitivity and hyperalgesic priming in a model of post-surgical pain (Tillu et al., 2012).

The aim of the present study is to establish AMPK activation as a bona-fide mechanism for the alleviation of post-surgical, and possibly other persistent pain states and to invent novel therapeutics and therapeutic strategies that employ this mechanism of action for use in humans. To do this, we utilized multiple AMPK activators, including resveratrol, metformin, and A-769662, which possess different mechanisms of AMPK activation to demonstrate a shared endpoint – inhibition of incision-induced mechanical hypersensitivity and hyperalgesic priming.

5.3. Materials and Methods

5.3.1. Experimental animals

Male ICR mice (Harlan, 20-25 g) were used for the study. All animal procedures were approved by the Institutional Animal Care and Use Committee of The University of Arizona and were in accordance with International Association for the Study of Pain guidelines.

5.3.2. Behavior testing

For the testing, animals were placed in acrylic boxes with wire mesh floors and allowed to habituate for approximately 1 h on all testing days. Paw withdrawal thresholds were measured using calibrated von Frey filaments (Stoelting, Wood Dale, IL) by stimulating the plantar aspect of left hind paw using the up-down method (Chaplan et al., 1994).

5.3.3. Plantar incision and behavioral testing

Prior to surgery all animals were assessed for paw withdrawal thresholds. A mouse model of incisional pain was used for this study (Banik et al., 2006). A 5 mm longitudinal incision was made with a number 11 blade through skin, fascia and muscle of the plantar aspect of the hindpaw in isoflurane-anesthetized rats. Sham controls underwent the same procedure but without the incision. The skin was apposed with 2 sutures of 5 mm silk. Animals received intraplantar injection of resveratrol or vehicle around the incision at times indicated after incision. Animals were allowed to recover for 24 hrs and then paw withdrawal thresholds were measured at 24 hrs, 48 hrs, 5 days, and 14 days post-surgery. For hyperalgesic priming experiments, the animals received an intraplantar injection of PGE_2 (100 ng in 25 μl) 14 days following incision or sham procedures. The paw withdrawal thresholds were again measured at 3 h and 24 h following the PGE_2 injection. Mice were randomly assigned to plantar incision or naive groups and subdivided into further groups that would receive either drug or vehicle. In all experiments lab personnel conducting the injections were blinded to the experimental conditions.

5.3.4. Tissue analysis

To determine re-epithelialization, plantar skin was excised from the left hind paw of mice either 3 days or 7 days following plantar incision surgery. The skin was immediately cryoprotected and frozen in O.C.T compound and sectioned (20 μm) on cryostat. The sections were then fixed in formalin and immersed in 0.1% hematoxylin for 3 minutes, washed in tap water, then

immersed in 0.1% eosin for 3 minutes, and dehydrated through graded ethanol (Protocol from University of Michigan Center for Organogenesis). Finally sections were coverslipped with Permount (Fisher, Pittsburgh, PA). The sections were imaged and the width between 2 epithelial edges was measured using an Olympus BX51 microscope.

5.3.5. Drugs and primary antibodies

Resveratrol was from Cayman Chemical; metformin from LKT laboratories and A769662 was from LC Laboratories. For the cream preparation with resveratrol, lyophilized resveratrol was serially dissolved in polyethylene glycol 400 (PEG 400) and solid PEG ointment to achieve a final concentration of 2 mg/ml. Hematoxylin Gill 2X, O.C.T. compound, Permount mounting medium and xylene were purchased from Fischer Scientific.

5.3.6. Statistical Analysis and Data Presentation

Data are shown as means and the standard error of the mean (± SEM) of 6 animals (for behavioral studies). Graph plotting and statistical analysis used Graphpad Prism Version 5.03 (Graph Pad Software, Inc. San Diego, CA, USA). For behavioral experiments, statistical evaluation was performed by one- or two-way analysis of variance (ANOVA), followed by appropriate post-hoc tests. For skin histology experiments, statistical evaluation was performed by an unpaired t-test. The a priori level of significance at 95% confidence level was considered at $p < 0.05$.

5.4. Results

5.4.1. Topical Resveratrol inhibits acute mechanical hypersensitivity and hyperalgesic priming induced by plantar incision

We have previously demonstrated that local injection of resveratrol, a potent AMPK activator, into the hind paw following plantar incision dose-relatedly reverses incision-mediated mechanical hypersensitivity as well as hyperalgesic priming induced by incision. Though resveratrol injection efficaciously blocked incision-induced allodynia, for clinical translatability there is a need of better route of administration that is convenient and causes minimal discomfort. To address this issue, we made a novel cream preparation with resveratrol which can be applied topically. Lyophilized resveratrol was serially dissolved in polyethylene glycol 400 (PEG 400) and solid PEG ointment to achieve a final concentration of 2 mg/ml. We utilized a mouse model of incisional pain to assess if topical application of resveratrol can prevent development of allodynia following the plantar incision (Brennan et al., 1996; Pogatzki and Raja, 2003). Animals received a plantar incision on the left hindpaw. Resveratrol (100 μg) or vehicle was applied on the paw on and around the incision either immediately following incision or immediately following incision and 24 hrs following incision. All mice were maintained under anesthesia with isoflurane for 1 hr until the cream containing drug or vehicle was absorbed. Mice with plantar incision that received vehicle displayed mechanical hypersensitivity lasting for at least 9 days. In contrast, animals that received topical resveratrol either once at the time of incision or twice significantly attenuated acute mechanical hypersensitivity induced by incision (Fig 5.1A). In this model, hyperalgesic priming can be revealed by a second intraplantar injection of

inflammatory mediator PGE_2 (100 ng) in the hindpaw, after the resolution of initial allodynia (Asiedu et al., 2011) following plantar incision. Topical application of resveratrol at the time of incision or at the time of incision and 1 day later both prevented the development of hyperalgesic priming precipitated by hind paw injection of PGE_2 following resolution of incision-induced mechanical hypersensitivity (Fig 5.IB and 5.1D). Thus, resveratrol not only blocks acute allodynia induced by plantar incision but it also blocks its transition to persistent nociceptive state. These results suggest that even topical application of resveratrol can be a potentially efficacious treatment for post-surgical pain.

5.4.2. Metformin inhibits acute mechanical hypersensitivity and hyperalgesic priming induced by plantar incision

Another therapeutic opportunity for activating AMPK is the prototypical AMPK activator, metformin. Metformin is already clinically available, safe, inexpensive and a well-tolerated drug. Moreover, metformin has a different mechanism of action in activating AMPK than resveratrol and hence can have differential efficacy in modulation of downstream targets. Metformin is known to activate AMPK through multiple indirect mechanisms including LKB1 stimulation (Shaw et al., 2005) and inhibition of AMP deaminase (Ouyang et al., 2011). In addition, in contrast to resveratrol which lacks bioavailability, metformin has a good bioavailability and thus can be given orally which is a preferred route of administration in humans. Hence, we investigated if systemic application of metformin can prevent development of incision-induced mechanical hypersensitivity and further hyperalgesic priming following the plantar incision. Animals received a plantar incision on the left hindpaw.

Metformin (30, 100, 150 or 200 mg/kg) or vehicle was injected intra-peritonially (i.p.) for 4 days starting 2 days prior to the surgery. Mice with plantar incision that received vehicle displayed long-lasting acute mechanical hypersensitivity (9 days) as well as hyperalgesic priming following PGE_2 lasting at least 24 hrs. In contrast, i.p. injection of maximal dose (200 mg/kg) of metformin prevented both plantar-incision induced mechanical hypersensitivity (Fig 5.2A and 5.2B) and the expression of hyperalgesic priming following PGE_2 injection on day 14 (Fig 5.3). Interestingly, the lower doses (30, 100 and 150 mg/kg) were not effective in blocking incision-induced acute mechanical hypersensitivity; however the dose of 150 mg/kg significantly blocked hyperalgesic priming.

