## *The mammalian target of rapamycin complex 1 (mTORC1) and the regulation of opioid efficacy*

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# The mammalian target of rapamycin complex 1 (mTORC1) and the regulation of opioid efficacy

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# List of abbreviations

| | |
|---|---|
| **4EBP1** | Eukaryotic translation initiation factor 4E-binding protein 1 |
| **AC** | Adenylate cyclase enzyme |
| **AD** | Alzheimer's disease |
| **ADHD** | Attention deficit hyperactivity disorder |
| **AMPA** | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| **AMPK** | Adenosine monophosphate-activated protein kinase |
| **ATP** | Adenosine triphosphate |
| **AWERB** | Animal Welfare and Ethical Review Body |
| **Akt** | Protein kinase B |
| **BBB** | Blood brain barrier |
| **CaM kinase II** | Calcium/calmodulin-dependent protein kinase II |
| **cAMP** | Cyclic adenosine monophosphate |
| **CCI** | Chronic constriction injury |
| **CGRP** | Calcitonin gene-related peptide |
| **CNS** | Central nervous system |
| **COX-2** | Cyclooxygenase 2 |
| **CPP** | Conditioned place preference |
| **CREB** | $Ca^{2+}$/cAMP response element binding protein |
| **CYP** | Cytochrome P450 enzymes |
| **CYP2D6** | Cytochrome P450 2D6 |
| **CYP3A4** | Cytochrome P450 3A4 |
| **DA** | Dopamine |
| **DRG** | Dorsal root ganglion |
| **eIF4E** | Eukaryotic translation initiation factor 4E |
| **eIF4F** | Eukaryotic initiation factor 4F |
| **eIF4G** | Eukaryotic translation initiation factor 4G |
| **ERK** | Extracellular signal-regulated kinase |
| **FDA** | Food and Drug Administration |
| **GABA** | g-aminobutyric acid |
| **GAP** | GTPase-activating protein |
| **GDP** | Guanosine diphosphate |
| **GI** | Gastrointestinal |

| | |
|---|---|
| **GP** | General Practitioner |
| **GPCR** | G protein-coupled receptors |
| **GRK** | G-protein receptor kinase |
| **GTP** | Guanosine triphosphate |
| **H** | Hydrogen |
| **ICSI** | Intracytoplasmic sperm injection |
| **IGF1** | Insulin-like growth factor |
| **iGluRs** | Ionotropic receptors |
| **JNK** | c-Jun N-terminal kinase |
| **LKB1** | Liver kinase B1 |
| **M3G** | Morphine-3-glucuronide |
| **M6G** | Morphine-6-glucuronide |
| **MAPK** | Mitogen-activated protein kinase |
| **mGluRs** | Metabotropic ligand sensitive receptors |
| **mTOR** | Mammalian target of rapamycin |
| **mTORC1** | Mammalian target of rapamycin complex-1 |
| **mTORC2** | Mammalian target of rapamycin complex-2 |
| **N** | Nitrogen |
| **NAc** | Nucleus accumbens |
| **NICE** | National Institute for Health and Care Excellence |
| **NIDDM** | Non-insulin-dependent diabetes mellitus |
| **NMDA** | N-methyl-D-aspartate |
| **NMDARs** | NMDA receptors |
| **nNOS** | Neuronal nitric oxide synthase |
| **NO** | Nitric oxide |
| **NRSF** | Neuron-restrictive silencer factor |
| **NSAIDs** | Nonsteroidal anti-inflammatory drugs |
| **OHSS** | Ovarian hyperstimulation syndrome |
| **OIH** | Opioid-induced hyperalgesia |
| **ORL1** | Opioid receptor like-1 |
| **OUD** | Opioid use disorder |
| **p70S6K** | 70 kDa ribosomal protein S6 kinase 1 |
| **PCOS** | Polycystic ovary syndrome |
| **PI3K** | Phosphatidylinositol-3 kinase |
| **PIKK** | Phosphoinositide 3-kinase (PI3K)-related kinase |

| | |
|---|---|
| **PKA** | Protein kinase A |
| **PKC** | Protein kinase C |
| **PNS** | Peripheral nervous system |
| **Rheb** | Ras homolog enriched in brain |
| **S6RP** | S6 Ribosomal Protein |
| **SNI** | Spared nerve injury |
| **SNL** | Spinal nerve ligation |
| **SNPs** | Single nucleotide polymorphisms |
| **TSC** | Tuberous sclerosis |
| **UGT** | Uridine diphosphoglucuronosyl transferase |
| **UGT2B7** | UDP-Glucuronosyltransferase-2B7 |
| **ULK1** | Protein kinase that initiates autophagy |
| **VLDL** | Very low-density lipoprotein |
| **VTA** | Ventral tegmental area |
| **WHO** | World Health Organisation |

## Statement of copyright

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# Abstract

Prolonged opioid administration leads to pharmacological tolerance that significantly restricts the clinical usefulness of opioids. In addition, the misuse of and addiction to opioids is a serious international crisis that affects public health as well as social and economic welfare. Thus, opioid-based treatments and research into understanding the mechanisms underlying the effects of opioids in chronic pain is warranted. Recently, there is growing evidence supporting the mammalian target of rapamycin complex 1 (mTORC1), a kinase which controls protein synthesis, as a regulator of opioid effectiveness, though the precise role of mTORC1 in the improvement of opioid-based treatments is uncertain. Therefore, this study aims to determine the extent to which alterations in mTORC1 activity within nociceptive pathways underlie the responsiveness to morphine. Specifically, by expanding upon studies investigating the role of mTORC1 in chronic pain, the goal was to define the importance of mTORC1 as a therapeutic target in mechanisms counteracting the analgesic effects of opioids in chronic pain leading to the development and maintenance of morphine-induced tolerance.

By using a combination of *in vivo* models of tolerance and neuropathic pain as well as by behavioural testing and tools of molecular biology, it has been shown that inhibition of mTORC1 activity blocked the development and maintenance of morphine-induced analgesic tolerance in *naïve* mice and in animals subjected to neuropathic pain. This approach also potentiates the analgesic efficacy of morphine in neuropathic pain. Interestingly, improvement of morphine-mediated analgesia was observed after peripheral administration that could minimise the risk of side-effects associated with systemic administration of opioids. Moreover, mTORC1 inhibition did not regulate the motivational properties of morphine potentially offering safe pain control. The originality of this study shows that all these effects were produced by the anti-diabetic drug metformin which is known to inhibit *in vivo* mTORC1 activity *via* activation of the adenosine monophosphate-activated protein kinase (AMPK) and has safer therapeutic profile compared to direct mTORC1 inhibitors.

To conclude, data presented here reveals that inhibition of mTORC1 activity improve morphine analgesic effectiveness and provided a novel insight into the complexity of the adaptive mechanisms that underlie opioid treatment and its side-effects in chronic pain. Importantly, this study shows that metformin may offer a novel and clinically relevant strategy for modulation of morphine efficacy in chronic pain, especially when prolonged opioid treatment is required.

# Chapter 1. Introduction

## 1.1 Pain

Pain is classified as a highly distressing sensation, typically caused by damage or intense stimuli, which manifests itself as a highly unpleasant feeling. Typically every person will experience some degree of pain at some point in their lives. It is the number one reason why people go and visit their general practitioner (GP). Pain has several causes and effects and is itself, a highly complex biological phenomenon. In the medical field, pain is described as a physical sensation that is regarded as a symptom of an underlying condition (Dekkers, 2017). The most frequently used definition of pain provided by the International Association for the Study states; 'Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described regarding such damage. However, this definition fails to mention that not all pain is elicited because of tissue damage in fact, many people report pain in the absence of tissue damage or experience pain with no explainable pathophysiological reasons. Pain is also a protective mechanism; pain allows us to move away from damaging stimuli, or to protect a body part/area while it is given time to heal. It also serves as a reminder to avoid similar damaging situations in the future (Loeser and Treede, 2008).

### 1.1.1 Types of pain

Classifying pain is essential to guide its assessment and treatment. There are many ways to classify pain and various classifications may overlap (Deardorff et al., 2011). The three well-recognised broad categories of pain are:

a) acute pain (nociceptive pain): this develops from a clearly defined cause e.g. surgery or from an injury, it is transmitted to the brain by the nervous system when the peripheral nociceptive nerve fibres are triggered by thermal, chemical or physical stimuli (Grichnik and Ferrante, 1991). Nociceptive pain is a type of pain that everyone is most familiar with, for example acute pain is associated with bee stings, sunburn or toe stubs to repetitive strain injury. Nociceptive pain typically changes with movement, position and load (Grichnik and Ferrante, 1991), and lasts less than three to six months (Treede et al., 2015).
b) chronic pain: this is defined as a recurrent or ongoing pain, lasting beyond the usual time course of injury healing, typically lasting for more than three to six months (Merskey, 1986). Chronic pain adversely affects the individual's daily functioning

and well-being (IASP, 1986). Patients affected by chronic pain report poorer quality of life compared with individuals affected by common chronic diseases of the lungs or heart, making chronic pain the most frequent reason for seeking a new medical approach (Alemzadeh-Ansari et al., 2017).

c) neuropathic pain (neuralgia): this develops from damage or malfunctioning of the somatosensory nervous system, in both the peripheral or central nervous system (Woolf and Mannion, 1999). There are various causes of neuropathic pain which include; disease, pinching or injury (Woolf and Mannion, 1999). Cases of the simplest neuropathies are mechanical upsets, such as hitting the sciatica or the funny bone. However, this is a broad category and comprises anything that has the potential to damage neurons. Examples include multiple sclerosis, chemotherapy, alcoholism and phantom limb pain (Baron et al., 2010). Many patients with neuropathic pain exhibit paroxysmal or persistent pain that is independent of a stimulus. It regularly feels like a stabbing, electrical or burning sensation (Treede et al., 2008). Neuropathic pain is most likely to lead to chronic pain (Treede et al., 2008). Peripheral nerve injury pain has two key features: allodynia and hyperalgesia (Kumar et al., 2018). Allodynia is a sensation of pain evoked by a non-painful stimulus and mediated by the action of low threshold of myelinated Aβ fibres on an altered signal of the CNS or by a reduction in the threshold of the peripheral nociceptor terminals (Woolf and Mannion, 1999). Hyperalgesia is a significant pain response to a suprathreshold noxious stimulus due to the irregular processing of nociceptor input (Costigan et al., 2009) and mediated by A- or C-fibre nociceptors.

### 1.1.2 Chronic pain mechanisms

Chronic pain is categorised as nociceptive or neuropathic, contingent on whether the integrity of the somatosensory nervous system is compromised by the basic disease (Merskey, 1986). Nociceptive pain arises from the activation of receptors (nociceptors) that are sensitive to noxious stimuli. Intense or prolonged exposure to these specific stimuli like chemical mediators released during inflammation, is known to increase the responsiveness of nociceptive nerve fibres (Woolf and Mannion, 1999). This process known as ‘peripheral sensitisation’, consists of a modification in the activation threshold of nociceptors and the upregulation of voltage-gated sodium channels. Peripheral sensitisation generates improved action, potential firing and transmitter release in the dorsal horn of the spinal cord, where somatosensory data is managed. In this case, the dorsal horn neurons become more excited

owing to the mounting input; a process that is termed ‘central sensitisation’ (Woolf and Mannion, 1999).

Heightened depolarisation causes N-methyl-D-aspartate (NMDA)-type glutamate receptors, NMDA to be engaged. Moreover, neuropeptide receptor activation generates a sudden increase in intracellular calcium, prompting signalling pathways and changes in gene expression which support a long-term shift in the activity of nociceptive circuits. To a certain extent, central sensitisation even appears like the long-term potentiation of excitatory transmission within the hippocampus. Additionally, central sensitisation produces a heightened response to painful stimuli (hyperalgesia) and promotes pain that is produced by typically nonpainful stimuli (allodynia) (Merskey, 1986).

Clinical results indicate that pain hypersensitivity creates gradual structural changes in the brain. Furthermore, these changes can be altered and relieved by means of pain relief. It is worth stating that the pathophysiology of neuropathic pain is essentially different (Costigan et al., 2009). Peripheral nerve lesions induce stimulus-independent (ectopic) activity in nerve fibres. Innate immune cells respond at the lesion site located in the dorsal root ganglion, where the cell bodies of peripheral somatosensory neurons are present, as well as in the dorsal horn of the spinal cord. Active microglia of the dorsal horn emits chemical mediators which control the activity of neurons in the vicinity (Costigan et al., 2009).

Brain-derived neurotrophic factor, which is a mediator, diminishes the inhibitory effect of g-aminobutyric acid (GABA) and glycine. Disinhibition releases polysynaptic connections in the dorsal horn, which are known to further enhance the abnormal input from the lesioned nerve. Additionally, central sensitisation occurs, which is similar to nociceptive pain. In addition, exacerbated by a relative shortfall in transmitter uptake, excitotoxic cell death is caused by improved glutamatergic transmission, which in turn reduces the number of inhibitory interneurons (Baron et al., 2010). This deficit and a change in descending modulatory pathways from the brainstem create a notable imbalance between inhibition and excitation. The complexity of chronic pain mechanisms presents a therapeutic challenge. Without biomarkers, it will continue to be problematic to create targeted strategies concerned with reducing pain or inhibition in the individual patient (Baron et al., 2010).

### 1.1.3 Pain pathways

Pain is an essential function of the nervous system, it allows the body to be aware of a potential or actual injury in relation to wellbeing and survival (Tracey, 2016). Without this

sensitivity and reactivity to noxious stimuli, the body has no means of preventing or minimising tissue injury (Tracey, 2016). The specialised sensory receptors (nociceptors) are responsible for the detection of noxious stimuli, such as temperature, pressure and injury related to chemicals (Basbaum et al., 2009). Nociceptors are excitatory neurons and produce several neurotransmitters, including; glutamate and other components such as peptides (e.g., substance P, somatostatin, calcitonin gene-related peptide [CGRP]) (Basbaum et al., 2009).

The peripheral terminal of the nociceptor is where the noxious stimuli are detected and transduced into electrical signals (Basbaum et al., 2009). When the electrical energy reaches a threshold level, an action potential is induced and driven towards the central nervous system (CNS), specifically to the thalamus and subsequently to the cortex and spinal dorsal horn (D'Mello and Dickenson, 2008). Nociceptors are generally electrically silent and transmit action potentials only when stimulated (Basbaum et al., 2009). Nociceptors in the peripheral nervous system (PNS) are pseudounipolar dorsal root ganglion neurons with thinly myelinated or unmyelinated axons which conduct pain signal at different velocities (Dubin and Patapoutian, 2010). The thinly myelinated fibres are referred to as Aδ-fibres, which conduct in a range of 2 m/s to 20 m/s (Watson et al., 2012). These fibres respond to high intensity mechanical stimulation and are termed high threshold mechanoreceptors. The non-myelinated axons are referred to as C-fibres; they are polymodal, responding to chemical, mechanical and thermal stimuli with conduction velocities of less than 2.5 m/s (Watson et al., 2012). In addition to the Aδ and C fibres, there are primary afferent Aβ fibres, highly myelinated that carry non-noxious stimuli and typically respond to light touch. Nociceptors are widely distributed throughout the body in the skin (cutaneous nociceptors) and other tissues, such as muscles, joints and meninges (visceral nociceptors). Nociceptive information is transmitted to the brain through the spinothalamic tracts (also known as anterolateral system or the ventrolateral system (see Figure 1.1 for details that illustrate the cutaneous and visceral nociceptive pathways). This ascending information can activate descending pathways, from the midbrain periaqueductal grey area, which exerts an inhibitory control over the dorsal horn.

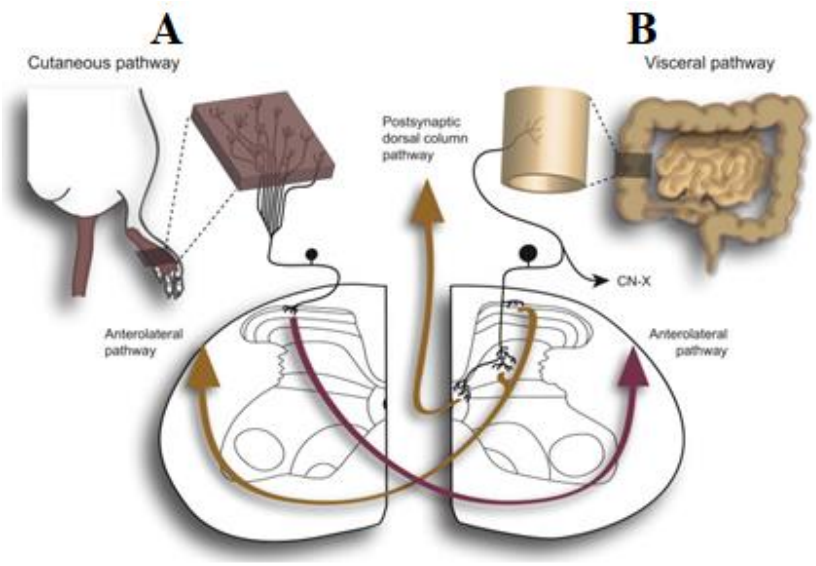


**Figure 1.1: Cutaneous and visceral nociceptive pathways. (A)** Cutaneous nociceptors C-fibre or Aδ-fibre convey information about noxious stimuli from the skin *via* spinal nerves into the spinal cord dorsal horn primarily in laminae I–II. **(B)** Visceral nociceptors are Aδ and C-fibres convey similar signals from gut structures *via* spinal or vagus nerves that terminate in laminae I, IV–VI and X in the spinal cord dorsal horn. Visceral information leaves the dorsal horn *via* anterolateral or dorsal column pathways, whereas cutaneous information can exit the dorsal horn *via* the anterolateral pathway (obtained from Watson et al., 2012).

### 1.1.4 Epidemiology of chronic pain in the UK and worldwide

There is a concern regarding the burden of pain in the UK, because chronic pain affects more than one-third of the UK population, which means that around 28 million adults are living with chronic pain. It is anticipated that chronic pain will affect one in three people over the next two decades (Fayaz et al., 2016). Additionally, studies revealed that 43% of the population experience chronic pain, with 14.3% living with chronic pain that is either severely or moderately disabling (Fayaz et al., 2016). Similarly, researchers determined that females are more likely to experience chronic pain than males, and prevalence of chronic pain is expected to increase with ageing. In one study, prevalence of chronic pain among those over the age of 75 was as high as 62% (Toye et al., 2017). There is a reluctance to discuss pain because it is not as visible or tangible as other conditions, even though it may have a devastating impact on quality of life (Fayaz et al., 2016).

Numerous studies also demonstrate the prevalence of chronic pain worldwide. One survey completed in 2009 that was carried out in various regions across the world, including North America, South America, Europe, Asia, the Middle East and Africa, discovered that the percentage of prevalence of chronic pain reached 37.3% in developed countries and 41.1% in developing countries with regards to the entire population (Tsang et al., 2008). These values indicate that chronic pain is a global health problem (Goldberg and McGee, 2011). A further study conducted in 2012 analysed 28 European countries and revealed that chronic

pain of moderate to severe intensity affects 25-30% of adult Europeans (Leadley et al., 2012). Of the one fifth of people who reported chronic pain, the majority were thought to suffer with neuropathic pain (Cohen and Mao, 2014). A different study conducted in Western Europe revealed that the presence of neuropathic pain was associated with a significantly increased disease burden in the chronic pain population. These negative effects were seen in terms of health status, employment experience and besides direct medical costs (Langley et al., 2013).

The World Health Organisation (WHO) estimates that by 2030, unipolar depression, coronary heart disease, cerebrovascular disease and road traffic accidents will be the four leading contributors to the global burden of disease (Van Hecke et al., 2013). Chronic pain is an important co-morbidity associated with all of these diseases or conditions (Van Hecke et al., 2013). Nonetheless, chronic pain is more than just the co-morbidity of other known diseases or injuries, as it is now classified as a pathological condition or illness (Simon, 2012). Moreover, the cost of treating chronic pain exceeds the costs of other chronic conditions including heart disease, respiratory disease or cancer (Disorbio et al., 2006). Approximately 30–40% of individuals with chronic pain experience inadequate pain relief and report high rates of dissatisfaction with their treatments (Müller-Schwefe, 2011).

### 1.1.5 Treatment options for chronic pain

Acute pain can be treated simply and inexpensively (Blondell et al., 2013) .The initial treatment may include physical therapy, such as therapeutic exercises with over-the-counter pain medicines. In contrast, the treatment of chronic pain is more complicated and involves more than one therapeutic modality (Peppin et al., 2015). Consequently, it is credited with a high cost (Turk, 2002). There are several options for the treatment of chronic pain that are available without a prescription. However, every chronic pain patient is different and they each respond differently to their medication. In 1990, the WHO introduced the pain relief ladder, this is a stepwise approach for pain management. Typically, the first step was to try a common oral pain medication, for instance nonsteroidal anti-inflammatory drugs (NSAIDs) and acetaminophen. If these medications do not control the pain, physicians prescribe other strong medication, for instance opioids and a variety of adjuvant agents (e.g., antidepressants, anticonvulsants) (Aronson, 1997).

Specific management of chronic pain is covered by the National Institute for Health and Care Excellence (NICE) guidelines, these guidelines provide information regarding the most

appropriate treatment regimens for different diseases in England and Wales. The NICE guidelines offer the tricyclic antidepressants (amitriptyline), serotonin noradrenaline reuptake inhibitors (duloxetine) and anti-convulsant drug (e.g., gabapentin or pregabalin) as initial treatment. If the initial treatment is not tolerated or is not effective for pain management, the NICE guidelines offer one of the remaining three drugs or a weak opioids, including dihydrocodeine and tramadol. If the second line treatment is not tolerated and pain persists or increases, the NICE guidelines consider a higher dose of weak opioid or strong opioid (e.g., morphine) or lacosamide, lamotrigine, levetiracetam, as a third line treatment. Typically, the choice of a drug is made by balancing the clinical efficacy of the drug, indications for treatment and its toxicity (Aronson, 1997). An understanding of the pharmacological action of these medications and mechanisms underlying chronic pain significantly helps to establish strategies for pain control.

Opioid analgesics continue to be the mainstream of pharmacologic treatment for acute and chronic pain even with the presence of other non-opioid pain medications being introduced into the market over the last few years (Wang et al., 2016). Compared with other medications, opioids typically have a quick onset concerning acute pain relief (Jefferies, 2010; Jena et al., 2016). However, prolonging administration of opioids creates opioid tolerance, opioid-induced hyperalgesia and other complications, for instance nausea, constipation and dyspepsia which have a negative effect on pain management (DuPen et al., 2007).

## 1.2 Opioids

### 1.2.1 History

Opium is the earliest form of pain relief in the history of human kind (Wang et al., 2016) and has been used since the Roman and Byzantine times (Bryan, 2018). Opium, the dried latex, was discovered in poppy seeds (*Papaver somnifrum*) for several thousand years (Kieffer, 1999). It is difficult to estimate when and where the opium poppy was first cultivated, but there is a general agreement that the ancient Sumerians grew poppies and extracted opium from seed capsules at the end of the third millennium B.C. (Brownstein, 1993). Opium was probably consumed by the ancient Egyptians and was known to the ancient Greeks as well (Brownstein, 1993).

In 1806, German medicinal chemist Friedrich Sertürner isolated the active ingredient from opium and named it morphine after the Greek god of dreams, Morpheus (Brownstein, 1993; Schmitz, 1985). In 1939, the first opiate with a structure entirely different to morphine was discovered and called meperidine (Brownstein, 1993). This discovery was followed by the development of additional synthetic compounds and a subsequent study on the role of opioids and their mechanisms of action (Brownstein, 1993). Despite this long history of opioid drug development and many important discoveries in the field of opioid analgesia, multiple fundamental questions concerning opioid effectiveness remain unanswered. Such questions are associated with mechanisms underlying opioid tolerance, dependence and opioid safety to prevent new populations of patients from becoming dependent on or addicted to opioids. The Food and Drug Administration (FDA) focuses on improvements in abuse deterrent strategies by increasing the safety of these medications, by means of future potential strategies, for instance additional legislative policies, physician education and public awareness (Jones et al., 2018).

### 1.2.2 Chemical structure and application

Opium and isoquinoline alkaloids like morphine, codeine, papaverine, noscapine and thebaine are isolated from the dried capsules of *Papaver somniferum* (Yoshimatsu and Shimomura, 1992); see Figure 1.2 for stages in opium extraction from the plant). Specifically, opium is extracted by air drying the white milky resin that is inside the seed pod, this resin quickly turns into a brownish gum like liquid or semisolid (Brook et al., 2017). Then it is further dried and ground up into a powder (Weid et al., 2004). Traditionally, the dry opium was used as an astringent, antispasmodic, diaphoretic, aphrodisiac, expectorant, narcotic, hypnotic and sedative (Lim, 2012). Poppy has also been used to treat coughs and toothache (Lim, 2012). Additionally, the ability of opium to serve as an analgesic is well known (Brook et al., 2017).

Pharmacologically, morphine alkaloids are the most important active ingredients within opium (Amabile and Bowman, 2006). The semisynthetic derivatives, such as hydromorphone, hydrocodone and oxycodone, which are produced by a minor chemical modifications of the natural opioids, while leaving the characteristic morphinan nucleus intact (Vardanyan and Hruby, 2014). Other opioids, such as (fentanyl), are classified as synthetic opiate agonists and lack the typical morphinan nucleus (Vardanyan and Hruby, 2014).

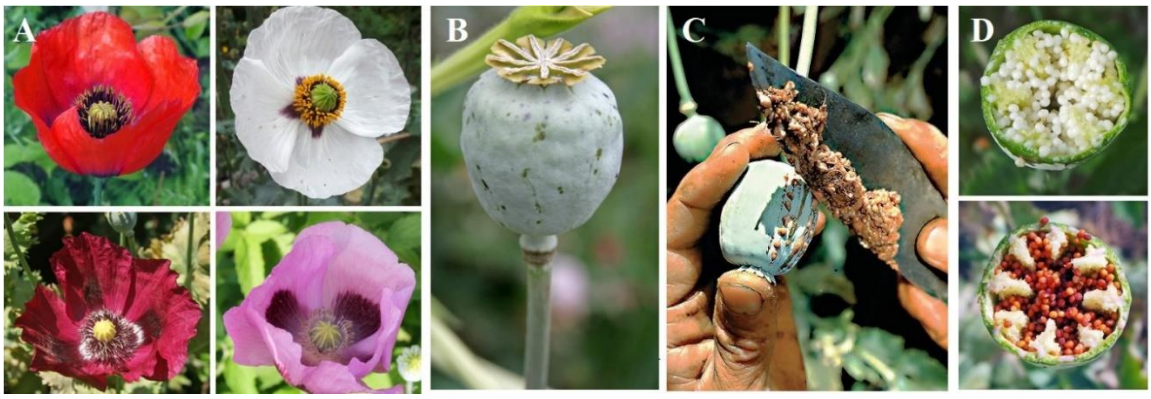


**Figure 1.2: *Papaver somniferum* plant and stages in opium extraction from the plant. (A)** *Papaver somniferum* is type of opium poppy whose flowers are single or double forms, with substantial variation of colour shape and arrangement of petals. **(B-C)** Unripe seedpods (capsules) of the opium poppy with latex sap dripping from a recent cut. The latex sap consist of a mixture of naturally-occurring narcotic alkaloids. **(D)** The seed inside the capsule turns from white (top) to brown (bottom), then black when it matures (adapted from Weid et al., 2004 and Meos et al., 2017).

The legal cultivating of opium for medicinal use currently takes place in several countries, including; Australia, Turkey (Jelsma, 2011), India (Lubbe and Verpoorte, 2011) Spain, France, Hungary and the UK (Malloch-Brown, 2008). However, the global demand for illicit more opium for recreational use has been to continued grow (Jelsma, 2011). Nowadays, Afghanistan dominates the global illicit production of almost two thirds of the total area under illegal opium cultivation (Zhaliapava, 2009). Approximately, two thousand tonnes of opium are produced annually. This supplies the world with the raw material required to make products for medicinal and research purposes (Maurer, 2017). Moreover, some poppy plants are grown in the private gardens of several countries as ornamental flowers (Meos et al., 2017).

Morphine is a white powder with the chemical formula $C_{17}H_{19}NO_3$ (Juda and Ulrich, 1957). It is the most considerable of opium's 24 alkaloids, accounting for 9 to 14% of opium-extract by mass (Brochmann-Hanssen, 1971). Morphine and its hydrated form $C_{17}H_{19}NO_3.H_2O$ are sparingly soluble in water (Brochmann-Hanssen, 1971), for example in five litres of water only one gram of the hydrate can dissolve (Roy and Flynn, 1989). For this reason, industrial pharmaceutical companies produce hydrochloride salts and sulphate of the drug (Roy and Flynn, 1989). Both of the modified compounds are over three hundred times more water-soluble than its original molecule (Roy and Flynn, 1989).

As illustrated in Figure 1.3, morphine consists of five condensed rings; three rings are roughly in the same plane including the aromatic rings (Braenden et al., 1955). The other two rings, including the nitrogen ring are each at right angles to the other trio of rings

(Braenden et al., 1955). In general, a ring is when a group of atoms bond to each other and form a closed figure with their bonds (Ritchie et al., 2011). An aromatic ring in general is a type of circle of atoms arranged, in which the bonds alternate between single bonds and double bonds (Ritchie et al., 2011).

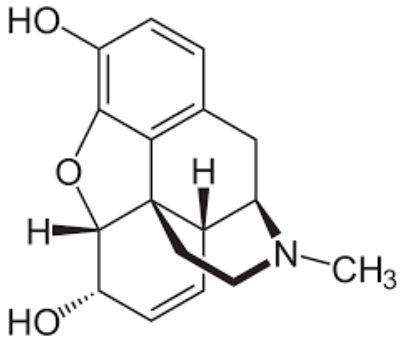


**Figure 1.3: Chemical structure of morphine**. It is the principal alkaloid in opium and the prototype opiate analgesic and narcotic. Molar mass: 285.34 g/mol (obtained from Gylbert, 1973).

This organised structure contains a quaternary carbon atom (is one that is bonded to four other carbon atoms), an aromatic ring and two carbon atoms that connect the quaternary carbon atom to a tertiary amine group. This is known as the 'morphine rule' (Randić et al., 1987). A primary amine group is made of a nitrogen (N) atom that contains a lone pair of electrons and is linked to two hydrogen (H) atoms and other variable groups, while the tertiary amino group is not linked to any hydrogen atoms. As a result of these bonds, the nitrogen atom has a slightly positive charge which contributes to its chemical function in the brain (Schiller et al., 2000). Opioids are a class of drugs resembling opium in physiological effects and the addictive propreties include the illegal drug heroin. Opioid analgesics, like morphine, codeine and structurally less similar drugs, for example, meperidine, all have an aromatic ring and quaternary carbon atom bound to a tertiary amine group by two other carbon atoms, which follows the morphine rule (morphinan nucleus) Their ability to relieve pain is from their fulfilment of the morphine rule (Fuller, 1997).

Morphine acts directly on the CNS to decrease pain perception (Botney and Fields, 1983). It can be prescribed for both acute and chronic pain (Hoskin and Hanks, 1991) and is frequently used to treat pain associated with cancer (Hanks et al., 2001) or severe chronic non-cancer pain, such as myocardial infarction (Weihrauch et al., 2005) and the pain experienced during labour (Scott et al., 1980).

Morphine can be given orally in a liquid form, as quick-acting tablets (Osborne et al., 1990) or as slow-release capsules and tablets (Heafield et al., 1999). It is also available as a

bioadhesive buccal tablet (Beyssac et al., 1998) or as an injection that is commonly administered in hospitals following surgical operations (postoperative pain) (Singelyn et al., 1998). Additionally, it can be injected into muscles (an intramuscularly) (Rawal et al., 1984), under the skin (subcutaneously) (Mellett and Woods, 1961), intravenously (Singelyn et al., 1998), into the subarachnoid space around the spinal cord (intrathecal) (Rawal et al., 1984) or it can be given rectally (Westerling, 1985).

### 1.2.3 Opioid action on opioid receptors

Opioids exert their complex pharmacologic effect by binding to opioid receptors (Arvidsson et al., 1995). Opioid receptors are found throughout the body within the CNS, PNS and in peripheral organs, such as the heart, liver and lungs, in addition to the gastrointestinal and reproductive tracts (Arvidsson et al., 1995). However, the distribution and expression of opioid receptors varies significantly between different organs, as well as between different animal species. These receptors belong to the superfamily of G-protein coupled receptors which contain seven-transmembrane regions with the amino (N-terminus) located outside the cell and the carboxy terminus located within the cell (Koneru et al., 2009). It involves activation of Gαi/o proteins and its role is to mediate neuronal inhibition (Chahl, 1996). There are three major types of opioid receptors (mu (μ), kappa (κ), and delta (δ) (Koneru et al., 2009)), that were cloned in early 1990s. Additionally, a fourth type of receptor family termed opioid receptor like-1 (ORL1), which is also known as nociceptin or the orphanin FQ receptor are the youngest members of the opioid receptor family and were added to the list in 1994 (Arvidsson et al., 1995). Table 1.1. presents opioid receptor types.

Opioid receptors are activated by naturally occurring endogenous peptides or ligands (e.g., dynorphins, enkephalins, endorphins, endomorphins and nociception) and synthesised by exogenous agonists which interact with opioid receptors to provide analgesia *via* action on antinociceptive pathway (Fields, 2011) because these receptors are expressed in areas of pain perception within the CNS and PNS. However, only agonists at μ-opioid receptors produce potent analgesia (Fields, 2011), these include heroin, morphine and oxycodone (Fields, 2011). The potency and efficacy of opioids vary at the different opioid receptors, in both the clinical and experimental models (Al-Hasani and Bruchas, 2011). The overall effect of a particular opioid is the combined pharmacological, physiological and adverse effects upon stimulation of all appropriate opioid receptors (Al-Hasani and Bruchas, 2011).

There are many possible undesired side-effects associated with μ-opioid receptors activation (Table 1.1), for researchers to investigate other opioid receptors *in vivo* and in clinical studies to find optimal opioid targets for pain management (Al-Hasani and Bruchas, 2011).

**Table 1.1: Opioid receptor type classification, locations, endogenous ligands and pharmacodynamic effects.** (Mansour et al., 1994; Dhawan et al., 1996; Mollereau and Mouledous, 2000; Walwyn et al., 2010).

| Opioid receptor class | Subtypes | Endogenous opioid peptide affinity (ligands) | Cellular expression | Physiological actions |
|---|---|---|---|---|
| mu Opioid Receptor (μ) | $\mu_1$<br>$\mu_2$<br>$\mu_3$ | β-endorphin (not selective)<br>enkephalins (not selective)<br>endomorphin-1<br>endomorphin-2 | **Brain**<br>• cortex (laminae III and IV)<br>• thalamus<br>• rostral ventromedial medulla periaqueductal gray (PAG)<br>**Spinal cord**<br>dorsal horn (lamina I and II)<br>**Other regions**<br>peripheral sensory neurons, immune cells, respiratory and gastrointestinal(GI) tracts | **$\mu_1$**: euphoria, confusion supraspinal analgesia by blocking all pain messages, nausea, low addiction potential.<br><br>**$\mu_2$:** respiratory depression, physical dependence, sedation, miosis, gastrointestinal effect by decrease bowel tone and contractility.<br><br>**$\mu_3$:** possible vasodilation |
| Delta Opioid Receptor (δ) | $\delta_1$<br>$\delta_2$ | enkephalins (not selective)<br>β-endorphin | **Brain**<br>• pontine nuclei<br>• amygdala<br>• olfactory bulbs<br>• deep cortex | **$\delta_1$**: analgesia, cardioprotection.<br><br>**$\delta_2$**: analgesia, cardioprotection thermoregulation. |
| Kappa Opioid Receptor (κ) | $\kappa_1$<br>($\kappa_{1a}$,$\kappa_{1b}$)<br>$\kappa_2$<br>($\kappa_{2a}$,$\kappa_{2b}$)<br>$\kappa_3$ | dynorphin A<br>dynorphin B<br>α-neoendorphin | **Brain**<br>• Hypothalamic nucleai<br>• PAG<br>• claustrum<br>**Spinal cord:**<br>Substantia gelatinosa<br>**Other regions**<br>Peripheral sensory neurons | **$\kappa_1$ ($\kappa_{1a}$,$\kappa_{1b}$)**: analgesia.<br><br>**$\kappa_2$ ($\kappa_{2a}$,$\kappa_{2b}$)**: analgesia, diuresis by reduction in secretion of antidiuretic hormone.<br><br>**$\kappa_3$**: spinal analgesia, peripheral effect. |
| Opioid receptor like-1 (ORL) | | Nociception or orphanin FQ | **Brain:**<br>• forebrain (cortical areas, olfactory regions, limbic structures, thalamus)<br>• brainstem (central periaqueductal gray, substantia nigra, several sensory and motor nuclei)<br>**Spinal cord:** dorsal and ventral horns horn | Modulation of pain, effects on locomotion, anxiety, stress, feeding (appetite), learning and memory, reward/addiction and urogenital activity. |

### 1.2.4 Pharmacological action of opioids

The pharmacological action of opioid drugs is produced by binding to opioid receptors that are located on neuronal cell membranes. Centrally acting opioid drugs have effects in many areas, including primary afferent neurons, the spinal cord, midbrain and thalamus (Arvidsson et al., 1995). In the PNS, opioids acts on both the myenteric plexus and submucous plexus in the wall of the gut (Chahl, 1996). In neurons, opioids act at two different sites; specifically the presynaptic nerve terminal and the postsynaptic ascending neuron and also activate descending inhibitory controls in the midbrain (Koneru et al., 2009). The presynaptic action of opioids is to inhibit neurotransmitter release including acetylcholine and the neuropeptide and substance P which is considered to be the main effect of opioids on the nervous system (Chahl, 1996). As mentioned above, the opioid receptor belongs to the superfamily of G-protein-coupled receptors which consist of three subunits α, β and γ. When the receptor is occupied the subunit is uncoupled and forms a complex which interacts with cellular systems to produce a response, which includes inhibitory effects in neurons (Chahl, 1996). Figure 1.4 below illustrates the function of G-proteins.

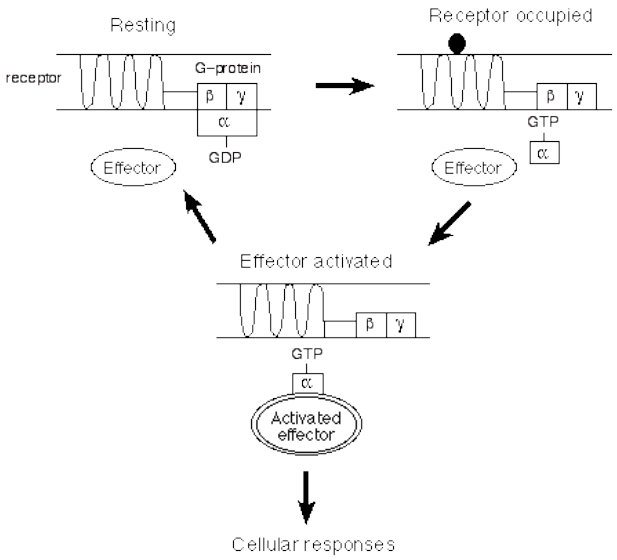


**Figure 1.4: The function of G-proteins**. Opioid receptors, μ, κ and δ, are coupled to guanosine diphosphate (GDP) nucleotide binding to Gi (inhibitory) protein that consists of three subunits (α, β and γ). When the opioid binds to the receptor, GDP dissociates from (α subunit), as well as from (β and γ) and guanosine triphosphate (GTP) takes its place. Thus, (α subunit) interacts with the system within the cell that produces the effect (the effector) (obtained from Chahl, 1996).

Opioids work in several ways, they can inhibit neurotransmitter release by inhibiting $Ca^{2+}$ entry into the presynaptic neuronal cell, or they can work directly by inactivating voltage-gated $Ca^{2+}$ channels or they can work indirectly, by enhancing the outward movement of $K^+$ ions *via* stimulation of the voltage-sensitive $K^+$ channels; thus, shortening the repolarisation the duration time of the action potential (Bovill, 1997). Opioids also inhibit the adenylate

cyclase (AC) enzyme, which is responsible for converting adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Chahl, 1996). Together all of these actions result in a reduction of neurotransmitter release; and therefore, decrease excitability along the cell membranes of neurons that are involved in pain pathways (DuPen et al., 2007). Figure 1.5 illustrates the mechanism of action of opioid.

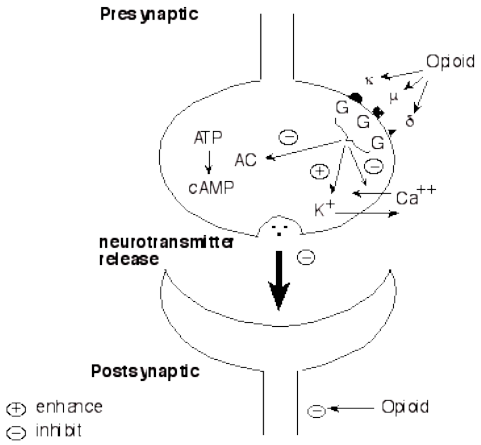


**Figure 1.5: Opioid mode of action**. All the three subtypes of opioid receptors, μ, κ and δ, are involved in antinociceptive and analgesic mechanisms at both spinal and supraspinal levels (obtained from Chahl, 1996).

A major advance in understanding pain mechanisms has been the growing recognition that the ongoing activity in nociceptive pathways may create profound changes in the levels of neurotransmitters in primary afferent neurons and alters sensitivity to opioid analgesia. Thus, neuropathic pain is associated with a decrease in opioid sensitivity (Chahl, 1996).