5.4.3. A769662 inhibits hyperalgesic priming in a model of post-surgical pain

Another strategy that might be employed to achieve effective AMPK activation is by utilizing compounds that activate AMPK directly thus eliminating the dependence on upstream kinases. One such direct activator is an investigational compound, A769662. A769662 appears to bind allosterically to AMPK presumably causing the γ subunit to assume a conformation that protects the α subunit from protein phosphatase activity thus inhibiting the dephosphorylation of AMPK on Thr-172 (Sanders et al., 2007). Thus, A769662 can offer a potent and potentially selective mechanism for pharmacological manipulation of AMPK without the requirement for stimulation of upstream kinases (Goransson et al., 2007). We investigated if A-769662 prevented the development of acute hypersensitivity and hyperalgesic priming following plantar incision. Animals received a plantar incision on the left hindpaw. A-769662 (30 mg/kg) or vehicle was injected i.p. for 4 days starting 2 days prior to

the surgery. Mice with plantar incision that received vehicle displayed long-lasting acute mechanical hypersensitivity as well as hyperalgesic priming following PGE_2 lasting at least 24 hrs (Fig 5.4A and 5.4B). Interestingly, systemic A-769662 was not effective in blocking incision-induced acute mechanical hypersensitivity (Fig 5.4A); however it significantly blocked hyperalgesic priming following PGE_2 injection (Fig 5.4B).

5.4.4. Co-treatment with systemic metformin and local resveratrol inhibits acute mechanical hypersensitivity and hyperalgesic priming in a model of post-surgical pain

It has been demonstrated above that metformin and resveratrol individually entirely attenuate the development of the incision-induced hypersensitivity and hyperalgesic priming at the respective maximal doses. However the maximal doses of these drugs may cause unwanted side-effects. To avoid those, an alternative treatment strategy could be the co-treatment with the two AMPK activators at sub-optimal dose especially if this co-treatment strategy is equally efficacious in attenuating acute hypersensitivity and hyperalgesic priming following plantar incision compared to the efficacy with individual maximal doses. Hence, we investigated if co-treatment of sub-efficacious dose of metformin and resveratrol can have additive effects on attenuation of incision-induced mechanical hypersensitivity and hyperalgesic priming compared to their individual effects. Animals received a plantar incision on the left hindpaw and received treatment of vehicle, resveratrol or metformin alone or a co-treatment of metformin and resveratrol. Metformin (100 or 150 mg/kg) was injected i.p. for 4 days starting 2 days prior to the surgery and resveratrol (1 or 3

μg) was injected in the left hindpaw around the incision on the day of the surgery and 24 hrs post-surgery. The mice with co-treatment of metformin and resveratrol received either 100 mg/kg metformin with 1 μg resveratrol or 150 mg/kg metformin with 3 μg resveratrol. Mice with plantar incision that received vehicle displayed long-lasting acute mechanical hypersensitivity as well as hyperalgesic priming following PGE_2 (Fig 5.5A and 5.6). When treated with sub-efficacious doses of both, metformin as well as resveratrol, the mice showed significantly reduced acute mechanical hypersensitivity compared to treatment with individual drugs suggesting potential additive effects of combined AMPK activator use (Figs 5.5A, 5.5B and 5.5C). Further, between the two dosages tested in this experiment, the higher dosage of 150 mg/kg metformin and 3 ug of resveratrol yielded significant response to the co-treatment compared to individual drugs suggesting that the 100 mg/kg metformin and 1 ug of resveratrol may be sub-optimal. Additionally, co-treatment with systemic metformin and local resveratrol at individually sub-efficacious doses at the time of incision blocked the development of hyperalgesic priming following PGE_2 injection (Fig 5.6).

5.4.5. AMPK activators do not affect wound healing

An important potential consideration in these experiments is that AMPK activators would be expected to reduce protein translation and therefore may negatively influence wound healing. This effect would have a negative impact on their clinical utility. To test if AMPK activators affect the wound healing, we utilized either vehicle or maximal doses of the AMPK activators (resveratrol or metformin) described in the previous experiments and assessed wound healing

with Hematoxylin & Eosin (H & E) staining (Lai et al., 2009). Skin samples were excised 3 and 7 days after plantar incision to assess healing and wound closure, respectively. For the animals treated with resveratrol, no differences were noted in the wound size between the resveratrol and vehicle treated groups on Day 3 (Fig 5.7A). By Day 7, the wound was completely closed in the animals in both groups receiving vehicle or resveratrol (Fig 5.7A). Similar results were obtained with intra-peritoneal metformin treatment (Fig 5.7B). Thus, AMPK activators do not influence wound healing following plantar incision suggesting they do not interfere with the wound healing process.

5.5. Discussion

The present findings establish AMPK activation as a bona-fide mechanism for the alleviation and/or prevention of a chronic post-surgical pain state. We had previously demonstrated that local injection of resveratrol at the time of incision alleviated incision-induced acute mechanical hypersensitivity as well as the development of hyperalgesic priming. In vitro studies exhibited that resveratrol potently activates AMPK in sensory neurons and inhibits mTORC1 and ERK activity leading to a reduction of cap-dependent translation. Since resveratrol is known to affect a multitude of signal transduction pathways the previous work posed a question whether the behavioral effects observed with local resveratrol injection were specific to AMPK mediated signaling or they were downstream effects of some other mechanism of action/ target (Korkina et al., 2013; Svajger and Jeras, 2012; Whitlock and Baek, 2012). To answer this question we utilized multiple AMPK activators which possess different mechanism of action to activate AMPK and to demonstrate that AMPK activation during plantar incision

is necessary to inhibit incision-induced acute mechanical hypersensitivity and also the development of hyperalgesic priming.

Further the study was also targeted towards development of a formulation of the AMPK activators in order to have better clinical translatability in humans. To that effect we invented a novel cream preparation with resveratrol which can be applied topically. Using the plantar incision model in mice, we demonstrated that topical application of resveratrol attenuates incision-induced mechanical hypersensitivity as well as the development of hyperalgesic priming precipitated by hind paw injection of PGE_2 following resolution of incision-induced mechanical hypersensitivity. In addition to formulating a topical cream, we tested metformin, another potent AMPK activator, which is clinically available and widely prescribed for diabetes but not currently used for the treatment of pain. There is already some indication that metformin might be effective for chronic pain states such as neuropathic pain based on the lower incidence of neuropathic diabetic pain in patients taking metformin (Pop-Busui et al., 2009) and case reports for other types of chronic pain (Labuzek et al., 2012; Labuzek et al., 2013). This study also demonstrates that systemic metformin injection in mice with plantar incision dose-dependently and efficaciously attenuates incision-induced mechanical hypersensitivity as well as the development of hyperalgesic priming precipitated by hind paw injection of PGE_2. To address the potential pitfall of the individual treatments in terms of side-effects, we also demonstrated that the co-treatment with systemic metformin and local resveratrol at individually sub-efficacious doses at the time of incision blocked acute hypersensitivity and hyperalgesic priming suggesting potential super-additive effects of combined AMPK activator use. Thus, the

therapeutic strategy with full clinical translatability for treatment of post-surgical pain developed in this study include topical administration (resveratrol cream), which prevents the occurrence of side effects due to systemic delivery; the use of a widely prescribed, safe, and well-tolerated drug (metformin) and a combination use of oral metformin and local resveratrol that we believe may have wide applications for prevention of chronic post-surgical pain.