### 1.2.5 Opioids metabolism

Metabolism refers to the chemical process of biotransformation, which is the mechanism by which drugs are broken down so that they can be eliminated by the body (Houston, 1994). Certain drugs perform their pharmacological effects and are then excreted from the body intact, without going through the biotransformation process. However, other drugs require metabolism to occur to enable them to be delivered to their target site and remain there for an adequate length of time, prior to being eliminated from the body (Mandel, 1971). All opioids are eliminated from the body *via* metabolism by drug-metabolising enzymes such as Cytochrome P450 enzymes (CYP) *via* phase I and uridine diphosphoglucuronosyl transferase (UGT) *via* phase II (Holmquist, 2009).

In phase I, oxidative biotransformation converts the drug to a more water soluble compound, enhancing its excretion by the kidney or typically precedes the compound for phase II either by hydrolysis, reduction, oxidation or conjugation of the metabolite with

glycine, glucuronide or sulphate with subsequent secretion into the bile (Armstrong and Cozza, 2003). Cytochrome P450 3A4 (CYP3A4) and the highly polymorphic Cytochrome P450 2D6 (CYP2D6), markedly affect the function of the opioid. In phase II, glucuronidation by glucuronic acid produces molecules that are highly hydrophilic and therefore easily excreted (Armstrong and Cozza, 2003). In several cases, these metabolites have activity comparable to or even more than the parent drug, resulting in opioid active metabolites. The best example is morphine being a metabolite of codeine (Drewes et al., 2013). The metabolism of opioids undergo varying degrees as it ranges between phase I and II (Smith, 2009). The process of metabolism ends when the molecules are sufficiently hydrophilic to be excreted from the body (Houston, 1994). Around 60% of morphine is glucuronidated to morphine-3-glucuronide (M3G), whereas 5-10% is glucuronidated to morphine-6-glucuronide (M6G). These reactions are primarily catalysed by UDP-Glucuronosyltransferase-2B7 (UGT2B7) in the liver (Thorn et al., 2009). Additionally, the clinical effects of (M3G) and (M6G) are dependent on the ability of the metabolites to reach their sites of action in the CNS by passing the blood brain barrier (BBB) (Carrupt et al., 1991). Due to their hydrophilic nature, highly polar glucuronides are generally not considered capable of crossing the BBB owing to its lipophilic composition (Bickel et al., 1996). However, particular animal studies have shown that glucuronides exert effects of their own and demonstrate that M6G may be a more potent analgesic than morphine (Paul et al., 1989; Frances et al., 1992; Sjogren et al., 1998; Lipkowski et al., 199).

Patients may need to trial various opioids before finding a drug that provides a powerful analgesia with acceptable tolerability (Smith, 2009). Reasons underlying this variability include many different factors, such as genes, race (Sadhasivam et al., 2012) and other less understood factors, for example allelic variants (gene polymorphism) which affect the binding between the opioid and receptor (Mroziewicz and Tyndale, 2010). Furthermore, metabolic variability may influence the efficacy and tolerability of the opioid (Smith, 2009). There are several factors that contribute to this metabolic variability, such as the risk of drug interactions with an opioid (Smith, 2009) and prior medical conditions, most notably kidney or liver disease (Gelot and Nakhla, 2014).

### 1.2.6 Overview of opioid-related conditions

Public health authorities have described a rapid and unprecedented increase in mortality and morbidity rates, associated with the use of opioid pain medication. This has resulted in efforts to address the opioid crisis and reduce the use of opioids for non-medical reasons.

Concerns related to safety, effectiveness and abuse accountability have developed over the last few decades, occasionally driving a greater restrictive perspective and sometimes leading to a higher level of willingness to endorse this therapy.

Opioid use disorder (OUD), is a condition characterised by the harmful consequences of repeated opioid administration leading to clinically significant impairment or distress. It is a pattern of compulsive opioid use and (occasionally) physiological dependence on opioids (Brezing and Bisaga, 2015). Most individuals with OUD have shown significant levels of tolerance and they experience withdrawal symptoms on abrupt discontinuation of opioid administration (Schuckit, 2016). Individuals with OUD regularly develop conditioned responses to drug-related stimuli (i.e., intolerable craving on seeing any white powdery substance resembling heroin cocaine or drug paraphernalia), which is a phenomenon that occurs with most drugs that cause infinitive psychological changes (Satel and Lilienfeld, 2014). These responses probably involve relapse, are difficult to extinguish and typically remain long after detoxification is complete (Schuckit, 2016).

Opioid tolerance is a condition that affects pain management therapy (Wilson-Poe et al., 2017). Tolerance is defined as a state of adaptation in which repeated exposure to a drug induces changes, these changes result in diminution of one or more of the drug's anti-nociceptive effects (Wilson-Poe et al., 2017). It is a significant clinical problem that limits the option of using opioids to treat pain (Wilson-Poe et al., 2017). Tolerance to the antinociceptive effects of opioid drugs is relatively easy to demonstrate in animal models in a variety of animal species (Collett, 1998). Consequently, after decades of research, numerous mechanisms at molecular, cellular and network levels account for the behavioural observation of opioid analgesic tolerance (Wilson-Poe et al., 2017). Additionally, there is accumulating evidence that indicate opioid treatment might not only be linked with the development of tolerance but also linked with increased sensitivity to pain; a condition referred to as opioid-induced hyperalgesia (OIH) (Richebe and Rivat, 2017).

OIH occurs with prolonged exposure to opioids, resulting in a paradoxical increase in atypical pain that doesn't appear to be related to the initial nociceptive stimulus (Richebe and Rivat, 2017). This was first observed in patients with an opioid addiction and is discussed in peer-reviewed literature by Andrews and colleagues (Andrews, 1943). Similarly, Tilson et al. (1973), first reported that abrupt, discontinuous treatment of opioids caused a decrease in the pain threshold of rats.

Opioid addiction is a significant issue in Europe and the United States (Häuser et al., 2017; Calcaterra et al., 2019), with prescription opioid addiction being one of the biggest drug problems today. Indeed, the number of prescriptions for strong opioids has increased in the United Kingdom in recent years, especially in more deprived communities (Mordecai et al., 2018), primarily because physicians are increasingly warned not to leave patients suffering. A recent study reported that prescriptions for non-cancer patients, increased approximately seven-fold between 2000-2010, with morphine being the most frequently prescribed drug (Zin et al., 2014). In England and Wales, morphine or heroin was a contributing factor in 1,200 registered deaths registered in 2015 (Martins et al., 2015), a 26% increase on the previous year and a massive 57% increase compared to 2013 (Martins et al., 2015). Thus, any long-term use of opioids places the patient at risk of addiction, even if the substance is used as prescribed. In contrast, the long-term administration of opioid drugs for chronic pain therapy continues to be controversial (Rosenblum et al., 2008).

### 1.2.6.1 Opioid tolerance pharmacological phenomena

Opioid tolerance is a significant clinical phenomenon required to increase doses of an opioid to achieve the same analgesic effect. It was first described by Light & Toorance in the 1920s; nevertheless, it is a poorly understood phenomenon. Figure 1.6 shows an idealised dose-response curve for an administered drug. As the drug dose increases, the observed effect of the drug increases. With repeated use of opioid, the curve shifts to the right, which is an indication of tolerance.

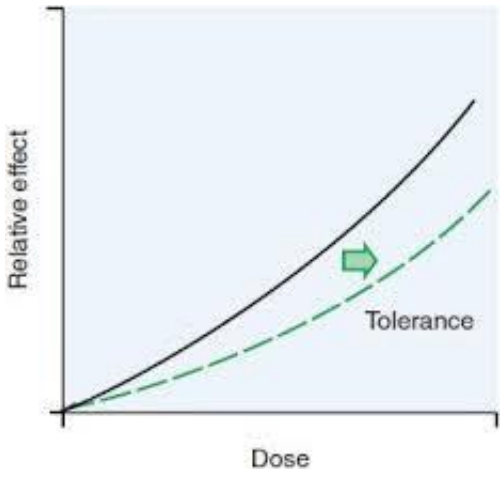


**Figure 1.6: Tolerance shifts the dose-response curve**. Upon chronic use of opioid, the curve shifts to the right such that doses higher than initial doses are required to achieve the same effects (adapted from Brunton, 2014).

### 1.2.6.2 Why is tolerance important?

Opioid therapy has long been feared by the general public and by doctors (Collett, 1998) due to concerns regarding addiction and tolerance. These are both common problems among patients receiving long-term opioid treatment. Importantly, tolerance develops to some pharmacological effects much more rapidly than to other effects from the same drug. This is termed 'selective tolerance' (Collett, 1998). For example, tolerance develops quickly to the sedative and euphoric effect produced by opioids such as heroin, besides drug abuse tends to increase their dose to re-experience that elusive feeling of "high." In contrast, tolerance to the gastrointestinal (GI) effects (constipation) and pupil (miosis) of opioids does not develop (Collett, 1998). The discrepancy between the development of tolerance to the euphorigenic effects (rapid) and tolerance to effects on vital functions (slow), for example blood pressure and respiration can lead to potentially fatal overdoses. In 2014, the WHO estimated that 69,000 people die from opiate overdose annually. Clinically, opioid dose escalations more than ten-fold the suggested dose are common in chronic pain management (Buntin-Mushock et al., 2005). Patients may develop opioid tolerance within several hours, even after a single bolus of opioid, and yet, numerous studies demonstrate that relatively stable doses of opioids can maintain pain relief for weeks or even years (Eisenberg et al., 2005; Farrar et al., 2010).

### 1.2.6.3 Opioid tolerance types and mechanisms

There are different types of opioid tolerance, as shown in Table 1.2. Tolerance can be separated into two main types: innate or acquired. Innate tolerance refers to a lack of opioid sensitivity due to inherited genetic differences (pharmacogenetic make-up) (Collett, 1998). In most cases, innate tolerance is observed after administration of the first dose. However, acquired tolerance is a consequence of repeated opioid exposure and can be subdivided into three general classifications based on the prevalent mechanism: pharmacokinetic, pharmacodynamic or learned tolerance (Dumas and Pollack, 2008).

**Table 1.2: Types of opioid tolerance** (Collett, 1998)**.**

- **Innate**
- **Acquired**
  1. Pharmacokinetic tolerance
  2. Pharmacodynamic tolerance
  3. Learned tolerance

**1. Pharmacokinetic tolerance** refers to changes in the metabolism or distribution of the drug after repeated or prolonged drug administration that resulted in reduced concentrations

in the blood and the sites of drug action (Dumas and Pollack, 2008). There are different pharmacokinetic mediators that can contribute to opioid tolerance:

A. *Metabolic and distributional mediators of tolerance*

One of the most important sources of extensive variability in drug concentration and consequently, response, within a population can be attributed to differences in drug absorption, distribution, metabolism and excretion (Wilkinson, 2005). Biotransformation and polymorphisms in the cytochrome CYP and the other enzymes will influence individual opioid disposition and response (Kadiev et al., 2008). Likewise, it may contribute to morphine tolerance. It has been shown that chronic treatment of rats with morphine caused a significant increase in enzyme activity, resulting in a reduction of morphine analgesia, due to drug tolerance (Vlaskovska et al., 1999).

B. *Metabolite contributions to opioid response and tolerance*

Tolerance also may occurs from accumulation of metabolites in the vascular system that occurs over a period of time (Smith, 2009). Specific opioids, for example codeine, morphine, hydromorphone, tramadol and oxycodone produce multiple active metabolites after administration (Reisfield et al., 2007). These metabolites interfere with the pharmacologic response either by competing for receptor binding with the active parent compound (e.g., antagonists or partial agonists) or by down-regulating the response of the receptor system (Smith, 2009; Dumas and Pollack, 2008). For example, morphine produces two non-opioid active metabolites; M3G and M6G (Klimas and Mikus, 2014). It is assumed that M6G is a strong μ-receptor agonist responsible for much of the pain-relieving effects than morphine itself, although the extent of its contribution remains unclear (Klimas and Mikus, 2014). In contrast, M3G has low affinity for the opioid receptors, therefore no analgesic effect is produced (Smith, 2009).

**2. Pharmacodynamic tolerance** occurs when the intrinsic response of the opioid receptor diminishes over time, due to changes in the receptor binding type and location, or due to alterations in signal transduction (Dumas and Pollack, 2008).

Animal studies have revealed that opioid tolerance can be acute or chronic (Wang and Ho, 1994). Acute opioid tolerance is predominantly mediated by pharmacodynamics mechanisms and may develop within four hours after a single bolus of fentanyl or morphine, and one hour after a single injection of alfentanil (Wang and Ho, 1994). This phenomenon is exemplified by nasal-administered cocaine (Jeffcoat et al., 1989). At first, the cocaine

concentration is proportionate to its effects. Nonetheless, over time, the euphoric response to cocaine decreased, despite continuing or even increasing the circulating concentration (Jeffcoat et al., 1989).

Chronic tolerance can be mediated by either pharmacodynamic or pharmacokinetic mechanisms, which may develop with an incubation period of 8-10 days and last for an extended period (Wang and Ho, 1994). In cases where chronic tolerance develops, cross-tolerance within the pharmacological class also may occur (Dumas and Pollack, 2008). The characteristics of acute opioid tolerance may differ from those of chronic opioid tolerance and the mechanisms underlying acute and chronic opioid tolerance are poorly studied and need to be further explored (Dumas and Pollack, 2008). Moreover, there are different pharmacodynamic mediators that can contribute to opioid tolerance:

A. *Opioid receptor-mediated changes*

The opioid analgesics must bind to specific sites (opiate receptors) to elicit its pharmacological responses (Arvidsson et al., 1995). Opioid tolerance is observed after protracted exposure and after acute treatment (acute tolerance), though it is not observed for all the pharmacological effects (Collett, 1998). There are different hypotheses linked directly to receptor regulation, such as down-regulation, phosphorylation, desensitisation, internalisation, endocytosis and G protein uncoupling.

*Down-regulation*: Down-regulation diminishes the quantity of the available opioid receptors that may result from internalisation followed by receptor degradation, or a decrease in receptor synthesis (Allouche et al., 2014). Down-regulation of opioid receptors is observed following chronic exposure to high-intrinsic-efficacy opioid agonists (e.g., etorphine), but not following low-intrinsic-efficacy agonists (e.g., morphine) (Gomes et al., 2002). However, in certain cases, tolerance occurs without receptor down-regulation (Whistler et al., 1999).

*Phosphorylation*: Receptor phosphorylation is the first step in the desensitisation and internalisation of the μ-opioid receptor (Dumas and Pollack, 2008). Phosphorylation is a key post-translational modification mechanism *via* protein kinase that controls the conformation and activity of many proteins (Wang and Wang, 2006). Growing evidence has identified the essential role of protein phosphorylation *via* multiple major protein kinases in promoting the

development and maintenance of opioid tolerance. Table 1.3 summarises the most recent publications on the role of protein kinase in the regulation of opioids.

**Table 1.3: Summary of studies in regards to tolerance models and protein kinase inhibitors.**

| Animal | Method to induce tolerance | Kinase inhibitor | Administration route | Key findings | References |
|---|---|---|---|---|---|
| Rats | Continuous intrathecal infusion of morphine (40 nmol/ul/h) | PKCα antisense | Intrathecal | Block of spinal morphine tolerance. | *Hua et al., 2002* |
| Rats | Chronic lumbar intrathecal infusion of morphine (20 nmol/ul/h) | PKC inhibitor: Chelerythrine GF109203X | Intrathecal bolus injection | Blocking the PKC activity prevents expression of the morphine tolerance. | *Granados-Soto et al., 2000* |
| Mice | Acute peripheral tolerance | Calphostin C Go-6976 | Intraplantar injection | Completely reversed morphine tolerance. | *Inoue and Ueda, 2000* |
| Mice | Acute peripheral tolerance | Protein kinase A inhibitor KT-5720 | Intraplantar injection | No effect on morphine tolerance. | *Inoue and Ueda, 2000* |
| Mice | Implantation of 75mg morphine pellets | PKC inhibitor: drug: Go-7874 sangivamycin | Intracerebrovent-ricular injections | Significantly reversed morphine tolerance. | *Smith et al., 2002* |
| Mice | Subcutaneous injection of morphine (100 mg/kg) | Calcium/calmodulin dependent protein kinase II (CaMKII) antagonist: Trifluoperazine | Intrapitonial injection | Significantly attenuated the development of antinociceptive tolerance. | *Tang et al., 2006* |

*Desensitisation*: Desensitisation and tolerance have a similar definitions, they both include the notion of a decreased response following continued or intermittent agonist treatment (Allouche et al., 2014). Nonetheless, it is necessary to mechanistically distinguish between these two terms. Desensitisation typically refers to molecular changes at the level of receptor signalling and can be homologous or heterologous (Williams et al., 2013). In homologous desensitisation the reduction in the effect is restricted to agonists acting at a specific receptor, while in heterologous desensitisation the effects of agonists acting through different receptors share a component of the signalling cascade (Williams et al., 2013). It has been reported that desensitisation can be employed only to describe acute loss of μ-effector coupling that occurs within minutes after initiation of exposure to opioid agonists (Williams et al., 2013). In many systems, reduced responsiveness to agonists has corresponded with the desensitisation of GPCRs (Guang et al., 2004). *In vitro* research evidence indicates that

this process (desensitisation) involves phosphorylation of GPCRs and subsequent binding of regulatory proteins termed β-arrestins which uncouple the receptor from G proteins (Tobin, 2008). This uncoupling event desensitises in the receptor result in attenuating the second messenger signal cascade, which reducing agonist efficacy (Raehal and Bohn, 2014). The role of β-arrestin in receptor desensitisation was characterized in β-arrestin knockout mice that displayed an enhancement of opioid analgesia with a lack of antinociceptive tolerance (Connor et al., 2015; Raehal et al., 2005).

*Internalisation*: this is the process that modulates the number and functional activity of opioid receptors (Keith et al., 1998). Internalisation is the rapid process of the receptor that occurs within several minutes after μ-opioid receptor or δ- receptor activation (Keith et al., 1998). Receptor phosphorylation is the first step in the internalisation and desensitisation of the μ-opioid receptor (Keith et al., 1998).

*Endocytosis*: this is the mechanism by which specific molecules are ingested into the cell. Following activation, most receptors undergo regulation by a cascade of events that induce receptor desensitisation and endocytosis (Koenig and Edwardson, 1997). Following endocytosis, most G protein coupled receptors are recycled to the plasma membrane and retained in an intracellular compartment, or targeted for degradation (Martini et al., 2007). Endocytosis is the first step for re-sensitising the μ-opioid receptor (Martini and Whistler, 2007). Thus, the desensitisation–endocytosis–resensitisation cycle serves as a fast and dynamic means to titrate signalling *via* the receptor (Martini and Whistler, 2007). However, not all agonist ligands at the μ-opioid receptor induce the same degree of receptor endocytosis and desensitisation (Martini and Whistler, 2007). For example, the endogenous peptide ligands at the μ-opioid receptor promote rapid desensitisation, endocytosis and recycling (Martini and Whistler, 2007). By contrast, morphine induces a weak or partial desensitisation and practically no endocytosis (Johnson et al., 2006).

The imbalance of the desensitisation, endocytosis and resensitisation cycle leads to several cellular adaptations of G-protein coupling receptor (Martini and Whistler, 2007). Recently, it has been reported that μ-opioid receptor initiated autophagy in hippocampal neurons due to prolonged morphine exposure (Zhao et al., 2010). Nevertheless, the role of autophagy in morphine tolerance remains unclear.

*G-protein uncoupling*: All four opioid receptors subtypes (μ, κ, δ and ORL1) are seven-transmembrane spanning proteins that couple to inhibitory G-proteins (George et al., 2000). Several protein kinases have been reported as mediators of phosphorylation at specific sites of opioid receptors, such as G-protein receptor kinase (GRK), calcium/calmodulin-

dependent protein kinase II (CaM kinase II), protein kinase A (PKA) and protein kinase C (PKC) (Borgland, 2001). When agonists like fentanyl or morphine bind to opioid receptors, a conformational change in the G-protein receptor occurs (Borgland, 2001). This conformational change causes G-protein activation, as the free G-protein βγ-subunits facilitate the translocation of the mediator (GRK) to the plasma membrane which then phosphorylate the sites of opioid receptors (Borgland, 2001). For example, the cellular protein β-arrestin has a high affinity for the phosphorylated opioid receptor. It moves from the cytoplasm to the plasma membrane and then binds to the opioid receptor, resulting in a phosphorylated receptor-arrestin complex (Raehal et al., 2005; Raehal et al., 2014). This complex disrupts G-protein binding and renders the receptor to transduce any further signal (Raehal et al., 2005; Raehal et al., 2014). Likewise, it has been shown that (PKA) and (PKC) protein levels increase during protracted opioid administration resulting in phosphorylation of G-protein-coupled receptors that may be involved in receptor desensitisation (Smith et al., 2003; Dalton et al., 2005).

Currently, the coupling of opioid receptors to the mitogen-activated protein kinase (MAPK) signalling pathway has been characterised (Duraffourd et al., 2014). Following the activation of G proteins, three pathways have been proposed to converge on the MAPK signalling, which results in mediation of the nuclear signalling pathway. Firstly, phosphorylation and internalisation of G-protein receptors can stimulate the MAPK signalling pathway. Secondly, activation of phosphatidylinositol-3 kinase (PI3K) *via* G-protein βγ-subunits which have been released from the activated receptor, led to a sequence of phosphorylation steps, which activate MAPK. Finally, MAPK can also be activated by (PKA) in the CNS. The activation of MAPK pathways may not be necessary for short term opioid exposure, but possibly plays an essential role during chronic opioid administration, as it is influenced by the upregulation of cAMP. Once MAPK signalling is activated, it can phosphorylate several targets in the cytoplasm, or it can be translocated into the nucleus to influence gene regulation of different transcription factors.

B**.** *μ-opioid receptor polymorphisms*

Opioid pharmacogenetics produces analgesic variability within users in the population (LaForge et al., 2000). In humans, there are more than one hundred identified single nucleotide polymorphisms (SNPs) in relation to the μ-opioid receptor. It has been reported that some of these nucleotides are thought to play an essential part in opioid sensitivity, abuse and tolerance (Kreek et al., 2005). The most well-known of these SNPs is the A118G

nucleotide substitution, which changes the amino acid sequence at position 40 from asparagine to aspartic acid (Bond et al., 1998). Clinically, this mutant reduces the analgesic effects of the binding affinity of morphine and M6G (Romberg et al., 2005) and leads to changes in receptor signalling (Beyer et al., 2004). These changes are not likely to be a consequence of altered binding affinity, because *in vitro* studies reported that the N40D substitution does not affect the binding of the endogenous opioids nor that of the exogenous opioids morphine, methadone or fentanyl (Bond et al., 1998).

C. *NMDA receptor contributions to opioid tolerance*

N-Methyl-D-aspartate (NMDA) are glutamate-gated cation channels with high $Ca^{2+}$ ion permeability that play a crucial role in higher organisms in different biological aspects (Parsons et al., 1998). Glutamate is one of the main excitatory neurotransmitters in the CNS and exerts its effects by binding to specific classes of receptors; specifically ionotropic and metabotropic receptors (Parsons et al., 1998). Ionotropic receptors (iGluRs) are voltage sensitive and couple to ion channels, which include NMDA, kainic acid and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Zhu and Barr, 2004; Niciu et al., 2012). Ionotropic receptors are fast acting and, once opened, can generate large changes in current flow, even if the voltage difference across the membrane is weak (Kam et al., 2010). The metabotropic glutamate receptors (mGluRs) are coupled to G proteins and slow acting, they exert their effects indirectly, typically by protein synthesis and gene expression (Kam et al., 2010). Those effects often improve the excitability of glutamate cells and regulate neurotransmission in neurons, which may be implicated in synaptic plasticity (Niciu et al., 2012).

NMDA receptors are essential for the development of the CNS, controlling the patterns of breathing, locomotion and the processes underlying synaptic plasticity and memory function (Blanke and Van Dongen, 2008). Thus, abnormal expression levels of glutamate will alter the function of NMDA receptors which has been associated with several neurological disorders and diseases (Blanke and Van Dongen, 2008).

Glutamate receptors are distributed throughout the brain and spinal cord particularly in neurons and glia cells (Niciu et al., 2012). When the presynaptic cells release glutamate into the synaptic cleft, glutamate binds to the postsynaptic AMPA causing the channels to open. Consequently, $Na^{+}$ flows into the postsynaptic cell resulting in depolarisation (Herman et al., 1995; Takumi et al., 1999). The AMPA receptors typically work in conjunction with NMDA receptors to produce the necessary depolarization to remove the $Mg^{2+}$ block into the

extracellular space resulting in $Ca^{2+}$ influx. Under normal physiological conditions, $Ca^{2+}$ influx allows for synaptic plasticity while, overactive NMDA receptors (excessive $Ca^{2+}$ influx) cause neurodegenerative diseases in addition to the death of the neuronal cell (Herman et al., 1995; Takumi et al., 1999; Mao, 1999). The role of glutamate in the development and maintenance of opioid tolerance has been well established (Inturrisi, 1997). Certain researchers have suggested that the interconnected relationship between the μ-opioid receptor and NMDA receptors is due to co-localization in central tissues (Gracy et al., 1997; Ko et al., 2008; Rodríguez-Muñoz et al., 2012), as prolonged morphine exposure causes enhancement of the function of NMDA receptors (Wang et al., 2015). In addition, NMDA receptors co-localize with group I mGlu receptors (mGluR1 and mGluR5 subtypes) on neurons (Fundytus et al., 2001). This colocalization results in an interaction between receptors which in turn activates PKC (Fundytus et al., 2001). Activated PKC has been known to phosphorylate opioid receptors and induce desensitization, as mentioned before. Thus, group I mGluRs antagonist has been showed to attenuate phosphorylation and desensitization of opioid receptors which potentially increase the efficacy of opioid analgesics (Osikowicz et al., 2008; Huang et al., 2019). Furthermore, it has been documented that Toll-like Receptor 4 mediates morphine tolerance *via* tumour necrosis factor (TNF) signalling and facilitates glutamatergic signalling (Eidson et al., 2017).

The role of AMPA and kainate receptors in opioid tolerance and psychological dependence appears to be more controversial. Various researchers have documented that treatment with AMPA/kainate antagonists, either directly or systemically into the central nervous system, improved opioid efficacy (Rasmussen et al., 1996; Watanabe et al., 2002; Akgün et al., 2018). However, other investigators have shown that systemic administration of AMPA/kainate antagonists reduced the development of tolerance and acute dependence, while there was no effect on the development of chronic dependence (McLemore et al., 1997). Additionally, systemic administration of a selective AMPA antagonist has been revealed to reverse the development of tolerance in morphine tolerant mice, although it failed to affect the development of antinociceptive tolerance to selective delta- or kappa-opioid receptor agonists (Kest et al., 1997).

In addition, there are other neuroadaptations due to chronic opioid exposure, including an increase in the $Ca^{2+}$/cAMP response element binding protein (CREB) (Bilecki and Przewlocki, 2000), upregulation of the cAMP pathway (Bilecki and Przewlocki, 2000), and increase of Fos-related antigens (Shoda et al., 2001). However, the mechanism behind the

connection between upregulation of these cellular elements and opioid tolerance remains unclear.

Overall, several mechanisms are recommended for opioid tolerance in the literature. Recently increasing evidence suggests that opioid-mediated effects are controlled by the mammalian target rapamycin complex 1 (mTORC1), a kinase that is involved in the control of protein synthesis and regulation of nociceptor sensitivity (Xu et al., 2014; Zhang et al., 2019). This will be discussed in more detail in the subsequent section of the introduction.

**3. Learned tolerance:** this is the last class of acquired tolerance and it is attributed to learning. Learned tolerance refers to a reduction in the effects of a drug as a result of compensatory mechanisms that are learned either behaviourally or conditioned (Collett, 1998). In behavioural tolerance, an individual learns to function despite repeated exposure to a drug. A typical example was reported among chronic alcohol abusers when they learned to walk in a straight line, despite their motor functions being in deficit because of alcohol intoxication (Dumas& Pollack., 2008). Being able to perform this task involves awareness in learning about their impairment and the acquisition of motor skills (Dumas & Pollack., 2008). Conditioned tolerance (situation-specific tolerance) is a particular case of behavioural tolerance (Collett, 1998). It is a prototypical learning design developed by Pavlov (1927), who suggested that conditioned tolerance develops when environmental cues are consistently paired with administration of the drug. Removal of these environmental cues will result in enhancement and improvement in pharmacological drug effect (Siegel, 1978). For example, when morphine-tolerant rats are placed in unusual circumstances and treated with morphine, antinociceptive tolerance is reduced and the drug's effect enhanced (Collett, 1998).

#### 1.2.6.4 Opioid-induced hyperalgesia pharmacological phenomena

Opioid-induced hyperalgesia is a phenomenon observed in patients treated with opioids. Those patients paradoxically demonstrate increased sensitivity to painful stimuli (Dumont et al., 2007). Figure 1.7 presents an idealised dose-response curve for an administered drug. As the drug dose increases, the observed effect of the drug increases. However, an OIH increase in opioids dose, shifts the curve to the left. Thus, chronic opioid exposure may produce two interrelated outcomes. Firstly, the desensitisation process which leads to the reduced clinical efficacy of opioids, and secondly, a sensitisation process which can facilitate nociception so that the opioid's analgesic effect is counteracted (Brunton, 2014).

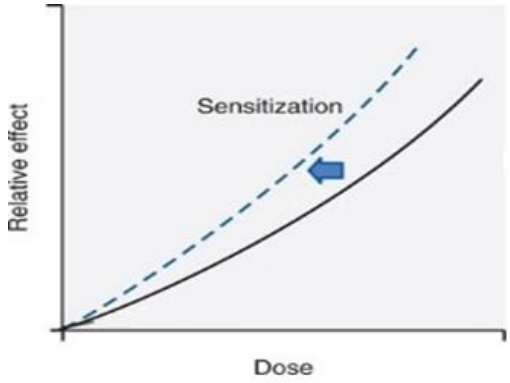


**Figure 1.7: Opioid-induced hyperalgesia shifts the dose-response curve.** Upon chronic use of opioid, the curve shifts to the left to facilitate nociception, counteracting the opioid's analgesic effect resulting in increased intensity of normally painful stimulus (adapted from Brunton, 2014).

Opioid-induced hyperalgesia can clinically demonstrate both hyperalgesia (increased response to painful stimuli) and allodynia (painful response to normally non-painful stimuli), together or separately. Patients typically described the pain in an anatomically distinct region, and moreover, it is a different quality than the original pain position (DuPen et al., 2007). The critical dilemma for clinicians making a diagnosis is to differentiate OIH from other phenomena, such as opioid tolerance, opioid withdrawal, opioid addiction, physical dependence, disease progression or pseudo addiction (Velayudhan et al., 2013). Table 1.4 presents an overview of opioid tolerance and OIH to assist in distinguishing these conditions.

**Table1.4: Differential diagnosis for OUD** (Silverman, 2009; Tordoff and Ganty, 2010).

| Condition | Clinical features | Onset | Response to opioid administration |
|---|---|---|---|
| **Opioid tolerance** | Characterised by persistent pain and is in distinct region (localized). Tolerance may appear due to therapeutic or side-effects. | Gradual | Pain improves |
| **Opioid-induced hyperalgesia (OIH)** | Characterised by an anomalous increase in pain associated with hyperalgesia and allodynia. Pain may be observed at a different location and can be distributed. It is usually poorly defined regarding quality and region. | Gradual or Abrupt | Excessive pain or pain worsens |
| **Opioid withdrawal** | Characterised in the acute phase (early symptoms) by watery eyes, sweating, poor sleep and Flu-like symptoms laterally associated with by adrenergic symptoms, such as an increase in heart rate (tachycardia) and blood pressure (hypertension) with abdominal pain (cramping) and diarrhoea also be observed. Pain sensitivity increases and can be widespread beyond that of the pre-existing position pain. | Abrupt | Pain improves |
| **Opioid addiction** | Characterised by behaviour that includes impaired control (over drug use) and compulsive strong desire to use opioids despite harm and craving. Pain may or may not be present. | Gradual | Pain may improve, but aberrant behaviour may worsen |
| **Physical dependence** | Characterised by a state of adaptation with prolonged opioid use, which results in tolerance and even physical withdrawal symptoms upon cessation of opioid or decrease in dosage. Pain presents at the original site. | Gradual | Pain improves |
| **Disease progression** | The pain gradually worsens even being on opioids. Pain may occur in other areas than the original site. | Gradual | Pain improves |
| **Pseudo addiction** | Characteristically occurs when pain is under-treatment, resulting in patients seeking opioids for relief of pain. It is usually mistaken for addiction. Pain presents at the original site. | Variable | Pain improves |

The phenomenon of OIH has been recognised for more than 100 years (Kayan et al., 1971). Studies of OIH in humans has been performed in different settings: in volunteers during short-term opioid infusions, in patients with chronic pain and in patients during perioperative exposure to opioids (Marion Lee et al., 2011). The exact mechanism of this paradoxical response is poorly defined, but it is possible that it is multifactorial. OIH is generally thought to result from neuroplastic changes in the CNS and PNS, which have induced sensitisation of the pronociceptive pathways (Brunton, 2014). There are many proposed mechanisms for OIH, such as involvement of the central glutaminergic system, spinal dynorphins, descending facilitation, decreased reuptake and enhanced nociceptive response, while genetic mechanisms have been described as significant mechanisms (Gardell et al., 2002; Liang et al., 2006; Colvin and Fallon, 2010). Of these mechanisms, the central glutaminergic system is considered the most common possibility for OIH (Roeckel et al., 2016). Furthermore, peripheral mechanisms may be implicated in the development of OIH which includes activation of serotonin receptors (5HT2 and 5HT3) in certain chronic pain states, by means of changing the balance from descending inhibitory control towards pro-

nociception. For example in animal models, ondansetron; a 5HT3 antagonist, can block the signs of OIH (Liang et al., 2011). In addition, alteration of cytokine production, activation of substance P and changes in calcium channels and nitric oxide synthesis, all have been involved in the development of OIH in various studies (Colvin and Fallon, 2010).

#### 1.2.6.5 Opioid tolerance and hyperalgesia: two sides of the same coin?

Clinicians have observed signs of tolerance in their patients for many years; however, the underlying molecular mechanisms remain poorly understood. Additionally, in the past decade, the phenomenon of OIH has been reported in animal models as well as in case descriptions (Hayhurst and Durieux, 2016). The difference between OIH and opioid tolerance is conceptually easy to understand, though both are clinically difficult to separate (Hayhurst and Durieux, 2016). There is a significant issue in relation to knowledge differentiating both conditions by clinicians, which may contribute to harm and discomfort in patients when they are not diagnosed appropriately (Hayhurst and Durieux, 2016). Although both phenomena, OIH and tolerance cause increased pain, with the usual consequence of escalating doses of opioids, they are nevertheless caused by two distinct mechanisms at the cellular level (Chu et al., 2008). Whether the increased opioid administration is caused by reducing the pain threshold, as in OIH, or by lowering the potency of the drug, as in tolerance, the clinical effect is the same. Both appear to have a dose-response relationship and as such, the consequence of OIH or tolerance in the setting of high-dose opiates is increased (Hayhurst and Durieux, 2016).

Hypothesised mechanisms that have been used to elicit OIH and tolerance are demonstrated in various animal behavioural research. By measuring the anti-nociceptive effectiveness of an opioid treatment versus baseline pain sensitivity over a period of time. For example, arrestin and G protein receptor kinase are recruited to μ-opioid receptor, leading to internalisation of receptors resulting in analgesic tolerance (Dumas and Pollack, 2008). Activation of the extracellular signal-regulated kinase (ERK) pathway will reduce pain thresholds and hence, induces hyperalgesia (Sanna et al., 2015). There is considerable evidence suggesting the common cellular mechanisms of OIH and tolerance. For instance, both conditions are associated with changes in NMDARs (Price et al., 2000; Chaplan et al., 1997). It is reasonable to expect that these findings might extrapolate to humans. From these similarities in cellular mechanisms, researchers suggest the use of various targeted therapies that can alleviate or reverse both phenomena, which have been revealed to be effective based on pre-clinical and clinical studies (Mao et al., 1995; Mao, 2002).

### 1.2.6.6 The neurobiology of opioid dependence and addiction

Opioid dependence, addiction and tolerance are manifestations of brain abnormalities resulting from long-term exposure to opioids, such as morphine, oxycodone, heroin and others of morphine-derived drugs. All of these compounds are the underlying causes of opioid addiction (intense drug craving and compulsive use) and dependence (the need to keep taking drugs to prevent withdrawal syndrome) (Bond et al., 1998; Christie, 2008).
The abnormalities that create dependence are well known scientifically. Several mechanisms have been proposed to explain opioid dependence include upregulation of cAMP levels beyond the control values and induction of PKA. Also chronic opioid exposure caused activation of PKC which can attenuate opioid receptor activity and affect ion conductance and changes in endogenous ligands which further leads to opioid dependence and withdrawal. Moreover, transitory change of glutamate (NMDA) receptor (Bond et al., 1998; Kosten and George, 2002; Christie, 2008). However, the effect of different environmental conditions on the responsiveness of a human to drug abuse and addiction, is still not fully understood.

Signs and symptoms of opioid deprndence appears to resolve within days or weeks of stopping or reducing the opioids such as irritability, severe body ache, diarrhoea, sleeping problems, high blood pressure, fever, anxiety and rapid mood changes (Kosten and George, 2002). It should be mentioned that defects in the brain, which induced addiction, are complex and long-lasting (Kosten and George, 2002). These abnormalities may involve or interact with environmental effects, such as the social context of initial opiate use and psychological conditioning. It was recently reported that environmental conditions play a significant role in rodents sensitivity to drug addiction (Holmes et al., 2005). Furthermore, the enriched environment can promote long-term modification in neural functions and might attenuate the occurrence of pathogenic behaviours (Xu et al., 2007).

When an opioid such as oxycodone or heroin travels through the circulation to the brain, the chemicals bind to μ-opioid receptors. This binding of these compounds with these receptors, targeted similar biochemical brain processes that reward humans and animals with feelings of pleasure (Le Merrer et al., 2009). The pleasurable effect with opioid use is defined as a 'liking' reaction to reward, whether or not it is explicitly felt (Le Merrer et al., 2009). Clinically opioids are prescribed for patients to alleviate or eliminate painful sensations. However, in the absence of significant pain, opioids can activate the reward pathway, which

motivate people to repeat the use of the drug simply for the feeling of pleasure (Le Merrer et al., 2009).

One of the brain circuits that is stimulated by opioids, is the mesolimbic (midbrain) reward system (Kosten and George, 2002). This system produces signals in a part of the brain called the ventral tegmental area (VTA), that result in liberating the neurotransmitter dopamine (DA) in an alternative another part of the brain; the nucleus accumbens (NAc) (Kosten and George, 2002). This release of DA into the NAc, creates feelings of pleasure (Kosten and George, 2002). In the brain, DA has several distinct pathways other than involved in reward-motivated behaviour (Luo and Huang, 2016). DA plays a significant role in motor control, which is accomplished by complex interactions between different groups of nerve cells in the CNS, that are located in the substantia nigra in the midbrain (Luo and Huang, 2016). Neurons of the substantia nigra communicate with neurons of the basal ganglia by releasing DA (Gerfen and Wilson, 1996). DA is involved in the development of numerous movement disorders and psychiatric disorders, such as Parkinson's disease, Huntington's disease, depression, schizophrenia, anxiety disorders, obsessive-compulsive disorder and attention deficit hyperactivity disorder (ADHD) (Graybiel et al., 1994). Both depression and anxiety have been linked to inactive DA receptors in the nucleus accumbens (an area within the basal ganglia that is associated with pleasure and reward) (Hayden et al., 2010). Likewise, researchers have ascertained that overactive DA pathways are involved in several neuropsychiatric disorders, including schizophrenia and drug addiction (Kesby et al., 2018).

### 1.3 The mammalian target of rapamycin (mTOR)

Target of rapamycin (TOR) is a highly conserved serine-threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKKs) family (Feng et al., 2005). It is a central signal integrator, that is responsible for regulating numerous major cellular processes, including; responding to signals arising from nutrients, growth factors and cellular energy (Feng et al., 2005). The activity of TOR ranging from worms to mammals has been recognised in a wide range of organisms (Wullschleger et al., 2006). Rapamycin (Sirolimus), is a macrolide used as an immunosuppressive and anti-proliferative drug (Martel et al., 1977). The genetic screens in *Saccharomyces cerevisiae* yeast identified TOR1 and TOR2 as mediators of the toxic effects of rapamycin on yeast in the early 1990s (Cafferkey et al., 1993; Kunz et al., 1993; Sabatini, 2017). The mammalian Target of Rapamycin (mTOR) is also documented as the mechanistic target of rapamycin and FK506-

binding protein, in humans it is encoded by the mTOR gene (Loewith and Hall, 2011; Laplante and Sabatini, 2012). In mammals, mTOR is a signalling kinase for every aspect of cellular life (Showkat et al., 2014). It plays a vital role in controlling most of the physiological processes that use or generate large amounts of energy like growth (mass accumulation) and cell proliferation in response to different environmental cues (Wullschleger et al., 2006). Nevertheless, mTOR deregulation is implicated in several pathological conditions like cancer (Pópulo et al., 2012), obesity (Cota et al., 2008) diabetes (Leibowitz et al., 2008) and neurodegeneration diseases (Francois et al., 2016). At the molecular biology level, the mTOR pathway is regulated by different kinase cascades including PI3K, PI3K-dependent kinase 1 and Akt. Likewise, the mTOR pathway is regulated by mediators of this cascade, consisting of PTEN and tuberous sclerosis (TSC) 1 and 2 (Feng et al., 2005; Wullschleger et al., 2006).

PTEN, TSC1, TSC2 and liver kinase B1 (LKB1), have been identified as negative regulators of mTOR (Feng et al., 2005; Jozwiak et al., 2005), alternately, Akt and PI3K are positive regulators (Hahn-Windgassen et al., 2005). Figure 1.8 illustrates mechanisms generating mTOR activation.

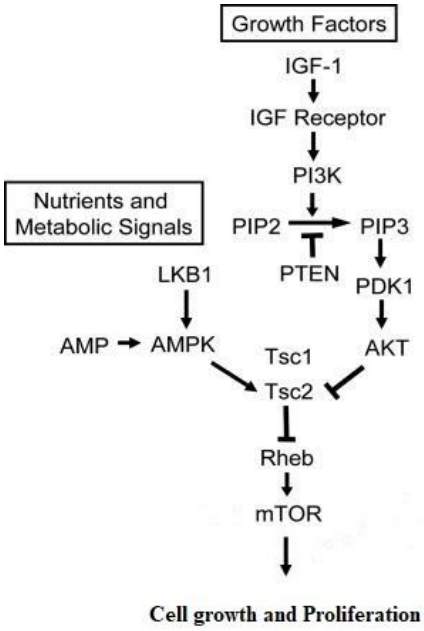


**Figure 1.8: Simplified diagram illustrating the signal transduction generating mTOR activation.** The heterodimer TSC1/2 transmits several upstream signals that trigger mTOR activation in response to many intracellular and extracellular cues. In the absence of amino acids and growth factors, the TSC1/TSC2 complex continues to inhibit Rheb, therefore, mTOR remains inactive. Insulin and insulin-like growth factor 1 (IGF1) induces phosphorylation of the TSC1/TSC2 complex through PI3K and Ras pathways, then mTOR can interact with GTP bound Rheb and become active. Moreover, in response to hypoxia or a low energy state, Adenosine monophosphate-activated protein kinase (AMPK) phosphorylates TSC2 increases its GAP activity towards Rheb, subsequently mTOR is inactivated. In turn, activation of the mTOR pathway generates cell growth and proliferation (adapted from Feng et al., 2005).

Biochemical and genetic analysis has revealed that mTOR is present in functionally multiprotein distinct complexes within the cell, known as mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2) (Zhou and Huang, 2010; Showkat et al., 2014). Both mTOR complexes are large and have different sensitivity to rapamycin upstream inputs and downstream outputs (Hay and Sonenberg, 2004). Figure 1.9 below illustrates different internal and external cues that activate mTORC1 and mTORC2.

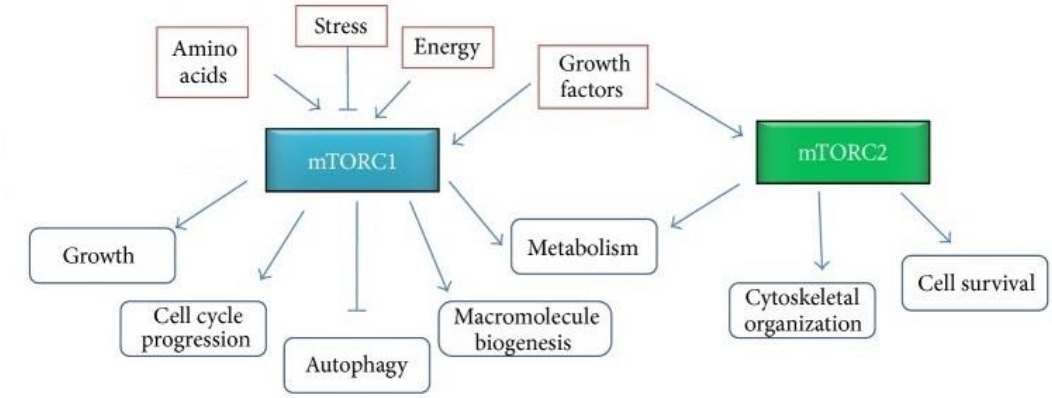


**Figure 1.9: mTORC1 and mTORC2 complexes, different interaction partners and cellular functions.** The mTORC1 integrated its activity from many environmental inputs comes from amino acids, growth factors, cellular energy status and stress. Thus, it plays a significant role in generating cell growth and proliferation *via* stimulating anabolic processes and inhibiting catabolic processes like autophagy. mTORC2 is regulated by a poorly identified mechanism *via* growth factors and, mTORC2 controls cell migration, survival and metabolism (adapted from Showkat et al., 2014).

Both complexes are formed by a catalytic mTOR subunit, as well as other unique shared components, such as deptor (mTOR indictor), mammalian lethal with sec-13 (mLST8, also known as GβL) and other scaffold proteins, that regulate the assembly and stability of each complex (Saleiro and Platanias, 2015; Foster and Fingar, 2010). Specifically, mTORC1 consists of six known proteins; mTOR, mlT8, raptor, pras40, deptor and Tti1/Tel2 complex (Grahammer et al., 2014). Moreover, the presence of the regulatory-associated protein of mammalian target of rapamycin (raptor) and proline-rich Akt substrate 40kDa (pras40) makes mTORC1 sensitive to rapamycin (Hay and Sonenberg, 2004).

In contrast, mTORC2 is insensitive to acute rapamycin treatment (Carayol et al., 2010), but with chronic exposure, it can disrupt its structure in specific cell types and tissues (Zhou and Huang, 2010). The action of rapamycin against mTORC1 or mTORC2 forms a gain-of-function complex with the 12-kDa FK506-binding protein (FKBPs) (Abraham and Wiederrecht, 1996). Rapamycin binding to either FKBP12 or FKBP51 forms a drug – receptor complex that can then act to inhibit mTORC1 and effectively inhibit its kinase activity (Abraham and Wiederrecht, 1996). Nonetheless, inhibition of mTORC2 activity by

rapamycin, is dependent upon a rapamycin-FKBP12 complex, that can prevent the formation of mTORC2 by binding to free mTOR (Zhou and Huang, 2010; Li et al., 2014). Figure 1.10 illustrates both mTOR complexes; mTORC1 and mTORC2, as well as the various signals that regulate them, including the action of Rapamycin on each.

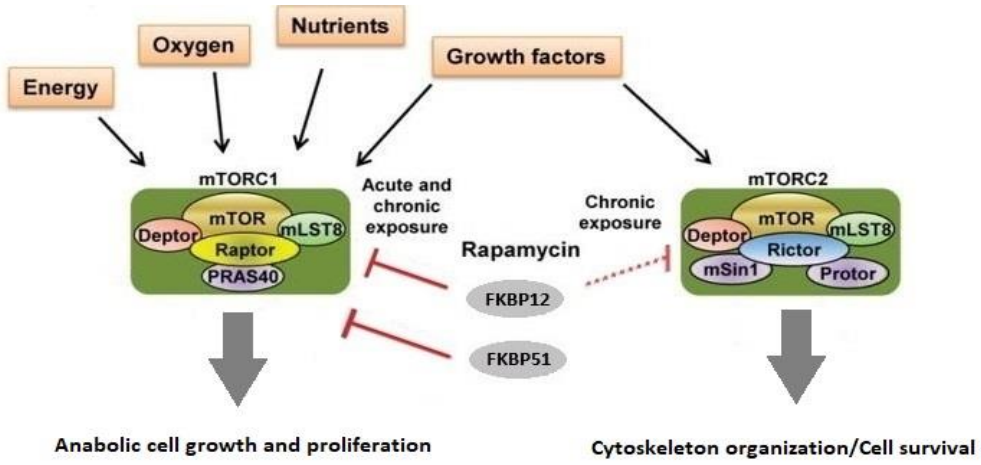


**Figure 1.10: mTOR complexes and the regulation of key cellular processes.** mTORC1 promotes cell growth and proliferation in response to different signals which comes from growth factors, oxygen, energy levels and nutrients. Whereas mTORC2 only responds to growth factors, in turn it regulates actin/cytoskeleton organisation and cell survival. Short-term exposure of rapamycin inhibits mTORC1, while chronic rapamycin can inhibit mTORC2 (adapted from Li et al., 2014).

### 1.3.1 The mammalian target of rapamycin complex 1 (mTORC1)

Despite it being 25 years since mTOR was discovered, the complexity of mTOR signalling is just beginning to be understood (Sabatini, 2017). By identifying the valuable role of mTOR in regulating the growth of organisms, cell survival and coordinating it with the availability of nutrients (Sabatini, 2017) and by recognising the catalytic subunit of mTOR (mTORC1 and mTORC2), the mTOR pathway becomes less elusory (Sabatini, 2017). It is well established that mTORC1 is the master regulator of host amino acid metabolism for cell growth and cell size (Grahammer et al., 2014; Li et al., 2014; Yang et al., 2014). It is predominantly responsible for regulating protein translation factors (Yang et al., 2014). When mTORC1 is activated, this leads to an increase in the phosphorylation levels of its downstream effectors, which in turn, is responsible for regulating multiple cellular processes (Lutz et al., 2015; Yang et al., 2014).

Accumulating evidence indicates that mTORC1 can be activated by a variety of upstream signals (Laplante and Sabatini, 2012; Dibble and Manning, 2013). The mTORC1 pathway integrates inputs from several extracellular and intracellular cues as described earlier (growth factors, energy status, amino acids stress and oxygen) (Laplante and Sabatini, 2012). These factors activate mTORC1 by way of different signalling pathways. For

example, growth factors such as insulin and insulin-like growth factor 1 (IGF1) bind to specific receptor tyrosine kinases, which lead to activation of the PI3K-Akt pathway. Akt activation results in phosphorylation of TSC-1/TSC-2 (suppresses TSC action) leading to the activation of mTORC1 (Sarbassov et al., 2004). Figure 1.11 displays a simple schematic depiction believed to be involved in the cell growth including PI3K-Akt, TSC-1/TSC-2 regulatory complex.

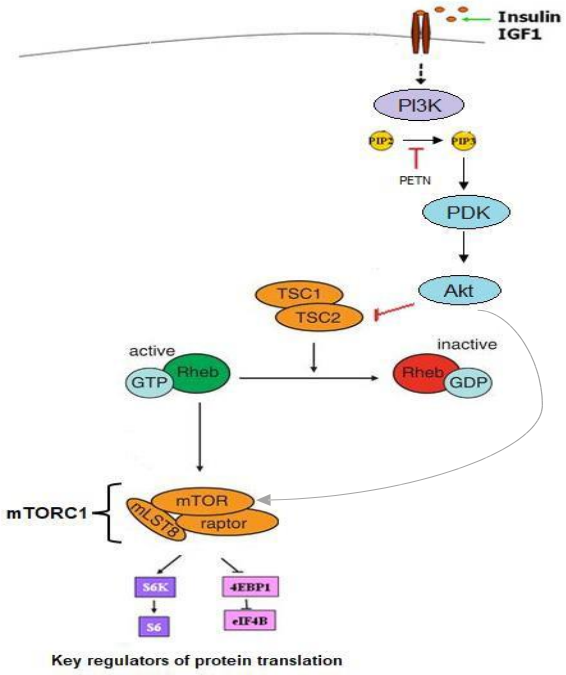


**Figure 1.11: Signal transduction pathways leading to mTORC1 activation.** Through a multiple-step process, growth factors stimulate the PI3K/Akt/mTOR pathway. **(1)** PI3K is responsible for the production of phosphatidylinositol (3, 4, 5)-triphosphate (PIP3) *via* phosphorylation of the biphosphate PIP2. **PTEN a (lipid phosphatase)** acts in opposition to PI3K. In response to growth factors e.g (insulin) PI3K stimulate and deplete the PTEN effect; thus, PIP3 binds to phosphoinositide-dependent protein kinase (PDK). PDK activates Akt, when Akt activates phosphorylate TSC2. (2) TSC acts as a GTPase-activating protein (GAP) for the small GTPase RAS homologue enriched in brain (Rheb), usually TSC1 and TSC2 are dimerized complex binds (Rheb). When TSC2 is phosphorylated, the GTPase Rheb is maintained in its GTP-bound state. In this case, GTP-bound Rheb activates mTOR. Active mTOR bound to Raptor protein forms mTORC1. Moreover, Akt can activate mTORC1 directly by phosphorylation at S2448 site (adapted from Öst et al., 2010 and van Veelen et al., 2011).

Furthermore, in response to hypoxia or a low energy state, adenosine monophosphate-activated protein kinase (AMPK), communicates directly with mTORC1 by phosphorylating TSC2 and increasing its GAP activity, in relation to Rheb (Laplante and Sabatini, 2012; Dibble and Manning, 2013). This will be discussed in detail later. In contrast, amino acids appear to regulate mTORC1, independent to the TSC-Rheb- pathway. Research suggests that leucine and arginine are the most well-known stimulators implicated in mTORC1 activation *via* a novel family of GTPases, termed Rags (Dibble and Manning, 2013). The Rag GTPases induced translocation of mTORC1 to the lysosomal surface. Subsequently,

mTORC1 comes into contact with Rheb resulting in activation of this kinase (Sancak et al., 2008). The activated complex produces increased protein synthesis and cell proliferation.

In general, proteins do most of the work inside the cells, as they are required for the function, structure and regulation of the body's tissues and organs (Lodish et al., 1995). Protein synthesis is one of the most fundamental biological processes by which individual cells create their specific proteins (Davis and Squire, 1984). Additionally, protein synthesis has a defining role in forming the proteome (complete set of proteins) and promotes cell growth (Conn and Qian, 2011; Breuza et al., 2016). However, failure in this process can lead to the formation of toxic aggregation or inactivation of functional proteins, leading to cell death, which is linked to specific neurodegenerative conditions (Ross and Poirier, 2004; Conn and Qian, 2011).

Basically, protein synthesis comprises two steps: transcription and translation (Davis and Squire, 1984). Most studies have highlighted that mTORC1 signalling controls the critical regulators of the translation step: ribosomal protein S6 kinase (also known as p70S6 kinase) (Yang et al., 2014) and eukaryotic translation initiation factor 4G (eIF4G), eIF4B and 4Ebinding protein 1/2 (4EBP1/2) (Yang et al., 2014). mTORC1 activation (Phosphorylation) inhibits 4EBP and activates p70S6K, both of which increased the level of mRNA translation (Yang et al., 2014).

The process of translation is the addition of one amino acid at a time to the end of the polypeptide that is being formed, which proceeds in three different phases; specifically initiation elongation and termination (Pain, 1996). This process takes place inside the ribosome (Pain, 1996). A ribosome is a complex molecular machine that consists of two subunits, a small 40S subunit and a large 60S subunit (Pain, 1996). Most mature eukaryotic mRNAs have a 7-methyl-guanine cap structure at the 5’-end that controls initiation of the translation (Shives et al., 2016). Normally, eIF4E remains bound to 4EBP1/2, which prevents the formation of the eIF4G initiation complex (Pain, 1996; Shives et al., 2016).

During the translation initiation step, the cap structure is recognised by the eIF4G initiation complex, which includes the eIF4E protein. Active mTORC1, phosphorylate 4EBP1/2, which produces changes in its shape and it releases its binding from eIF4E, allowing the formation of the functional eIF4G initiation complex, to initiate cap-dependent translation (Shives et al., 2016). Additionally, eIF4B needs to be phosphorylated by p70S6K 1/2 to

associate with the translation initiation complex, whilst mTORC1 activation also induces phosphorylation of p70S6K1, thereby activating the protein translation process. Importantly, recruitment of eIF4B in relation to the initiation complex, is mTOR-dependent (Shives et al., 2016). Similarly, phosphorylation of p70S6K 1 by mTORC1 stimulates the translation rate of mRNAs, containing the 5'-oligopyrimidine tract, which is involved in the translation process, like elongation factors (Yang et al., 2014; Lutz et al., 2015; Shives et al., 2016). Thus, mTORC1 activation increases the translation capacity of the cell, p70S6K controls cell size, whereas 4EBP controls cell proliferation (Yang et al., 2014). Figure 1.12 illustrates the mTORC1 signalling pathway and translation initiation.

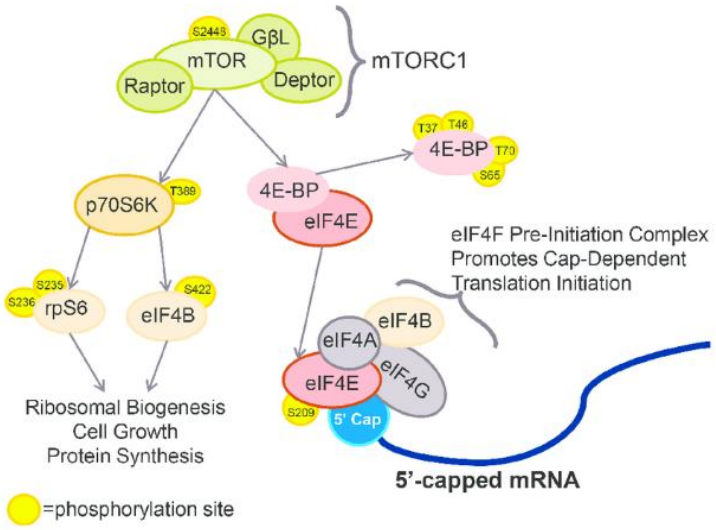


**Figure 1.12: The mammalian target of rapamycin complex 1 (mTORC1) pathway and translation initiation.** Activated mTORC1 phosphorylates its downstream effectors: **(1)70 kDa ribosomal protein S6** kinase (p70S6K) and (2) eukaryotic initiation factor 4E binding protein (4EBP). The phosphorylation of 4EBP cause the release of eIF4E which then combine with the translation initiation complex, resulting in increased protein translation. Phosphorylated p70S6K phosphorylates both eIF4B and ribosomal protein S6 (S6RP), also increased protein translation (adapted from Shives et al., 2016).

It is a delicate balance between maintaining normal translation and upregulation of protein synthesis in the cell. Any alterations linked to the rate of protein synthesis causes a vast array of illnesses (Costigan et al., 2009).

### 1.3.2 The role of the mTORC1 pathway in pain

Recent researches has focused on the role of mTORC1 in pain, particularly in neuropathic pain. Studies have suggested that neuropathic pain is intimately linked to neuronal plasticity, identified by structural changes in the nervous system (Costigan et al., 2009). The synapse is the essential cellular unit of electrochemical communication between neurons; these connections or communication are 'plastic' (Hoeffer and Klann, 2010). In other words, the physiological responsiveness of the synaptic connection is modifiable (Kerchner and Nicoll,

2008). Neuroplasticity allows the neurons in the brain to compensate for injury and to adjust their activities in response to new situations (Shaw et al., 1994). Essentially, neuroplasticity plays a significant role in multiple neurodegenerative and neuropsychiatric diseases (Shaw et al., 1994). Synaptic plasticity is also defined temporally or as short-term alterations, as some changes last only for a few seconds, whereas other changes persist over a lifetime. Generally in animals, long-term alterations in synaptic plasticity is frequently measured as long-term depression or long-term potentiation or both (Hoeffer and Klann, 2010; Kerchner and Nicoll, 2008).

Neuropathic pain is the result of lesions in the somatosensory nervous system, which alters its function and structure (Shaw et al., 1994). In turn, these functional and structural alternations integrate to cause spontaneous and amplified pain (Shaw et al., 1994). The maladaptive plasticity in the neurons of this neuropathic condition produces a series of changes that include ectopic generation of action potentials, modulation of the ion channel, facilitation and disinhibition of synaptic transmission, which ultimately led to the formation of new synaptic circuits (Costigan et al., 2009; Colombo et al., 2010). Unfortunately, all these synaptic alternations usually result from changes in protein transcription and translation (Costigan et al., 2009). In fact, mTORC1 signalling pathways are crucial for protein synthesis as mentioned previously. Several molecular biological studies have revealed that the expression of phosphorylated counterparts of mTORC1, 4EBP1 and p70S6K were upregulated in pain (Géranton et al., 2009; Liang et al., 2013; Xu et al., 2011). The active mTORC1 phosphorylates 4EBP1/2 and p70S6K 1/2, leading to the initiation of protein translation (Beretta et al., 1996). Thus, mTORC1 and its downstream pathway signifies a key player in the mechanism governing neuroplasticity in chronic pain. It is also well established that long-lasting synaptic plasticity is regulated by local protein synthesis in axons and dendrites (Sutton and Schuman, 2006), which contain all the necessary components for local protein synthesis, including mRNAs and ribosomes (Wang et al., 2010). The contribution of mTORC1 signalling to local protein synthesis is also reported (Jiménez-Díaz et al., 2008). In Table 1.5, the summary of various interesting studies explains the contributions of mTORC1 signalling pathways in neuropathic pain.

A greater understanding of the role of mTORC1 in neuropathic pain will be a valuable tool in developing the next generation of therapies for the modulation of pain in human disease states.

**Table 1.5: Summary of various recent studies about mTORC1 pathway contributions to neuropathic pain.**

| Animal | Method to induce pain | mTORC1 inhibitors | Administration route | Key findings | Reference |
|---|---|---|---|---|---|
| Male Sprague Dawley rats | Neuropathic pain induced by spared nerve injury (SNI) | Rapamycin | Intraplantar injection | -Rapamycin decreased the sensitivity of a population of myelinated nociceptors which are known to be important for the increased mechanical sensitivity that follows injury.<br>-Local treatment with rapamycin significantly attenuated persistent pain that follows tissue injury, but not acute pain. | *Jiménez-Díaz, et al., 2008* |
| Male Sprague-Dawley rats | Neuropathic pain induced by spared nerve injury (SNI) | Rapamycin | Intrathecal injection | -Reduced the thermal sensitivity test.<br>-Rapamycin decrease neuropathic pain by acting on an mTORC1path way. | *Géranton et al., 2009* |
| Male Sprague Dawley rats | Neuropathic pain induced by spinal nerve ligation (SNL) | Temsirolimus (CCI-779) | Intrathecal injection | -CCI-779 exerts strong inhibitory effects on SNL-induced behavioural hypersensitivity compared to saline.<br>-Confirm that mTORC1 pathways are vital in nociception and persistent pain like states. | *Asante et al ., 2010* |
| Adult male C57BL/6 J mice | Neuropathic pain induced by spared nerve injury (SNI) | Temsirolimus (CCI-779) | Intrapitonial injection | -Reduced the response to mechanical and cold stimuli in neuropathic mice.<br>-mTORC1 signalling pathway is a potential target for therapeutic intervention, particularly in chronic pain. | *Obara et al., 2011* |
| Male ICR mice | Interleukin-6 (0.1 ng) was injected into the plantar surface of the left hind paw in a volume of 25 µl | Resveratrol is A activator of AMPK which profoundly inhibits ERK and mTOR signalling | Intraplantar injection | -Resveratrol completely blocks the development of persistent nociceptive sensitisation consistent with the blockade of the transition to a chronic pain state. | *Tillu et al., 2012* |
| Adult male Sprague-Dawley rats | Neuropathic pain induced by injury to the sciatic nerve branches | Rapamycin | Infuosion to insular cortex | -Rapamycin reduced mechanical allodynia.<br>-Decreased neural excitability in the insular cortex (IC), thereby inhibiting neuropathic pain-induced synaptic plasticity. | *Kwon et al., 2017* |
| Adult female C57BL/6 J mice | Neuropathic pain induced by Spinal Cord Contusion Injury (SCI) | Rapamycin | Intraperitoneal injection | -Reduced secondary neural tissue damage.<br>-Suppressed the microglial activation in the lumbar spinal cord and attenuate the development of neuropathic pain after SCI. | *Tateda et al., 2017* |

### 1.3.3 The role of the mTORC1 pathway in the regulation of opioid effects

Evidence has revealed that opioid-induced tolerance and hyperalgesia may be linked to changes in the entire transcription and translation process of different critical tolerance-associated proteins in the dorsal neuronal horn like NOS, PKCγ and CaMKIIα and activation of glial cell and cytokine release in the CNS and in PNS along the sensory afferent fibres (Lutz et al., 2015). Interestingly, the development of opioid disorders like tolerance and hyperalgesia, and also acute chronic pain transition, may be linked by common or similar cellular mechanisms in the primary afferent (Joseph et al., 2010). Given that mTORC1 promoted the protein translation process that is needed for the initiation and maintenance of chronic pain (Jiang et al., 2016), it is reasonable to assume that mTORC1 is involved in the development and maintenance of opioid disorders (tolerance, hyperalgesia and dependence) (Sun et al., 2016).

A group of researchers used a different animal model of opioid tolerance and hyperalgesia to explore the role of mTORC1 inhibitors for the prevention or reduction of opioid tolerance in chronic pain management. For instance, Xu et al. (2014), reported that intrathecal injection of rapamycin, a classic mTORC1 inhibitor, into morphine tolerant rats, resulted in attenuating the development of morphine-induced tolerance in their behavioural tests (mechanical threshold and thermal latency). Furthermore, the expression of the immunoreactivities of mTORC1 and its downstream effectors is activated by chronic morphine injections by way of the μ-opioid receptor-triggered P13K/Akt pathway in dorsal horn neurons of the spinal cord (Mazei-Robison et al., 2011; Xu et al., 2014; Sun et al., 2016). However, under normal conditions, the P13K/Akt pathway is found to be inactive (Vanhaesebroeck and Alessia, 2000; Lutz et al., 2015). Figure 1-13 depicts the proposed model for the involvement of spinal cord mTORC1 in chronic opioid tolerance.

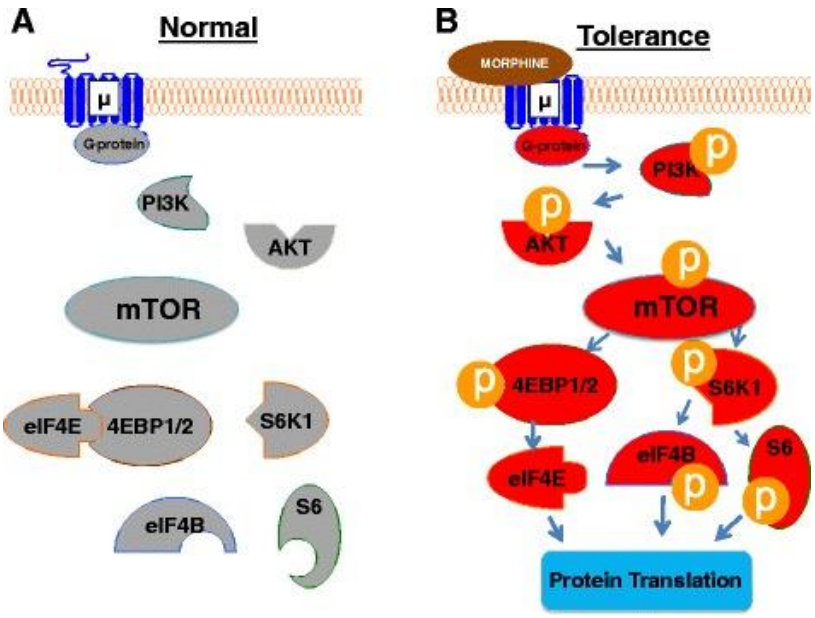


**Figure 1.13: Proposed model for the involvement of spinal cord mTORC1 in opioid tolerance. (A)** Under normal physiological conditions mTORC1 is inactive thus 4EBP1/2 binds to eIF4E preventing its associations with eIF4G resulting in silencing of the protein translation due to the absence of cap-dependent translation eIF4F. **(B)** Repeated binding of morphine to μ-opioid receptors resulted in phosphorylation of PI3K and Akt pathways, which in turn phosphorylated the mTOR/p70S6K 1/2,4EBP1/2 cascade, resulting in the initiation of mRNA translation (optioned from Lutz et al., 2015).

Even though it is well documented, that repeated and long-term exposure to opioids like morphine causes opioid receptor-mediated adaptive changes within the nervous system, it is still ineffectively managed by current therapy in the clinic.

Neuroplasticity also plays a crucial role in modulating the rewarding value of abused drugs. It has been shown *in vivo* that mTORC1 signalling was increased in ventral tegmental area (VTA) but not in nucleus accumbens (NAc), in response to chronic morphine administration for the reason that elevated levels of phospho-S6 and phospho-4EBP were detected (Mazei-Robison et al., 2011). This suggests that there is some specificity of opioid-induced alternation within the brain reward circuit. Additionally, chronic morphine exposure induces phenotypic changes in VTA dopaminergic neurons which is characterised by a reduction in the size of the soma and increased cell excitability (Mazei-Robison et al., 2011).

### 1.3.4 mTORC1 inhibitors in clinical use

Rapamycin (sirolimus) is a natural macrolide that was discovered in the early 1970s (Vezina et al., 1975). It is a specific inhibitor of mTORC1, and is produced by bacterium; *Streptomyces hygroscopicus*, which was cultured from a soil sample isolated on Rapa Nui, better known as Easter Island (Vezina et al., 1975). Rapamycin was first found to inhibit the growth of yeast with antifungal properties (Manning, 2017). The anti-fungal and antimicrobial use of rapamycin was temporarily abandoned after the discovery of its strong

immunosuppressive activity (Lopez et al., 2014), due to its potent action in blocking T-cell activation, which significantly reduced acute graft rejection in liver, kidney and heart transplant patients (Watson et al., 1999; Kahan and Group, 2000). In addition, rapamycin has demonstrated several other interesting effects, including cytostatic (suppress cell growth) and antiproliferative properties, which expands its potential clinical applications to oncology (Molina et al., 2012). Rapamycin is poorly water soluble and has a high percentage of protein binding (approximately 92%), which affects its bioavailability (Palavra et al., 2017). Consequently, pharmaceutical development focused on improving its pharmacokinetics features and stability. All rapalogs or analogues (temsirolimus, everolimus and ridaforolimus) are created by replacing the hydrogen at the C-40-O position with different moieties, as rapamycin requires two sides for binding with FKBP12 and mTORC1 to provide its pharmacological action so that there is limited room for further modification (Zheng and Jiang, 2015). Figure 1.14 represents the molecular structure of rapamycin and its rapalogs.

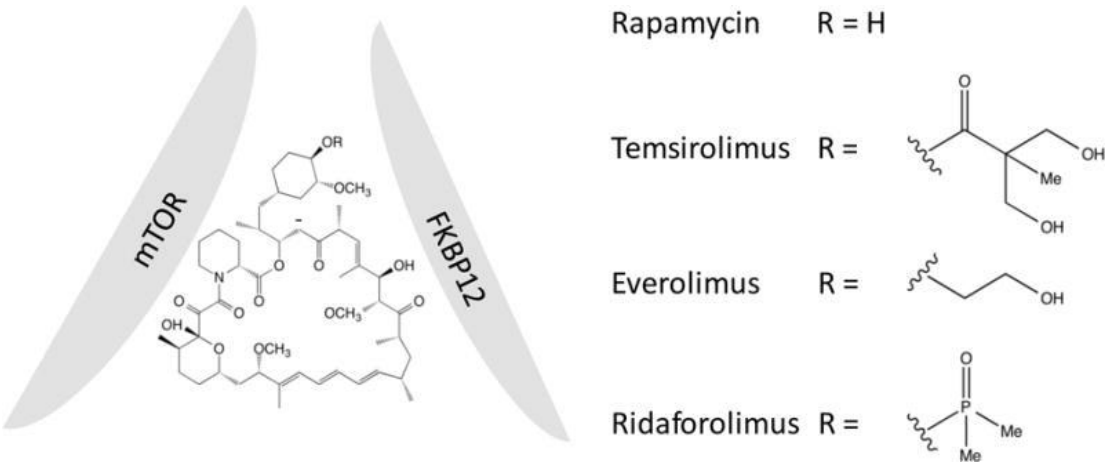


**Figure 1.14: Molecular Structure of rapamycin and its rapalogs.** They all share the same central macrolide chemical structure and have a unique R group at the C-40 position (obtained from Zheng and Jiang, 2015).

Despite the slight differences at C 40 between the sirolimus and rapalogs, they have significant clinical implications, specifically due to distinct pharmacokinetic properties, particularly bioavailability and half-life (Zheng and Jiang, 2015). Temsirolimus (CCI-779), is formulated or designed to overcome the poor solubility of rapamycin, which undergoes extensive first-pass metabolism leading to low bioavailability and potentially variable absorption and exposure (Zheng and Jiang, 2015). Temsirolimus exhibits better solubility and an elevated volume of distribution that allows extensive delivery into peripheral tissues. This drug is metabolised by CYP3A4 and is primarily excreted by the faeces (around 82%), having a terminal half-live of between 9 and 27 hours (MacKeigan and Krueger, 2015).

Rapamycin and its derivatives are used as a novel therapeutic in cancer treatment (Yee et al., 2006; Molina et al., 2012). In 2007, Temsirolimus became the first rapalog to be approved by the FDA for cancer treatment (Kwitkowski et al., 2010). The most frequent adverse effects associated with rapamycin and its rapalogs include stomatitis, mouth ulcers, infections, marrow suppression, hypercholesterolemia, as well as other metabolic disturbances (Kaplan et al., 2014). However, in the longer duration regimens and larger dosage of rapamycin and its rapalogs, there is a greater elevation of total cholesterol (Morrisett et al., 2002) and occurrences of thrombocytopenia and leukopenia (Hong and Kahan, 2000). These results suggest that sirolimus changes the insulin signalling pathway to decrease lipoprotein lipase activity and increase adipose tissue lipase activity, resulting in an increased hepatic synthesis of triglyceride, increased secretion of very low-density lipoprotein (VLDL) and increased hypertriglyceridemia (Morrisett et al., 2002). One study undertaken demonstrated that hypercholesterolaemia and hypertriglyceridemia occurred in approximately half the patients on the higher dose of rapamycin, compared with roughly one-fifth in the placebo group (Sacks, 1999).

The therapeutic application of rapamycin and its derivatives with regards to attenuating opioid tolerance have been significantly hindered due to the potential for serious side-effects in humans, which limits its use (Sacks, 1999). Similarly, longer term treatment with rapamycin causes an engagement of feedback signalling through p70 S6 Kinase (p70S6K) in neurons that produces stimulation of the ERK pathway and upregulation of Akt phosphorylation resulting in augmented neuronal excitability (Wan et al., 2007; Melemedjian et al., 2013). Recently, a second generation of mTOR inhibitors (known as mTOR kinase inhibitors) has also been developed. The second generation directly inhibit the mTOR by blocking the ATP catalytic site rather than linking FKBP12 which repress both mTORC1 and mTORC2 activity making them more effective than rapalogs in a variety of preclinical cancer models (Zheng and Jiang, 2015; Luo and Wang, 2017). The use of the second generation of mTOR inhibitors may overcome a number of the limitations associated with long-term treatment of rapalogs (Rodrik-Outmezguine et al., 2011).

## 1.4 5'Adenosine monophosphate-activated protein kinase (AMPK)

### 1.4.1 AMPK signalling pathway

Nutrients (food intake) provide the body with the energy that is essential for all adult physiological processes and moreover, are an essential requirement for healthy growth

during development (Heidrich et al., 2010). Organisms need to couple nutrient sensing mechanisms to signalling pathways, in order to adapt and accommodate to every change in the environment to allow survival *via* conditions of nutrient stress (Lage et al., 2008), consequently, maintaining healthy growth and development (Lage et al., 2008).

The CNS plays an essential role in maintaining energy balance. It does this by controlling energy intake, energy expenditure and energy storage (Lage et al., 2008). The energy obtained from nutrients is used for regular physiological activities in the whole body in the form of adenosine triphosphate (ATP) (Lage et al., 2008). Adenosine triphosphate is the primary source of energy for all cellular metabolic activities (Bonora et al., 2012), with excess energy being stored in the form of fat, protein and glycogen (Bonora et al., 2012). An imbalance in energy expenditure and energy consumption stimulates the CNS and the peripheral metabolic system, to initiate metabolic processes in order to restore energy homeostasis (Roh et al., 2016).

AMPK is a critical player in peripheral and central energy regulation (Roh et al., 2016). AMPK is a highly preserved sensor of intracellular adenosine nucleotide levels which integrates nutritional and hormonal signals to maintain cellular energy balance (Mihaylova and Shaw, 2011). AMPK exists in most species as an obligate heterotrimer containing α catalytic subunit (α1 and α2) and two other subunits, a scaffolding β-subunit (β1 and β2) and a regulatory γ-subunit (γ1, γ2 and γ3) (Mihaylova and Shaw, 2011). AMPK is distributed in the tissues of different mammals including the brain, liver and skeletal muscle (Kelly et al., 2004; Mihaylova and Shaw, 2011). It is activated when there is an even moderate decrease in ATP production, resulting in relative increases in adenosine monophosphate (AMP) or adenosine diphosphate (ADP) (Mihaylova and Shaw, 2011). The cellular energy levels are influenced by a variety of conditions, such as nutrient starvation (primarily glucose), heavy exercise, hypoxia and other pathological conditions (Dziurla et al., 2010; Sun and Zhu, 2017). In response to the low energy conditions, AMPK promotes or activates catabolic pathways and inhibits anabolic pathways to generate more ATP (Mihaylova and Shaw, 2011), differentiation and metabolism (Mihaylova and Shaw, 2011).

Under lower intracellular ATP levels, both AMP and ADP can directly bind to the γ regulatory subunits, resulting in a conformational change in its structure which protects the activating phosphorylation of AMPK (Heidrich et al., 2010). Likewise, it can be activated in response to calcium flux *via* CAMKK2 (CAMKKβ) kinase, which appears to be primarily

in neurons and T cells (MacDonald et al., 2018). Moreover, studies have revealed that the majority of AMPK activation is mediated *via* LKB1, in every tissue type examined to date (MacDonald et al., 2018). In addition to physiological elevation, AMP/ADP and AMPK can be activated in response to several pharmacological agents, such as metformin, AICAR, biguanides and A769662 (Kim et al., 2016).

Upon activation of AMPK, many signalling pathways that restore cellular ATP supplies are coordinated (Mihaylova and Shaw, 2011). These signalling pathways have the potential to influence the metabolism of carbohydrate, fat and protein (Viollet and Andreelli, 2011; Mihaylova and Shaw, 2011). Additionally, AMPK activates autophagy directly by phosphorylation of ULK1 (protein kinase that initiates autophagy) and indirectly owing to its ability to inactivate mTORC1 (Kim et al., 2011), in addition to the decrease in protein synthesis associated with the activation of AMPK due to the inhibition of the key proteins involved in the regulation of translation initiation (4EBP1 and p70S6K) (Shackelford and Shaw, 2009; Kim et al., 2011). Recently, evidence has revealed that AMPK participates in the regulation of non-metabolic processes, for instance cell cycle progression, cell growth and organisation of the cytoskeleton (Williams and Brenman, 2008; Viollet et al., 2010). Figure 1.15 illustrates the AMPK signalling pathway.

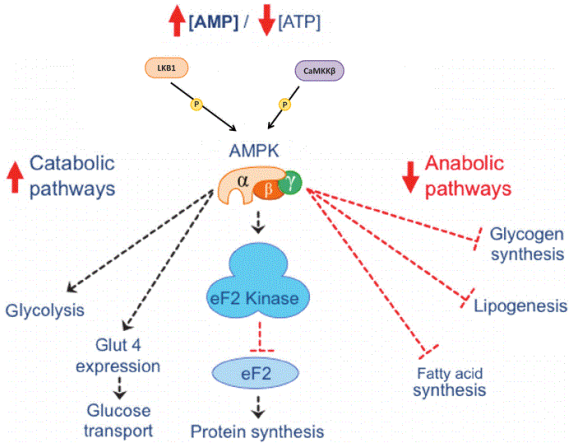


**Figure 1.15: AMPK signalling pathway.** Allosteric activation of AMPK occurs *via* an increase in the intracellular AMP/ATP ratio due to various physiological stresses, such as hypoglycaemia and hypoxemia that allows upstream kinases like LKB1 to phosphorylate Thr172 in AMPK α-catalytic subunit. CaMKKβ activates AMPK in response to the calcium increase. Catabolic pathways, such as glycolysis are activated by AMPK and the anabolic pathways, for example fatty acid synthesis, mediated by ACC1 are inhibited. Likewise, AMPK inhibits protein synthesis by down regulation of mTORC1, a key modulator of protein synthesis (adapted from Pamenter, 2014).

Given the numerous roles attributed to AMPK in maintaining the balance between anabolic and catabolic pathways, AMPK dysregulation participates in several pathological states. It is involved in increasing the prevalence of various metabolic disorders, such as type II

diabetes (non-insulin-dependent diabetes mellitus (NIDDM)), obesity, coronary heart disease, Alzheimer's disease and cancers (Luo et al., 2010; Salminen et al., 2011; Xu et al., 2014). Thus, AMPK is considered to be a significant therapeutic target for the treatment of a disease where AMPK can play a significant pathological role (Asiedu et al., 2016); examples include mitochondrial disorders (Wu et al., 2014), cardiovascular diseases (Shirwany and Zou, 2010), neurodegenerative pathologies (Marinangeli et al., 2016) and specific types of cancer, for instance liver and colon cancer concerning the importance of AMPK as a suppressor of cell proliferation (Qiu et al., 2014). Moreover, there is a wealth of emerging evidence that AMPK activation plays an essential role in neuronal processes and transmission (Giuditta et al. 2002; Klann et al. 2004; Sutton and Schuman 2005; Jung et al., 2012).