There are multiple mechanisms through which AMPK can be modulated pharmacologically. As noted above, although the prototypical AMPK activator metformin attenuated development of hyperalgesic priming in mouse model of post-incisional pain, it is not a direct AMPK activator and lacks potency (Zhou et al., 2001). Rather metformin interferes with mitochondrial function leading to activation of LKB1 and subsequent AMPK phosphorylation (Shaw et al., 2005). Similarly activation of AMPK by resveratrol requires upstream LKB1 and calcium/calmodulin-dependent protein kinase kinase β (Baur et al., 2006; Dasgupta and Milbrandt, 2007). Upstream mechanisms to engage AMPK activation pharmacologically lead to differential efficacies in modulation of downstream AMPK targets. Therefore one strategy that can be employed for better control of the activation of AMPK would be to utilize pharmacological agents that activate AMPK in a direct mechanism thus by-passing the upstream kinases and exhibit much better potency than indirect activators. Therefore we utilized A769662 which is a direct positive allosteric modulator of AMPK and prevents the kinase from dephosphorylation by binding to the γ2 subunit (Carling et al., 2012; Cool et al., 2006). Interestingly systemic A769662 injection did not reverse the acute hypersensitivity following incision but efficaciously blocked development of hyperalgesic priming. One explanation is that the dose

of systemic dose A769662 might not be high enough to reach therapeutic levels at the site of incision. Similar results were observed with low dose of metformin (150 mg/kg). These findings suggest that although the sub-maximal inhibition of mTORC1 and ERK pathways at the time of incision is not enough to alleviate the acute hypersensitivity following incision, it can efficaciously prevent the development of hyperalgesic priming. These findings show potential for AMPK activators to be important co-therapeutics for post-surgical pain even at sub-efficacious doses.

During a surgical procedure trauma to the nervous system can result in nerves being partially or completely severed, crushed or compressed (Parsons et al., 2013). Thus chronic pain following a surgical intervention can be sometimes associated with lesions of the central or peripheral aspects of nervous system making post-surgical pain partially neuropathic in nature (Wajer, 1989; Wildgaard et al., 2009). Previously we have demonstrated that in an animal model of neuropathic pain, treatment with AMPK activators led to a decrease in allodynia induced by peripheral nerve injury (PNI). Mice and rats were treated with AMPK activators, metformin or A769662, 14 days following PNI for 7 days. Both metformin and A769662 significantly reduced nerve injury-induced allodynia after 2–3 days of systemic treatment. Remarkably a 7 day treatment with either of these compounds leads to a complete resolution of neuropathic allodynia that is not reversed following cessation of treatment, even in animals with long-standing PNI (Melemedjian et al., 2011; Melemedjian et al., 2013). Based on the preclinical findings discussed above, we propose that there are several important avenues for targeting AMPK and AMPK activators can have

wide applications for prevention of chronic post-surgical pain and potentially other chronic pain states.

In conclusion, this study provides clear evidence and establishes AMPK activation as a mechanism to alleviate post-surgical acute and chronic pain. An important aspect which still needs to be explored is the site of action of AMPK. AMPK can mediate its effect by acting either peripherally at the site of primary afferents or centrally at spinal and supraspinal level but the exact site of action still remains unclear. The results that not only the topical application of resveratrol but also the additive effect of local resveratrol and systemic metformin alleviated incision induced pain strongly suggest that these AMPK activators act peripherally in producing this effect. These findings demonstrate potential of AMPK activators as effective treatment for chronic pain and the subsequent chapter 6 explores different avenues and strategies of recruiting AMPK activators clinically.

5.6. Figures

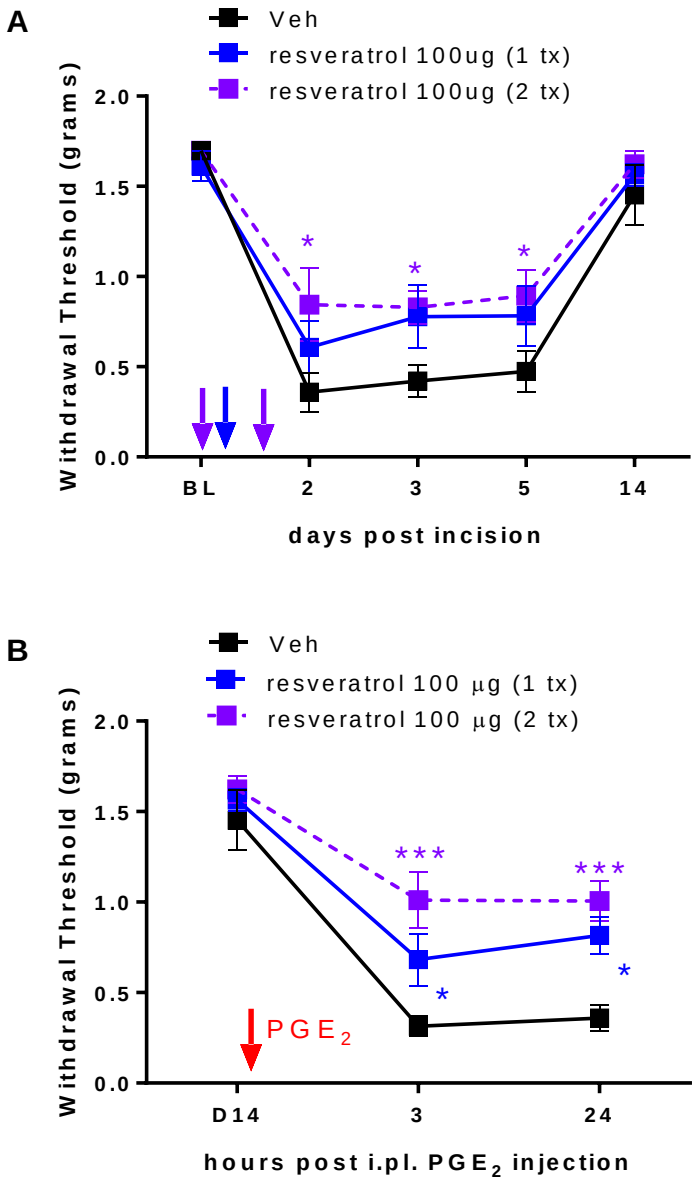

Figure 5.1: Topical Resveratrol inhibits acute mechanical hypersensitivity and hyperalgesic priming induced by plantar incision. Animals received a plantar incision on the left hind paw. Resveratrol (100 µg) or vehicle was applied on the paw on and around the incision either once, immediately following incision or twice, immediately following incision as well as 24 hrs following incision (n = 6 per group). Mice were maintained under anesthesia with isoflurane for 1 hr until the drug was absorbed. **A)** Single topical resveratrol (100 µg) application

immediately following incision resulted in blunted incision-induced acute hypersensitivity. Topical resveratrol (100 μg) application twice significantly blocked plantar incision-induced acute hypersensitivity. **B)** Topical application of resveratrol (100 μg) either once or twice following incision abolished plantar incision-induced hyperalgesic priming precipitated by PGE_2 injection on day 14 after incision. Colored stars denote significant differences between vehicle and treatment groups. * $p < 0.05$, *** $p < 0.001$; two way anova with Bonferroni's multiple comparisons test.