### 1.4.2 The role of AMPK in pathological pain

A harmful injury to peripheral nerves or peripheral tissues changes the sensitivity of nociceptive afferents leading to an increase in neuronal excitability (Dubin and Patapoutian, 2010). The induction of this neuronal excitability can occur rapidly after cell damage and it is mediated by different endogenous substances that act on nociceptors (Dubin and Patapoutian, 2010). These rapid changes in neuronal excitability are generally attributed to the phosphorylation that occurs in receptor/ion channels, or the phosphorylation of regulatory proteins that alter the intrinsic functional properties in both the primary sensory and the dorsal horn neurons (Woolf and Salter, 2000). In some cases, these short-term or rapid changes in the sensory neuron may often resolve after the stimulus is removed (Reichling and Levine, 2009; Price and Inyang, 2015). In 2000, the concept of AMPK as a regulator of ion channel activity emerged by way of the discovery made by of Hallows and co-workers, who showed that direct phosphorylation of the chloride channel (CFTR) *via* AMPK, inhibits the channel's activity by reducing its open probability (Hallows et al., 2000).

In cases where pain becomes pathological, it is likely that some specific signalling events change the transcription (DNA to RNA) or translation (RNA to protein) levels, which in turn affects gene expression and thus creates neuronal plasticity (Obata and Noguchi, 2004; Obara et al., 2012; Price and Dussor, 2013). These long-term changes potentially affect the function or phenotype of peripheral nociceptors, resulting in permanent alterations in pain sensitivity, which is associated with local activity-dependent translation (Price and Inyang, 2015). Multiple lines of evidence have indicated that mRNA translation can occur at distal sites (axons or dendrites) in neurons under the control of mTORC1 and the extracellular

signal-regulated kinase (ERK) signalling pathways (Jung et al., 2012). Consequently, local activity-dependent translation plays a vital role in changing the excitability of nociceptors (Jung et al., 2012; Obara et al., 2012). Notably, protein synthesis consumes a great deal of cellular energy and by knowing that AMPK is an energy sensor that regulates cellular metabolism, it is likely that AMPK decrease the neuronal excitability by modulating the protein synthesis within the cell.

#### 1.4.2.1 AMPK as a key regulator of ion channel activity

Living cells use energy to move ions or substances, against a concentration or electrochemical gradient, to maintain the correct concentrations of ions and molecules inside the cell. It is critical to couple ion transport processes within the cell to the metabolic state (Whittam and Wheeler, 1970). Additionally, in response to painful stimuli, the nociceptive neurons generate a train of action potentials, due to ions suddenly flowing in and out of the neurons (Xu et al., 2008). Indeed, the frequency of firing signals the intensity of pain sensation (Momin and McNaughton, 2009). Furthermore, continuous action potentials into the nociceptor, can lead to biochemical alternation *via* activation of the MAPK signalling pathways which in turn change gene expression and cell functional phenotype (Cheng and Ji, 2008; Dubin and Patapoutian, 2010).

Sodium ($Na^+$) and potassium ($K^+$) ions are used in the cell systems for action potentials. The voltage-gated $Na^+$ channels regulate the generation and propagation of action potentials in excitable cells and thus, are important to determine the excitability in primary sensory neurons. Therefore, $Na^+$ channels play a fundamental role in the detection and conduction of harmful stimuli in the PNS and the establishment of chronic pain disorders (Mantegazza and Catterall, 2012). There are different types of $Na^+$ channels in the cells membrane that have been identified over the years. The voltage-dependent $Na^+$ channels subtype Nav 1.7 and Nav 1.8 are expressed in peripheral nociceptive pathways, which are located in the dorsal root ganglia (Cummins et al., 2007). Numerous evidence reported that AMPK activation mediates inhibition of the epithelial sodium channel ENaC expression in an indirect manner *via* phosphorylation of ubiquitin ligase Nedd4-2, which resulted in decreased ENaC expression and ubiquitination of ENaC (Carattino et al., 2005; Bongiorno et al., 2011). Furthermore, AMPK activation generates enhanced $K^+$ channel activity which result in a robust decrease in neuronal excitability (Ikematsu et al., 2011).

#### 1.4.2.2 AMPK role in inflammatory pain

Evidence gathered reveals that all MAP kinases pathways contribute to pain sensitisation that is responsive to tissue or nerve damage in endothelial cells *via* distinct molecular and cellular mechanisms, such as extracellular signal-regulated kinases (ERK), p38 and c-Jun N-terminal kinase (JNK) (Ma and Quirion, 2002; Jin et al., 2003; Zhao et al., 2007). Generally, ERK is involved in the regulation of growth factor that leads to cell proliferation, while p38 and JNK are known to stress-induced cell death (cell apoptosis). Under different pain conditions, activation of MAPKs pathways results in the initiation and maintenance of pain hypersensitivity by way of non-transcriptional and transcriptional regulation (Johnson and Lapadat, 2002). However, after persistent pain conditions, ERK, p38 and JNK are differentially activated in CNS particularly in spinal glial cells, leading to the synthesis of proinflammatory/pronociceptive mediators that enhance and maintain the pain (Gao and Ji, 2008; Ji et al., 2009). For example, activation of ERK in spinal cord dorsal horn neurons by using different second messenger pathways play an essential role in the central sensitisation by regulation of the activity of glutamate receptors, $K^+$ channels and induction gene transcription (Ji et al., 2009).

Moreover, activation of ERK in sensory neurons, such as amygdala (amygdala neurons: located in the brain and associated with emotional stimulation), is also required as an inflammatory pain mediator. An emerging body of evidence indicates that AMPK activation inhibits ERK, p38 and JNKs kinases *via* negative regulation of small GTPases (hydrolase enzymes) that are upstream of the MAPK signalling cascade (Melemedjian et al., 2011; Tillu et al., 2012). Similarly, activating AMPK may inhibit cyclooxygenase II (COX-2) resulting in decreased formation of prostanoids; one of the critical pro-inflammatory mediators. While there are several COX-2 inhibitors available in the clinic, the use of these selective inhibitors is limited due to concerns about their side-effects, for example heart attacks (Lee et al., 2009; Al-Fayez et al., 2006). Interestingly, different animal models have demonstrated that inhibition of MAPK pathways by AMPK activators attenuates both inflammatory and neuropathic pain (Carrasquillo and Gereau, 2007). Thus, AMPK activation may provide a novel treatment for inflammatory pain.

#### 1.4.2.3 AMPK role in cancer and chemotherapy-induced peripheral neuropathy

The energy demands or consumption of energy by cancer cells are elevated, due to rapid cell growth and high cell proliferation rate. By recognising that AMPK is a primary regulator of

energy metabolism (Mihaylova and Shaw, 2011), this enzyme could be a potential therapeutic target for tumour cell treatment.

Most cancer cells display high levels of mTORC1 expression, which in turn can positively regulate cell growth, *via* activation of its downstream effectors; 4EBP1 and p70S6K, as discussed previously. Indeed, activation of the AMPK pathway negatively influence the signalling pathways of both the mTORC1 and MAPK kinases. These are implicated in a series of biological signalling cascades which play an essential role in the regulation of cell proliferation. Activation of AMPK inhibits the metastatic potential of some tumour cells, such as malignant melanoma, due to decreases in ERK and COX-2 signalling activity and causes a reduction in the level of protein synthesis by stimulating the induction of autophagy and apoptosis through the AMPK/ mTORC1 or AMPK/JNK signalling pathway (Lee et al., 2009).

It should be mentioned that there are certain types of cancer in which activation of AMPK may promote tumour cell growth. A recent study has shown that AMPK agonist metformin, possesses both tumour suppressing and tumour-promoting abilities, as when AMPK is activated it could stimulate PI3K-Akt signalling by negative mechanisms relating to feedback loop regulation (Martin et al., 2012). Activation of AMPK specifically inhibits mTORC1 but not mTORC2. Hence, it can activate the PI3K-Akt signalling pathway, which can promote tumour survival. In general AMPK activation in cancer therapy can be cell type and context dependent. Thus, using AMPK activator in cancer therapy will be one of the most challenging obstacles to address in future researches (Li et al., 2015).

Chemotherapy-induced peripheral neuropathy is a serious side-effect, which comes from using chemotherapeutic agents. It is one of the major causes of persisting pain in cancer patients (Starobova et al., 2017), characterised by the gradual onset of signs. Firstly, the pain appears in the lower limbs, then advances to the hands (Starobova et al., 2017). Patients primarily complain of numbness, hypersensitivity to mechanical stimuli and severe pain in the affected areas (Starobova et al., 2017). The histopathological changes associated with this type of neuropathy commonly involve deficits or changes in Aβ, Aδ and C afferent fibres as well (Griffith et al., 2014). The mechanisms behind chemotherapy-induced peripheral neuropathy are not entirely understood. Nonetheless, many factors have been indicated to be involved in this type of pain, like cytokines, chemokines and growth factors, such as NGF,

which act on sensory neurons as a result activation of mTORC1 and MAPK signalling (Li et al., 2015; Asiedu et al., 2016).

#### 1.4.2.4 AMPK role in neuropathic pain

Injury to the PNS is caused by numerous things, for instance metabolic diseases such as diabetes, trauma or exposure to specific drugs like chemotherapeutics agents, which leads to the development of neuropathic pain. Indeed, *in vivo* studies have shown an activation of mTORC1 and MAPK pathways and their downstream counterparts in both neurons and axons in neuropathic rodent models. Several studies have shown that activation of AMPK with AICAR, metformin and A-769662, attenuates hyper-excitability in sensory neurons associated with neuropathic pain. In Table 1.6, the researcher reviewed a number of recent publications on AMPK activators.

**Table 1.6: Summary of several recent studies on different neuropathic pain models and AMPK activators.**

| Animal | Method to induce neuropathic pain | AMPK activator | Behavioural assessment | Key findings | Reference |
|---|---|---|---|---|---|
| Male ICR mice<br><br>Adult male Sprague–Dawley rats | Neuropathic pain induced by using the Spared Nerve injury surgery model (SNI) in mice and Spinal nerve ligation (SNL) was done on rats. | Intraperitoneal injection of:<br>• Metformin (200 mg/kg)<br>• A769662 (30 mg/kg) | Mechanical sensitivity by: von Frey filaments. | Metformin and A769662, nascent protein synthesis resulting in a resolution of neuropathic allodynia. | *Melemedjian et al., 2011* |
| Adult male Sprague–Dawley rats | Diabetic neuropathy induce by streptozotocin injection | Intraperitoneal injection of:<br>• Metformin (200,500 mg/kg)<br>• AICAR (160 mg/kg) | Mechanical sensitivity by: von Frey filaments.<br>Heat nociception: a paw thermal stimulation system.<br>Cold allodynia | Metformin and AICAR is able to attenuate diabetes induced hyperalgesia and allodynia. | *Ma et al., 2015* |
| Male ICR mice | Neuropathic pain induced by using the Spared Nerve injury surgery model (SNI) | Intraperitoneal injection of:<br>• metformin (200 mg/kg) | Mechanical sensitivity by: von Frey filaments.<br>Cold allodynia | Metformin treatment successfully decreased the hypersensitivity. | *Inyang et al., 2016* |
| Adult male Sprague–Dawley rats | Trigeminal neuropathic pain induced by chronic constriction injury (CCI) of the infraorbital nerve. | Orally administration of:<br>• resveratrol | Mechanical sensitivity of the whisker pad by : von Frey filaments. | Resveratrol resolves trigeminal neuropathic allodynia and decreases sensory neuron excitability. | *Yang et al., 2016* |
| Adult male Sprague–Dawley rats | Diabetic neuropathy induce by streptozotocin injection | Intraperitoneal injection of:<br>• A769662 (30 mg/kg) | Mechanical sensitivity by: von Frey filaments.<br>Thermal hyperalgesia to both hot (45 °C) and cold (10 °C) water. | A769662 treatment significantly improved mechanical/thermal hyperalgesia threshold. | *Yerra and Kumar, 2017* |
| Adult male Sprague-Dawley rats | Peripheral nerve injury induced by chronic constriction injury (CCI) | Peri-sciatic nerve administration:<br>• Ozone<br>Intramuscular injection of :<br>• AICAR (0.25 mg/kg)<br>• A769662 (0.25 mg/kg) | Mechanical allodynia by: an electro von Frey.<br><br>Thermal hyperalgesia by foot withdrawal latency. | Direct injection of AMPK agonist and ozone have anti-nociceptive effect on CCI rats. | *Lu et al., 2017* |

### 1.4.3 Cross-talk between AMPK and mTORC1 pathway in spinal synaptic plasticity

In mammals, low intracellular ATP levels promote AMPK activation by stimulating phosphorylation of the catalytic α subunit. Activated AMPK, as mentioned previously, facilitates catabolic pathways to increase ATP production and turns off the synthetic pathways which consume ATP like protein synthesis (Ke et al., 2018). *In vitro*, an increase in mTORC1 signalling pathways is associated with high cellular ATP levels, thus mTOR activity is assumed to serve as an intracellular ATP sensor (Dennis et al., 2001). In mammals, nutrient availability results in enhancement of mTORC1 expression; thus, energy status determines the rate of both cell growth and cell proliferation in the said cell (Dennis et al., 2001). The interplay between mTORC1 and AMPK provides an exact mechanism to coordinate the cell, with changes to the surrounding environment like fasting, hypoxia and some pathological conditions (Xu et al., 2012; Dalle Pezze et al., 2016). Studies have shown that AMPK regulates the activities of both p70S6K and 4EBP1 (Goncharova et al., 2002; Kimura et al., 2003; Sofer et al., 2005), indicating a convergence of AMPK signalling and mTORC1 signalling pathways. Interestingly, the downstream targets of AMPK lie within the mTORC1 signalling pathway and the upstream target of mTORC1 coordinates with AMPK (Xu et al., 2012). Figure 1-16. illustrates the signals cross-talk between mTORC1 and AMPK.

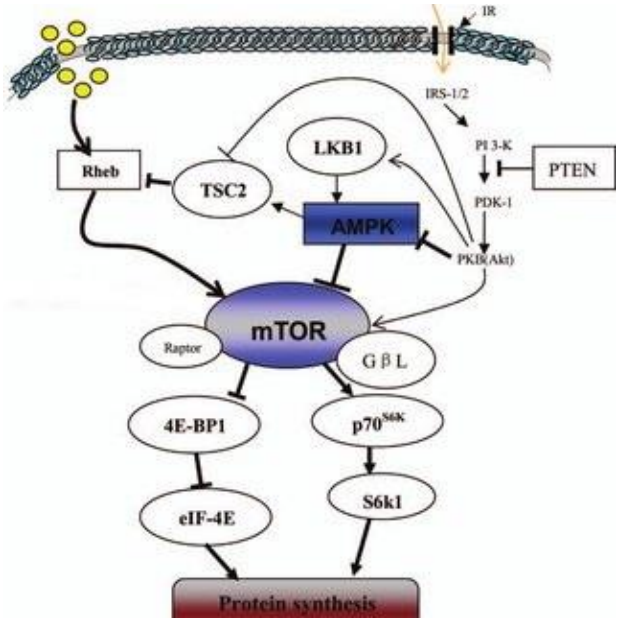


**Figure 1.16: A schematic diagram of the AMPK and mTORC1 signalling network in mammalian cells.** AMPK inhibits mTORC1activation *via* the phosphorylation and inactivation of TSC2 by means of several ways (adapted from Xu et al., 2012).

Molecular mechanism studies documented two obvious upstream targets of mTORC1 lie within the AMPK signalling pathway. These targets were identified as tuberous sclerosis complex (TSC) and mTOR (Goncharova et al., 2002; Cantó and Auwerx, 2010). Studies on small GTPase Rheb and TSC have reported that the AMPK-dependent inhibition of mTOR signalling is by means of targeting TSC2, an essential mediator of mTOR activity (Montagne et al., 2001). When AMPK is activated, direct phosphorylation of TSC2 on T1227 and S1345 is obtained, enhancing the stability of the complex (TSC1-TSC2) and therefore inhibiting mTORC1 signalling (Inoki et al., 2003). TSC2 act as GTPase-activating protein for (Rheb) (Long et al., 2005). Rheb binds directly to the kinase domain in mTORC1 and induces conformational changes to generate active mTORC1. Following activation, mTORC1 phosphorylates its downstream targets 4EBP1 and p70S6K, resulting in the upregulation of protein synthesis (Long et al., 2005). The small GTPase Rheb, in its GTP-bound state, is an essential and potent mediator of mTORC1 kinase activity in a GTP dependent manner (Long et al., 2005; Xu et al., 2012). In addition, the LKB1 and Akt are upstream kinases of AMPK related to mTORC1 (Xu et al., 2012).

Historically, growth factors mediate the activation of mTORC1 *via* Akt pathway (Inoki et al., 2002), Akt stimulates mTORC1 activity through direct phosphorylation of both; TSC2 and pras40 (the proline-rich Akt substrate, a component of mTORC1) (Sancak et al., 2007; Acosta-Jaquez et al., 2009). Activation of the Akt pathway reduces AMPK activity by maintaining a high level of intracellular ATP associated with a concomitant reduction in the AMP/ATP ratio. This could be particularly essential at the organismal level and in the cancer cell. However, the link between Akt and AMPK activity remains unknown (Xu et al., 2012). Currently, a study examining lung carcinomas demonstrated that cell proliferation required inhibition of LKB1/AMPK signalling and it is associated with activation of the Akt/mTOR/p70S6K pathway (Huypens, 2007).

Moreover, the tumour suppressor LKB1 (also termed serine/threonine kinase 11) and Akt are upstream kinases of mTORC1 (Xu et al., 2012). LKB1 increases AMPK activity *via* phosphorylation of Thr172 in α catalytic subunit (Shackelford and Shaw, 2009) and subsequently reduces phosphorylation of p70S6K and 4EBP1 (Boudeau et al., 2003). Recent studies in cell culture have demonstrated that LKB1 is required for mTORC1 inhibition under low ATP conditions (Shaw et al., 2004). A study established in transgenic mice, revealed that a deficiency of LKB1 in heart and skeletal muscle, resulted in a nearly complete loss of AMPK activity (Sakamoto et al., 2005). Similarly, lack of LKB1 in the liver prevents

AMPK activation (Shaw et al., 2005). Likewise, in autosomal dominant Peutz-Jeghers syndrome which is characterised by multiple hamartomas (similar to the benign tumours seen in tuberous sclerosis), LKB1 is inactivated (Boudeau et al., 2003). Taken together, these findings suggest that LKB1 is a critical physiological upstream kinase for AMPK and the regulation of mTORC1 by the tumour suppressors. Thus, LKB1 could be an interesting target to establish the role of mTORC1 inhibition in the pathogenesis of human tumours.

### 1.4.4 The role of AMPK in regulation of opioid effects

Opioids such as morphine are powerful analgesic drugs prescribed for a variety of painful conditions (Pajohanfar et al., 2017). Despite opioids efficacy in providing acute pain relief, long-term exposure to opioids produces a reduction in their analgesic efficacy owing to tolerance. Therefore, dose escalation is required to manage the elevated pain (Pajohanfar et al., 2017). However, this need to escalate the opioid dose increases the risk of respiratory depression, sedation and constipation, and in turn these side-effects potentially reduce the patient's quality of life (Xu et al., 2014).

The mechanisms underlying opioid tolerance are not fully understood. Over the years researchers suggested several mechanisms such as loss of μ-opioid receptor signalling besides other factors that were mentioned previously (Corder et al., 2018). Significantly, the MAPK signalling pathway, including (ERK), p38, c-Jun N-terminal kinase are involved in morphine-induced neuroinflammation and tolerance (Chen and Sommer, 2009). Additionally, neuropathic pain and opioid-induced tolerance share various similar mechanism profiles (Watkins et al., 2005).

Based on recent evidence that suggests the activation of AMPK inhibits the MAPK signalling pathway and that AMPK agonist reduce both acute and neuropathic pain, Han et al. (2014), demonstrated for the first time that AMPK activation suppresses morphine-induced tolerance. They investigated that resveratrol, an AMPK agonist, directly attenuated morphine-induced p38/NF-kB signalling in microglia cell. This effect was achieved by activation of AMPK (AMPK –dependent) (Han et al., 2014). The same research group determined that AMPK activation by resveratrol blocks acute and chronic morphine tolerance in both male and female mice (Han et al., 2014). This study creates a rationale for further investigation of AMPK agonists as adjuvants with opioids treatment, so as to limit the development of side-effects that reduce opioid efficacy and the patient's quality of life.

### 1.4.5 AMPK activators

Many types of AMPK agonists have been identified over the years and studied. Targeting AMPK *via* an agonist agent can be performed directly or indirectly (Kim et al., 2016). Direct activation of AMPK causing allosteric modulation, which promotes phosphorylation of Thr172 and/or inhibition which causes dephosphorylation of Thr 172 like thienopyridine (compound A-769662), 5-aminoimidazole-4-carboxamide riboside (AICAR) and benzimidazole (Compound 911**)** (Kim et al., 2016). The indirect activation of AMPK comes from increasing the AMP: ATP and ADP: ATP ratios by inhibiting mitochondrial intracellular ATP production. Consequently, AMPK is activated by AMP and ADP, such as metformin, resveratrol and thiazolidinediones (Kim et al., 2016).

Metformin (dimethylbiguanide) is one of the most important compounds within the guanidine derivatives (Ruggiero-Lopez et al., 1999), and is extensively used for the management of diabetes mellitus (Graham et al., 2011). Currently, metformin is reputed to be the most commonly prescribed agent in the treatment of type II diabetes in obese or overweight patients who are usually above 40 years old (Klonoff et al., 2008). Metformin's history can be traced back to the use of Galega officinalis Linn as a botanical medicine in medieval Europe (Bailey and Day, 2004). It is a perennial plant found in most tepid regions, including Britain with white, purple or blue flowers that grow over two feet high (Bailey and Day, 2004).

**Figure 1.17: The *Galega officinalis* plant, commonly known as, French lilac.** These plant are borne in ladder formation and are decorated with short spikes of lilac, pea-like leaves (obtained from Bailey and Day, 2004).

The extract contains guanidine and galegine as major chemical components (Palit et al., 1999). It is well known for its hypoglycaemic effect on animals, though it was too toxic for clinical use (Palit et al., 1999). Chemists bound two guanidines together, forming a biguanide which is found to be more tolerable and safe (Patade and Marita, 2014). In 1929, metformin was synthesised and then clinically developed in the 1950s by the French physician Jean Sterne, who launch it as Glucophage ("glucose eater") (Gottlieb and Auld, 1962). Despite

the long history of metformin, it has witnessed a considerable renewal of interest in recent years.

### 1.4.5.1 Metformin's chemistry

Chemically, metformin is 1,1 dimethyl-biguanide hydrochloride with a mechanism of action and uses similar to other biguanides (Mubeen and Noor, 2009). Figure 1.18 illustrates the chemical structure of metformin.

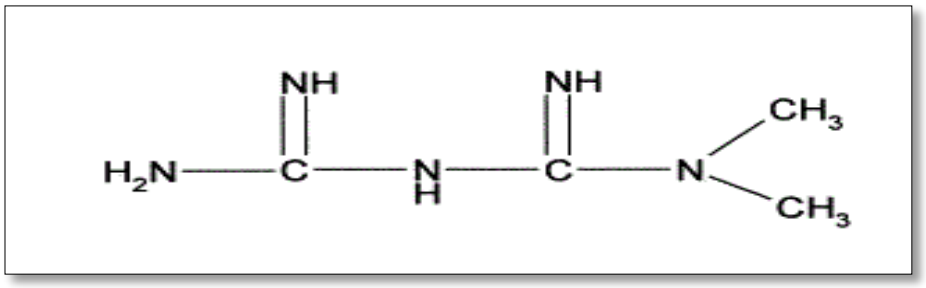


**Figure 1.18: Chemical structure of metformin**. It is a white, hygroscopic free-flowing crystalline solid with a bitter taste. Its molecular formula is C4H11N5. This small molecule is freely soluble in water (obtained from Ruggiero-Lopez et al., 1999).

It should be pointed out that metformin's structure was generally represented in an erroneous tautomeric form (electronic-structure) for several years, but that was corrected in 2005 by a group of chemists from India (Bharatam et al., 2005).

### 1.4.5.2 Metformin's mechanism of action

Metformin has been implicated as a significant contributor to glucose-lowering efficacy (Zhou et al., 2001; Phung et al., 2010). Additionally, metformin has beneficial effects on circulating lipids (Hundal et al., 2000), which are linked to an increase the risk cardiovascular disease (Nagi and Yudkin, 1993). However, the mechanisms by which metformin produces these effects on glucose and the metabolism of lipids remains a paradox (Ramachandran and Saraswathy, 2014). At the molecular biology level, the most recognised mechanism in relation to metformin is to activate AMPK (Pryor and Cabreiro, 2015; Kim et al., 2016). Targeting AMPK provides the beneficial therapeutic effects of metformin on glucose and the metabolism of lipids (Pryor and Cabreiro, 2015). Evidence suggests that the most significant effect of metformin is in glucose utilisation (Klip and Leiter, 1990), as metformin reduces glucose production in the liver (Perriello et al., 1994) *via* inhibition of the mitochondrial glycerophosphate dehydrogenase enzyme (Hur and Lee, 2015). Likewise, metformin decreases glucose absorption in the small intestine (Ikeda et al., 2000) and increases glucose uptake into skeletal muscle and adipose tissue by acting on the essential

critical components of the insulin-signalling pathway (Klip and Leiter, 1990). It should be mentioned that metformin does not increase insulin levels in the circulation; in fact, it improves insulin sensitivity and decreases insulin resistance. Therefore, an understanding of the molecular basis of metformin's action on glucose and lipid homeostasis is a critical issue that enables researchers to avoid any impaired metabolic handling which promotes metabolic disturbance and the risk of serious side-effects like fatal and non-fatal lactic acidosis (Salpeter et al., 2010). A strong association was established between using metformin and acidemia in patients, as metformin administration increases lactic acid level in the body, resulting in the development of lactic acidosis (Connelly et al., 2017). However, this relationship is evident in patients typically diagnosed with acute kidney injury (Connelly et al., 2017).

It is reported that metformin inhibits the activity of mitochondrial complex I (Hur and Lee, 2015). This inhibition might be a mechanism of metformin-induced in direct AMPK activation (Zhang et al., 2012; Hardie et al., 2001). By the inhibition of mitochondrial complex I activity, the intracellular ATP levels are reduced (Zhou et al., 2001) and AMP levels are increased *via* the action of adenylate kinase converting two molecules of (ADP) to AMP and ATP (Dzeja and Terzic, 2009) (Figure 1.19). AMPK is assumed to be activated by a two-pronged approach, either by AMP or ADP which directly binds to both sites at the γ regulatory subunit creating conformational changes that allosterically activate AMPK or AMP binding at Thr172 in the activation loop, which lies in the activation segment of the N-terminal kinase domain of the α regulatory subunit, leading to increased AMPK phosphorylation (Dzeja and Terzic, 2009). Several research groups have demonstrated that the LKB1 directly mediates AMPK phosphorylation (Dzeja and Terzic, 2009; Mihaylova and Shaw, 2011). Additionally, AMPK can be phosphorylated independently to LKB1 by metabolic hormones including adiponectin and leptin in response to changes in the intracellular calcium *via* CAMKK2 (CAMKKβ) kinase (Zhou et al., 2001; Wen et al., 2013; Pamenter, 2014).

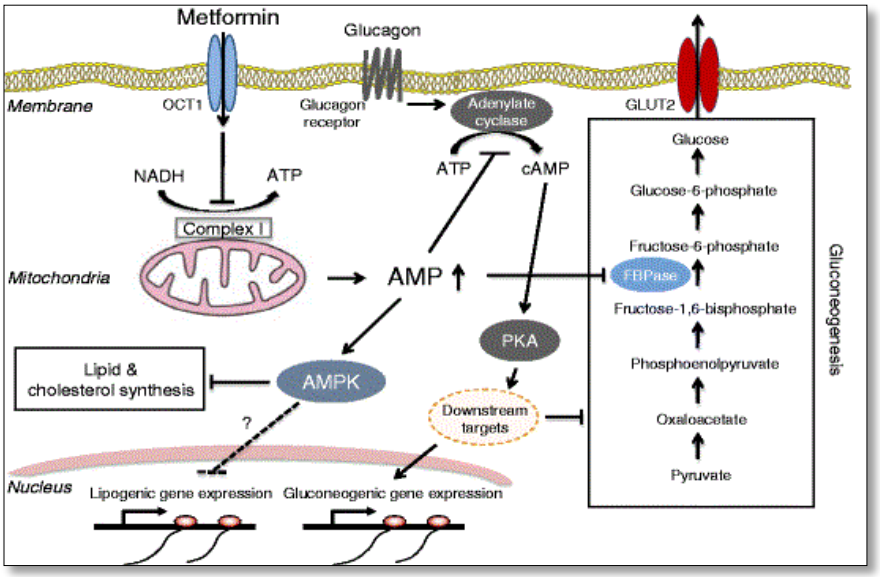


**Figure 1.19: Schematic diagram of the anti-hyperglycaemic action of metformin on the liver cell.** Metformin is transported into the liver cell (hepatocytes) *via* the portal vein through organic cation transporter 1 (OCT1) located in the plasma membrane. Inside the cell, by means of an unknown mechanism(s), metformin inhibits the mitochondrial respiratory chain (complex I) resulting in a shortage of energy production in the liver cell achieved by a reduction in ATP and a concomitant increase in AMP levels which directly activate AMPK and inhibit gluconeogenesis. A high AMP concentration function is an essential mediator to suppress adenylate cyclase resulted in inhibition of 3′-5′-cyclic adenosine monophosphate (cAMP) production, therefore leading to decrease the expression of gluconeogenic enzymes such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase. Together, these results cause the inhibition of gluconeogenesis and lipid/cholesterol synthesis (obtained from Rena et al., 2013).

### 1.4.5.3 Metformin's safety and tolerability

The incidence of lactic acidosis has been associated with the use of metformin since its introduction (Connelly et al., 2017), however, it is highly controversial. Metformin is associated with a very low or (rare) occurrences of lactic acidosis (Nishihama et al., 2017), in a different way to other early biguanides, phenformin and buformin (Williams and Palmer, 1975). Both biguanides, phenformin and buformin were initially used in clinics but because they were closely associated with fatal lactic acidosis, they were withdrawn from the market in most countries (Williams and Palmer, 1975). In 1976, the use of phenformin was discontinued in the United States (Gan et al., 1992).

Metformin is the most widely prescribed drug for type II diabetes and is taken by an estimated one hundred and fifty million individuals worldwide (Pryor and Cabreiro, 2015). According to current studies, the prevalence of metformin-associated lactic acidosis is quoted as 3.0 to 16.7 cases per 100000 patient-years (Rüegg and Caduff, 2017). A recently published study demonstrated that there was no significant difference in the incidence of lactic acidosis and hyperlactatemia between patients with and without metformin therapy (Lee et al., 2017).

However, another study revealed that a clear association was found between metformin and the development of lactic acidosis in acute kidney injury patients (Connelly et al., 2017). In addition, an evaluation of more recent data on the incidence of lactic acidosis related to metformin therapy, suggested that this risk is low or negligible, if care is taken when prescribing the drug to patients with suspected clinical risks to lactic acidosis (Lee et al., 2017). Metformin is currently the drug of choice for managing type II diabetes as recommended by the American Diabetes Association and the European Association for the Study of Diabetes (Inzucchi et al., 2015).

Additionally in relation to lactic acidosis, metformin administration associated with gastrointestinal intolerance symptoms include nausea, diarrhoea, bloating, flatulence, anorexia, a metallic taste and abdominal pain (Lashen, 2010), which is often associated with high-dose initiation and rapid titration of metformin therapy (Scarpello and Howlett, 2008). The gastrointestinal intolerance remains a clinical problem to be addressed by taking metformin with meals or transferring the patient to a prolonged-release formula (Scarpello and Howlett, 2008). The gastrointestinal intolerance symptoms occur with patients to varying degrees and in most cases it is resolved spontaneously (Lashen, 2010).

#### 1.4.5.4 Views on the clinical uses of metformin

Metformin has been used globally for the treatment of diabetes mellitus type II throughout the last five decades (Krosnick, 2002). It improves blood glucose levels by enhancing insulin sensitivity in liver and muscle, while at the same time not being associated with hypoglycaemia (Krosnick, 2002) at any reasonable dose, unlike other antidiabetic drugs (Wadher et al., 2011). Hence, it is known as an antihyperglycaemic rather than a hypoglycaemic drug (Melchior and Jaber, 1996). However, hypoglycaemia may occur in some patients associated with strenuous physical activity or fasting (Bodmer et al., 2008).

Publications reported that metformin regularly promotes body weight loss in patients with obesity specifically, who are diagnosed with type II diabetes mellitus (Lee and Morley, 1998). Several studies tested the effect of metformin on satiety and its efficacy in inducing weight loss (Chapman et al., 2005; DeFronzo et al., 2005). In Polycystic Ovary Syndrome (PCOS), metformin was the first insulin sensitising drug to be used to evaluate the role of insulin resistance in different diseases (Velazquez et al., 1994). PCOS is a common endocrine disorder (Balen and Michelmore, 2002) that affects approximately 15

million women, which is roughly 5-10% of the female population in the reproductive ages 13-45 (Jaganmohan et al., 2017).

Randomised trial data analysis demonstrate that metformin should be considered as first-line treatment for non-obese women with anovulatory infertility due to PCOS (Johnson et al., 2011). Furthermore, Tang et al. (2006), presented that short-term or co-treatment with metformin in female patients diagnosed with PCOS undergoing *in vitro* fertilisation (IVF) or Intracytoplasmic sperm injection (ICSI) cycles, significantly decrease the risk of ovarian hyperstimulation syndrome (OHSS) and improves the pregnancy outcome (Tang et al., 2006). In contrast, a recent study published revealed that metformin does not play a decisive role in the treatment of PCOS patients (Kalem et al., 2017). Despite, the controversy concerning the role of metformin in the treatment of infertility in patients with PCOS, it appears that for obese women, this treatment has a beneficial effect (Morin-Papunen et al., 2012). A large-scale randomised-controlled study is necessary to ascertain the effects of metformin in PCOS (Chen et al., 2017).

Epidemiological and clinical studies with experimental evidence suggest a relationship between Alzheimer's disease (AD) and type II diabetes (Chen et al., 2009). Insulin controls the metabolism of β -amyloid precursor protein in neurons, thus, decreasing the intracellular accumulation of β-amyloid peptides, which are pivotal in AD pathogenesis (Chen et al., 2009). Studies by Li et al. (2012), demonstrated that metformin attenuates AD-like biochemical changes in the brains of mice. Furthermore, a recent randomised placebo-controlled crossover study suggested that metformin is associated with improved patient executive function and improvements in learning, memory and attention skills (Koenig et al., 2017). Despite the strong connection between AD and DM, the association between the neuropathology of AD and DM is less evident (Shinohara and Sato, 2017).

Epilepsy is a common neurological disorder with a lifetime prevalence of 7.6 per 1000 persons (Fiest et al., 2016). Considerable evidence has indicated that metformin treatment could facilitate seizure termination (Yang et al., 2017; Nielsen et al., 2013). Several observational studies have suggested that metformin treatment appears to be associated with a lower risk of developing various types of cancer and cancer-related mortality among patients with type II diabetes (DeCensi et al., 2010). A meta-analysis study conducted by Zhang et al. (2012) that reviewed the current available evidence to examine the potential role of metformin in chemoprevention for liver cancer in patients with type II diabetes, confirmed

that metformin is effective in reducing the risk of liver cancer (Zhang et al., 2012). Additionally, multiple studies reported an association between metformin use and lower breast cancer risk (Zakikhani et al., 2007; Hadad et al., 2014; Iglesias, 2017). Furthermore, a meta-analysis by Hou et al. (2017), indicates that metformin therapy is correlated with a significant decrease in the risk of colorectal adenoma and advanced adenoma in patients with type II diabetes. Numerous randomised trials specifically designed to evaluate the efficacy of metformin as an antineoplastic agent in preclinical and clinical studies are warranted (Hamieh et al., 2017).

Cardiovascular disease includes diseases of the heart and circulation. The 2013 Global Burden of Disease Study reported that approximately 30% of all deaths worldwide were caused by cardiovascular disease (Bhatnagar et al., 2014). According to the British Heart Foundation, cardiovascular disease accounted for more than 26 % of all deaths in the U.K in 2017. Recently, a significant reduction in the incidence of major cardiovascular events has been observed in safety trials with some glucose-lowering drugs but not with other agents in patients with type II diabetes and cardiovascular disease (Gerstein et al., 2011; Boussageon et al., 2011; Ferrannini and DeFronzo, 2015). A randomised controlled trial at the Glasgow Clinical Research Facility determined that metformin administration could decrease cardiovascular event in patients with type II diabetes and might decrease cardiovascular risk for non-diabetic persons *via* mechanisms independent of glucose-lowering (Preiss et al., 2014). Several studies regarding *in vivo* and *in vitro* research refer to specific evidence on the antiatherogenic properties of metformin (Mamputu et al., 2003; Zang et al., 2006). For example, it has been reported that metformin as an activator of AMPK decreases endothelial cell damage caused by oxidative stress due to hyperglycaemic conditions (Cai et al., 2016).

Studies have also demonstrated the anti-inflammatory properties of metformin which are exerted in both diabetic populations (Balducci et al., 2010), irrespective of diabetes status (Cameron et al., 2016).

Recently published research provides potential evidence that the use of metformin may be associated with an improvement in chronic pain, in both animals and humans. A study completed by Melemedjian et al. (2011), investigating the effect of metformin administration on neuropathic pain in a mouse model, resulted in a complete reversal of tactile allodynia. Moreover, this finding established that metformin (AMPK activators), may be a potential efficacious class of drugs for the treatment of peripheral nerve injury induced neuropathic

pain. A different study reported that the analgesic effect of metformin, by activating the AMPK pathway, had an effect on decreasing paw withdrawal latency to heat and paw withdrawal latency to cold stimuli in diabetic rats (Ma et al., 2015). Likewise, a retrospective study on metformin use in radiculopathy pain patients showed that metformin had a possible positive impact on decreasing the severity of lumbar radiculopathy pain (Taylor et al., 2014). Recently, a study examined the interaction between metformin and several conventional or adjuvant analgesic drugs; specifically aspirin, ibuprofen, pregabalin and tramadol in a rat model of somatic inflammatory hyperalgesia. The study revealed that metformin significantly reduced hyperalgesia in those rats (Pecikoza et al., 2017). Moreover, the same research team suggested that patients who are already using metformin at lower doses of these analgesia might be sufficient for achieving satisfactory pain relief (Pecikoza et al., 2017). Generally, metformin may be a helpful antiproliferative and anti-inflammatory agent in the treatment of many diseases.

### 1.4.6 Cross-talk between metformin and mTORC1 Pathway in opioid efficacy for neuropathic pain management

The therapeutic limitations of opioid medications in neuropathic pain management, show a clear demand to understand the molecular mechanisms and initiate rapid development of opioid- induced tolerance. It is evident that neuroplasticity in the form of adaptive changes in protein transcription and translation is involved in the development of both neuropathic pain and opioid-induced tolerance (Mayer et al., 1999). However, the mechanisms and signalling pathways are not yet fully understood. Nonetheless, mTORC1 is a key player in the mechanism governing and regulating neuroplasticity in both conditions (Xu et al., 2014) and malfunction of mTORC1 signalling is associated with a number of human pathological diseases, due to mTORC1, which has a central role in regulating cell growth, proliferation and metabolism, as mentioned previously. The regulation of mTORC1 is accomplished by the integration of several inputs, including those of mitogens, nutrients and energy (Xu et al., 2014). The role of the mTORC1 pathway, in response to energy depletion, is important for elucidating the effect of metformin. Activation of AMPK *via* metformin cause inhibition of mTORC1 in a TSC1/2-dependent manner. However, recent evidence established that metformin acts to inhibit mTORC1 signalling in an AMPK-independent manner *via* suppressing Rag function (Kalender et al., 2010). The ability of Rag GTPases to activate the mTORC1 signalling pathway is based on their capacity to induce translocation of the diffused mTOR from cytoplasm to the perinuclear intracellular compartment that contains its activator Rheb (Sancak et al., 2008). Biguanides administration inhibits the function of

Rag which disperses mTORC1 throughout the cytoplasm, inhibiting mTORC1 signalling *via* the Rag GTPases is independent with regard to AMPK activation (Kalender et al., 2010). Figure 1.20 represents a model of biguanides, metformin and phenformin action on mTORC1.

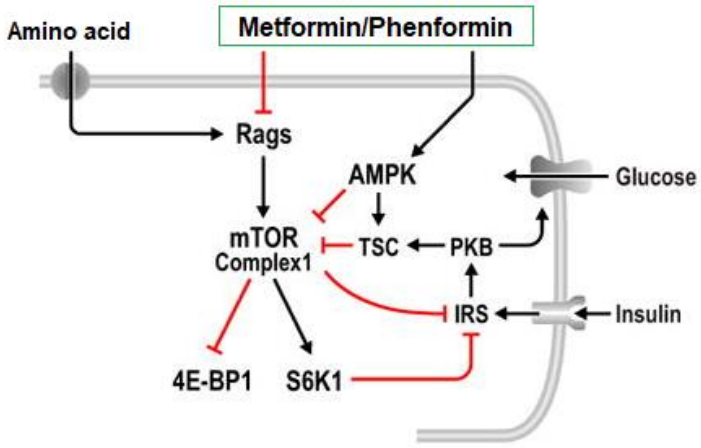


**Figure 1.20: Model for metformin/phenformin action on mTORC1**. The anti-diabetic biguanides, inhibit mTORC1 through indirect AMPK activation by increasing the cellular AMP/ATP ratio leading to phosphorylates TSC2 and activates the TSC. Additionally, by inhibition of Rag GTPases (adapted from Kalender et al., 2010).

From this point, the dual action of biguanides inhibiting the mTORC1 signalling pathway, gives metformin a novel target, concerning opioid efficacy. Consequently, co-administration of metformin with morphine may possibly be a beneficial approach for increasing the clinical use of morphine and other opioids in treating neuropathic pain and attenuating tolerance, following repetitive morphine treatment. Nonetheless, the safety of metformin is even more important in treating non-diabetic patients.

## 1.5 Hypotheses and aims

As identified in the introduction, the management of chronic pain is unsatisfactory and opioid-based treatments need research into understanding the mechanisms underlying the effects of opioids in chronic pain. Therefore, I hypothesised that the mammalian target of rapamycin complex 1 (mTORC1), a kinase which controls protein synthesis, which represents a novel and tractable target for the improvement of opioid analgesic efficacy in chronic neuropathic pain was hypothesised. Specifically, the inhibition of the mTORC1 pathway that may block the establishment and maintenance of morphine-induced tolerance and potentiate the analgesic efficacy of morphine in neuropathic pain was postulated.

Importantly, these effects may be achieved by using the clinically utilised and relatively safe anti-diabetic drug metformin validating a novel and clinically viable avenue for the direct translational application of this strategy in humans. The overall aim of this research was to provide a basis to further explore the role for mTORC1 in the regulation of opioid systems, particularly in reversing opioid-induced side effects.

This study addressed the following aims:

**1:** *to identify whether mTORC1 inhibition leads to alterations in the development and maintenance of morphine-induced tolerance in naïve mice and in animals subjected to neuropathic pain,*

***2:*** *to identify whether mTORC1 inhibition leads to alterations in the analgesic efficacy of morphine in neuropathic mice,*

**3:** *to identify whether mTORC1 inhibition leads to alterations in the regulation of the rewarding and motivational properties of morphine,*

**4:** *to determine mechanisms that drive the additive analgesic benefits on morphine treatment related to the effects produced by mTORC1 inhibition,*

**5:** *to identify the effect of mTORC1 inhibitors on the activity of mTORC1 pathway in spinal cord.*

# Chapter 2. General Methods

This chapter describes the general methods applicable to the *in vivo* and *in vitro* experiments, including the behavioural tests and the molecular biology techniques used within this thesis. More specific details concerning the materials employed in each experiment with the study protocols are described in the methods section of the relevant chapters.

## 2.1 Animals and ethics

All the protocols relating to the *in vivo* experiments reported in this thesis were completed in accordance with the terms and conditions approved by the Animal Welfare and Ethical Review Body (AWERB) of Durham and University , consistent with the guidelines provided by the UK Animals (Scientific Procedures) Act 1986 together with the ARRIVE guidelines under the Home Office. All the experiments were performed in laboratory environments approved by the Life Science Support Unit (LSSU), Durham University and the Comparative Biology Centre (CBC) at Newcastle University. All attempts were made to reduce animal suffering and to reduce the number of animals used in the study.

## 2.2 Subjects

Adult male C57BL/6J mice (8 weeks of age; 20-25 g; Charles River, Harlow, UK) were housed in standard polyethylene cages (2-5 per cage), controlled for temperature (22 ± 2°C) and humidity (55%) under a regular 12-h day/night cycle (lights on at 8:00 A.M.; lights off at 8:00 P.M.). The animals were allowed to acclimatise to the colony room within the Life Science Support Unit, Durham University, UK or the Comparative Biology Centre (CBC) at Newcastle University, for at least 7 days after arrival. Standard laboratory rodent food and water were available *ad libitum*. The animals were habituated to testing procedures for at least 3-4 days, prior to the conducting the experiments. The handling and testing of the animals was conducted during the light phase, between 9:00 A.M. and 16:00 P.M.

## 2.3 Neuropathic pain model

Animal models play a central role in all areas of biomedical research (Franco, 2013). A number of different animal models have been established to model the diverse aetiology and manifestations of neuropathic pain. Models include peripheral nerve injury and central pain neuropathy (Jaggi et al., 2011). These different models broaden our knowledge of the

mechanisms that are involved in neuropathic pain and thus, help to evaluate the potential analgesic effect of novel pharmacotherapies for treating patients (Jaggi et al., 2011). In this study, spared nerve injury (SNI) model of peripheral neuropathic pain was used.

### 2.3.1 Spared nerve injury (SNI)

The SNI model induced by a partial sciatic nerve injury, as described by Decosterd and Woolf (2000). In brief, the surgery was performed under isoflurane inhalation anaesthesia (1-3% isoflurane with oxygen as the carrier gas for maintenance; up to 5% for induction). The mice were carefully monitored under anaesthesia to avoid excessive depression of cardiac and respiratory functions. Body temperature was maintained throughout the procedure by using a battery operated heating pad. Sedation was monitored using a gentle toe pinch withdraw reflex during the surgical procedure. The lateral surface of the left thigh was prepared by shaving the hair with a razor, followed by use of a surgical scrub with alcohol. The skin was then incised, and a section was made directly through the biceps femoris muscle (BFM) exposing the sciatic nerve and its terminal branches, the sural, common peroneal and tibial nerves as depicted in Figure 2.1.

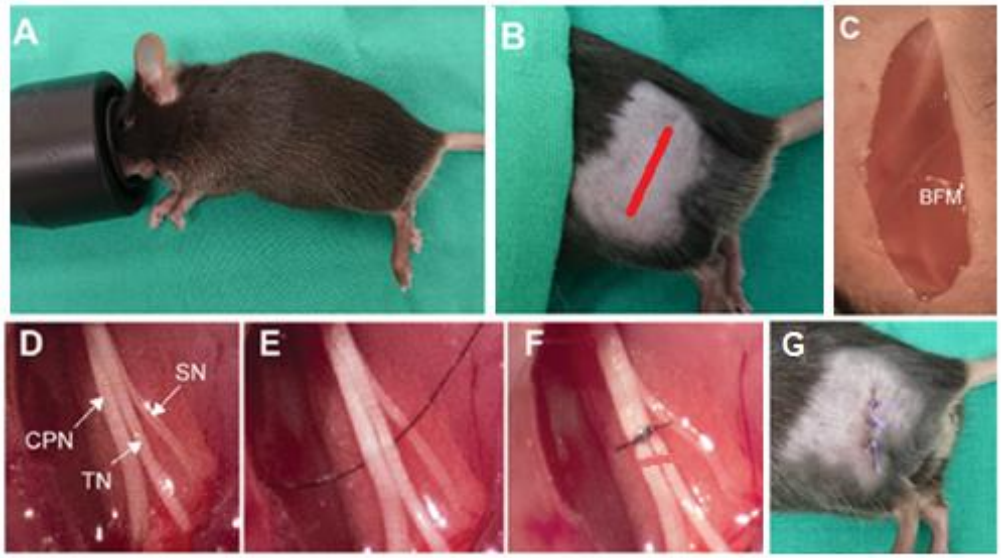


**Figure 2.1: Spared nerve injury surgical procedure to induce neuropathic pain in mice. (A)** The mouse was anesthetised under inhalation anaesthetic *via* nose mask and positioned lying flat. Then the surgical area was shaved and disinfected using alcohol swabs. **(B)** Red line indicates the incision site at the left hind limb of mouse leg. **(C)** A careful, blunt dissection was made through muscle (red line) to expose the trifurcation of the sciatic nerve. **(D)** Showed the exposure of the sciatic nerve and it terminal peripheral branches; tibial nerve (TN), common peroneal nerve (CPN) and sural nerve (SN). **(E)** Following exposure of the nerve a 4.0 silk suture was passed under the tibial nerves and common peroneal. **(F)** Ligation of the common peroneal and tibial nerves was performed *via* a single surgical knot then followed by cutting of the tibial and common peroneal nerves while leaving the sural nerve intact. **(G)** Muscles were reapproximated in the midline with a coated vicryl suture if necessary, then the skin was closed with a 0.6 nylon suture with at least 3-5 individual knots along the incision (adapted from Cichon et al., 2018).

The procedure involved tight-ligation with 4.0 metric silk of the sciatic nerve followed by cutting of the tibial and common peroneal nerves while leaving the sural nerve intact. Moreover, considerable care was taken to avoid any contact with or to stretch the intact sural nerve. The muscle and skin incisions were then sutured with the appropriate suture silk. Following surgery, the mice were moved to a warmed recovery cage and monitored closely until they began to move around individually. The mice were then returned to their routine holding area and monitored.

### 2.4 Behavioural testing

In all experiments, mice were habituated to a plexiglas behaviour chamber under ambient light for 2-3 days before the beginning of the experiment. The experimenter remained blind to the treatment during the testing procedure.

#### 2.4.1 Nociceptive thresholds (tail-flick test)

The tail-flick test, first described in 1941, involves application of a heat stimulus to the rodent's tail. The time taken for the tail to "flick" or twitch is recorded (D'Amour and Smith, 1941). Thus, the basal pain threshold to a thermal stimulus in *naïve* and SNI mice was assessed by tail-flick latency induced by a noxious hot stimulus (radiant heat), as determined with a tail-flick analgesic meter (Analgesia Meter, Ugo Basile, Italy; Figure 2.2).

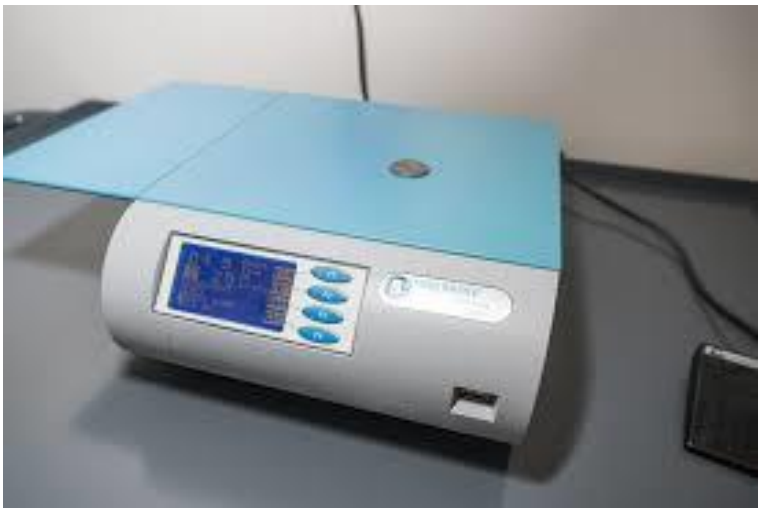

**Figure 2.2: Tail-flick test (radiant heat) apparatus.** Radiant heat is generated from an infrared source (50W bulb). This system can automatically start/stop timing when the mouse tail-flicks. When the mouse feels pain and flicks its tail, a sensor detects it, stops the timer and switches off the bulb. The reaction time of the mouse in s is determined and automatically recorded (Ugo Basile, Italy).

In this test, the dorsal surface; 2 cm from the tip of the mouse tail was exposed to a radiant heat source and the temperature of the beam of light was adjusted to 55 ± 1°C. The mice

were gently wrapped in a soft cloth such that their tails were exposed, and the response was defined as the removal of the tail from the heat source in a second (s). The cut-off time was 9 s to avoid tail skin injury. The test was repeated 2 to 3 times on the same mouse with a minimum resting time between each measurement of 2-3 min.

### 2.4.2 Mechanical stimulation (von Frey test)

Mechanical sensitivity was assessed by measuring the withdrawal threshold of the paw ipsilateral or contralateral to the site of nerve injury in response to mechanical stimuli using von Frey filaments (Stoelting, Wood Dale, IL, USA; Figure 2.3 C). Mice were placed in plastic cubicle cages (10 × 6 × 6 cm; l× w × h) with a metal mesh floor and were allowed to habituate for at least 10-15 min before testing began (Figure 2.3 A). A set of calibrated ascending force of nylon monofilaments, were applied to the lateral surface of the mouse's hind paw through the mesh floor starting with the lowest filament of 0.04 g. Each monofilament was applied five times at an interval of 2-3 s. The threshold was taken as the lowest force that induced a rapid response by the withdrawal of the paw. Filaments ranged from 0.04 to 2 g and were used as described by Sommer and Schäfers, (1998).

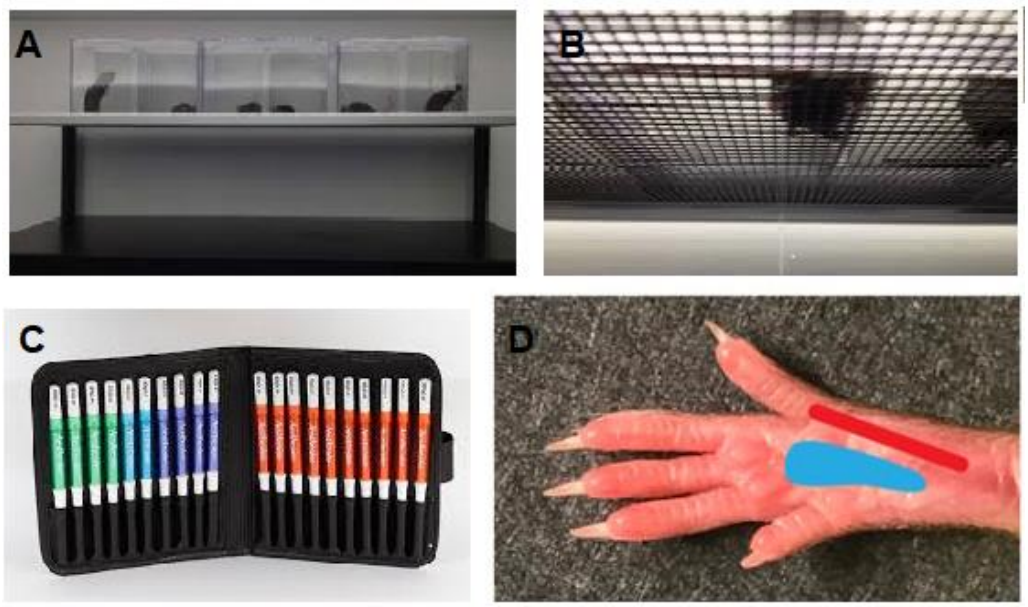


**Figure 2.3: von Frey test. (A)** Mice were placed individually in plexiglass cages on an elevated mesh platform for paw access**. (B)** Metal platform: a mesh-like open grid of square holes 5X5 mm. **(C)** von Frey filaments. Set of monofilaments made of nylon calibrated from 0.04 g to 2 g. **(D)** Plantar view of the left hindlimb paw. The red area in the image corresponds to the sural nerve skin territory that was tested with the von Frey hair, while the blue area corresponds to the tibial nerve skin territory which was denervated from SNI surgery and must not be tested during the test.

### 2.4.3. Cold hypersensitivity (acetone evaporation test)

Acetone evaporation test is a technique first described in 1994 to measure aversive behaviours triggered by evaporative cooling. It is typically considered as a measure of cold allodynia (Carlton et al., 1994; Yoon et al., 1994). A volume of 50µl of acetone was directly applied on to the lateral part of the plantar surface of the hind paw *via* a blunt needle connected to a syringe, without touching the skin and avoiding spraying the acetone (Figure 2.4). Sensitivity to cold was recorded by quantifying the duration of nocifensive responses, as the duration of time that the mouse spent lifting, licking or shaking the hind paw. The cut-off latency for this test was 20 s. Data was collected from the hind paw ipsilateral or contralateral to the nerve injury.

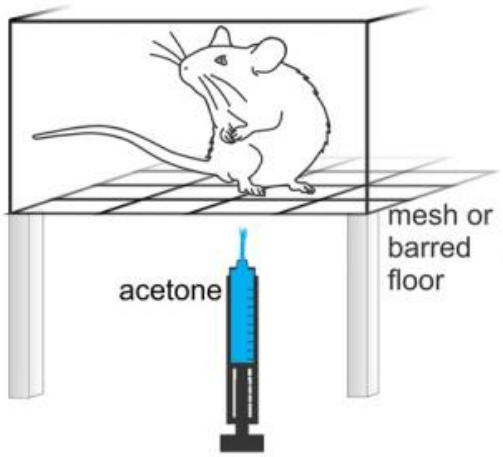


**Figure 2.4: Acetone evaporation test.** The mice were placed individually in plexiglas cages (10 × 6 × 6 cm; l× w × h) on an elevated mesh platform to allow paw access. Acetone is applied in a volume of 50µl to the lateral hind paw *via* a blunt needle. Attempts were made to make a consistent application of acetone for each of the mice (obtained from Deuis et al., 2017).

### 2.4.4 Conditioned place preference (CPP) test

Conditioned place preference (CPP) is a learned behaviour shown in many vertebrates, including humans. Place conditioning is a form of Pavlovian conditioning to evaluate the motivational properties, such as the rewarding and aversive effects of drugs and natural substances. CPP occurs when a subject prefers one place more than others because the preferred location has been paired previously with rewarding events. The CPP paradigm is routinely used to measure the rewarding or aversive motivational effects of objects or to explore the reinforcing effects of natural and pharmacological stimuli, including drugs of abuse. It is a standard preclinical behavioural model used to study the rewarding and psychoactive (affective) properties of drugs in animals (Prus et al., 2009). The basic characteristics of this task involves the association of a particular place with drug treatment, followed by the association of a different place with the absence of the drug (i.e., the drug's

vehicle or placebo). A common CPP apparatus consists of three-compartments. These compartments have a distinctly different sign, typically in texture or colour (Figure 2.5).

### 2.4.4.1 CPP apparatus

As illustrated in Figure 2.5, the CPP apparatus consists of three connected boxes. Two main conditioning chambers had different visual and tactile cues, including wall colour and floor texture and were joined by a smaller middle compartment (neutral chamber) that had no unique characteristics and was not paired with a drug or vehicle. The CPP apparatus was provided with two automatic guillotine sliding doors between the compartments that could be opened to allow mice to pass freely between them. In addition to the sliding doors, the CPP apparatus had lights and infrared photobeam detectors above the chamber floor to automatically record compartment residence times and movements within and between the three compartments.

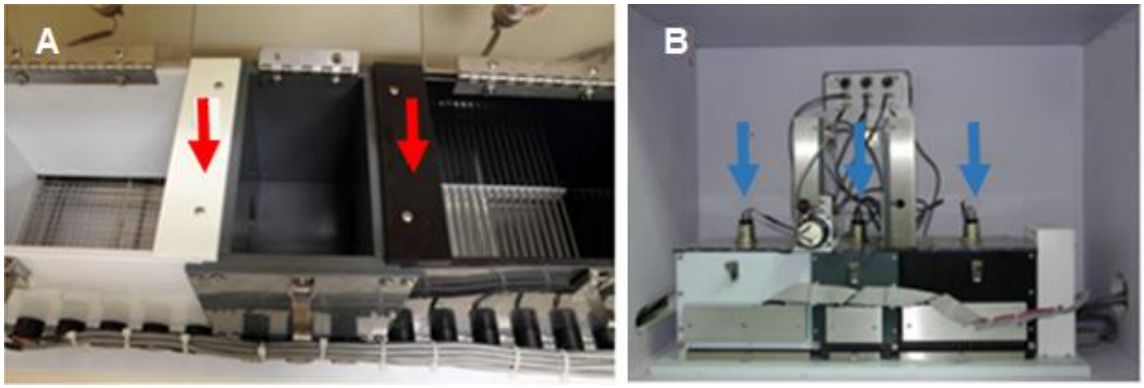


**Figure 2.5: CPP apparatus. (A)** The CPP apparatus consisted of three connected boxes: a central grey one with normal flooring, one on the left, white-walled with a mesh grating as the floor and one on the right, black-walled with steel bars on the floor with special guillotine metal trap doors (red *arrows* in the picture) that could be opened or closed so that mouse was allowed to either explore the entire apparatus or be confined to one of the boxes. **(B)** The CPP apparatus also provided with individual lamps on the top of the transparent surface facing door (blue arrows in the picture) supplied varying light levels.

### 2.4.4.2 Training and testing

The conditioning schedule consisted of three phases: preconditioning habituation (one session), conditioning phase (multiple sessions) and place preference test post conditioning sessions (one session; Figure 2.6).

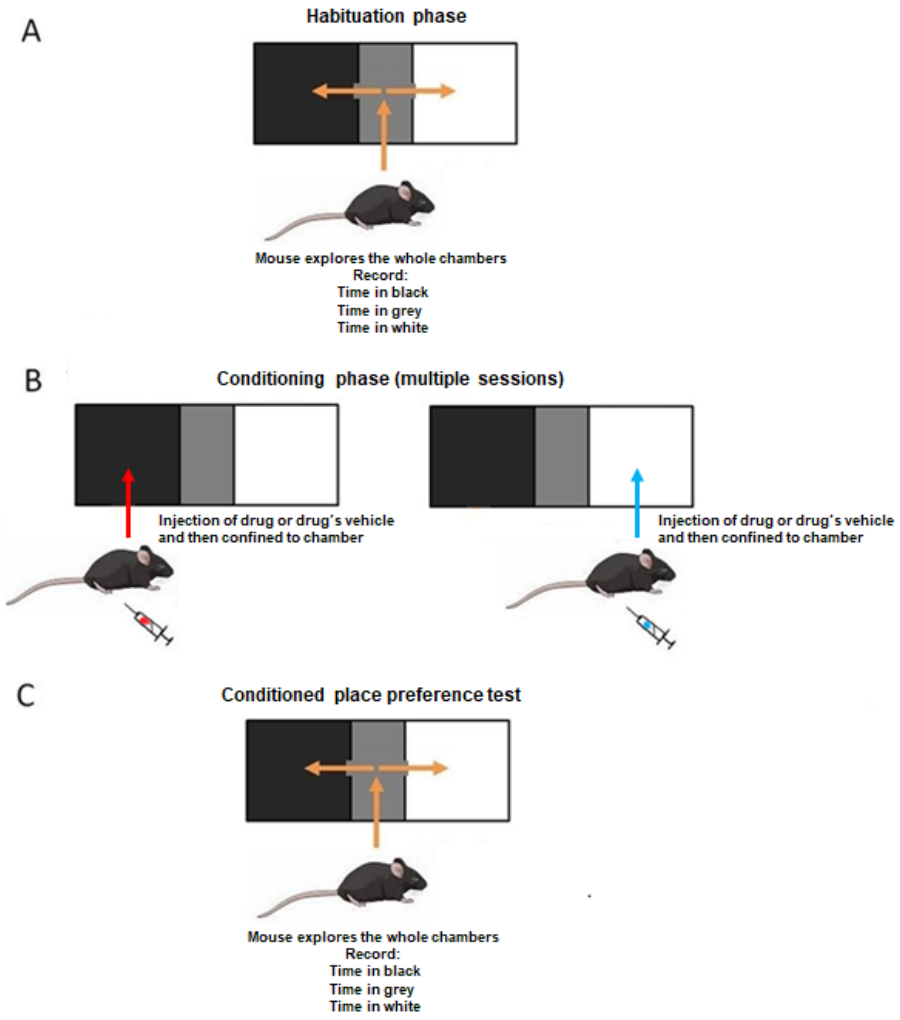


**Figure 2.6: Conditioned place preference paradigm.** In the CPP assay, mice learned the association between a specific context and a rewarding stimulus. It involved three phases. **(A)** Habituation phase: mice, were initially placed in the middle compartment (grey box) and allowed to explore all the apparatus boxes; black, white and the grey for 15 min. **(B)** Conditioning phase: mice were treated with a drug (e.g. a drug with rewarding properties, the unconditioned stimulus) or vehicle. They were always placed in either the black or white box without access to the other boxes. This conditioning phase consisted of 8 consecutive sessions for 45 min, during which drug sessions or vehicle sessions were randomly conducted. Thus, during the conditioning phase, one chamber became associated with the drug's effects and the other chamber associated with the vehicle. **(C)** Place preference test: mice were placed in the middle chamber (grey box) again and allowed to access both the drug-associated and the vehicle-associated chambers for 15 min. The relative amount of time spent in the drug-associated chamber was considered a measure of the drug's reinforcing effects.

*The Preconditioning phase (habituation)*: a mouse was placed in the central compartment (grey box) to reduce the stress and novelty associated with handling for 5 min. After 5 min of acclimatisation, the guillotine metal doors were raised and the mouse was allowed to explore all three interconnected chambers (black, white and grey boxes) for 15 min. The time spent in each chamber was recorded.

*Conditioning phase*: mice were randomly assigned to a black or white test box which served as the drug-paired chamber, i.e. the chamber where the animal received the treatment (e.g., a drug with rewarding properties, unconditioned stimulus). In this phase, the mouse was

forced to stay in the selected chamber for the entire session (45 min). In that way the features of the box (wall colour and floor texture) became associated with the unconditioned stimulus. Thereafter, the mice were returned to their home cages. On alternate days, the mouse would be given an equivalent vehicle solution without drug and placed in the alternative box, vehicle-paired chamber for the same amount of time (45min). One conditioning session with drug occurred each day for 8 days. The drug-paired chambers were assigned in such a way that vehicle and drug groups were counterbalanced and unbiased towards contextual cues.

*Place preference test*: a mouse was placed in the central compartment (grey box) for 5 min, the trap doors were raised, and the mouse was allowed to explore all three chambers of the apparatus (black, white and grey boxes) for 15 min once again. The mouse was conditioned to expect the drug in either the white or black chamber and, given the choice, preferred to spend time in that chamber in anticipation of the drug. The time spent in each chamber was recorded.

## 2.5 Biochemical assays

### 2.5.1 Immunoblotting

Western blotting is an essential technique in cell and molecular biology used to identify and quantify the expression of extracted proteins. Specifically, a complex mixture of proteins is separated based on its molecular weight, and thus by type, *via* gel electrophoresis. These results are subsequently transferred to a specific membrane producing a band for each peptide or protein.

#### 2.5.1.1 Sample preparation

Upon completion of experimental protocols mice were sacrificed by cervical dislocation. In *naïve* mice, the spinal cord segment corresponding to the lumbar area (L4-L6) was rapidly removed and frozen in dry ice (frozen carbon dioxide). In SNI mice, the ipsi- and contra-lateral dorsal horn quadrants (L4-L6) were quickly dissected out and frozen in dry ice. All tissue was stored at -80°C until further processing. For protein extraction, each sample was manually homogenised in 150 µl lysis buffer (1% Np-40, 20 mM Hepes pH7.4, 100 mM NaCl, 100 mM NaF, 1 mM $Na_3VO_4$, 5 mM EDTA with 1× protease inhibitor cocktail (SIGMA); 1× phosphatase inhibitor cocktail I and II (Sigma-Aldrich)) and incubated on ice for 120 min to solubilise proteins and give the highest yield. The homogenised samples were

then clarified by centrifugation at 21,000 rpm for 15 min at 4°C to precipitate the membrane and tissue debris. The supernatant containing most of the soluble proteins was collected and stored at -20°C. Total protein concentration was assessed using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) prior to each preparation of protein samples.

#### 2.5.1.2 Loading and running the gel

After determination of the total protein concentration, tissue extracts (15-30 µg f proteins per well) were loaded with 2x concentrate of loading solution (Sample Buffer, Laemmli 2x; Cat. No. S3401, (Sigma-Aldrich)) to create the loading buffer. The sample was boiled at 95°C for 5 min. This procedure was used to decrease the strength of the protein-protein complex and at the same time to unfold any protein enabling access of the antibody-antigen reaction as the antibodies typically recognise a small portion of the protein of interest. A volume of ~15µl per well of the sample was run on 4-20% Criterion TGX Stain-Free Precast gels (Bio Rad, Hemel Hempstead, UK) in Tris/Glycine/SDS running buffer (25Mm Tris, 192Mm Glycine, 0.1% (w/v) SDS, Ph8.3) (Bio Rad, Hemel Hempstead, UK) at 200 (V) for 30 min using the Bio Rad PowerPac system. Separated proteins were transferred to midi-sized PVDF membranes (Bio Rad Hemel Hempstead, UK) using the Bio Rad Trans-Blot Turbo Transfer system which was run at 2.5 ampere (A) and 25 (V) for 7 min. Membranes were then blocked for 60 min at room temperature using either 4% non-fat milk in TTBS (is a mixture of tris-buffered saline (TBS) and Tween 20(Sigma)) or in 10 mM Tris-HCl pH 5 7.5, 150 mM NaCl, 0.05% Tween 20 (Sigma) with 0.24% I-Block (Applied Biosystems, Paisley, UK) to prevent the nonspecific binding of the antibodies and subsequently the blot was probed with a primary antibody overnight to 48 (h) at 4°C.

#### 2.5.1.3 Antibody labelling

Different primary antibodies were applied in this study to specifically bind the protein of interest as listed in Table 2.1. Membranes were then washed in phosphate-buffered saline (PBS) 3 x 10 min at room temperature and then incubated in host-dependent secondary antibody (anti-mouse or anti-rabbit) horseradish peroxidase (HRP) (goat anti-mouse IgG (H+L)-HRP conjugate; Cat. No. 170-6516, Bio Rad, USA) and (Goat anti-rabbit IgG (H+L)-HRP conjugate; Cat. No. 170-6515, Bio Rad, USA) for 60 min at room temperature. Secondary antibodies were used at dilution of 1:1000 in either 0.24% I-block or 4% non-fat milk. Subsequently, incubation membranes were washed (PBS) 3 x 10 min at room temperature. HRP activity was visualised by applying Bio-Rad Clarity Western ECL

substrate (Clarity western lumiol/enhancer solution and peroxide solution at ratio of 1:1, Hemel Hempstead, UK) and using ChemiDoc MP (Biorad Hemel Hempstead, UK) imaging system. Membranes were then washed and incubated with GAPDH antibody overnight at 4˚C and further processed as described above.

**Table 2.1: Western blotting primary antibodies used in the experiments.**

| Antibody | Source | Molecular Wt. (kDa) | Dilution | Purification | Catalogue number | Supplier | Incubation |
|---|---|---|---|---|---|---|---|
| mTOR | Rabbit | 289 | 1:500 | Polyclonal | 2972 | Cell signalling Technology (Beverly, MA, USA) | 48 h 4°C |
| Phospho – mTOR (Ser2448) | Rabbit | 289 | 1:500 | Polyclonal | 2971 | Cell signalling Technology (Beverly, MA, USA) | 48 h 4°C |
| p70 S6 Kinase | Rabbit | 70 to 85 | 1:500 | Polyclonal | 9202 | Cell signalling Technology (Beverly, MA, USA) | 48 h 4°C |
| Phospho-p70 S6 Kinase (Thr389) | Rabbit | 70 to 85 | 1:500 | Monoclonal | 9234 | Cell signalling Technology (Beverly, MA, USA) | 48 h 4°C |
| S6 Ribosomal protein (5G10) | Rabbit | 32 | 1:500 | Monoclonal | 2217 | Cell signalling Technology (Beverly, MA, USA) | 48 h 4°C |
| Phospho - S6 Ribosomal protein (Ser235/236) | Rabbit | 32 | 1:500 | Polyclonal | 2211 | Cell signalling Technology (Beverly, MA, USA) | 48 h 4°C |
| 4EBP1 | Rabbit | 15 to 20 | 1:500 | Polyclonal | 9452 | Cell signalling Technology (Beverly, MA, USA) | 48 h 4°C |
| Phospho - 4EP1 (Thr37/46) | Rabbit | 15 to 20 | 1:500 | Polyclonal | 9459 | Cell signalling Technology (Beverly, MA, USA) | 48 h 4°C |
| GAPDH (Loading control) | Mouse | 37 | 1:1000 | Monoclonal | AB2302 | EMD Millipore (Burlington, MA, USA) | 24 h 4°C |

### 2.5.2 Immunohistochemistry

Immunohistochemistry is a powerful method from sample preparation (fixation) to sample labelling for visualising cellular components. It is based on the principle of antibodies binding specifically to antigens in biological tissues. The Fixation aims to preserve tissue in a life-like state by directly perfusing fixative chemical such as (4 % paraformaldehyde) through the natural vascular system of a deeply anaesthetised animal (Gage et al., 2012).

Perfusion fixation is the optimal method of tissue preservation before autolysis begins (Gage et al., 2012).

#### 2.5.2.1 Sample preparation

Mice were deeply anaesthetised with sodium pentobarbital (100 mg/kg, i.p.) prior to beginning the transcardial perfusion and the withdrawal reflex in each pelvic limb was checked. The animal was then placed on its back on a shallow tray filled with crushed ice. A midline incision was made through the integument and abdominal wall just underneath the rib cage. The sternum was lifted away, and any tissue connected to the heart was trimmed. Next, a small incision was made in the apex of the left ventricle to insert the needle toward the aorta. Similarly, the right atrium was cut to allow the perfusate to exit the circulation. The mouse was perfused firstly with 30 ml of ice-cold 0.9% saline (0.9% NaCl; Fresenius Kabi Ltd, UK) containing 5000IU/mL heparin (5000IU Heparin Sodium; Leo Pharmaceuticals Products, Denmark) until the saline flushed out all the mouse's blood. Saline perfusion was followed by ice-cold 4% paraformaldehyde (Sigma-Aldrich) in a 0.1M phosphate buffer (PB), pH 7.4 (Sigma; St Louis, MO) containing 0.05M sodium fluoride (~200mL per mouse) (Sigma-Aldrich) *via* a peristaltic pump for 3 min to allow complete fixation of the tissue. The entire segment of the spinal cord was dissected from the body and immediately placed in ice-cold fixative without exposing the spinal cord. The tissue was allowed a further 2-4 h of postfixation at 4°C and subsequently transferred to 30% sucrose (Sigma-Aldrich) solution in PB containing 0.01% sodium azide (Sigma-Aldrich). The lumbar (L4–L6) spinal cord was carefully isolated from the vertebral column (Figure 2.7). To prepare cryostat sections, tissue was first embedded in TissueTek (Sakura) prior to freezing and transverse sections of 40 µm thickness were cut at -20°C using the CryoJane Tape-Transfer System (Leica Microsystems) on a Leica CM1900 cryostat and using low-profile disposable blades 819 from Leica Biosystems). Samples were collected as free floating sections in a 6-well plate and directly used for staining or stored at 2-8°C in 5% (w/v) sucrose solution until time of use (Obara et al., 2011; Obara et al., 2015).

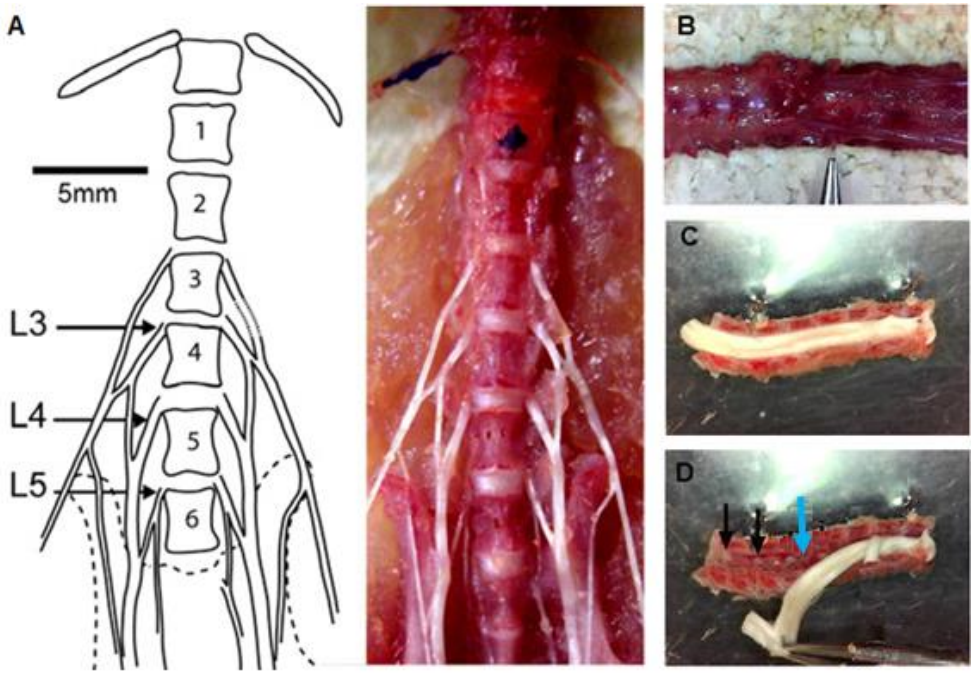


**Figure 2.7: Mouse spinal cord exposure. (A)** C57BL/6J spinal cord anatomy. **(B)** Muscles, fat and other soft tissues were cut from the spinal column using curved scissors. **(C)** Spinal cord was carfefully exposed using forceps and small scissors. **(D)** Then the spinal cord was gently peeled from the column (the black *arrows* show a part of the spinal cord that can be touched with tweezers and removed, while the blue arrow indicates the area of interest) (adapted from Rigaud et al., 2008 and Sleigh et al., 2016).

### 2.5.2.2 Labelling protocols

Sections of lumbar (L4–L6) spinal cord were blocked in PB saline containing 0.2% Triton X-100 and 5% normal goat serum for 1h at room temperature. Sections were then left to incubate with primary antibodies for 3 days at 4°C (Table 2-1). A tyramide signalling amplification (TSA) based protocol was used to amplify the P-mTOR and P-S6 ribosomal protein signals (Obara et al., 2011; Obara et al., 2015). Briefly, appropriate biotinylated secondary antibodies were used at a concentration of 1:400 for 90 min. Sections were then incubated with avidin-biotin complex (ABC Elite, Vector Laboratories, Burlingame, CA) (1:250 Vectastain A+1:250 Vectastain B) for 30 min followed by a signal amplification step with biotinylated tyramide solution (1:75 for 7 min; PerkinElmer, Boston, MA). Finally, samples were incubated with FITC-avidin (1:600 for 120 min; Vector Laboratories, Burlingame, CA). The sections were then reprobed with a second primary antibody to determine cellular colocalisation. The appropriate directly labelled secondary antibody (Alexafluro 594 goat anti-rabbit; Cat. No. A-11037, Thermo Fisher Scientific Life Technologies, UK) was applied at a concentration of 1:500 and incubated in darkness for 120 min. All sections were mounted on slides and coverslipped with Gel Mount Aqueous Mounting Medium (Sigma-Aldrich) to prevent photobleaching and preserve samples for long-term storage. Slides were stored in dark boxes at 4°C. Tables 2.2 and 2.3 highlight all antibodies used in the experiments. Table 2.2 comprises a list of primary antibodies, while

Table 2.3 summarises secondary antibodies employed for colocalisation in immunofluorescence studies.

**Table 2.2: Primary antibodies used in immunohistochemistry experiments.**

| Antibody | Source | Dilution | Purification | Catalogue number | Supplier | Incubation |
|---|---|---|---|---|---|---|
| Phospho - S6 Ribosomal protein (Ser235/236) | Rabbit | 1:1000 | Polyclonal | 2211 | Cell Signalling Technology (Beverly, MA, USA) | 24 h 4°C |
| Phospho – mTOR (Ser2448) | Rabbit | 1:1000 | Polyclonal | 2971 | Cell Signalling Technology (Beverly, MA, USA) | 24 h 4°C |

**Table 2.3: Second primary antibodies used in immunohistochemistry experiments.**

| Antibody | Source | Dilution | Purification | Catalogue number | Supplier | Incubation |
|---|---|---|---|---|---|---|
| Anti-Glial fibrillary acidic protein (GFAP) | Rabbit | 1:1000 | Polyclonal | NB300-141 | Novus Biologicals (Littleton, CO, USA) | 72 h 4°C |
| Anti-NeuN | Rabbit | 1:500 | Polyclonal | ABN78 | EMD Millipore (Burlington, MA, USA) | 72 h 4°C |
| Anti- Ionized calcium binding adaptor molecule 1 ( Iba-1) | Rabbit | 1:1000 | Polyclonal | 019-19741 | Wako Chemicals (Düsseldorf, Germany) | 72 h 4°C |

# Chapter 3. The effect of the mammalian target of rapamycin complex 1 (mTORC1) inhibitors on the development and maintenance of morphine tolerance in *naïve* mice

## 3.1 Introduction

Opioids are considered the most effective treatment for acute pain. Unfortunately, opioid use is limited because of the associated side-effects that can occur when opioids are used long-term or in high doses (Collett, 1998). Examples of typical side-effects include respiratory depression, sedation and constipation (Collett, 1998). A more serious concern with opioid use is the development of pharmacological tolerance, addiction and dependence which further limits the clinical use of these drugs (Collett, 1998). Opioid analgesic tolerance is characterised by a decrease in analgesic effectiveness of opioid drug (Dumas and Pollack, 2008). Specifically, long-term use of opioid, e.g., chronic morphine treatment, will typically result in the need to use higher doses to achieve adequate pain relief.

Growing evidence shows that the neurobiological mechanisms of opioid-induced analgesic tolerance associated with adaptive neuroplastic changes in protein translation in the nervous system are thought to promote opioid tolerance (Xu et al., 2014). However, morphine-induced analgesic tolerance observed in patients is still ineffectively managed by available drugs, in part because these therapeutic options target a single mechanism or produce several side-effects (Xu et al., 2014; Simpson and Jackson, 2016).

As mentioned in Chapter 1, mTORC1 governs most protein translation (Wang and Proud, 2006) which is activated after repeated or prolonged morphine administration (Xu et al., 2014). Additionally, it was reported that mTORC1 activation was triggered *via* the μ-opioid receptor and mediated by intracellular PI3K/Akt signalling pathway (Jiang et al., 2016). Moreover, mTORC1 and its downstream effectors, p70S6K and 4EBP1, were expressed in the mammalian nervous system, particularly in the spinal cord dorsal horn in the cytoplasm of neurones as well as in the peripheral axons that are critical for pain transmission (Géranton et al., 2009). Thus, this cellular localisation of the mTORC1 pathway further supports potential involvement of this kinase in the regulation of opioid-induced analgesic tolerance.