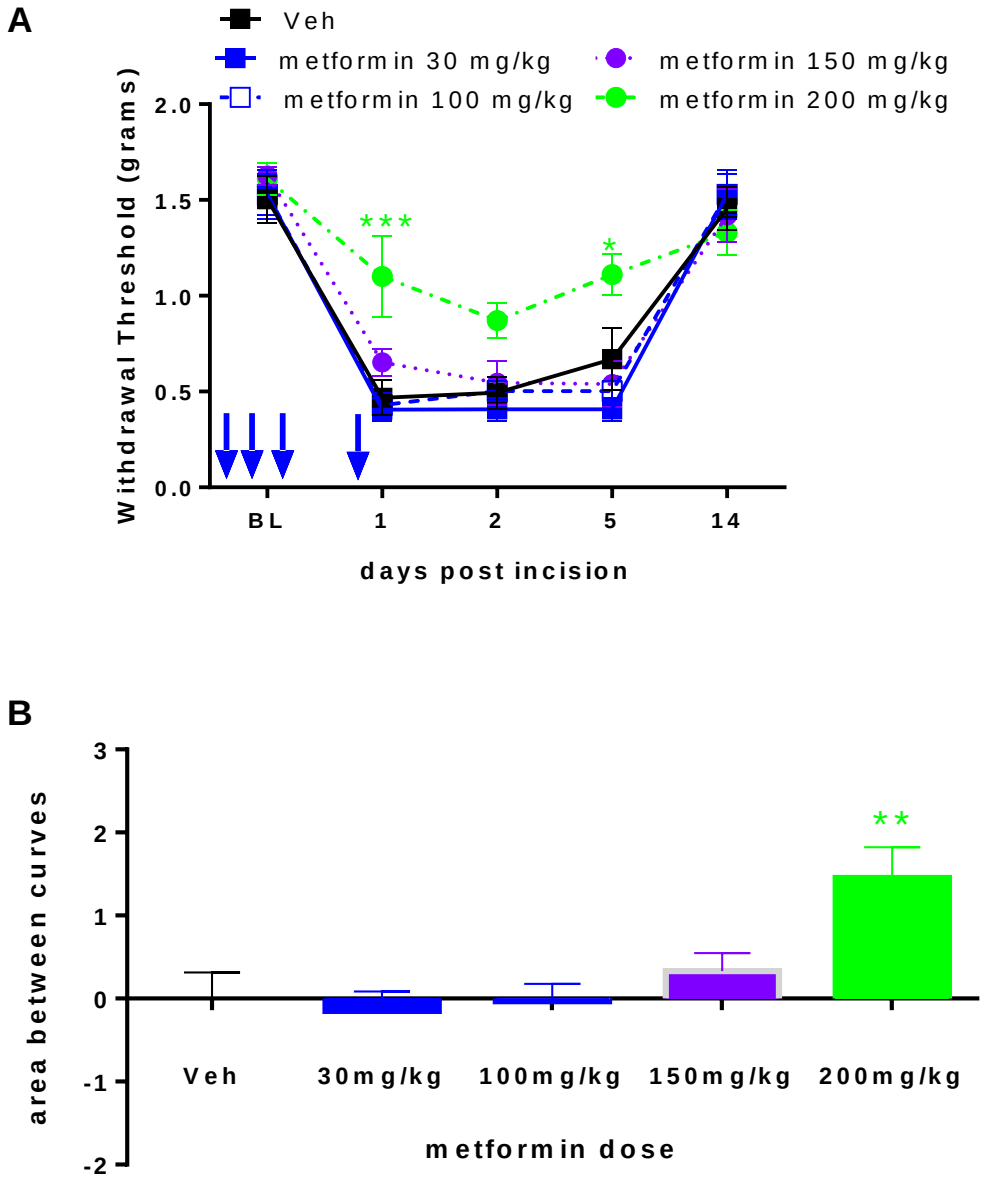

Figure 5.2: Metformin inhibits acute mechanical hypersensitivity induced by plantar incision. Animals received a plantar incision on the left hind paw. Metformin (30, 100, 150 or 200 mg/kg) or vehicle was injected intra-peritonially for 4 days starting 2 days prior to the surgery (n = 6-8 per group). **A)** Metformin (30, 100, 150 or 200 mg/kg) injection significantly blocked plantar incision-induced acute hypersensitivity. * $p < 0.01$, *** $p < 0.001$; two way anova with Bonferroni's multiple comparisons test.

B) Area under the curve (AUC) analysis showing dose-related effects in A. Colored stars denote significant differences between vehicle and treatment groups. ** $p < 0.01$; one way anova with Dunnet's multiple comparisons test.

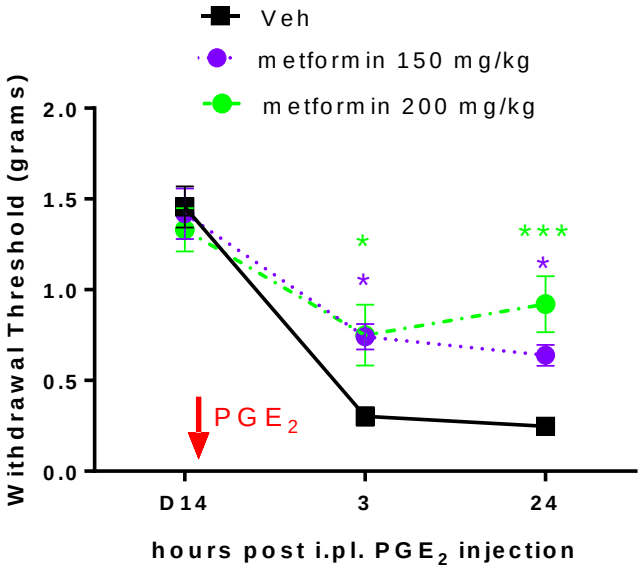

Figure 5.3: Metformin inhibits plantar incision-induced hyperalgesic precipitated by PGE_2 injection. Animals received a plantar incision on the left hind paw. Metformin (30, 100, 150 or 200 mg/kg) or vehicle was injected intraperitonially for 4 days starting 2 days prior to the surgery. Hyperalgesic priming was precipitated by a second intraplantar injection of inflammatory mediator PGE_2 (100 ng) in the hind paw on day 14 after incision (n = 6-8 per group). Metformin (150 or 200 mg/kg) injection significantly blocked plantar incision-induced hyperalgesic priming precipitated by PGE_2 injection on day 14 after incision. Colored stars denote significant differences between vehicle and treatment groups. * $p < 0.05$, *** $p < 0.001$; two way anova with Bonferroni's multiple comparisons test.

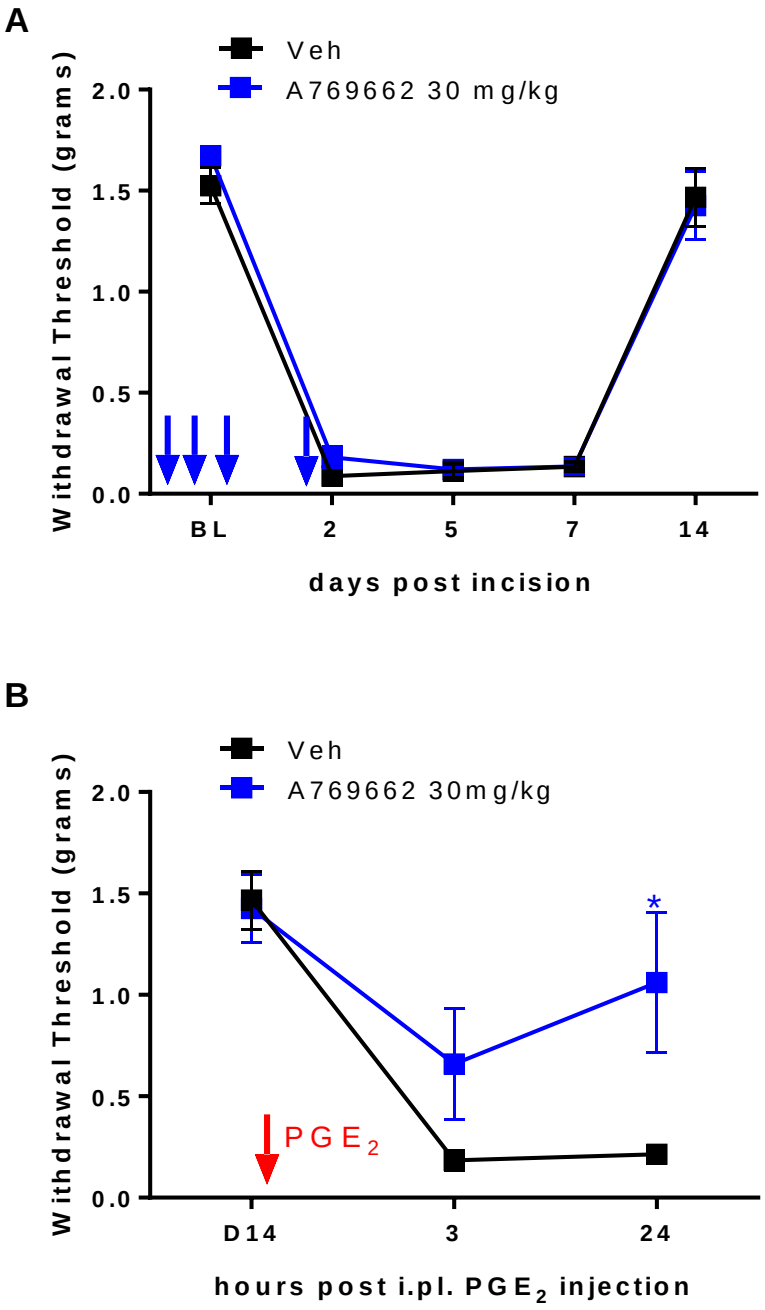

Figure 5.4: A-769662 does not inhibit acute mechanical hypersensitivity induced by plantar incision but blocks the hyperalgesic precipitated by PGE_2 injection. Animals received a plantar incision on the left hind paw. A-769662 (30 mg/kg) or vehicle was injected intra-peritonially for 4 days starting 2 days prior to the surgery. **A)** A-769662 (30 mg/kg) injection did not block plantar incision-induced acute hypersensitivity (n = 9 per group). **B)** A-769662 (30 mg/kg)

injection significantly blocked plantar incision-induced hyperalgesic priming precipitated by PGE_2 injection on day 14 after incision Colored stars denote significant differences between vehicle and treatment groups. * $p < 0.05$; two way anova with Bonferroni's multiple comparisons test.

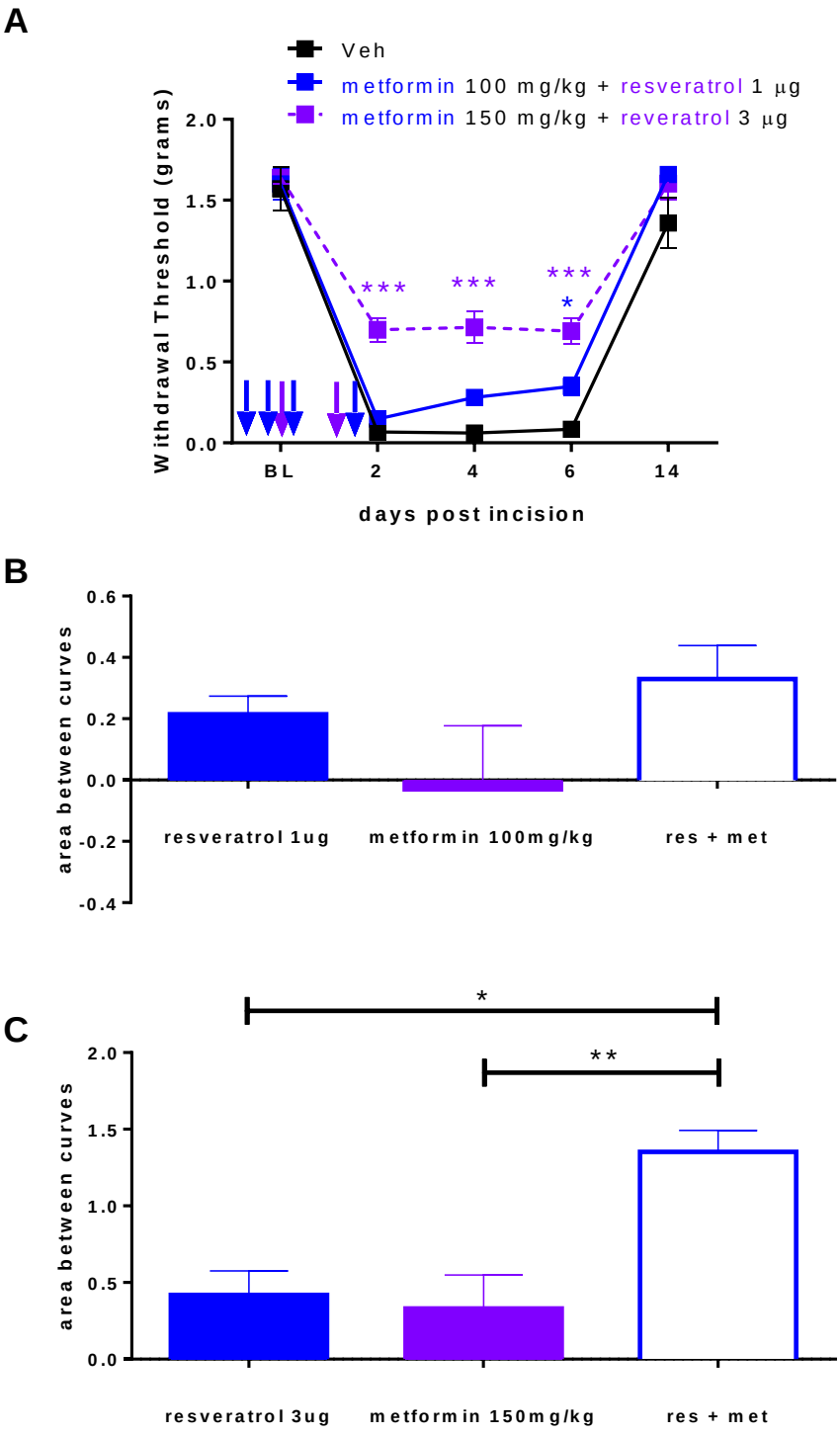

Figure 5.5: Co-treatment with systemic metformin and local resveratrol inhibits acute mechanical hypersensitivity induced by plantar incision.

Animals received a plantar incision on the left hind paw. Metformin (100 or 150 μg) was injected intra-peritonially for 4 days starting 2 days prior to the surgery and resveratrol (1 or 3 μg) was injected in the left hind paw around the incision on the day of the surgery and 24 hrs post-surgery (n = 5-6 per group). **A)** Co-treatment with lower dose of systemic metformin (100 μg) and local resveratrol (1 μg) did not block the acute hypersensitivity induced by plantar incision but the co-treatment with higher dose, i.e. systemic metformin (150 μg) and local resveratrol (3 μg), significantly blocked plantar incision-induced acute hypersensitivity. Colored stars denote significant differences between vehicle and treatment groups. * $p < 0.05$, *** $p < 0.001$; two way anova with Bonferroni's multiple comparisons test. **B &C)** Area under the curve (AUC) analysis showing dose-related effects in A compared to systemic metformin (100 or 150 μg) or local resveratrol (1 0r 3 μg) alone. Black stars denote significant differences between metformin or resveratrol alone and co-treatment groups. * $p < 0.05$, ** $p < 0.01$; one way anova with Dunnet's multiple comparisons test.