It was therefore hypothesised that inhibition of mTORC1 signalling pathway may lead to inhibition of the development and maintenance of morphine-induced analgesic tolerance, besides this strategy may restore the analgesic effectiveness of morphine in *naïve* mice. This

study employed the anti-diabetic biguanide metformin which was shown to inhibit the mTORC1 pathway (Melemedjian et al., 2011), as well as a selective and direct mTORC1 inhibitor CCI-779. These drugs were injected systemically (i.p.) prior to morphine administration, in order to assess the role of mTORC1 in the development and maintenance of morphine tolerance and moreover, to determine whether acute i.p. administration of metformin and CCI-779 can counteract the loss of analgesic potency of morphine in morphine tolerant *naïve* mice.

## 3.2 Material and methods

### 3.2.1 Subject

Adult male C57BL/6J mice (8 weeks of age, Charles River, UK) weighing 25-30 g at the beginning of the study were used. Mice were housed 4 per polyethylene cage on sawdust bedding under standard conditions (12 h light/dark cycle, lights on from 8:00 am) with food and water available *ad libitum*. Experimental protocols were approved by the AWERB Committee at Durham and Newcastle University and were consistent with the guidelines provided by the UK Animals (Scientific Procedures) Act 1986. For more details see Chapter 2.

### 3.2.2 Preparation and administration of drugs

***Morphine***

To induce analgesic tolerance, morphine (morphine sulphate salt pentahydrate; Sigma-Aldrich, UK) was prepared in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK) immediately prior to injection. Mice were weighed and then injected intraperitoneally (i.p.) with morphine (20 mg per kg body weight) or equivalent vehicle (saline) solution without morphine, as a control group, twice daily for 9 consecutive days, in a volume of 4ml per kg body weight.

***Metformin***

For systemic (i.p.) administration, metformin (metformin hydrochloride; Cat. No. 2864; Tocris Bioscience, UK) was prepared in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK) immediately prior injections. Mice were weighed and then injected i.p. with metformin (200 mg per kg body weight) or equivalent vehicle (saline) solution without metformin as a control group. Metformin/vehicle was administered i.p. 20 h before i.p. first injection of morphine was given and then i.p. administration of metformin/vehicle was continued for 8

consecutive days. The timing and concentration of metformin injections were based on previously published research using metformin (Melemedjian et al., 2011; Obara et al., 2015).

***CCI-779***

For systemic (i.p.) administration, CCI-779 (temsirolimus; Cat. No. T-8040; LC Laboratories, USA) was prepared in pure ethanol as a stock solution at 60mg/mL on the day of experiment and diluted to 2.5 mg/mL in 0.15M NaCl, 5% polyethylene glycol 400, 5% Tween 20 (Ravikumar et al., 2004; Obara et al., 2015) immediately before injection. Mice were weighed and then injected intraperitoneally (i.p.) with a 1% v/w solution of CCI-779 (25 mg per kg body weight) or the equivalent vehicle (0.15M NaCl, 5% polyethylene glycol 400, 5% Tween 20) solution without CCI-779 as a control group. CCI-779/vehicle was injected 20 h before the first i.p. injection of morphine was given and then i.p. administration of CCI-779/vehicle was continued for 8 consecutive days. The timing and concentration of CCI-779 injections were based on previously published research using CCI-779 (Obara et al., 2011; Obara, 2015).

### 3.2.3 Nociceptive threshold in *naïve* mice

The pain threshold to a thermal stimulus in *naïve* mice was assessed by tail-flick latency (Analgesia Meter, Ugo Basile, Italy) as described in detail in Chapter 2. Briefly, tail-flick latency to noxious heat was determined by applying a heat stimulus to the dorsal tail surface approximately 2 cm from the tip of the tail. The cut off time for the tail-flick latency was set to 9 s. Mice were tested for their baseline latencies before first i.p. administration of morphine and then on the morning of each testing day, they were tested for analgesia 30 min after i.p. morphine administration. Mice were also monitored daily for any signs of heat-induced skin lesions resulting from repeated tail-flick testing. No animal developed injuries that may affect their nociceptive threshold to tail-flick stimulation.

### 3.2.4 Experimental design

To establish the role of mTORC1 inhibition on morphine-induced tolerance in *naïve* mice, two experiments were conducted:

*Experiment I*: On day 0 (Table 3.1, Figure 3.1) *naïve* mice were injected i.p. with metformin or CCI-779 20 h prior i.p. administration of morphine and then the injections were repeated

for 8 consecutive days in order to assess the role of mTORC1 in the development and maintenance of morphine analgesic tolerance.

*Experiment II*: On day 7 (Table 3.1, Figure 3.1) *naïve* morphine tolerant mice were injected i.p. with metformin or CCI-779 20 h prior i.p. administration of morphine to determine whether a single injection of mTORC1 inhibitors influenced and restored the morphine analgesic effect in morphine tolerant mice. The i.p. injection of mTORC1 inhibitors was also repeated on day 8 to determine whether any following injection of mTORC1 inhibitors potentiated the effect resulting from the first injection.

Table 3.1 and Figure 3.1 below summarises the experimental design of both experiments.

**Table 3.1: A summary of experiments I and II illustrating schedule of chronic and acute administration of both mTORC1 inhibitors.**

| Days | **Experiment I** | | **Experiment II** | |
|---|---|---|---|---|
| | Chronic metformin or CCI-779 | | Acute metformin or CCI-779 | |
| | Morning | Evening | Morning | Evening |
| -1 | TF | | TF | |
| 0 | TF → D | | TF | |
| 1 | TF → M → TF → D | M | TF → M → TF | M |
| 2 | M → TF → D | M | M → TF | M |
| 3 | M → TF → D | M | M → TF | M |
| 4 | M → TF → D | M | M → TF | M |
| 5 | M → TF → D | M | M → TF | M |
| 6 | M → TF → D | M | M → TF | M |
| 7 | M → TF → D | M | M → TF → D | M |
| 8 | M → TF → D | M | M → TF → D | M |
| 9 | M → TF | Tissue collection | M → TF | Tissue collection |

**TF**: tail-flick test
**D**: drug; metformin (200mg/kg, i.p.) or CCI-779 (25mg/kg, i.p.)
**M**: morphine (20mg/kg, i.p.)
**Chronic administration**: metformin (200mg/kg, i.p.) or CCI-779 (25mg/kg, i.p.) injected 20 h before first morning morphine i.p. injection; then repeated for 8 consecutive days
**Acute administration**: metformin (200mg/kg, i.p.) or CCI-779 (25mg/kg, i.p.) injected on day 7 20 h before morning morphine i.p. injection on day 8. Also, another systemic injection of metformin (200mg/kg, i.p.) or CCI-779 (25mg/kg, i.p.) was injected on day 8 20 h prior to the morning morphine i.p. injection on day 9. After completion of chronic and acute administrations, the mice were sacrificed for tissue collection (spinal cord) to determine changes in the activity of the mTORC1 pathway.

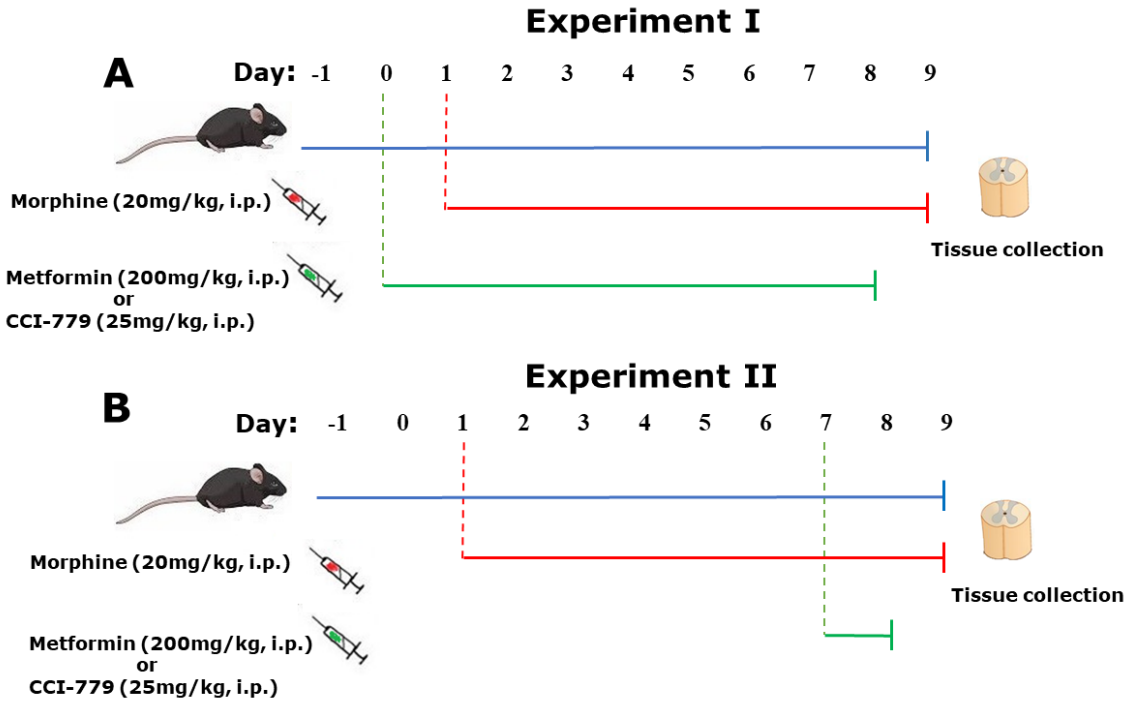


**Figure 3.1: A schematic illustration of experiment I and II. (A; experiment I)** Represents the chronic administration of metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) schedule where these drugs were injected 20 h before first morphine i.p. injection and then repeated subsequently for 8 consecutive days (once daily after morning morphine injection). **(B; experiment II)** Represents the acute administration of metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) schedule where these drugs were injected into morphine tolerant mice on day 7 (first injection) and day 8 (second injection) 20 h before morning morphine i.p. injection on day 8 and day 9. The blue line represents pain assessment using the tail-flick test. The red line represents morning and evening injections of morphine (20 mg/kg, i.p.). The green line represents i.p. mTORC1 inhibitors administrations. The final step involved tissue collection for biochemical analysis.

### 3.2.4.1 Design of experiment I

*Naïve* mice were divided into 5 different experimental groups as indicated in Table 3.2. Group 1 (n = 12) consisted mice receiving morphine (20 mg/kg, i.p.) twice daily at 12 h intervals (8 am/8 pm) for 9 consecutive days (day1-9 in Table 3.1), group 2 (n= 6) received saline under identical conditions and served as controls. To determine the influence of mTORC1 inhibitors on morphine analgesic effects, mice were injected with metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) 20 h prior the morning morphine (20 mg/kg, i.p.) on each testing day (day 0–8; group 3, n= 5-6). To determine the effect of mTORC1 inhibitors without morphine, mice were injected i.p. with metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) 20 h prior to the morning saline i.p. injection for 8 following days (group 4, n= 6). Finally, in group 5 (n= 6), mice were injected i.p. with an equivalent vehicle solution without mTORC1 inhibitors (controls).

**Table 3.2: Design of experiment I.**

| Group | Morphine (20mg/kg, i.p.) | Saline (i.p.) | Chronic administration of mTORC1 inhibitors | | Vehicle (i.p.) | Number of animals |
|---|---|---|---|---|---|---|
| | | | Metformin (200mg/kg, i.p.) | CCI-779 (25mg/kg, i.p.) | | |
| 1 | + | | | | | 12 |
| 2 | | + | | | | 6 |
| 3 | + | | + | | | 5-6 |
| 4 | | + | + | | | 6 |
| 5 | | | | | + | 6 |

*Naïve* mice were randomly assigned into 5 groups and received different treatment: (1) i.p. morphine twice daily; morning injections were given between 9:00 am and 10:00 am, and evening injections were given between 8:00 pm and 9:00 pm; (2) controls; i.p. saline twice daily between 9:00 am and 10:00 am and between 8:00 pm and 9:00 pm; (3) morphine + mTORC1 inhibitors; morphine twice daily (as above) followed by i.p. mTORC1 inhibitors (given between 1:00 pm and 2:00 pm) and then morphine evening injection;.(4) i.p. mTORC1 inhibitors were given once daily (as above);(5) controls; i.p. vehicle once daily between 1:00 pm and 2:00 pm.

### 3.2.4.2 Design of experiment II

*Naïve* mice were divided into 5 different experimental groups as depicted in Table 3.3. A paradigm to induce morphine analgesic tolerance was identical as mentioned above. Briefly, morphine at a dose of 20 mg/kg was administered i.p. every 12 h (group 1, n = 12). Control mice (group 2, n = 6) were injected with saline under identical conditions. Mice were rendered tolerant to morphine after 6 days of treatment. On day 7, mice were injected with metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) (group 3, n= 6) 20 h prior to the morning morphine i.p. injection on day 8. The injection was repeated 20 h after (on day 8). For testing of the action of mTORC1 inhibitors without morphine, the control mice were injected i.p. with metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) (group 4, n= 6) 20 h prior to the morning saline i.p. injection on day 8 and day 9. In group 5 (n= 6), mice were injected i.p. with equivalent vehicle solution without mTORC1 inhibitors (controls).

**Table 3.3: Design of experiment II.**

| Group | Morphine (20mg/kg, i.p.) | Saline (i.p.) | Acute administration of mTORC1 inhibitors | | Vehicle (i.p.) | Number of animals |
|---|---|---|---|---|---|---|
| | | | Metformin (200mg/kg, i.p.) | CCI-779 (25mg/kg, i.p.) | | |
| 1 | + | | | | | 12 |
| 2 | | + | | | | 6 |
| 3 | + | | + | | | 6 |
| 4 | | + | + | | | 6 |
| 5 | | | | | + | 6 |

*Naïve* mice were randomly assigned into 5 groups and received different treatment: (1) i.p. morphine twice daily; morning injections were given between 9:00 am and 10:00 am, and evening injections were given between 8:00 pm and 9:00 pm; (2) controls; i.p. saline twice daily between 9:00 am and 10:00 am and between 8:00 pm and 9:00 pm; (3) morphine + mTORC1 inhibitors; morphine twice daily (as above) followed by i.p. mTORC1 inhibitors(given between 1:00 pm and 2:00 pm) on day 7 and day 8, and then morphine evening injection; (4) i.p. mTORC1 inhibitors were given once daily on day 7 and day 8; (5) controls; i.p. vehicle once daily between 1:00 pm and 2:00 pm.

### 3.2.5 Statistical data analysis

Data analysis and statistical comparisons were performed using GraphPadPrism version 8.01 for Windows (GraphPad Software, CA, www.graphpad.com). Tail-flick latencies were expressed in seconds and presented as the means ± SEM. Each group included 5 to 12 mice. Comparisons between groups were performed using analysis of variance for ordinary measurements (two-way ANOVA) followed by Bonferroni's multiple comparison post-hoc test and Student's t-test, used when two groups were compared. A value of $P<0.05$ was considered statistically significant.

## 3.3 Results

### 3.3.1 The mammalian target of rapamycin complex 1 (mTORC1) inhibitors blocked the development and maintenance of morphine analgesic tolerance in *naïve* mice

As illustrated in Figure 3.2, administration of morphine (20 mg/kg, i.p.) to *naïve* mice produced a significant and potent analgesic effect on the first day of morphine treatment when compared to saline injected mice (day 1: 8.81 ± 0.14 s *vs.* 2.91 ± 1 0.19 s). For the next 5 days of morphine administration (20 mg/kg, i.p.; twice daily), morphine consistently produced a significant analgesia effect when compared to saline-injected mice, although the effect decreased gradually on the following days of chronic morphine administration. On day 7, morphine antinociceptive effect significantly decreased when compared to its efficacy on day 1 (day 7: 4.83 ± 0.29 s *vs.* day 1: 8.81 ± 0.14 s) and there was no significant difference in comparison to saline treated mice (day 7: 4.83 ± 0.29 s *vs.* 3.621 ± 0.28 s, respectively), indicating the development of morphine-induced analgesic tolerance. Morphine-induced analgesic tolerance was observed until day 9 (treatment: $F_{(1,124)}= 192.50$, $P<0.0001$).

Interestingly, administration of metformin (200 mg/kg, i.p., once daily) 20 h before morphine administration significantly prevented the development and maintenance of morphine analgesic tolerance as measured 30 min after morning morphine administration by means of the tail-flick latency in *naïve* mice (Figure 3.3 A; treatment effect: $F_{(3,269)}= 352.00$, $P<0.0001$). Statistically a significant difference in response to tail-flick stimulation between the morphine-treated group and mice administered with metformin prior to morphine was observed on day 4 of the experiment (day 4: 7.32 ± 0.41 s *vs.* 8.45 ± 0.41 s,). This positive influence of metformin on the analgesic effect of morphine continued throughout the tested days. Results were as follows: morphine + metformin *vs.* morphine day 5: 8.74 ± 0.17 s *vs.* 5.77 ± 0.49 s; day 6: 7.68 ± 0.60 s *vs.* 5.21 ± 0.39 s; day 7: 7.68 ± 0.60 *vs.* 4.83 ± 0.29 s; day

8: 7.90 ± 0.56 s *vs.* 4.20 ± 0.36 s; day 9: 7.69 ± 0.50 s *vs.* 4.06 ± 0.16 s. Similarly to the effect produced by metformin, also repeated administration of CCI-779 (25 mg/kg, i.p., once daily) 20 h prior to morphine injection displayed higher latency compared to the morphine treated group (Figure 3.3 B; treatment effect: $F_{(4,322)}$= 455.10, P<0.0001). Specifically, the tail-flick latencies measured on days 4-9 were statistically significant when compared with latencies after morphine was treated alone. Results were as follows: morphine + CCI-779 *vs.* morphine day 4: 8.85 ± 0.09 s *vs.* 7.25 ± 0.36 s; day 5: 8.20 ± 0.29 s *vs.* 6.13 ± 0.14 s; day 6: 8.50 ± 0.29 *vs.* 5.45 ± 0.39 s; day 7: 8.13 ± 0.27 s *vs.* 5.37 ± 0.20 s; day 8: 7.91 ± 0.29 s *vs.* 4.88 ± 0.41 s; day 9: 8.04 ± 0.39 s *vs.* 4.93 ± 0.24 s. In addition, statistical analysis revealed a significant interaction between morphine and mTORC1inhibitors (morphine x metformin: $F_{(30,269)}$= 13.60, P< 0.0001; morphine x CCI-779: $F_{(40,322)}$= 17.51, P< 0.0001 ).

Control animals injected with vehicle or saline did not show any significant change in their response to tail-flick stimulation throughout the 9 days of testing (Figure 3.3). In addition, metformin (200 mg/kg, i.p., once daily; Figure 3.3 A) or CCI-779 (25 mg/kg, i.p., once daily; Figure 3.3 B) administered alone for 9 consecutive days had no significant effect on tail-flick latency when compared to the latency values of the vehicle/saline-treated group (P>0.05).

### 3.3.2 The mammalian target of rapamycin complex 1 (mTORC1) inhibitors restored the analgesic effect of morphine in *naïve* mice

As illustrated in Figure 3.4, administration of two consecutive doses of metformin (200 mg/kg, i.p., once daily; Figure 3.4 A) or CCI-779 (25 mg/kg, i.p., once daily; Figure 3.4 B) on day 7 and then on day 8 of morphine treatment (when tolerance to the analgesic effect of morphine was established) resulted in fully restoring morphine's analgesic effectiveness. Specifically, after 6 days of morphine administration (20 mg/kg, i.p., twice daily) mice were tolerant to the morphine analgesic effect as measured by the tail-flick test (day 1: 8.81 ± 0.14 s *vs.* day 6: 5.45 ± 0.39 s). On day 7 of morphine treatment, a single injection of metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) 20 h prior to morphine injection on day 8, fully restored morphine analgesic efficiency (Figure 3.4). The tail-flick latencies measured 30 min after morphine injection on day 8, preceded by administration of metformin and CCI-779, significantly increased when compared to the morphine treated group (morphine + metformin *vs.* morphine, day 8: 7.54 ± 0.49 s *vs.* 4.20 ± 0.36 s, treatment effect: $t_{(10)}$= 5.42, P<0.05) and (morphine + CCI-779 *vs.* morphine, day 8: 7.01 ± 0.89 *vs.* 4.88 ± 0.41 s, treatment: $t_{(9)}$= 2.51, P<0.05). Additional systemic injection of metformin or CCI-779 on

day 8, prior to morphine injection on day 9, resulted in the maintenance of the analgesic effect of morphine (morphine + metformin *vs.* morphine, day 9: 6.86 ± 0.87 s *vs.* 4.06 ± 0.16 s, treatment effect $t_{(10)}$= 3.18, P<0.05) and (morphine + CCI-779 *vs.* morphine, day 9: 7.85 ± 0.39 *vs.* 4.93 ± 0.24 s, $t_{(9)}$= 6.62, P<0.05).

None of the administered mTORC1 inhibitors alone showed antinociceptive properties as the tail-flick latency did not vary significantly from the respective control values in mice chronically injected with saline/vehicle (P>0.05).

### 3.3.3 The mammalian target of rapamycin complex 1 (mTORC1) inhibitors in combination with morphine induced body weight loss in *naïve* mice

Repeated administration of morphine (20mg/kg, i.p., twice daily) for 9 consecutive days induced body weight loss when compared to the values measured on day 0 in both experiments (Figure 3.5 A and B). The statistically significant difference in body weight was recorded on day 2 in *naïve* mice co-treated with morphine and metformin (200 mg/kg, i.p., once daily) or CCI-779 (25 mg/kg, i.p., once daily) when compared with saline treated animals (treatment effect for metformin + morphine: $F_{(3,248)}$= 30.74, P<0.0001; treatment effect for CCI-779 + morphine: $F_{(4,303)}$= 22.19, P<0.0001). Administration of metformin (200 mg/kg, i.p., once daily), CCI-779 (25 mg/kg, i.p., once daily) or vehicle did not affect the mouse's body weight when compared with saline treated animals (P>0.05).

It should be noted that despite the treatment having some effect on the animal's body weight, all animals subjected to experimental testing remained healthy and did not show any signs of unexpected distress.

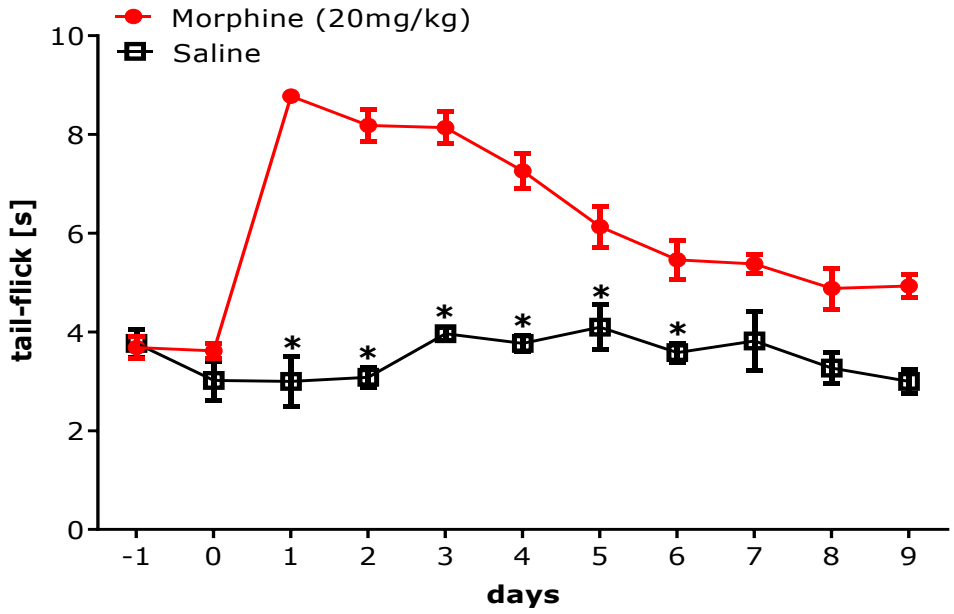


**Figure.3.2:.Repeated administration of morphine resulted in analgesic tolerance in *naïve* mice.** Administration of morphine (20 mg/kg, i.p., twice daily) produced a strong analgesic effect on day 1 followed by a gradual decrease in the analgesic effect over the following days, as measured by the tail-flick test. On day 7, mice developed tolerance to the analgesic effect of morphine as indicated by a lack of significant difference to saline controls. The data are presented as mean ± SEM, n= 6-12; **vs.* morphine; *P<0.05 (two-way ANOVA followed by Bonferroni's test).

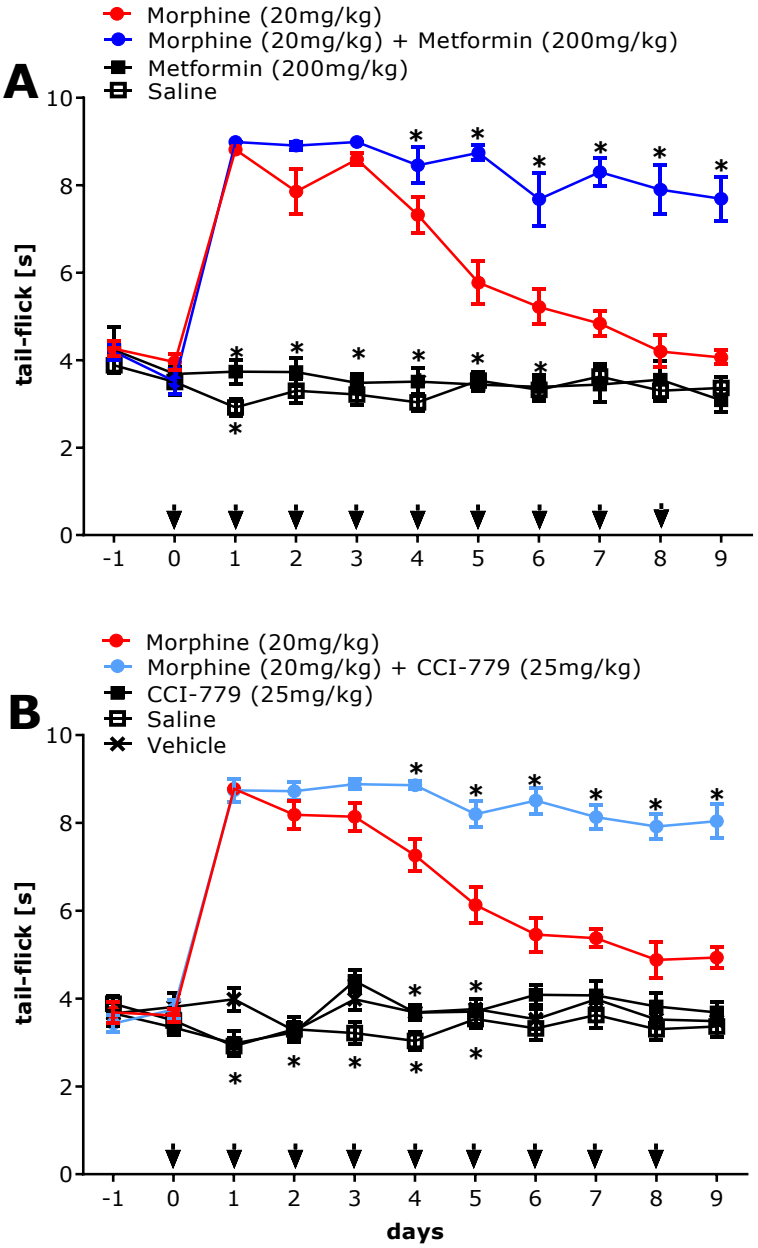


**Figure 3.3: Metformin (A) and CCI-779 (B) blocked the development and maintenance of morphine-induced analgesic tolerance in *naïve* mice.** Tolerance was induced by morphine (20 mg/kg, i.p., twice daily) administered for 9 consecutive days. Repeated systemic administration of metformin (200 mg/kg, i.p., A) and CCI-779 (25 mg/kg, i.p., B) 20 h before first morphine injection and then subsequently for 8 days (once daily after morning morphine injection) blocked the development and maintenance of morphine-induced tolerance, as measured by the tail-flick test conducted daily 30 min after morning morphine administration. The data are presented as mean ± SEM, n= 5-12; **vs.* morphine; *P<0.05 (two-way ANOVA followed by Bonferroni's test). Arrows indicate metformin/CCI-779 injections.

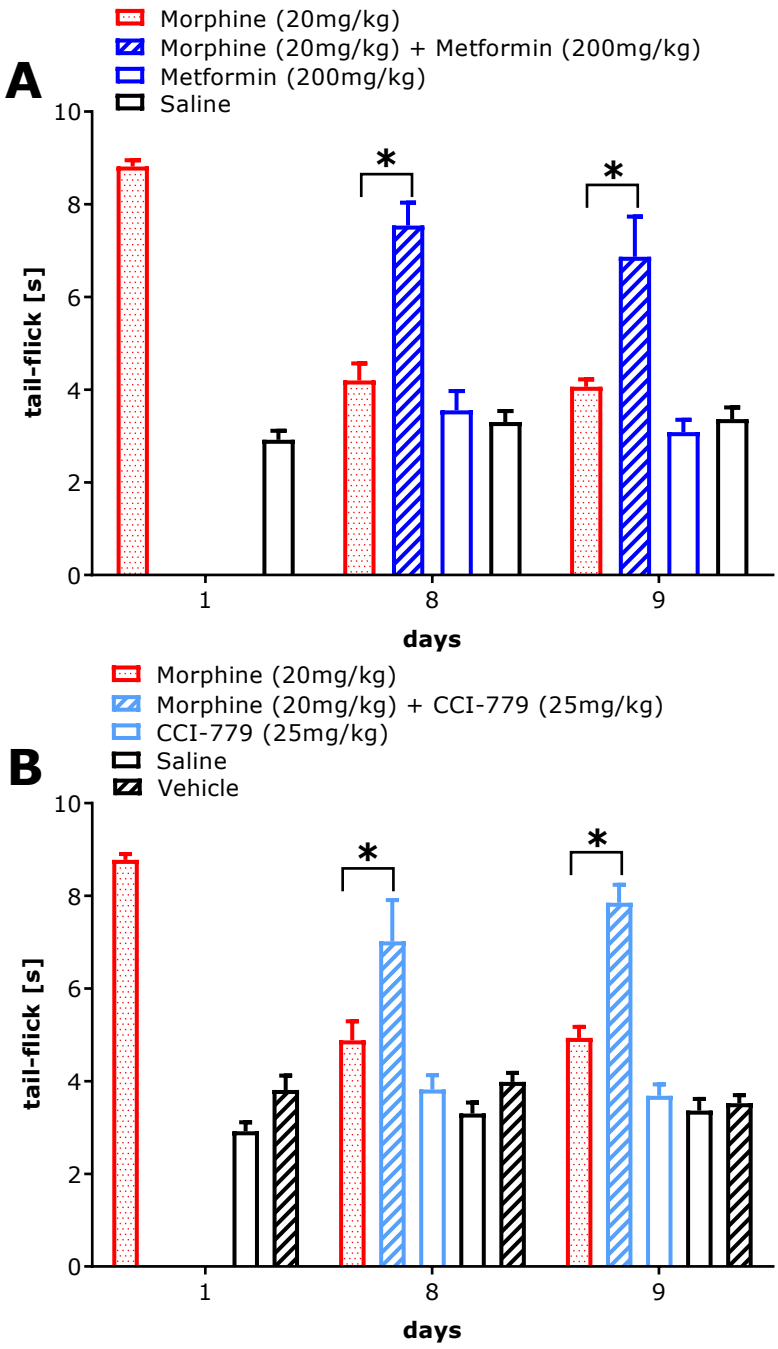


**Figure 3.4: Metformin (A) and CCI-779 (B) restored the analgesic effect of morphine in morphine tolerant *naïve* mice.** Tolerance was induced by morphine (20 mg/kg, i.p., twice daily) administered for 9 consecutive days. On day 7, when morphine tolerance was developed, a single systemic administration of metformin (200 mg/kg, i.p., A) or CCI-779 (25 mg/kg, i.p., B) 20 h before next morning's morphine injection restored the analgesic effect of morphine on day 8, as measured by the tail-flick test conducted 30 min after morning morphine. Additional systemic injection of metformin (200 mg/kg, i.p., A) and CCI-779 (25 mg/kg, i.p., B) on day 8 20 h before next morning's morphine maintained the analgesic effect of morphine on day 9. The data are presented as mean ± SEM, n= 6-12; **vs.* morphine; *P<0.05 (un-paired Student's t-test).

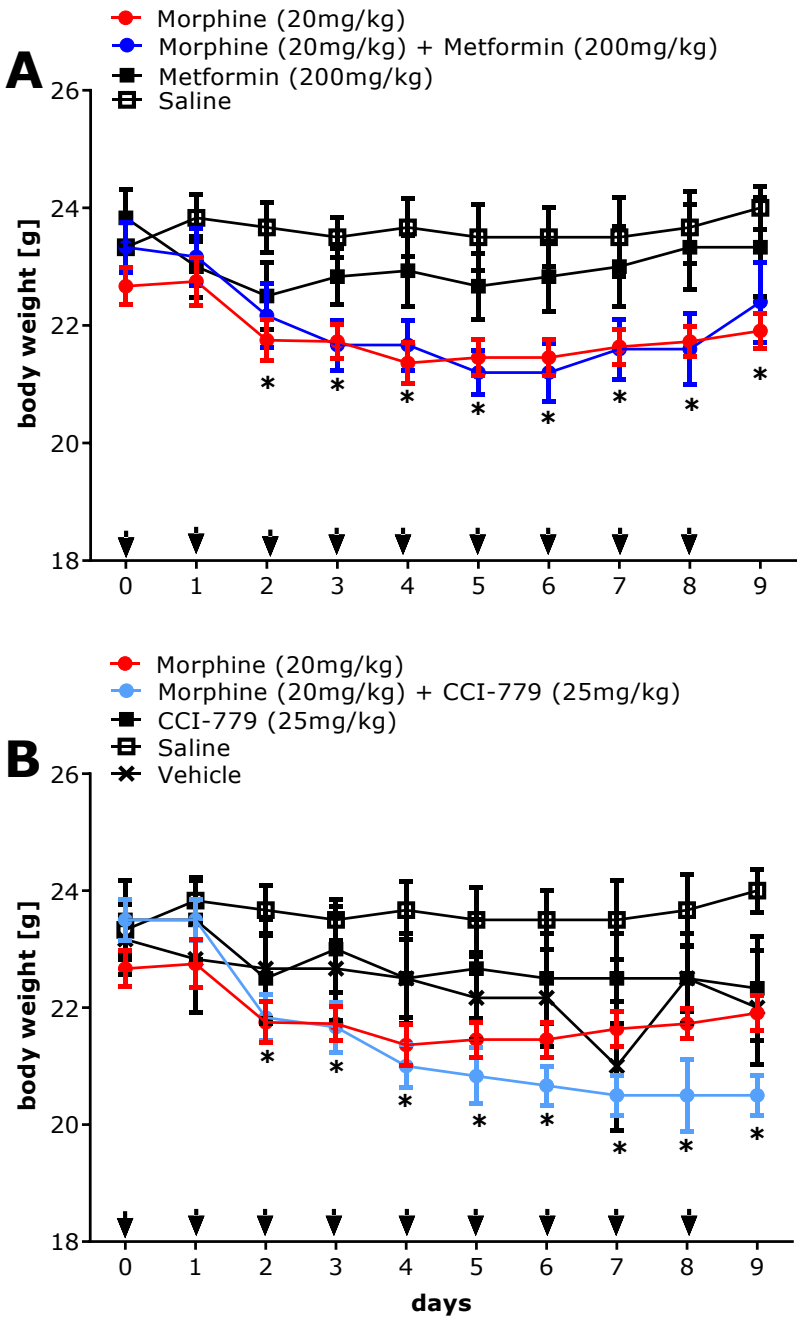


**Figure 3.5: Metformin (A) and CCI-779 (B) in combination with morphine induced body weight loss in *naïve* mice.** Repeated administration of morphine (20 mg/kg, i.p., twice daily) for 9 consecutive days induced significant body weight loss observed on day 2 when compared with saline treated mice. However, chronic administration of metformin (200 mg/kg, i.p., A) or CCI-779 (25 mg/kg, i.p., B) alone did not affect body weight when compared with saline controls. The data are presented as mean ± SEM, n= 5-6 mice per group; **vs.* saline; *P<0.05 (two-way ANOVA followed by Bonferroni's test). Arrows indicate metformin/CCI-779 injection time.

### 3.4 Discussion

In this chapter, systemic administration of mTORC1 inhibitors, metformin and CCI-779, inhibited the development and maintenance of antinociceptive tolerance to morphine in *naïve* mice was demonstrated. This was evident in sustained responses to morphine in the presence of metformin or CCI-779 for 9 days when compared to morphine tolerant animals not being exposed to mTORC1 inhibitors. This observation may have an important clinical implication as inhibition of the mTORC1 pathway could be used as an adjuvant with morphine treatment to improve its analgesic effect, especially when prolonged opioid treatment is required.

Similarly to this current observation, studies of others *in vivo* using the animal model of opioid tolerance discovered also that inhibition of the mTORC1 pathway improved morphine analgesia as it enhanced the behavioural pain threshold in the tested animals. A recent study by Zhang indicated the involvement of the mTORC1 pathway in morphine tolerance in *naïve* mice. It has been documented that repeated systemic administration of rapamycin (a selective inhibitor of mTORC1) to *naïve* mice blocked the development of morphine analgesic tolerance and furthermore, that this treatment attenuated morphine-induced cold and mechanical allodynia (Zhang et al., 2019). Specifically, the opioid tolerance model in their research involved C57BL/6J mice exposed to subcutaneous morphine injection at a dose of 20 mg/kg twice daily for 5 days (Zhang et al., 2019). They showed a significant difference in morphine's maximal possible analgesic effect on day 5 of the i.p. injection of rapamycin, suggesting that systemic administration of rapamycin had a beneficial effect on morphine induced tolerance in *naïve* mice (Zhang et al., 2019). Likewise, the same research group examined the reversal effect of systemic rapamycin on morphine analgesic tolerance, as they injected rapamycin on day 6 when morphine tolerance was fully established (Zhang et al., 2019). They found a marked or significant difference in morphine's maximal possible analgesic effect with systemic administration of rapamycin (Zhang et al., 2019). Furthermore, they observed that i.p. injection of rapamycin attenuated the development and maintenance of morphine-induced cold allodynia which is associated with opioid-induced hyperalgesia (Zhang et al., 2019).

In line with this observation, likewise Xu et al. (2014), observed that intrathecal rapamycin attenuated the development of morphine tolerance and hyperalgesia in *naïve* rats, as the co-administration of rapamycin blocked morphine-induced decreased in hind paw withdrawal threshold and latency to mechanical stimuli in *naïve* rats (Xu et al., 2014). Additionally, they

investigated the reversal effect of mTORC11inhibitors on morphine tolerance as they documented that a single intrathecal injection of rapamycin significantly restored the analgesic effect in *naïve* rats (Xu et al., 2014). Thus, preclinical studies indicated that mTORC1 is implicated in morphine induced tolerance and hyperalgesia and that targeting this pathway *via* rapamycin attenuated these deleterious effects associated with long-term morphine exposure.

An advantage of this current study is related to the use of CCI-779 which is the soluble ester of rapamycin; CCI-779 has a more aqueous solubility and better chemical stability in solution compared with rapamycin.

In this study, chronic systemic administration of CCI-779 produced an equivalent behavioural effect on morphine-induced analgesic tolerance when compared to results obtained from intrathecal rapamycin administration (Xu et al., 2014) and systemic rapamycin injection (Zhang et al., 2019). However, in the experiment where CCI-779 was administered once to reverse morphine tolerance, CCI-779 restored morphine antinociception in *naïve* mice 20 h following its admiration, whereas Zhang et al. (2019), reported that the reversal of morphine tolerance was identified after administration of two consecutive doses of rapamycin. It could be that CCI-779 is more potent than rapamycin in blocking the mTORC1 pathway as it reversed morphine tolerance within 20 h or is due to the difference in the experimental design relating to both studies, as the tail-flick test was carried out daily in this current study, while in Zhang et al. (2019), the test was conducted every other day.

However, the important factor behind the effect observed in this current study is the fact that CCI-779 inhibited the mTORC1 signalling pathway. This is in line with Obara et al. (2011), who revealed that CCI-779 at a dose of 25 mg/kg inhibited the mTORC1 pathway at the spinal cord level in *naïve* mice. Moreover, a study by Podsypanina et al. (2001) showed that CCI-779 at a dose of 20 mg/kg attenuated mTORC1 activity in mice with endogenous tumours. Hence, a dose of 25 mg/kg of CC-779 was capable of targeting the mTORC1 pathway.