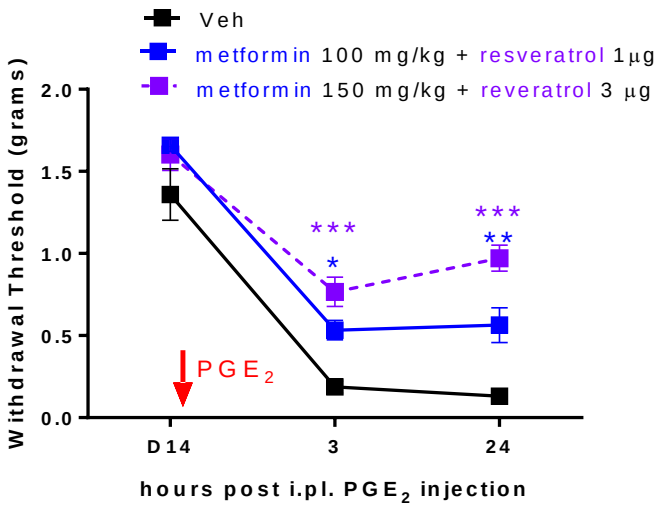

Figure 5.6: Co-treatment with systemic metformin and local resveratrol inhibits plantar incision-induced hyperalgesic precipitated by PGE_2 injection. Animals received a plantar incision on the left hind paw. Metformin (100 or 150 µg) was injected intra-peritonially for 4 days starting 2 days prior to the surgery and resveratrol (1 or 3 µg) was injected in the left hind paw around the incision on the day of the surgery and 24 hrs post-surgery (n = 5-6 per group). Co-treatment with systemic metformin and local resveratrol significantly blocked plantar incision-induced hyperalgesic priming precipitated by PGE_2 injection on day 14 after incision. Colored stars denote significant differences between vehicle and treatment groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two way anova with Bonferroni's multiple comparisons test.

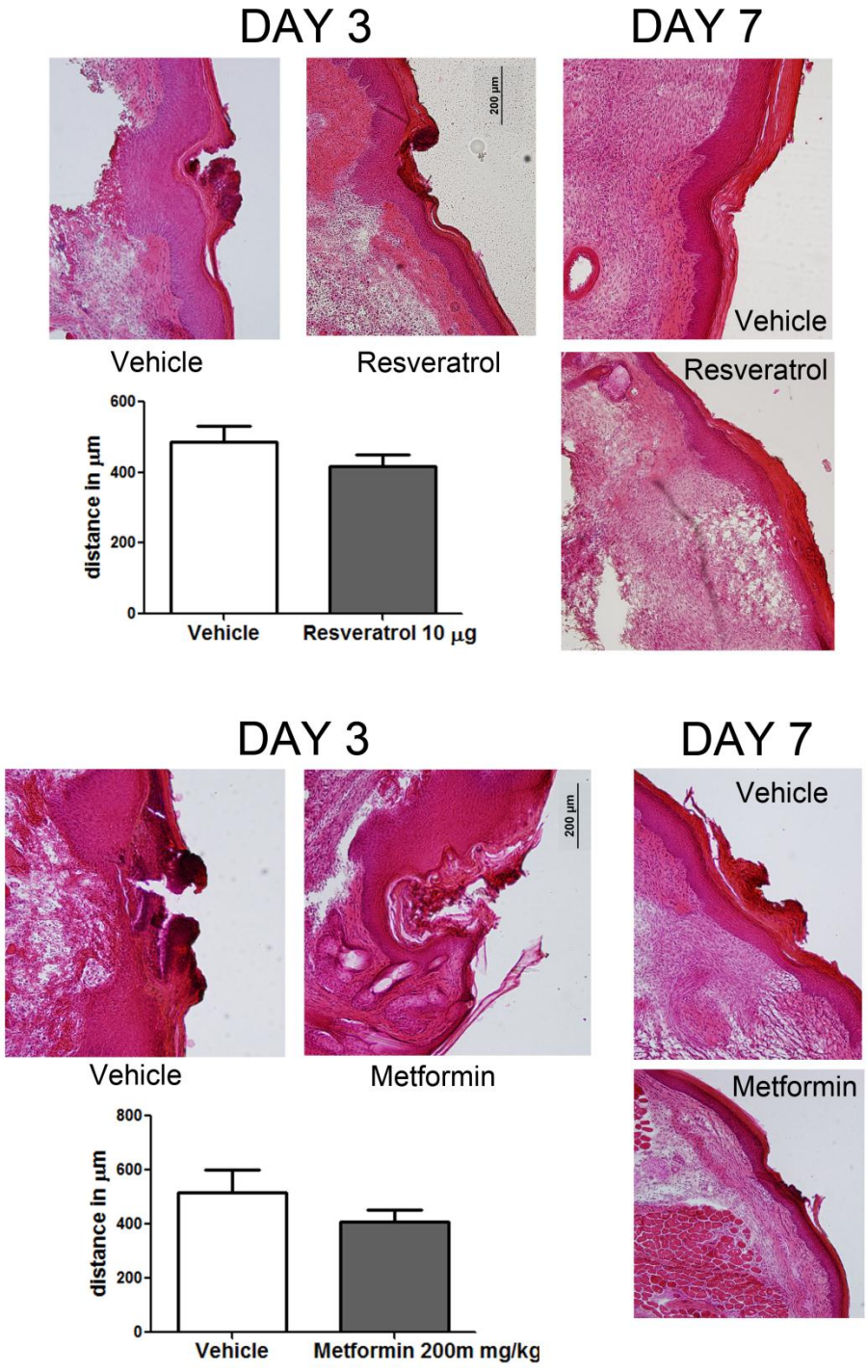

Figure 5.7: AMPK activators don't affect wound healing negatively in a model of post-surgical pain. Animals received a plantar incision on the left hind paw.

Skin from the left hind paw was excised 3 and 7 days after plantar incision and wound healing and wound closure was assessed using Hematoxylin & Eosin (H & E) staining. **A)** Resveratrol (10 μg) or vehicle was injected in the left hind paw around the incision on the day of the surgery and 24 hrs post-surgery. There was no difference in the wound size between the resveratrol and vehicle treated groups on Day 3. The wound was completely closed by Day 7 in the animals in both groups receiving vehicle or resveratrol. **B)** Metformin (200 mg/kg) or vehicle was injected intra-peritonially for 4 days starting 2 days prior to the surgery. No differences were noted in the wound size between the metformin and vehicle treated groups on Day 3. The wound was completely closed by Day 7 in the animals in both groups receiving vehicle or metformin.

CHAPTER VI

DISCUSSION

6.1. AMPK activators for other chronic pain conditions

Translation control of gene expression is a key factor in initiation and maintenance of pain hypersensitivity in a wide range of preclinical pain models (Bogen et al., 2012; Ferrari et al., 2013; Geranton et al., 2009; Jimenez-Diaz et al., 2008; Melemedjian et al., 2010; Melemedjian et al., 2011b; Price and Geranton, 2009; Price et al., 2007; Tillu et al., 2012). Activity-dependent protein translation, either controlled by mTORC1 or ERK/MNK/eIF4E contributes to the basal sensitivity of a subset of nociceptors. (Jimenez-Diaz et al., 2008) More importantly it leads to local changes in gene expression following injury to promote nociceptive hypersensitivity developing in the nociceptive system in a variety of chronic pain conditions and therefore can provide therapeutic opportunities for pharmacological intervention (Sonenberg and Hinnebusch, 2009). The work presented in this dissertation focused on assessing the role of AMPK activators in regulation of activity-dependent translation and blocking the feedback signaling mechanisms activated by targeting individual kinases. This work demonstrated that AMPK activators reduce peripheral nerve injury-induced allodynia and decrease excitability of sensory neurons in vitro (Melemedjian et al., 2011a). Moreover resveratrol, a natural product and activator of AMPK, reduces incision-induced allodynia in mice via an AMPK-mediated mechanism (Tillu et al., 2012). This approach was also validated by demonstrating the efficacy of multiple indirect and direct activators of AMPK such as metformin and A769662 in regulating chronic pain. These findings

provide strong evidence for utilization of AMPK activators for the treatment of a variety of chronic pain states. Bolstering this approach, Ellen Niderberger's group has recently confirmed that metformin and AICAR decreased the nociceptive behavior in two different mouse models of inflammatory nociception. They also showed that the AMPKα2 knockout mice showed increased nociceptive responses that could not be reversed by AMPK activators, suggesting that an AMPK-mediated mechanism is necessary and sufficient for alleviation of inflammatory pain. In addition, there has been renewed interest in the mechanism of action of some of the oldest drugs known to humans - salicylates, such as aspirin which have been used to reduce pain and inflammation for decades. Recently, they have been shown to mediate some of their effects in an AMPK dependent mechanism. Aspirin contributed to activation of AMPK in colorectal cancer cell lines even at low millimolar concentrations, a concentration which is readily reached in the plasma of humans being treated for rheumatoid arthritis with high doses of either aspirin (Preston et al., 1989; Steinberg et al., 2013) or salsalate (Fleischman et al., 2008; Steinberg et al., 2013). Interestingly, salicylate appears to bind at the same site as A-769662 and this binding event results in inhibition of Thr-172 dephosphorylation of the α subunit of AMPK. Our findings, along with the evidence from these studies, further emphasize the importance of AMPK activators for treatment of other chronic pain states.

6.2. AMPK mediated inhibition of protein translation and effect on wound healing

AMPK activators inhibit signaling involved in protein translation in trigeminal neurons and treatment with AMPK activator like metformin reduces

the PNI-induced increase in nascent protein synthesis in a neuropathic pain model. However since protein translation is necessary for nerve and tissue regeneration, it is important to consider whether AMPK activation leads to delayed wound healing or decelerated tissue repair (Zhou et al., 2013). Although the data presented in this dissertation suggests that AMPK activators can inhibit protein synthesis, it does not infer that the AMPK stimulation with metformin or other AMPK activators leads to a complete block of protein synthesis in peripheral nerves or other tissues. In the context of protein synthesis, metformin merely blocks the activity dependent protein translation and normalizes the protein synthesis rates in the uninjured nerves (Melemedjian et al., 2011b). Proteomic profiling of injured sciatic nerves after PNI indicates that metformin changes the proteomic profile of the injured nerve such that certain gene clusters associated with increased excitability are decreased whereas clusters associated with regeneration are increased (Melemedjian et al., 2013). In fact two groups of researchers have demonstrated that the local or systemic administration of resveratrol significantly accelerated skin regeneration and repair by increasing keratinocyte proliferation in models of skin wound and incision (Spallotta et al., 2013; Yaman et al., 2012). Another study provided evidence that daily administration of metformin accelerated epidermal regeneration and decreased the gastric ulcer area in diabetic rats in AMPK dependent mechanism (Baraka and Deif, 2011). Although the understanding of the role of AMPK activation in neuroprotection is still emerging, this may associate AMPK to wound healing. Recently it has been shown that a protein called apolipoprotein E (ApoE) is increased during nerve injury and further increased by metformin treatment. In fact, in naïve mice metformin treatment increases ApoE expression in the

peripheral nervous system (Melemedjian et al., 2013). Since ApoE is associated with recovery of peripheral nervous system function after injury (Li et al., 2010), this finding may suggest that in addition to metformin's anti-allodynic effects the drug may also promote functional recovery after PNI. A study in diabetic mice and rats supports this hypothesis as well. Diabetes leads to functional changes in sensory neuronal mitochondria, which will further decrease in AMPK activity in sensory neurons. AMPK activation with resveratrol improves mitochondrial function in sensory nerves form diabetic animals and enhances neurite outgrowth, which may be associated with enhanced epidermal nerve fiber density in the setting of diabetes.

6.3. Clinical pharmacological opportunities

Based on the preclinical findings presented in previous chapters, there are several opportunities for assessing AMPK as a target in the clinic for the treatment of chronic pain. One of the easiest and relatively straightforward routes is to design a retrospective study to evaluate the incidence and severity of pain in patients who are on metformin. Metformin is currently the most widely used drug for Type II diabetes, as well as some diseases related to metabolic syndrome (Hardie, 2008). About 50% of patients with diabetes are affected with painful diabetic peripheral neuropathy (Tesfaye and Selvarajah, 2012) and a lower incidence and severity of pain in patients on metformin would give an indication of potential of metformin as a treatment for pain. There is already some evidence that AMPK activators might be effective for diabetic neuropathic pain based on the lower incidence of neuropathic diabetic pain in patients taking metformin (Pop-Busui et al., 2009) and also for other types of chronic pain

(Labuzek et al., 2012; Labuzek et al., 2013). Further retrospective studies exploring this idea need to be conducted to elucidate the effectiveness of AMPK activators in treating diabetic neuropathic pain.

Based on the evidence that metformin reverses neuropathic pain in preclinical models of neuropathy in mice and rats, we can hypothesize that metformin should alleviate pain in patients with other types of peripheral neuropathies as well. A similar approach is to design and conduct prospective clinical trials for various other peripheral neuropathic pain states such as chemotherapy induced neuropathic pain and post-herpetic neuralgia. Although diabetic neuropathy is distinct from other neuropathic conditions they also share several similar features and mechanisms that might be alleviated by AMPK activation with metformin. Chemotherapy-induced peripheral neuropathy (CIPN) is a major dose-limiting adverse effect of many anticancer drugs such as platinum salts (cisplatin, carboplatin and oxaliplatin), spindle poisons (taxanes and vinca alkaloids), bortezomib and thalidomide (Balayssac et al., 2011; Ferrier et al., 2013). Several therapeutic strategies have been proposed to prevent the neurotoxic effects of these anti-cancer drugs, but with very limited success and CIPN is considered to be resistant to most of first-line treatments for neuropathic pain (Dworkin et al., 2010; Ferrier et al., 2013). Dose reduction or treatment discontinuation is the only recourse of the oncologists to limit the apparition of neuropathic symptoms. Hence novel therapeutic strategies need to be explored for the pain management in CIPN. Based on the rationale presented in this work, AMPK activators can be explored for potential treatment of CIPN induced pain. AMPK activators have also exhibited a role in tumor-suppression and have been associated with decreased cancer risk. A recent meta-analysis of diabetic

epidemiologic studies indicated an overall reduction of 30% in cancer risk in subjects taking metformin compared to other anti-diabetic drugs, with specific risk reductions being found for colon and liver cancers (Decensi et al., 2010; Hardie, 2013). These findings combined together provide an ideal opportunity for designing a prospective clinical trial in patients with colon or liver cancer on chemotherapeutics. Based on the rationale presented above we hypothesize that metformin treatment for 4-6 weeks will prevent the development of neuropathy in patients who were treated with chemotherapeutics. We also predict that metformin and will have a positive impact on pre-existing neuropathies in patients previously treated with chemotherapeutic agents.