An interesting aspect in this current research is related to the use of metformin. There are *in vivo* studies that exhibited the effect of metformin (antihyperglycemic drug used to treat type II diabetes) as a novel pharmacological treatment to reduce the decreased analgesia resulting from chronic morphine exposure. For example, it has been reported that repeated systemic

administration of metformin in *naïve* mice at doses of 50, 100, 200 mg/kg significantly attenuate the development of allodynia and hyperalgesia resulting from chronic morphine administration. This effect was observed in a dose-dependent manner (Pan et al., 2016). The same research group proposed that the beneficial effect of metformin on morphine induced tolerance is associated with increasing AMPK activation which is a negative regulator of mTORC1 (Pan et al., 2016). In addition, it has been documented that activated AMPK led to a reduction in the mTORC1 signalling cascade (Hardie, 2007; Melemedjian et al., 2011). Furthermore, a different research group demonstrated that 7 days of metformin treatment significantly attenuated the development of morphine tolerance in *naïve* rats (Fatemi et al., 2018). They demonstrated that chronic oral metformin treatment with 50 mg/kg significantly increased the analgesic latency and prevented the induction of morphine antinociceptive tolerance (Fatemi et al., 2018). However, they also revealed that acute oral treatment with metformin at doses of 5-50 mg/kg did not inhibit the expression of morphine tolerance in tolerant rats (Fatemi et al., 2018). This observation is in contrast to this current study, as *naïve* mice showed reversal of morphine tolerance demonstrated by an increase in sensitivity to morphine upon acute systemic metformin administration. The difference between both studies appears to be due to a pharmacodynamics issue. Oral administration of metformin may require additional time to regulate the cellular and molecular mechanisms, or a higher metformin concentration is required to attenuate morphine tolerance, as Fatemi tested the effect of 5-50 mg/kg of metformin administered only 45 min before morphine injection (Fatemi et al., 2018). In this current study, *naïve* tolerant mice were injected with approximately four-fold higher dose of metformin 20 h prior to the morphine injection. Thus, sufficient time and suitable concentration achieved in this present study resulted in the reduced morphine analgesic efficacy being overcome. This discrepancy further indicates and confirms that in pre-clinical studies, different factors could significantly interfere with the effect of a particular drug. These factors could be related to route of administration, experimental design, dose regimen and animal species.

Despite considerable research on opioid tolerance, the exact mechanisms of morphine tolerance are not clear. The influence of different compounds that reverses the antinociceptive effect of morphine tolerance was studied using different nociceptive tests in mice as well as rats. These studies identified multiple factors and/or drug targets that can significantly attenuate morphine tolerance *in vivo*. For example, Gilron et al. (2003), reported that gabapentin was capable of reversing morphine antinociceptive tolerance tested by tail-flick tests and paw-pressure in *naïve* tolerant rats, showing that opioid tolerance is

potentially mediated by glutamate and glutamate receptors (Gilron et al., 2003). Moreover, gabapentin decreases the dynorphin-induced allodynia, as chronic treatment with morphine produces an increase in dynorphin expression (Gilron et al., 2003). Similarly, it has been recently documented that systemic administration of diazepam at a dose of 0.5 mg/kg was capable of reversing antinociceptive tolerance to morphine in the mouse tail immersion assay (Gonek et al., 2017). In addition, several published papers revealed that PKC inhibitors reverse morphine tolerance. Smith and colleagues (1999), showed that injection of ET-18-OCH3 into morphine tolerant mice significantly reversed morphine antinociception measured by tail-flick, indicating a potential role for PKC in tolerance (Smith et al., 1999). It has also been reported that intrathecal injection of a melanocortin 4 receptor antagonist was capable of restoring morphine analgesia in tolerant rats using tail-flick assay. Therefore, emphasised that melanocortin 4 receptor is involved in the development of morphine tolerance (Starowicz et al., 2005).

One of the most recently identified targets involved in the regulation of morphine efficacy is mTORC1 signalling pathway. At the molecular level, numerous investigators have studied the role of the mTORC1 pathway in chronic morphine tolerance and hyperalgesia which is consistent with the finding of the current behavioural studies as described in detail above.
It is worth adding that based on the data presented here metformin and CCI-779 did not alter the pain threshold or the initial morphine-induced analgesia in *naïve* mice, which was in agreement with Melemedjian et al. (2011) and Obara et al., (2011). Moreover, metformin and CCI-779 did not alter the body weight of the mice. In line with this current observation, it has been documented that C57BL/6J mice treated with metformin in the drinking water at a dose of 2mg/ml for 4 weeks had no effect on mass body weight (Li et al., 2019). Additionally, it has been recognised that systemic administration of CCI-779 at different doses (10, 25 and 50mg/kg) for 4 days did not change the body weight of *naïve* C57BL/6J mice (Obara et al., 2011). However, repeated administration of morphine produced a reduction in body mass in all morphine treated animals. This observation is consistent with Papaleo et al., (2006). They confirmed that the gender difference in C57BL/6J mice is associated with this particular deleterious effect of morphine administration, observed as exposure to morphine induced consistent body weight loss in both male and female mice (Papaleo et al., 2006). Moreover, male mice treated with a higher dose of morphine ranging between 20–100 mg/kg for 6 consecutive days displayed a greater reduction in body weight than those treated with 10–50 mg/kg, although female mice produced a similar body weight loss in both morphine regimen treatments (Papaleo et al., 2006). Moreover, mice displayed

body weight loss induced by morphine administration in dose an age related manner (Koek et al., 2014). It was observed that intermittent morphine administration at doses range of 10–100 mg/kg was at least 10-fold more potent at producing body weight loss in adults than in adolescent mice (Koek et al., 2014). It is also recognised that loss of body mass is a prominent somatic sign of chronic opioid users due to nausea and vomiting (Colameco et al., 2009). However, some individuals may experience weight gain, given that opioid abuse frequently leads to physical inactivity.

In fact, there are side-effects which are relatively rare and temporarily associated with using metformin such as nausea, diarrhoea and bloating (Tomkin et al., 1971). When metformin is prescribed as a drug for long-term use, side-effects mediated by this drug should be taken into consideration. It was suggested that long-term use of metformin may result in vitamin B12 deficiency (Tomkin et al., 1971). However, a recent study from Qatar comparing non-metformin to metformin users, found that the serum B12 levels were similar between both groups (Elhadd et al., 2018). In addition, further studies from Brazil (Damião et al., 2016) and Pakistan (Khan et al., 2017), showed that metformin use is not associated with B12 deficiency in patients. In contrast to metformin, clinical development of CCI-779 and its analogues has revealed that these drugs produce numerous side-effects that can be serious and debilitating (Pallet and Legendre, 2013). These side-effects are regularly unpredictable and may frequently preclude the efficiency of mTOR inhibitors (Pallet and Legendre, 2013). Despite the fact that CCI-779 has been approved by the FDA as an immunosuppressive drug and featured in several clinical trials for cancer treatment, it potentially possesses a significantly higher risk concerning long-term treatment than metformin. Therefore, this current study may potentially offer a novel strategy for the improvement of opioid treatment by extending upon ongoing studies on mTORC1 in chronic pain and by using relatively safe and accessible medication.

In summary, this study reported that chronic use of mTOR inhibitors blocked and attenuated morphine analgesic tolerance in *naïve* mice. Moreover, it has been shown that mTOR inhibitors displayed ability to restore the analgesic effect in morphine-tolerant mice. An interesting aspect of this study is related to the effects produced by metformin, a widely used anti-diabetic drug that inhibits mTORC1 through activation of AMPK. This drug is relatively safe and in contrast to CCI-779 may potentially allow long-term treatment with fewer side-effects. In the following chapter, (Chapter 4), the tissue samples (L4-L6 of the spinal cord) collected at the end of the behavioural experiment were used for western blotting to

determine the extent to which metformin and CCI-779 alter the key signalling molecules of mTORC1, S6RP, 4EBP1/2 and p70S6K and their phosphorylated (activated) counterparts in pain-related regions. In addition, immunohistochemistry technique were used to determine the involvement of both neuronal and non-neuronal mechanisms in the spinal dorsal horn involved in opioid analgesia.

# Chapter 4. The effect of the mammalian target of rapamycin complex 1 (mTORC1) inhibitors on the activity of mTORC1 pathway in spinal cord

## 4.1 Introduction

Morphine is a highly effective drug against acute to moderate pain. However, long-term exposure to morphine leads to pharmacological antinociceptive tolerance resulting in morphine therapy being ineffective and therefore discontinued. Notwithstanding, that cellular and molecular mechanisms underlying tolerance induced by morphine is not fully understood.

It is well known that morphine exerts its analgesic function predominantly by activating the μ-opioid receptor in humans as well as animals. Additionally, the analgesic effect of morphine was absent when the μ-opioid receptor was mutated *in vivo* (μ-knockout mice) (Law et al., 2003). This result indicated that morphine tolerance could be mediated by activation of the μ-opioid receptor (Law et al., 2003; Xu et al., 2014). It has been reported that under chronic morphine exposure, the binding of morphine to the μ-opioid receptor activates PI3K/Akt, which directly phosphorylates TSC2 and releases the suppression of TSC1/TSC2 in the dorsal horn leading to activation of the mTORC1 signalling cascade resulting in initiation of protein synthesis which in turn produced more proteins implicated in the development of morphine tolerance (Xu et al., 2014).

Active mTORC1 activates its downstream effectors p70S6K and 4EBP, resulting in the initiation of mRNA translation leading to adaptive changes in protein translations (Xu et al., 2014). mTORC1 and its downstream effectors are distributed in different parts in the mammalian nervous system including the dorsal horn (Xu et al., 2010; Xu et al., 2014), dorsal root ganglion (Xu et al., 2010; Obara et al., 2011), ventral tegmental area (Mazei-Robison et al., 2011), hippocampus (Cui et al., 2010; Obara et al., 2011; Lyu et al., 2013;) and myelinated peripheral fibres (Jimenez-Diaz et al., 2008; Obara et al., 2011). Given this distribution, mTORC1 plays a vital role in several sensory and cognitive processes, such as learning, memory and perception, as mentioned in Chapter 1.

The dorsal horn of the spinal cord is strongly implicated in the generation of opioid tolerance associated with chronic morphine administration (Trang et al., 2015). In this research, it was postulated that activation of the mTORC1 pathway may possibly be a key factor in the

opioid-induced adaptive changes that occur in protein translation and contribute to the development and maintenance of opioid-induced tolerance under normal and neuropathic pain conditions. However, the intracellular processes that initiate the translation of multiple proteins after opioid stimulation remain elusive. Thus, in this current chapter western blotting analysis and the TSA-enhanced immunofluorescence technique were used to show that mTORC1 and its downstream effectors are present and regulated by metformin and CCI-779 in the spinal dorsal horn of *naïve* mice under chronic morphine administration. Likewise, in perfusion-fixed tissue, phospho-S6RP (P-S6RP; downstream effector of mTORC1) was co-stained with the neuronal marker NeuN, and non-neuronal the astrocytic marker GFAP and the microglial marker Iba1 to visualise the identity, distribution and co-localization of neuronal/non-neuronal cells with P-S6RP in the spinal cord dorsal horn that is involved in nociception and regulation of morphine tolerance.

## 4.2 Material and methods

### 4.2.1 Subject

Adult male C57BL/6J mice (8 weeks of age, Charles River, UK) weighting 25-30 g at the beginning of the study were used. Mice were housed 4 per polyethylene cage on sawdust bedding under standard conditions (12 h light/dark cycle, lights on from 8:00 am) with food and water available *ad libitum*. Experimental protocols were approved by the AWERB Committee at Durham and Newcastle Universities and were consistent with the guidelines provided by the UK Animals (Scientific Procedures) Act 1986. For more details see Chapter 2.

### 4.2.2 Preparation and administration of drugs

***Morphine***

To induce analgesic tolerance, morphine (morphine sulphate salt pentahydrate; Sigma-Aldrich, UK) was prepared in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK) immediately prior to injection. Mice were weighted and then injected intraperitoneally (i.p.) with morphine (20 mg per kg body weight) or equivalent vehicle (saline) solution without morphine, as a control group, twice daily for 9 consecutive days, in a volume of 4 ml per kg body weight.

***Metformin***

For systemic (i.p.) administration, metformin (metformin hydrochloride; Cat. No. 2864; Tocris Bioscience, UK) was prepared in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK) immediately prior injections. Mice were weighed and then injected i.p. with metformin (200 mg per kg body weight) or equivalent vehicle (saline) solution without metformin as a control group. Metformin/vehicle was administered i.p. 20 h before i.p. first injection of morphine was given and then i.p. administration of metformin/vehicle was continued for 8 consecutive days. The timing and concentration of metformin injections were based on previously published research using metformin (Melemedjian et al., 2011; Obara et al., 2015).

***CCI-779***

For systemic (i.p.) administration, CCI-779 (temsirolimus; Cat. No. T-8040; LC Laboratories, USA) was prepared in pure ethanol as a stock solution at 60mg/mL on the day of experiment and diluted to 2.5 mg/mL in 0.15M NaCl, 5% polyethylene glycol 400, 5% Tween 20 (Ravikumar et al., 2004; Obara et al., 2015) immediately before injection. Mice were weighed and then injected intraperitoneally (i.p.) with a 1% v/w solution of CCI-779 (25 mg per kg body weight) or equivalent vehicle (0.15M NaCl, 5% polyethylene glycol 400, 5% Tween 20) solution without CCI-779 as a control group. CCI-779/vehicle was injected 20 h before first i.p. injection of morphine was given and then i.p. administration of CCI-779/vehicle was continued for 8 consecutive days. The timing and concentration of CCI-779 injections were based on previously published research using CCI-779 ( Obara et al., 2011; 2015).

### 4.2.3 Experimental design

To establish the role of mTORC1 inhibition on morphine-induced tolerance in *naïve* mice, two experiment were conducted:

*Experiment I*: On day 0 (Table 4.1) *naïve* mice were injected i.p. with metformin or CCI-779 20 h prior i.p. administration of morphine and then for the injections were repeated for 8 consecutive days. Also, on day 7 and 8 *naïve* mice were injected i.p. with metformin or CCI-779 20 h prior i.p. administration of morphine. Upon completion of the experimental protocol *naïve* mice were sacrificed and tissue was collected (L4-L6) of the spinal cord in order to assess the role of mTORC1 in the development and maintenance of morphine analgesic tolerance.

*Experiment II*: On day 0 (Table 4.2) *naïve* mice were injected i.p. with metformin or CCI-779 20 h prior i.p. administration of morphine and then for the injections were repeated for 8 consecutive days in order to assess the role of mTORC1 in the development and maintenance of morphine analgesic tolerance. Upon completion of experimental protocols *naïve* mice were sacrificed and tissue was collected from L4-L6 segment of the spinal cord in order to assess the role of mTORC1 in the development and maintenance of morphine analgesic tolerance.

#### 4.2.3.1 Design of experiment I (western blotting)

*Naïve* mice were divided into 5 different experimental groups as indicated in Table 4.1. Group 1 (n= 5) consisted mice receiving morphine (20 mg/kg, i.p.) twice daily at 12 h intervals (8 am/8 pm) for 9 consecutive days, group 2 (n= 5) received saline under identical conditions and served as controls. To determine the influence of mTORC1 inhibitors on morphine analgesic effects, mice were injected with metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) 20 h prior the morning morphine (20 mg/kg, i.p.) on each testing day (day 0–8; group 3, n= 5). Mice were rendered tolerant to morphine after 6 days of treatment. On day 7, mice were injected with metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) (group 4, n= 5) 20 h prior to the morning morphine i.p. injection on day 8. The injection was repeated 20 h after (on day 8). Finally, to determine the effect of mTORC1 inhibitors without morphine, mice were injected i.p. with metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) 20 h prior to the morning saline i.p. injection for 8 following days (group 5, n= 5). Tissue was taken 1-2 h after the last morning morphine injection on day 9. The western blotting analysis was carried out with soluble fractions of spinal cord, specifically the lumber area of L4-L6. All details of each step from sample preparation to antibody labelling were mentioned in Chapter 2.

**Table 4.1: Design of experiment I.**

| Group | Morphine (20mg/kg, i.p.) | Saline (i.p.) | Administration of mTORC1 inhibitors Metformin (200mg/kg, i.p.) or CCI-779 (25mg/kg, i.p.) | | Number of animals |
|---|---|---|---|---|---|
| | | | Chronic 9x mTORC1 inhibitors | Acute 2x mTORC1 inhibitors | |
| 1 | + | | | | 5 |
| 2 | | + | | | 5 |
| 3 | + | | + | | 5 |
| 4 | + | | | + | 5 |
| 5 | | + | + | | 5 |

*Naïve* mice were randomly assigned into 5 groups and received different treatment: (1) i.p. morphine twice daily; morning injections were given between 9:00 am and 10:00 am, and evening injections were given between 8:00 pm and 9:00 pm; (2) controls; i.p. saline twice daily between 9:00 am and 10:00 am and between 8:00 pm and 9:00 pm; (3) morphine + mTORC1 inhibitors (chronic); morphine twice daily (as above) followed by i.p. mTORC1 inhibitors(given between 1:00 pm and 2:00 pm) and then morphine evening injection; (4) morphine + mTORC1 inhibitors (acute) ; morphine twice daily (as above) followed by i.p. mTORC1 inhibitors (given between 1:00 pm and 2:00 pm) on day 7 and 8, and then morphine evening injection; (5) i.p. mTORC1 inhibitors were given once daily (as above). Tissue was taken 1-2 h after the last morning morphine injection on day 9.

### 4.2.3.2 Design of experiment II (Immunohistochemistry)

*Naïve* mice were divided into 4 different experimental groups as indicated in Table 4.2. Group 1 (n= 5) consisted mice receiving morphine (20 mg/kg, i.p.) twice daily at 12 h intervals (8 am/8 pm) for 9 consecutive days (day1-9 in Table 4.1) To determine the influence of mTORC1 inhibitors on morphine analgesic effects, mice were injected with metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) 20 h prior the morning morphine (20 mg/kg, i.p.) on each (day 0–8; group 2, n= 5). To determine the effect of mTORC1 inhibitors without morphine, mice were injected i.p. with metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) 20 h prior to the morning saline i.p. injection for 8 following days (group 3, n= 5). Finally, in group 4 (n= 5), mice were injected with saline twice daily. i.p. and with an equivalent vehicle solution without mTORC1 inhibitors (controls). Tissue was taken 1-2 h after the last morning morphine injection on day 9, the details of each step of sample preparation and antibody labelling were mentioned in chapter 2.

**Table 4.2: Design of experiment II.**

| Group | Morphine (20mg/kg, i.p.) | Saline (i.p.) | Administration of mTORC1 inhibitors | | Vehicle (i.p.) | Number of animals |
|---|---|---|---|---|---|---|
| | | | Metformin (200mg/kg, i.p.) | CCI-779 (25mg/kg, i.p.) | | |
| 1 | + | | | | | 5 |
| 2 | | + | | | + | 5 |
| 3 | + | | + | | | 5 |
| 4 | | + | + | | | 5 |

*Naïve* mice were randomly assigned into 4 groups and received different treatment: (1) i.p. morphine twice daily; morning injections were given between 9:00 am and 10:00 am, and evening injections were given between 8:00 pm and 9:00 pm; (2) controls; i.p. saline twice daily between 9:00 am and 10:00 am and between 8:00 pm and 9:00 pm; (3) morphine + mTORC1 inhibitors; morphine twice daily (as above) followed by i.p. mTORC1 inhibitors (given between 1:00 pm and 2:00 pm) and then morphine evening injection;.(4) i.p. mTORC1 inhibitors were given once daily (as above).

### 4.2.4 Statistical data analysis

Data analysis and statistical comparisons were performed using GraphPadPrism, version 8.01 for Windows (GraphPad Software, CA, www.graphpad.com). Comparisons between groups were performed using analysis of variance (one-way ANOVA) followed by Bonferroni's multiple comparison post-hoc test. A value of $P<0.05$ was considered statistically significant.

*Experiment I*: Images were visualised using chemiluminescent substrate and BioRad Chemidoc. The optical density of the bands was then quantified using Image J (an open source Java-based image processing program) to calculate the intensity of the bands. Immunoblotting data is expressed as a mean ratio of phosphor/total protein expression ± SEM graphs generated on a Graphpad Prism.

*Experiment II*: Stained sections were examined and photographed using a CCD Microscope (DFC365 FX; Leica, Mannheim, Germany) equipped with 1.4 MP monochrome digital camera. Images were assembled using Image J. Digital images were analysed using Volocity 3D image analysis software (Pan et al., 2016), mean ± SEM graphs generated on a Graphpad Prism.

## 4.3 Results

### 4.3.1 The mammalian target of rapamycin complex 1 (mTORC1) inhibitors decreased phosphorylation of downstream targets of mTORC1 in the dorsal spinal cord of *naïve* mice

As illustrated in Figure 4.1, administration of metformin (200 mg/kg, i.p., once daily; Figure 4.1 A) or CCI-779 (25 mg/kg, i.p., once daily; Figure 4.1 B) reduced phosphorylation of the downstream targets of mTORC1, confirming that mTORC1 activity was inhibited by chronic and acute administration of both inhibitors, comparing with morphine treated animals. Statistical analysis of western blot showed a significant decrease in protein expression levels of phospho-p70S6-Kinase (P-p70S6K) and total p70S6K in the spinal cord after metformin treatment (treatment effect: $F_{(4,14)}= 4.47$, $P= 0.01$). Similarly, statistical analysis showed a significant decrease in protein expression levels of phospho-S6RP (P-S6RP) and total S6RP in spinal cord after CCI-779 treatment (treatment effect: $F_{(4,14)}= 7.33$, $P<0.05$).

### 4.3.2 The mammalian target of rapamycin complex 1 (mTORC1) inhibitors decreased immunoreactivity of the downstream target of mTORC1 in the spinal cord of *naïve* mice

As illustrated in Figure 4.2, treatment with morphine (20 mg/kg, i.p. twice daily) for 9 consecutive days increased the immunoreactivity of P-S6RP in dorsal horn of lumbar spinal cord. Statistical analysis of quantitative images revealed that co-administration of metformin (200 mg/kg, i.p., once daily; Figure 4.2 A) or CCI-779 (25 mg/kg, i.p., once daily; Figure 4.2 B) with morphine significantly reduced the immunoreactivity of P-S6RP in dorsal horn of lumbar spinal cord, comparing with morphine treated animals (metformin effect: $F_{(3,11)}= 6.73$, $P<0.05$; CCI-779 effect: $F_{(3,12)}= 67.73$, $P<0.0001$) confirming that mTORC1 activity was inhibited by chronic administration of metformin and CCI-779.

### 4.3.3 Morphological identification of cells expressing mTORC1 downstream targets in *naïve* mice

*Co-localization with NeuN*

As illustrated in Figure 4.3 and Figure 4.4, mice treated with chronic morphine (20 mg/kg, i.p. twice daily) for 9 consecutive days showed increase of P-S6RP immunoreactivity in dorsal horn of lumbar spinal cord and P-S6RP was co-localized with NeuN for neurons. Co-dministration of metformin (200 mg/kg, i.p., once daily; Figure 4.5) or CCI-779 (25 mg/kg,

i.p., once daily; Figure 4.6) did not affect or change the distribution of neurons in the dorsal, but both inhibitors decreased the immunoreactivity P-S6RP in the dorsal horn neurons.

*Co-localization with GFAP*

As illustrated in Figure 4.5 and 4.6, mice treated with chronic morphine (20 mg/kg, i.p. twice daily) for 9 consecutive days increased the immunoreactivity of P-S6RP in dorsal horn of lumbar spinal cord while chronic morphine administration had a slight increase in GFAP positive cells compared to saline treated mice. Co-administration of metformin (200 mg/kg, i.p., once daily; Figure 4.5) or CCI-779 (25 mg/kg, i.p., once daily; Figure 4.6) did not have an effect in decreasing the amount of GFAP positive cells. Indicating that metformin or CCI-779 treatment did not have an influence on astrocyte hypertrophy.

*Co-localization with Ib-a1*

As illustrated in Figure 4.7 and 4.8 mice treated with chronic morphine (20 mg/kg, i.p. twice daily) for 9 consecutive days increased the immunoreactivity of P-S6RP in dorsal horn of lumbar spinal cord while chronic morphine administration had no effect in Iba-1 positive cells compared to saline treated mice. Co-administration of metformin (200 mg/kg, i.p., once daily; Figure 4.7) or CCI-779 (25 mg/kg, i.p., once daily; Figure 4.8) did not have an effect in decreasing the amount of Iba-1 positive cells. Indicating that metformin or CCI-779 treatment did not have an influence on Iba-1 proliferation.

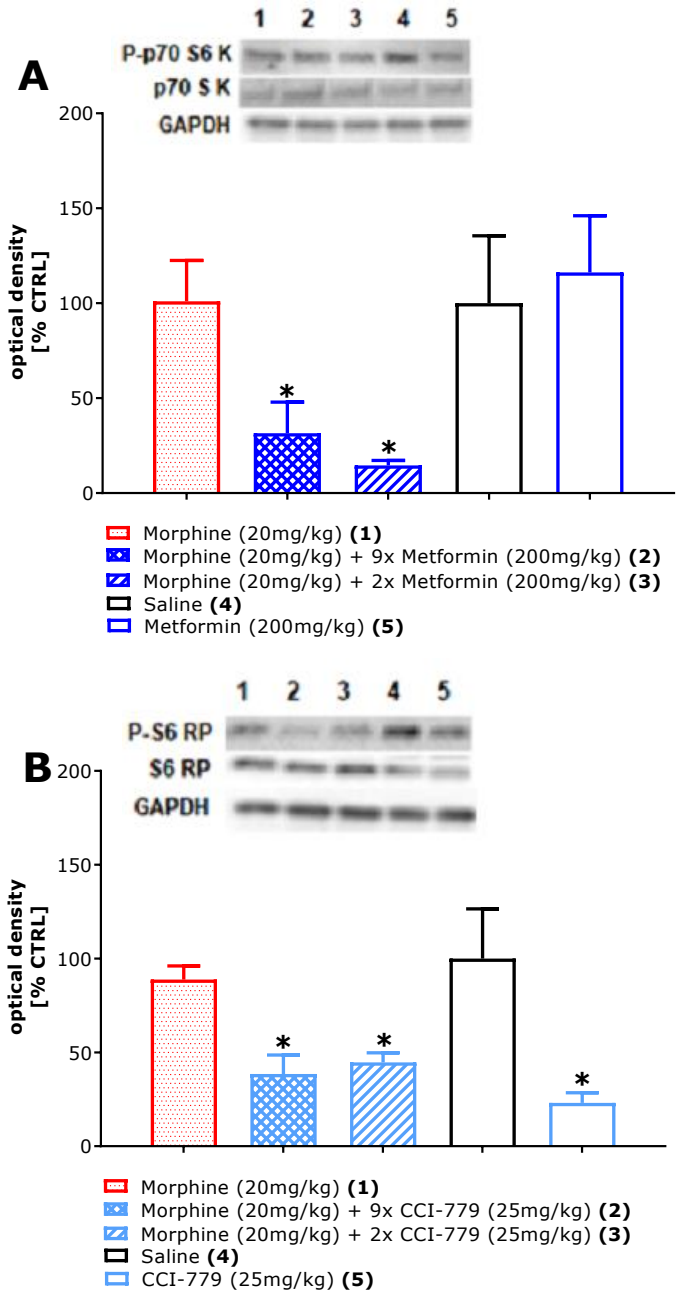


**Figure 4.1: Metformin (A) and CCI-779 (B) decreased phosphorylation of downstream targets of mTORC1 in the spinal cord of *naïve* mice.** Tolerance was induced by morphine (20 mg/kg, i.p.) administered twice daily for 9 consecutive days. Immunoblots probed with anti-phospho-p70S6 kinase (P-p70S6K), anti-phospho-S6RP (P-S6RP), anti-p70S6K and anti-S6RP antibodies after gel electrophoresis of lysates from L4-L6 of the spinal cord. The intensity of the bands for each antibody was normalized with the intensity of the GAPDH signal. **(A)** Administration of chronic metformin (200 mg/kg, i.p.) 20 h before first morning morphine injection and then repeated for 8 consecutive days or acute administration on day 7 and 8, 20 h before morning morphine on day 8 and 9 significantly reduced protein expression levels of P-p70S6K in spinal cord. Results are expressed as ratio P-p70S6K/p70S6K. **(B)** Administration of chronic CCI-779 (25 mg/kg, i.p.) 20 h before first morning morphine injection and then repeated for 8 consecutive days or acute administration on day 7 and 8 20 h before morning morphine on day 8 and 9 significantly reduced protein expression levels of P-S6RP in spinal cord. Results are expressed as ratio P-S6RP/S6RP. The data are presented as mean ± SEM, n= 4-5; **vs.* saline; *P<0.05 (one-way ANOVA, followed by Bonferroni's test). Representative immunoblots are shown above the appropriate graph bar and numbers indicate respective experimental groups.

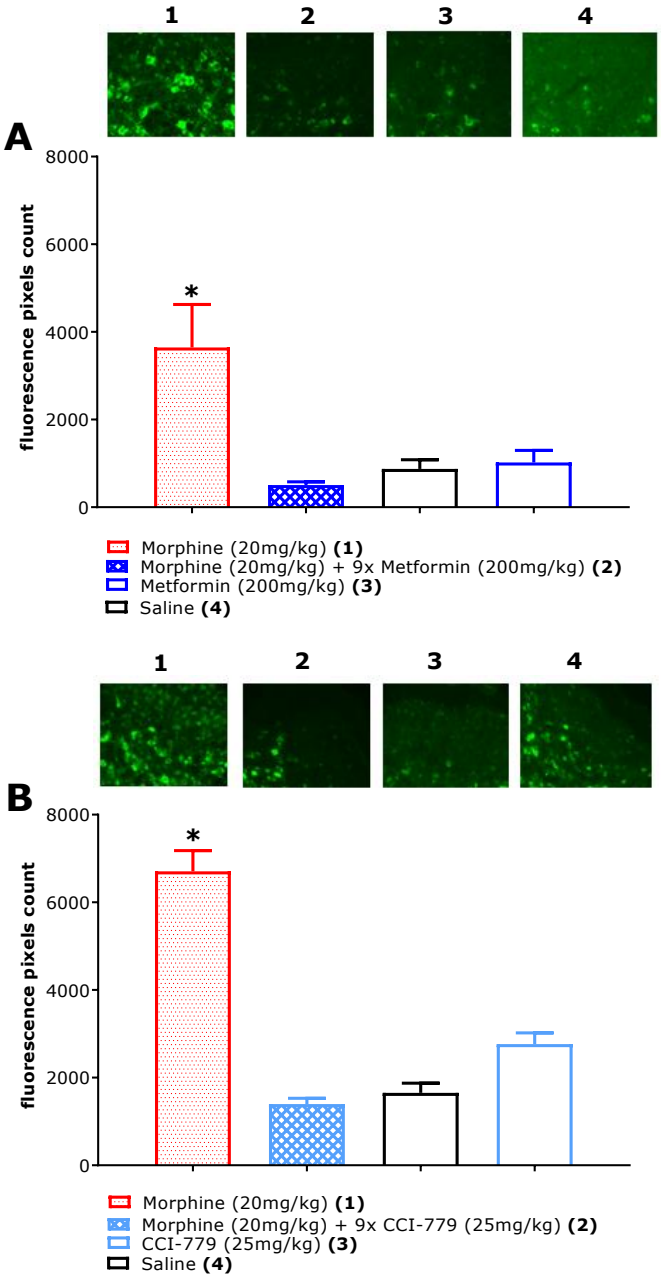


**Figure 4.2: Metformin (A) and CCI-779 (B) decreased the immonoreactivity of mTORC1 effector in the dorsal spinal cord of *naïve* mice.** Tolerance was induced by morphine (20 mg/kg, i.p.) administered twice daily for 9 consecutive days. Chronic morphine administration increased the immonoreactivity of phospho-S6 ribosomal protein (P-S6RP) in the in the dorsal horn of the spinal cord of *naïve* mice. **(A)** Administration of chronic metformin (200 mg/kg, i.p.) 20 h before first morning morphine injection and then repeated for 8 consecutive days decreased the expression of P-S6RP induced by chronic morphine injection. **(B)** Administration of chronic CCI-779 (25 mg/kg, i.p.) 20 h before first morning morphine injection and then repeated for 8 consecutive days decreased the expression of P-S6RP induced by chronic morphine injection. Quantification of P-S6RP immunofluorescence were represented as mean ± SEM of fluorescence pixels count in the superficial dorsal horns (n=3-5, 4 images/section) *vs. saline; *P<0.05 (one-way ANOVA, followed by Bonferroni's test).

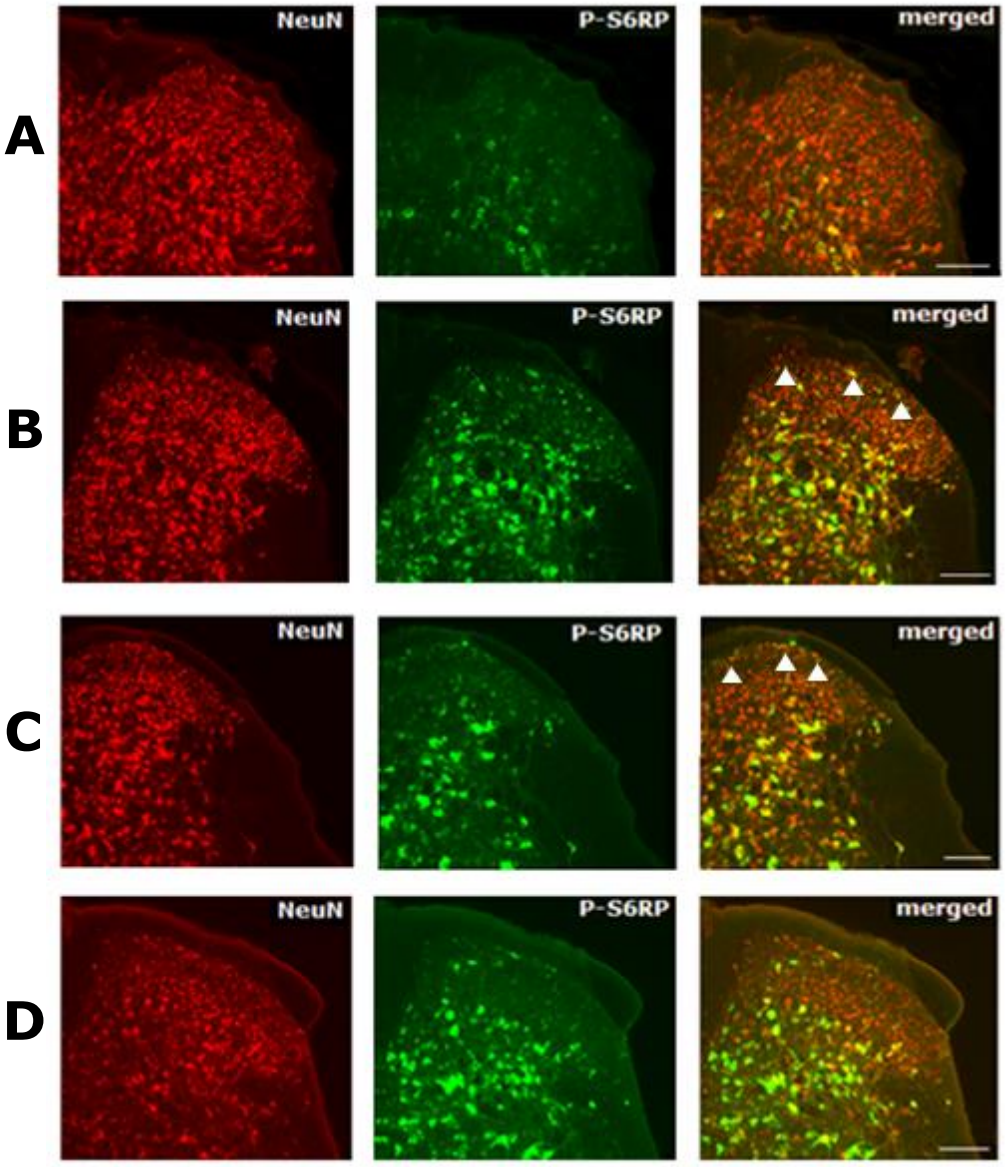


**Figure 4.3: Double labelling of mTORC1 effector and NeuN in lumbar spinal cord in *naïve* mice.** Immunofluorescence images showed the activation of phospho-S6 ribosomal protein (P-S6RP) after morphine administration in the in dorsal horn neurons. Chronic co-administration of metformin (200 mg/kg, i.p.) inhibited P-S6RP immunoreactivity induced by chronic morphine administration. Fluorescent images of P-S6RP (green) showed co-localization with NeuN (neuronal marker, red) n=3-5 as indicated by arrow heads. The single staining for each antibody and the merged image are shown from left to right and double staining appears in yellow. Scale bars 100 µm. **(A)** saline + vehicle, **(B)** morphine, **(C)** morphine + metformin, **(D)** metformin + saline.

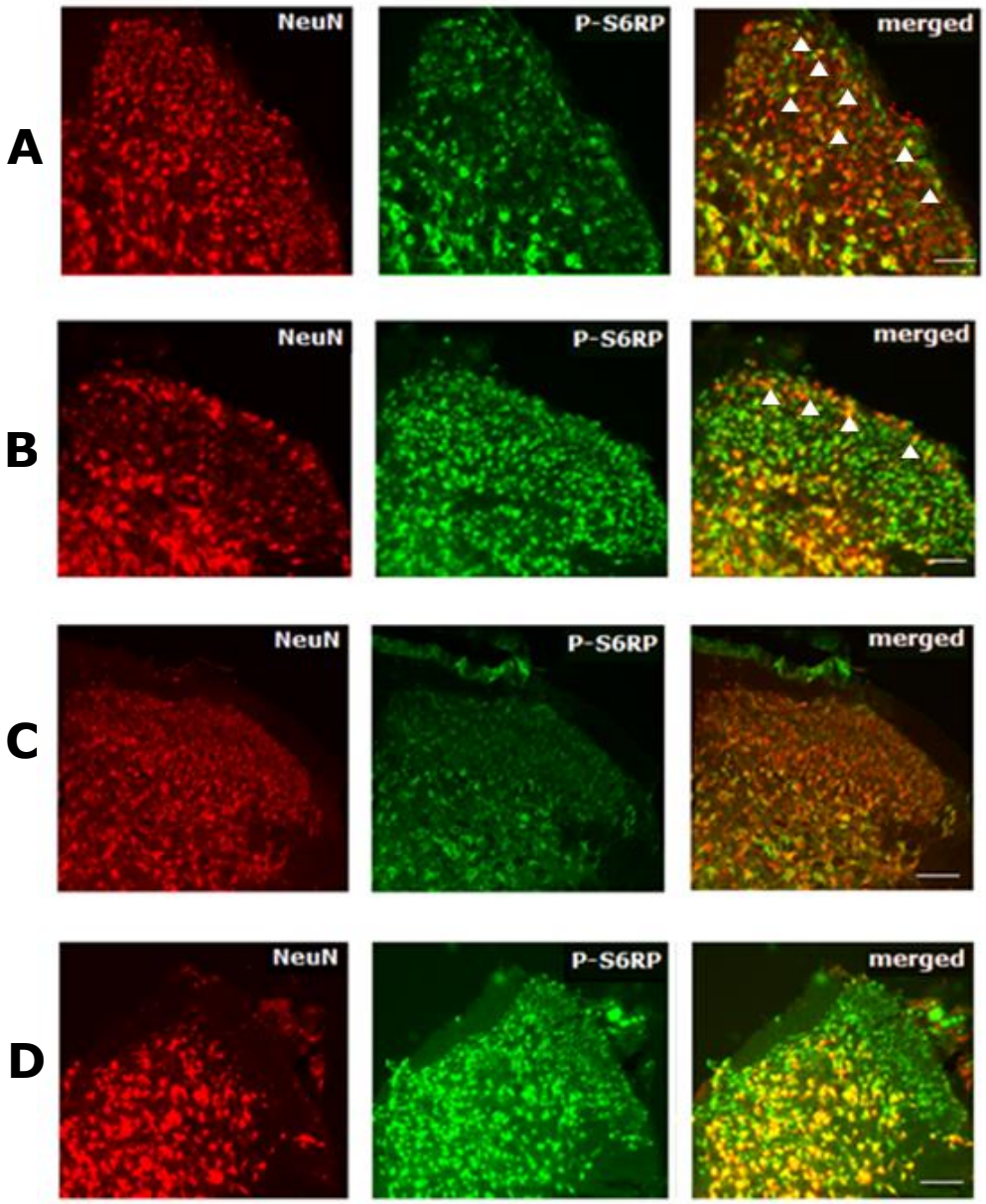


**Figure 4.4: Double labeling of mTORC1 effector and NeuN in lumbar spinal cord in *naïve* mice.** Immunofluorescence images showed the activation of phospho-S6 ribosomal protein (P-S6RP) after morphine administration in the in dorsal horn neurons. Chronic co-administration of CCI-779 (25 mg/kg, i.p.) inhibited P-S6RP immunoreactivity in chronic morphine administration. Fluorescent images of P-S6RP (green) showed co-localization with NeuN (neuronal marker, red) n=3-5 as indicated by arrow heads. The single staining for each antibody and the merged image are shown from left to right and double staining appears in yellow. Scale bars 100 μm. **(A)** saline + vehicle, **(B)** morphine, **(C)** morphine + CCI-779, **(D)** CCI-779 + saline.