Post-herpetic neuralgia which is a complication of shingles caused by herpes zoster virus is a common peripheral neuropathic condition associated with intractable debilitating pain along a dermatome and sensory abnormalities. Although several analgesics have been tried for the treatment of pain associated with post-herpetic neuralgia, it is still resistant to most pharmacological and interventional therapies. There is a clear need for development of effective treatment strategies. Although diabetic neuropathy and post-herpetic neuralgia are distinct symptomatically, both conditions are characterized by dysfunctional sensory neuronal mitochondria; a change that is linked to a decrease in AMPK activity in sensory neurons. Additionally both conditions are characterized by an abnormal nerve fiber density. There is evidence that AMPK activation with resveratrol improves mitochondrial function in sensory nerves form diabetic animals and enhances neurite outgrowth (Roy Chowdhury et al., 2012). This data combined with the evidence of lower incidence of neuropathic diabetic pain in patients taking metformin provides a substantial justification for designing a

clinical trial with AMPK activators for treatment of pain associated with post-herpetic neuralgia. Nonetheless these trials should be designed carefully by excluding patients with active herpes zoster lesions or dermatitis at the affected site to avoid confounding parameters. We hypothesize that long term treatment with AMPK activators will alleviate the pain associated with post-herpetic neuralgia.

Post-surgical pain, another major cause of chronic pain affecting up to 50% of patients undergoing some form of surgery, is another opportunity for targeting AMPK in the clinic. There is compelling evidence from this work suggesting AMPK activators can alleviate acute sensitization induced by incision and also prevent the development of chronic pain without affecting wound healing process negatively. In fact, the evidence that daily administration of metformin can accelerate epidermal regeneration and wound healing provides support the pre-clinical rationale for clinical trials with AMPK activators for treating post-surgical pain. This dissertation work demonstrated that even local injection of resveratrol at the site of incision or topical application prevented the development of acute sensitization and hyperalgesic priming in a model of post-surgical pain. Currently opioids are the mainstay treatment of post-surgical pain. Though opioids can be efficacious in providing pain relief acutely, persistent use of opioids can induce delayed hypersensitivity due to reduction in their analgesic efficacy and development of tolerance. Thus in addition to their potential for abuse liabilities, they also pose a serious side-effect of potential for exacerbation of chronic pain following surgery. A prospective study involving topical application of resveratrol cream daily in addition to oral dose of opioids is an ideal clinical trial for assessing the effectiveness of AMPK activators in

humans. If successful, this strategy offers an added advantage of restricting the use of opioids only for acute post-surgical pain thus minimizing their side-effects.

6.4. Pharmacological synergism of AMPK activators

There are multiple mechanisms through which AMPK can be modulated pharmacologically. Although the prototypical AMPK activator metformin reversed neuropathic pain and attenuated development of hyperalgesic priming in mouse model of post-incisional pain, it is not a direct AMPK activator and lacks potency (Zhou et al., 2001). Similarly although resveratrol efficiently prevented the development of acute allodynia and hyperalgesic priming in post-incisional pain model, it requires upstream LKB1 and calcium/calmodulin-dependent protein kinase kinase β (Baur et al., 2006; Dasgupta and Milbrandt, 2007). Upstream mechanisms to engage AMPK activation pharmacologically lead to differential efficacies in modulation of downstream AMPK targets. Therefore one strategy that can be employed for greater control over activation of AMPK would be to utilize positive allosteric modulators such as A-769662 and OSU-53 which activate AMPK directly thus bypassing the upstream kinases. Direct activators are also significantly more potent and efficacious compared to the indirect activators such as metformin (Carling et al., 2012; Giordanetto and Karis, 2012). In addition, because of their property to target specific subunits (e.g. γ2 for A769662) it is theoretically easy to achieve strong AMPK activation in certain tissues while avoiding potentially negative effects in other tissues and also predict the effects of the drug depending on the subunit expression in the tissue (Sanders et al., 2007a; Sanders et al., 2007b). Intriguingly during this work

systemic A769662 injection did not reverse the acute hypersensitivity following incision but efficaciously blocked development of hyperalgesic priming in post-incisional pain model. A-769662 has exhibited efficacy in neuropathic pain model as well (Melemedjian et al., 2011b). Thus a good strategy would be to invest more time and efforts in development of direct, highly specific AMPK activators. Recently several potential positive allosteric modulators of AMPK utilized in several patents have been published (Giordanetto and Karis, 2012). Although there is limited literature available about the efficacy and safety of these compounds, this class of compounds has a great potential in treating pain based on the data in this work. This knowledge should be harnessed to fully comprehend and utilize the opportunities linked to AMPK activation in pain research.

In addition to investigation for better compounds to activate and regulate AMPK, there are several strategies that might be employed to achieve better control over the mechanism of AMPK activation in the clinic for the alleviation of chronic pain. Although developing novel direct and positive allosteric modulators is promising, it is possible that allosteric activators of AMPK will have dose-limiting toxicities due to the ubiquitous nature of AMPK (Price and Dussor, 2013). To minimize the side-effects and toxicities of positive allosteric modulators it is possible that combining indirect and direct AMPK activators at sub-efficacious doses will achieve pharmacological synergism. Indirect activators enhance AMPK phosphorylation whereas direct allosteric activators protect the kinase from dephosphorylation via γ or β subunits (Price and Dussor, 2013). Based on this mechanistic understanding it is possible that combining upstream LKB1 or CaMKKβ activation with indirect activators leading to enhanced AMPK

phosphorylation will synergize with protection from dephosphorylation with direct activators in terms of enhancement of AMPK activity (Price and Dussor, 2013). While the exact idea has not been tested, Chapter 5 provides preliminary evidence that combining two AMPK activators at sub-efficacious doses results in super-additive effects suggesting pharmacological synergism. Co-treatment with systemic metformin and local resveratrol at individually sub-efficacious doses at the time of incision blocked acute hypersensitivity and hyperalgesic priming suggesting potential super-additive effects of combined AMPK activator use while sparing AMPK activation systemically that might lead to undesired side effects.

6.5. Conclusions

While there has been tremendous progress in our understanding of the physiology and pharmacology of AMPK in the past several years, the key role of AMPK and its downstream signaling pathways regulating sensitization of pain pathways in chronic pain conditions has not been systematically explored. First and foremost, this work elucidated the key role of protein translation signaling pathways in sensitizing pain pathways following incision and peripheral injury. These same pathways may also play a key role in the driving other chronic pain states. Further it was demonstrated that these signaling pathways are under the control of a single endogenous ubiquitous kinase AMPK. Activation of this kinase AMPK can result in negative regulation of multiple signaling pathways and is enough to attenuate acute sensitization and prevent transition to chronic pain in at least two animal models of chronic pain. Therefore, the evidence presented in this work suggests AMPK activators present a novel class of drugs

with utility for better treatment of post-surgical pain, neuropathic pain and more importantly for prevention of the transition to chronic pain in other chronic pain states. A key challenge is to identify and develop highly potent, efficacious and specific activators of AMPK which can be applied locally to prevent side-effects and dose-related toxicities. Future studies should be aimed at translating the knowledge gained from biochemical studies and the animal models to human chronic pain conditions in the clinic.

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