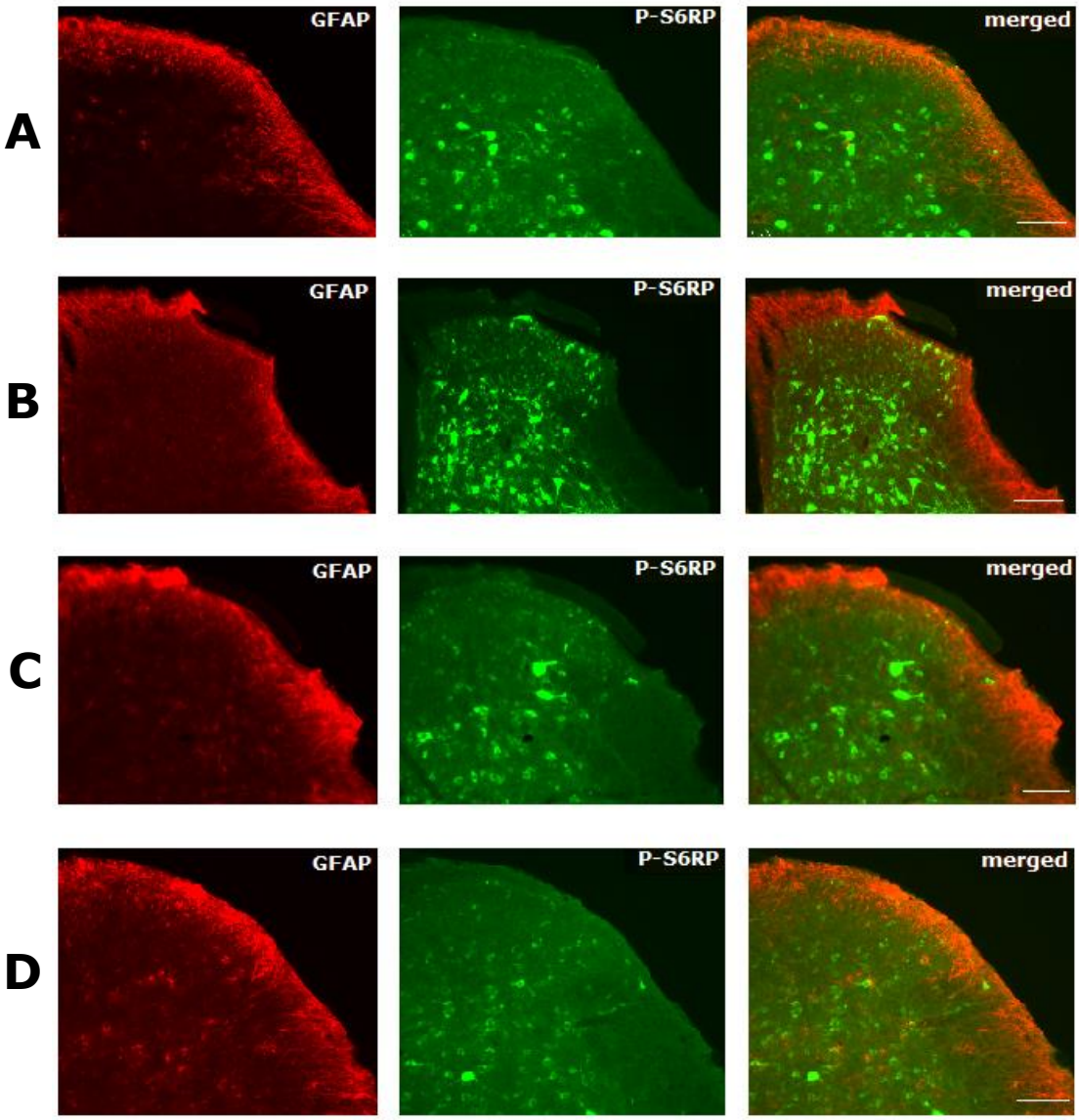


**Figure 4.5: Double labeling of mTORC1 effector and GFAP in lumbar spinal cord in *naïve* mice.** Immunofluorescence images showed the activation of phospho-S6 ribosomal protein (P-S6RP) after morphine administration in the in dorsal horn neurons. Chronic co-administration of metformin (200 mg/kg, i.p.) inhibited P-S6RP immunoreactivity in chronic morphine administration. Fluorescent images of P-S6RP (green) did not show co-localization with GFAP (astrocytic marker, red) n=3-5. Scale bars 100 µm. The single staining for each antibody and the merged image are shown from left to right and double staining appears in yellow. Scale bars 100 µm. **(A)** saline + vehicle, **(B)** morphine, **(C)** morphine + metformin, **(D)** metformin + saline.

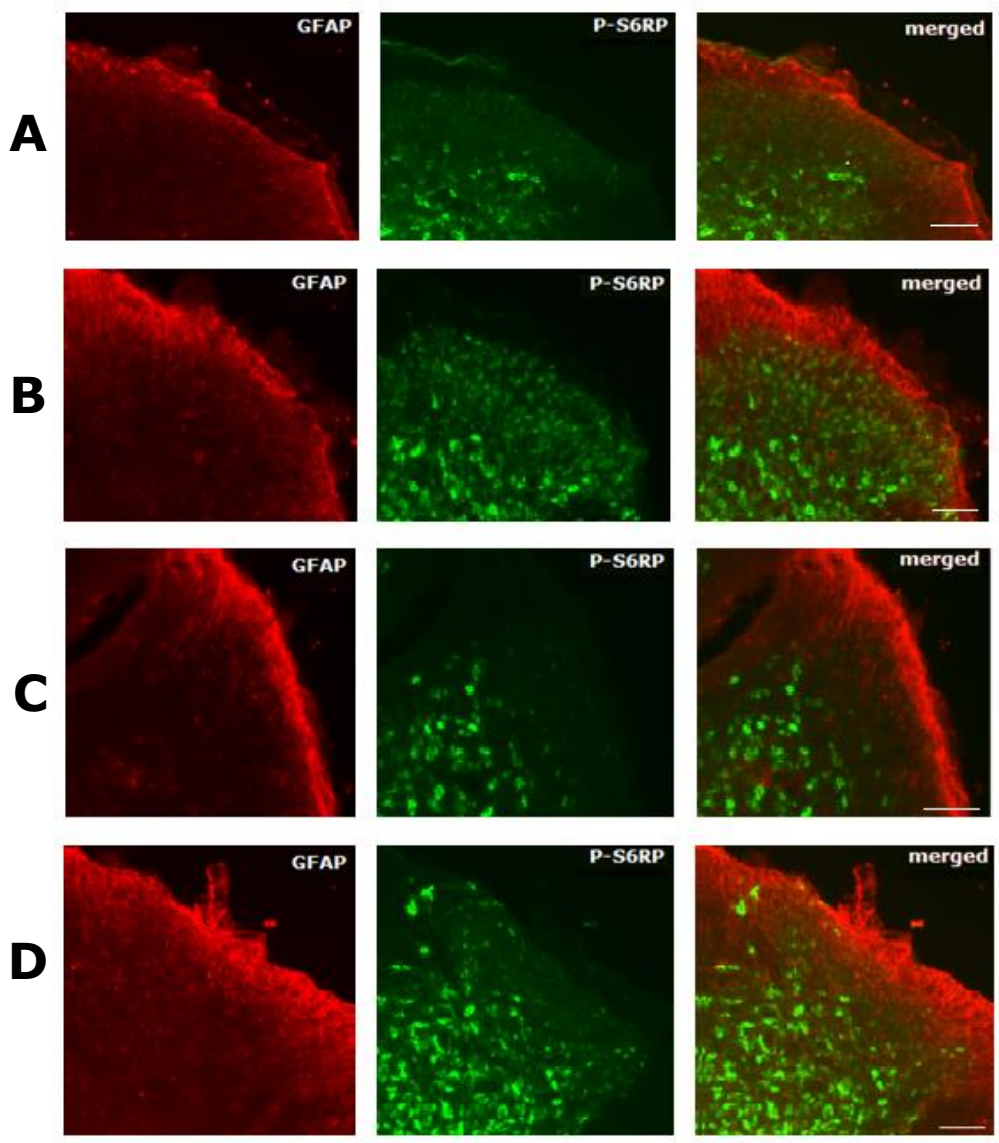


**Figure 4.6: Double labeling of mTORC1 effector and GFAP in lumbar spinal cord in *naïve* mice.** Immunofluorescence images showed the activation of phospho-S6 ribosomal protein (P-S6RP) after morphine administration in the in dorsal horn neurons. Chronic co-administration of CCI-779 (25 mg/kg, i.p.) inhibited P-S6 RP immunoreactivity in chronic morphine administration. Fluorescent images of P-S6RP (green) did not show co-localization with GFAP (astrocytic marker, red) n=3-5. Scale bars 100 μm. The single staining for each antibody and the merged image are shown from left to right and double staining appears in yellow. Scale bars 100 μm. **(A)** saline + vehicle, **(B)** morphine, **(C)** morphine + CCI-779, **(D)** CCI-779 + saline.

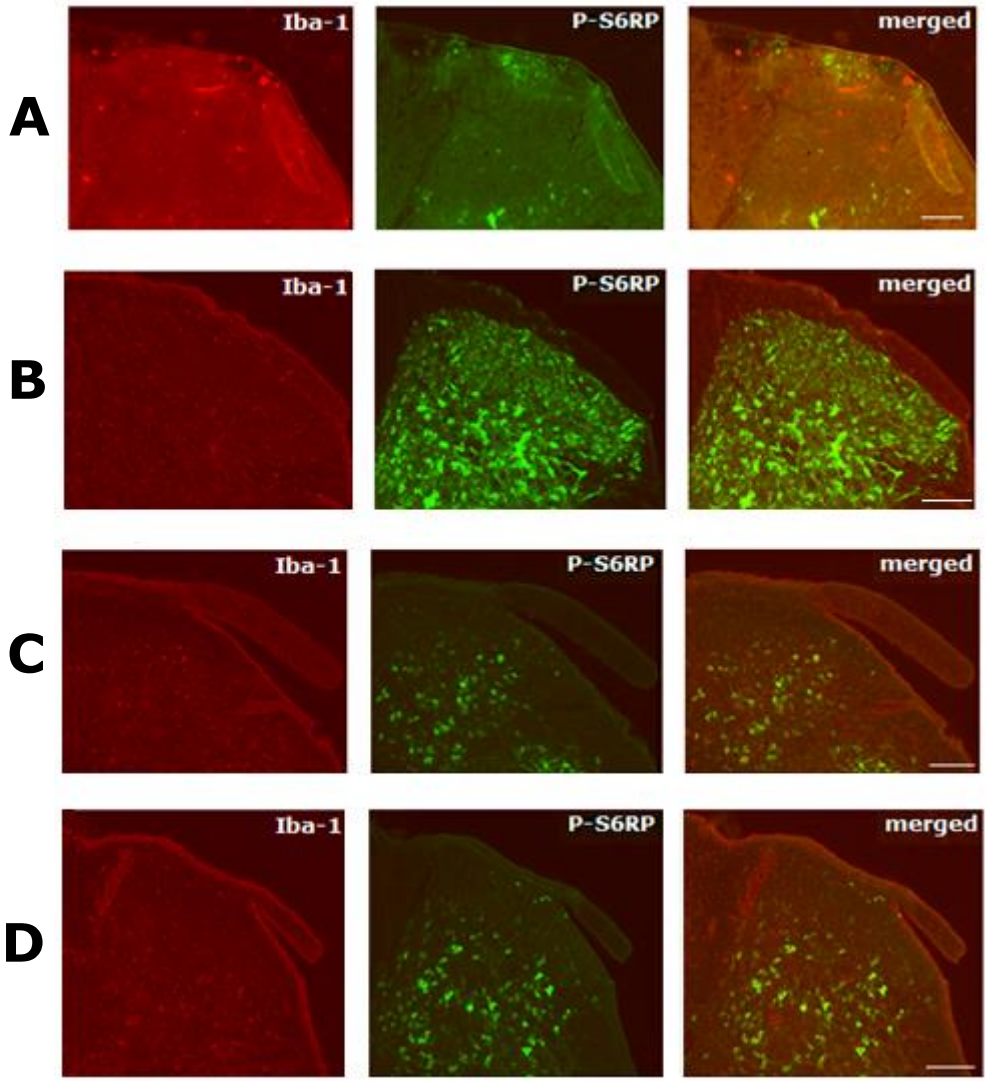


**Figure 4.7: Double labelling of mTORC1 effector and Iba-1 in lumbar spinal cord in *naïve* mice.** Immunofluorescence images showed the activation of phospho-S6 ribosomal protein (P-S6RP) after morphine administration in the in dorsal horn neurons. Chronic co-administration of metformin (200 mg/kg, i.p.) inhibited P-S6 RP immunoreactivity in chronic morphine administration. Fluorescent images of P-S6RP (green) did not show co-localization with Iba-1 (microglial marker, red) n=3-5. Scale bars 100 μm. The single staining for each antibody and the merged image are shown from left to right and double staining appears in yellow. Scale bars 100 μm. **(A)** saline + vehicle, **(B)** morphine, **(C)** morphine + metformin, **(D)** metformin + saline.

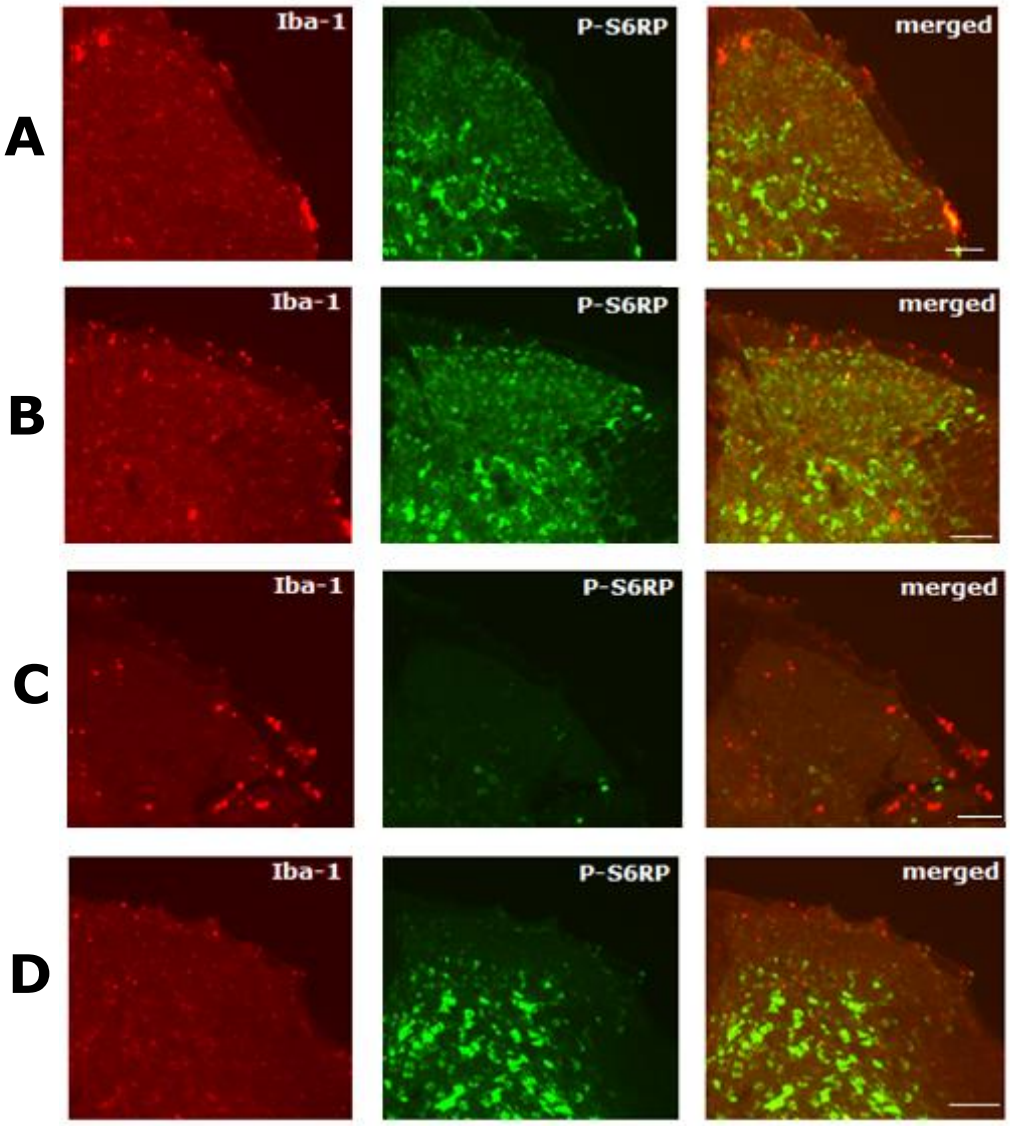


**Figure 4.8: Double labelling of mTORC1 effector and Iba-1 in lumbar spinal cord in *naïve* mice.** Immunofluorescence images showed the activation of phospho-S6 ribosomal protein (P-S6RP) after morphine administration in the in dorsal horn neurons. Chronic co-administration of CCI-779 (25 mg/kg, i.p.) inhibited P-S6RP immunoreactivity in chronic morphine administration. Fluorescent images of P-S6RP (green) did not show co-localization with Iba-1(microglial marker, red) n=3-5. Scale bars 100 μm. The single staining for each antibody and the merged image are shown from left to right and double staining appears in yellow. Scale bars 100 μm. **(A)** saline + vehicle, **(B)** morphine, **(C)** morphine + CCI-779, **(D)** CCI-779 + saline.

## 4.4 Discussion

In this chapter, systemic administration of mTORC1 inhibitors, metformin and CCI-779 reduced mTORC1 activity in *naïve* mice exposed to chronic morphine was demonstrated. The data obtained from western blotting and immunohistochemistry analysis strongly supports the behavioural results confirming my hypothesis about the involvement of the mTORC1 pathway in the regulation of opioid efficacy as presented in Chapter 3. In particular, this data emphasise the significant role of the mTORC1 pathway in morphine-induced tolerance highlighting the fact that metformin or CCI-779 improved morphine analgesia through blocking the activity of the mTORC1 pathway. Thus, the inhibition of spinal mTORC1 is a potential therapeutic target for prolonged opioid efficacy.

The activity of the mTORC1 pathway could be measured by the changes in the protein expression levels of its downstream effectors. It is well documented that the activation of mTORC1 triggers the phosphorylation of its downstream effectors 4EBP1 (cap-dependent translation) and p70S6K (regulate the translation of oligopyrimidine a class of mRNA transcripts), leading to initiation of the protein translation process which results in the biosynthesis of new proteins implicated in the modification of synaptic plasticity and the development of neurons (Jefferies et al., 1997). In brief, phosphorylation of 4EBP1 by mTORC1 releases eIF4E that is integrated with the free eIF4G to form eIF4F complex that initiates the protein translation process. Phosphorylation of p70S6K by mTORC1 kinase directly modulates S6RP which regulate the cellular translational capacity (Beretta et al., 1996). In this current study the activity of mTORC1 was assessed by measuring the phosphorylation level and expression of two downstream effectors 4EBP1 and S6RP in the spinal cord of *naïve* mice. mTORC1 and its activated counterparts were shown to be expressed in the mammalians nervous system, in particular within the dorsal root ganglion and superficial dorsal horn in lamina I and lamina II projection neurons (Geranton et al., 2009; Xu et al., 2010) and this current immunohistochemistry study further supports this observation. It was also showed that repeated morphine administration (subcutaneously at a dose of 20 mg/kg for 5 consecutive days) in *naïve* mice induced an increase in P-mTOR, P-p70S6K and P-4EBP1 expression levels in the dorsal horn neurons (Xu et al., 2014). Likewise, it was documented that the lumbar segments of the spinal cord in mice displayed significant increases in the phosphorylated counterparts of mTORC1 and 4EBP1 in mice treated with subcutaneous morphine twice daily at a dose of 20 mg/kg for 5 consecutive days (Zhang et al., 2019) suggesting that spinal mTORC1 may play a vital role in morphine tolerance. The immunofluorescence presented in this study further supports this claim as a

significant increase in the expression of P-S6RP was detected during chronic morphine administration. Interestingly, Xu et al. (2014) also showed that P-mTOR was not detected in μ-opioid receptors knockout animals suggesting a potential interaction between those two systems. In addition, since it has been reported that the superficial lamina I and lamina II is occupied by a great density of μ-opioid receptors that are implicated in nociception and morphine analgesia (Kumamoto et al., 2011) and mTORC1 and its activated counterparts were shown to be expressed there as well (Geranton et al., 2009; Xu et al., 2010), it may be possible that the anatomical localization may be interrelated to morphine induced tolerance as those two molecules may directly interact and this interaction may result in the regulation of opioid system by the changes in the activity of mTORC1. To support this, Xu et al. (2014) showed that μ-opioid receptor co-localized with mTOR and P-mTOR after twice daily intrathecal morphine injection at a dose of 10 μg for 6 consecutive days in *naïve* mice. This indicates that prolonged morphine administration induced μ opioid activation of the PI3K/Akt pathway in the dorsal horn neurons resulting in activation of mTORC1 signalling and its downstream effectors that are implicated in the development and maintenance of analgesic tolerance to morphine due to changes in neuroplasticity in the dorsal horn of the spinal cord *via* protein translation (Xu et al., 2014).

However, presented here immunoblotting analysis showed that repeated systemic morphine treatment did not affect the protein expression level of mTORC1 effectors when compared to the saline treated controls. It is possible that the difference in the observed effects between this current study and available literature is related to time-points during opioid exposure when the tissue samples were collected suggesting that time was potentially an important factor in relation to detecting the changes in the downstream targets of mTORC1. Indeed, Xu and colleagues (2014) showed that there is a time-dependent increase in the phosphorylated mTOR, p70S6K and 4EBP1 expression, although not in the total mTOR, p70S6K and 4EBP1 expression, after intrathecal morphine injection (Xu et al., 2014). They established that the maximum protein expression level for mTOR, p70S6K and 4EBP1 was detected after 7 days of treatment even though the experiment lasted 12 days (Xu et al., 2014). Furthermore, also *in vitro* study using HEK293 cells transfected with μ-opioid receptor reported time-sensitive increase in the mTOR phosphorylation level that began 5 minutes after morphine exposure and lasted for 2 hours (Wang et al., 2015). Thus, the discrepancy observed in this present study in morphine treated animals, could be linked to the time the tissue was collected, as it was harvested on day 9 of morphine administration or due to the delay associated with the last morning morphine injection.

Importantly, the present immunoblotting study revealed a significant decrease in protein levels of two mTORC1 markers, P-S6RP and P-p70S6K, after chronic and acute systemic co-administration of metformin or CCI-779 with morphine, implying that mTORC1 inhibitors potentially suppressed the protein synthesis apparatus associated with protein translation activated during chronic morphine exposure. Thus, this current finding strongly supports the idea that inhibition of the development and maintenance of morphine-induced tolerance and fully restoring the analgesic effect of morphine in mice is associated with inhibition of mTORC1 activity in spinal cord dorsal horn resulting from chronic co-administration of metformin and CCI-779 with morphine. This suppressing effect of systemic metformin and CCI-779 on the activity of mTORC1 in the dorsal horn was also observed by others. For example, Zhang et al. (2019) showed that systemic administration of rapamycin at a dose of 2.5 mg/kg resulted in a significant decrease in the expression of P-mTOR and other downstream effector P-4EBP1 in the spinal dorsal horn in comparison to morphine and vehicle groups of *naïve* mice. Importantly, the effect of the systemic administration of metformin and CCI-779 presented here is also consistent with the results obtained from the intrathecal rapamycin injection experiments (Géranton et al., 2009; Xu et al., 2014). Specifically, Géranton and co-workers (2009) reported that intrathecal rapamycin reduced the P-4EBP1/2 and P-p70S6K in the dorsal horn in *naïve* rats, signifying that rapamycin blocked spinal mTORC1 activity (Géranton et al., 2009). Similarly, intrathecal rapamycin was also showed to block the upregulation of P-mTOR, P- p70S6K and P-4EBP1 in the spinal dorsal horn of *naïve* rats (Xu et al., 2014), suggesting that spinal mTORC1 inhibition with rapamycin blocked the initiation of mRNA translation in the dorsal horn which contributed to morphine tolerance (Xu et al., 2014).

Additionally, the immunoblotting analysis presented here showed a significant decrease in the phosphorylation level of downstream targets of the mTORC1 after chronic CCI-779 treatment, but not metformin, showing that the repeated CCI-779 injection alone reduced the level of P-S6RP/S6RP in the spinal cord. This finding is in agreement with other studies that revealed that CCI-779 reduced the level of the downstream effectors of mTORC1, particularly P- p70S6K /p70S6K and P-4EBP1/4EBP1 in the spinal cord (Obara et al., 2011). The fact that metformin alone did not affect the expression level of mTORC1 could be related the pharmacodynamic and selectivity differences between metformin and CCI-779 as CCI-779 is a direct and selective mTORC1 inhibitor while metformin inhibits mTORC1 *via* activation of the adenosine monophosphate-activated protein kinase (AMPK; Kim, 2016). Given that both metformin and CCI-779 are well distributed and can easily cross the

brain-blood barrier, it should be also pointed out that systemic metformin and CCI-779 administrations may target areas other than the spinal cord which are associated with pain, such as the dorsal root ganglion and various regions of the brain (Łabuzek et al., 2010; Kuhn et al., 2007). It has been documented that mTORC1 and p70S6K are activated in the dorsal root ganglion resulting in neuronal plasticity during chronic inflammatory pain (Liang et al., 2013), cancer pain conditions (Shih et al., 2012), while rapamycin can alleviate pain by blocking mTORC1 activation in the dorsal root ganglion in addition to the dorsal horn (Géranton et al., 2009). Thus, the dorsal root ganglia may be a target for systemic administration of metformin and CCI-779 as well and further studies are required to confirm this. It could be possible that while changes in the activity of mTORC1 after metformin administration were not detected in the spinal cord, they may occur in the dorsal root ganglia and this site of metformin action may still regulate opioid efficacy. Moreover, it has been documented that mTORC1 signalling has been detected in the hippocampus, amygdala and ventral tegmental areas that are important areas for morphine-induced conditioned place preference and memory (Costa-Mattioliel et al., 2009; Cui et al., 2010; Mazei-Robison et al., 2011). This indicates that mTORC1 signalling has a broad function in the nervous system and systemic metformin and CCI-779 administrations could target regions other than the dorsal horn and these other sites of action should be taken into investigation when considering targeting of mTORC1 for the improvement of opioid efficacy.

Multiple studies showed the role of both neuronal and non-neuronal cells in the regulation of morphine tolerance (e.g., Song et al., 2001; Mao et al., 2002; Horvath et al., 2010; Fukagawa et al., 2013). It is evident that long-term morphine treatment activates the astrocytes and microglia cells (Garrido et al., 2005). Activation of these non-neuronal cells releases pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα), that influence synaptic transmission and that are involved in the development of morphine tolerance (Kao et al., 2012; Hua et al., 2016; Pan et al., 2016). Notably, it has been documented that both astrocytes and microglia cells did not overlap with μ-opioid receptors in the rat's spinal cord, either in normal conditions or after repeated morphine injections suggesting that glial cells are only indirectly activated by repeated morphine injections (Kao et al., 2012).

Immunofluorescence images in this present study showed an increase in GFAP expression, although not in Iba-1, in the spinal cord after repeated systemic morphine injections in *naïve* mice. It is well established that either systemic or intrathecal repeated morphine injection increase microglial activation (Johnston et al., 2004; Raghavendra et al., 2002; Hutchinson

et al., 2008) thus this current observation is unexpected. The reason behind this difference may lay in the technical differences and imaging methods.

Immunohistochemical analysis of the dorsal horn sections showed that P-S6RP did not co-localize with either GFAP or Iba-1 in the morphine-treated group or saline-treated group across all the tested sections, though it was detected in neurons. This result is consistent with other reports that showed P-mTOR, P- p70S6K and P-4EBP1 in neurons but not in astrocytes or microglia of the spinal dorsal horn (Xu et al., 2014). Additionally, it has been documented that the upsteam effectors of mTORC1, such as Akt and P-Akt, did not co-localize with markers of astrocytes and microglia cells in *naïve* rats or under inflammatory conditions induced by carrageenan injection (Choi et al., 2010; Xu et al., 2011). In contrast, however, P-mTOR was detected in the spinal astrocytes of rats after ischemic injury (Codeluppi et al., 2009). It could be that co-localization of the mTORC1 and its downstream effectors with glial cells depends on the condition that has driven the change in the mTORC1 activity.

Co-injection of mTORC1 inhibitors, metformin and CCI-779 with morphine, did not change the GFAP and Iba-1 expression in the spinal dorsal horn of *naïve* mice. Given that μ-opioid receptors were shown not to co-localize with glial cells (Kao et al., 2012) and P-S6RP did not co-localize with these types of cells in this current study, it is possible that the effectiveness of the systemic administration of metformin or CCI-779 in attenuating morphine tolerance which was observed in the behavioural study is due to the mTORC1 signalling pathway being blocked within other cells (presumably neurons) and this effect is not dependent on the inhibition of astrocytes and microglia cytokines production. Additionally, it has been recognised that systemic CCI-779 at a dose of 25mg/kg for 4 days demonstrated no significant difference in astrocyte levels in the contralateral side of the spinal dorsal horn in mice exposed to SNI as well as systemic CCI-779 administration did not change microglia expression and the TNF-α protein level in the spinal dorsal horn following SNI compared with vehicle treated animals thus indicating that astrocytes and microglia cells were unaffected by repeated systemic injection with mTORC1 inhibitor CCI-779 (Obara et al., 2011).

Interestingly however, Pan and co-workers (2016) reported that metformin at a dose of 200mg/kg., i.p. significantly reduced spinal microglial activation after chronic morphine administration and reduced morphine-induced TNF-α and IL-1β protein expression in the spinal cord suggesting that metformin attenuated morphine tolerance by inhibiting the upregulation of microglial induced by chronic morphine *via* AMPK activation (Pan et al., 2016). This is a single observation that requires further confirmation however this discrepancy in the metformin results may directly reflect the difference in animal species

used (CD-1 *vs.* C57BL/6J) and the timing of the injection (15 min prior to the morphine injection *vs.* 20 h prior to the morphine injection). The effect of mTORC1 inhibitor on glia activity may also result from affinity and selectivity of the inhibitor. It was shown recently, that administration of the second generation of mTORC1 inhibitor KU0063794 in model of spinal cord injury showed more potent effect in attenuating GFAP expression than rapamycin or CCI-779 (Cordaro et al., 2017). Additionally, the western blot analysis revealed a marked decrease in the expression of IL-1β and TNF-α production in mice spinal tissues treated with KU0063794 in comparison to rapamycin or CCI-779 treated mice (Cordaro et al., 2017). Another factor that could be taken into consideration that may have an effect on the mTORC1 inhibitors on glia activity is the type of condition under which the interaction between mTORC1 and glia is investigated. For example, it has been documented that cultured astrocytes isolated from the spinal cord of a rat required mTORC1 activity and that the culture did not grow when treated with rapamycin. Implication of this effect was shown in in spinal cord injury model where rapamycin showed beneficial therapeuthic effect associated reducing astrocyte proliferation (Codeluppi et al., 2009)

In conclusion, the western blotting and immunohistochemistry data in this current study demonstrated that systemic metformin or CCI-779 administration with morphine resulted in the significant inhibition of mTORC1 activity in the spinal cord. This inhibition was manifested as a reduction in P-p70S6K/p70S6K and P-S6RP/S6RP protein levels and reduced the p-S6RP fluorescence density or intensity in the dorsal horn of the spinal cords of *naïve* mice. The immunolabelling for p-S6RP showed co-localization mostly with NeuN but not GFAP or Iba-1 in morphine tolerance animals. Thus P-S6RP are expressed in neurones of the spinal dorsal horn and it is up-regulated in repeated morphine exposure. This suggests participation of the mTORC1 signalling pathway contributes to morphine tolerance in *naïve* mice. In the following chapter, (Chapter 5) aimed to confirm the precious effect of mTORC1 signalling cascade inhibition on the analgesic effect of morphine.

# Chapter 5. Determination of mechanisms that underlay the additive analgesic benefits of morphine treatment resulting from the effects produced by mTORC1 inhibitors

## 5.1 Introduction

Opioid-induced tolerance continues to be a challenging pharmacological effect associated with long-term treatment of opioids, even though intensive research into the neurobiological mechanisms of opioid-induced tolerance has been extensively conducted. It is acknowledged that constant and long-term exposure to opioids produces mediated adaptive changes within opioid receptors, comprising internalisation, desensitisation, phosphorylation, in addition to downregulation (Dumas and Pollack, 2008; Ueda, 2016). Additionally, it should be noted that chronic opioid exposure generates adaptive changes in various neuronal circuits, as well as activation of anti-opioid systems (Ueda, 2016). Each of these specific changes most probably arise by means of the translational modulation of numerous individual proteins, counteract opioid analgesia in addition to contributing to the mechanisms of opioid-induced tolerance (Xu et al., 2014). Recent research states that rapamycin-sensitive pathways are a significant factor in the mechanism governing neuroplasticity in opioid-induced tolerance (Xu et al., 2014: Zhang et al., 2019).

It is essential to mention that the magnitude of mTORC1 in relation to health and diseases has encouraged the synthesis of compounds that inhibit mTORC1 signalling. These include a family of rapalogs (temsirolimus known as CCI-779, sirolimus, deforolimus and everolimus) that build a complex with FK506-binding protein 12 (FK-BP12) that inhibits mTOR activity (Li et al., 2014). Conversely, these drugs, used as immunosuppressants or anti-cancer medications, caused several side-effects that could be serious or debilitating (Sacks, 1999; Morrisett et al., 2002). In contrast, AMPK activator metformin, a negative regulator of mTORC1, belongs to a biguanide class of antidiabetic drugs which are acknowledged to have a good safety profile, although there may possibly be a number of unwanted side-effects such as diarrhoea, nausea and dyspepsia in roughly 30% of people who use it (McCreight et al., 2016). Thus, metformin was employed in this research, as a novel target concerning the treatment of morphine tolerance *via* targeting mTORC1.

Remarkably, data found in previous chapters revealed that metformin blocked morphine induced tolerance in *naïve* mice and potentiated morphine analgesia in the equivalent with the results taken from the parallel study with CCI-779. This suggested that these studies were

associated with inhibition of the mTORC1 signalling cascade that is involved in nociceptor regulation and protein translational changes. To confirm involvement of AMPK, mTORC1 and protein translation-related mechanisms in observed behavioural effects three separate investigations have been designed in this chapter. The first experiment provides further evidence of the AMPK-mediated effect in suppression of morphine-induced tolerance. This was achieved by injecting mice with a direct activator (A-769662) that is known to produce mTORC1 inhibition. The second investigation was conducted to prove the involvement of inhibition of protein translation in the effects underlying mTORC1 inhibition and observed in morphine-induced tolerance. This was achieved by injecting mice with anisomycin (known protein synthesis inhibitor). Finally, an additional investigation was performed to confirm the specificity of the action of CCI-779 on mTORC1 by injected mice with an analog of FK520 (ascomycin) which binds to FKBP12 but does not inhibit mTORC1.

## 5.2 Material and Methods

### 5.2.1 Subject

Adult male C57BL/6J mice (8 weeks of age, Charles River, UK) weighing 25-30 g at the beginning of the study were used. Mice were housed 4 per polyethylene cage on a sawdust bedding under standard conditions (12 h light/dark cycle, lights on from 8:00 am) with food and water available ad libitum. Experimental protocols were approved by the AWERB Committee at Durham and Newcastle University and were consistent with the guidelines provided by the UK Animals (Scientific Procedures) Act 1986 (For more details see Chapter 2).

### 5.2.2 Preparation and administration of drugs

***Morphine***

To induce analgesic tolerance, morphine (morphine sulphate salt pentahydrate; Sigma-Aldrich, UK) was prepared in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK) immediately prior to injection. Mice were weighed and then injected intraperitoneally (i.p.) with morphine (20 mg per kg body weight) or equivalent vehicle (saline) solution without morphine, as a control group, twice daily for 9 consecutive days, in a volume of 4ml per kg body weight.

***Compound A-769662***

To target AMPK pathway directly, A-769662 (Cat. No. 3336; Tocris Bioscience, United Kingdom) was prepared in a solution that consists of three solvents 1% DMSO,30% polyethylene glycol 400, 1% Tween 80 (Cool et al., 2006) immediately before injections. Mice were weighed and then injected intraperitoneally (i.p.) with a 1% v/w solution of A-769662 (30 mg per kg body weight) or equivalent vehicle solution without A-769662 as a control group. A-769662/vehicle was administered i.p. 20 h before first i.p. injection of morphine was given and then i.p. administration of A-769662/vehicle was continued for 8 consecutive days. The timing and concentration of A-769662 injections were based on previously published research using A-769662 (Melemedjian et al., 2011).

***Anisomycin***

To impair protein synthesis, anisomycin (Cat. No. 1290; Tocris, Bioscience, United Kingdom) was prepared in a vehicle of saline containing 10% DMSO immediately before injection (Chuang et al., 2012). Mice were weighed and then injected i.p. with a 1% v/w solution of anisomycin (150 mg per kg body weight) or equivalent vehicle solution without anisomycin as a control group. Anisomycin/vehicle was administered i.p. 20 h before first i.p. injection of morphine was given and then i.p. administration of anisomycin/vehicle was continued for 8 consecutive days. The concentration of anisomycin injections was based on previously published research using anisomycin (Zhao et al., 2001; Hong et al., 2007; Wanisch and Wotjak, 2008).

***Ascomycin (FK520)***

FK520 which binds to FKBP12 and does not inhibit mTOR activity, was used as a negative control for systemic (i.p.) administration. Ascomycin (FK520; Cat. No.4210; Tocris, United Kingdom) was prepared in a solution consisting of 10% ethanol, 40% polyethylene glycol 400 in saline (Mollison et al., 1998) immediately before injection. Mice were weighed and then injected i.p. with a 1% v/w solution of FK520 (10 mg per kg body weight) or equivalent vehicle solution without FK520 as a control group. FK520/vehicle was was administered i.p. 20 h before first i.p. injection of morphine was given and then i.p. administration of ascomycin/vehicle was continued for 8 consecutive days. The concentration of ascomycin injections was based on previously published research using this drug (Hatanaka et al., 1988; Mollison et al., 1998).

### 5.2.3 Nociceptive threshold in *naïve* mice

The pain threshold to a thermal stimulus in *naïve* mice was assessed by tail-flick latency (Analgesia Meter, Ugo Basile, Italy) as described in details in Chapter 2. Shortly, tail-flick latency to noxious heat was determined by applying a heat stimulus to the dorsal tail surface approximately 2 cm from the tip of the tail. The cut off time for the tail-flick latency was set to 9 s. Mice were tested for their baseline latencies before first i.p. administration of morphine and then, in the morning of each testing day, they were tested for analgesia 30 min after i.p. morphine administration. Mice were also monitored daily for any signs of heat-induced skin lesions resulting from repeated tail-flick testing. No animal developed injuries that may affect their nociceptive threshold to tail-flick stimulation.

### 5.2.4 Experimental design

To establish the role of mTORC1 inhibition on morphine-induced tolerance in *naïve* mice, two experiments were conducted:

*Experiment I*: On day 0 (Table 5.1, Figure 5.1), *naïve* mice were injected i.p. with A-769662 or anisomycin or FK520 20 h prior i.p. administration of morphine and then the injections were repeated for 8 consecutive days in order to assess the role of A-769662 or anisomycin or FK520 in the development and maintenance of morphine analgesic tolerance.

*Experiment II*: On day 7 (Table 5.1, Figure 5.1), *naïve* morphine tolerant mice were injected i.p. with A-769662 or anisomycin or FK520 20 h prior i.p. administration of morphine to determine whether a single injection of A-769662 or anisomycin or FK520 influenced and restored the morphine analgesic effect in morphine tolerant mice. The i.p. injection of A-769662 or anisomycin or FK520 was also repeated on day 8 to determine whether any following injection of A-769662 or anisomycin or FK520 potentiated the effect resulting from the first injection.

Table 5.1 and Figure 5.1 below summarize the experimental design of both experiments.

**Table 5.1: A summary of experiments I and II illustrating schedule of the chronic and acute administration of 769662 or Anisomycin or FK520.**

| Days | Experiment I | | Experiment II | |
|---|---|---|---|---|
| | Chronic A-769662 or Anisomycin or FK520 | | Acute A-769662 or Anisomycin or FK520 | |
| | Morning | Evening | Morning | Evening |
| -1 | TF | | TF | |
| 0 | TF → D | | TF | |
| 1 | TF → M → TF → D | M | TF → M → TF | M |
| 2 | M → TF → D | M | M → TF | M |
| 3 | M → TF → D | M | M → TF | M |
| 4 | M → TF → D | M | M → TF | M |
| 5 | M → TF → D | M | M → TF | M |
| 6 | M → TF → D | M | M → TF | M |
| 7 | M → TF → D | M | M → TF → D | M |
| 8 | M → TF → D | M | M → TF → D | M |
| 9 | M → TF | | M → TF | |

**TF**: tail-flick test
**D**: drug; A-769662 (30 mg/kg, i.p.) or anisomycin (150mg/kg, i.p.) or FK520 (10mg/kg, i.p.)
**M**: morphine (20mg/kg, i.p.)
**Chronic administration**: A-769662 (30 mg/kg, i.p.) or anisomycin (150 mg/kg, i.p.) or FK520 (10 mg/kg, i.p.) injected 20 h before first morning morphine i.p. injection; then repeated for 8 consecutive days
**Acute administration**: A-769662 (30 mg/kg, i.p.) or anisomycin (150 mg/kg, i.p.) or FK520 (10 mg/kg, i.p.) injected on day 7 20 h before morning morphine i.p. injection on day 8. Also, another systemic injection of metformin A-769662 (30 mg/kg, i.p.) or anisomycin (150 mg/kg, i.p.) or FK520 (10 mg/kg, i.p.) was injected on day 8 20 h prior to morning morphine i.p. injection on day 9. After completion of chronic and acute administrations mice were sacrificed.

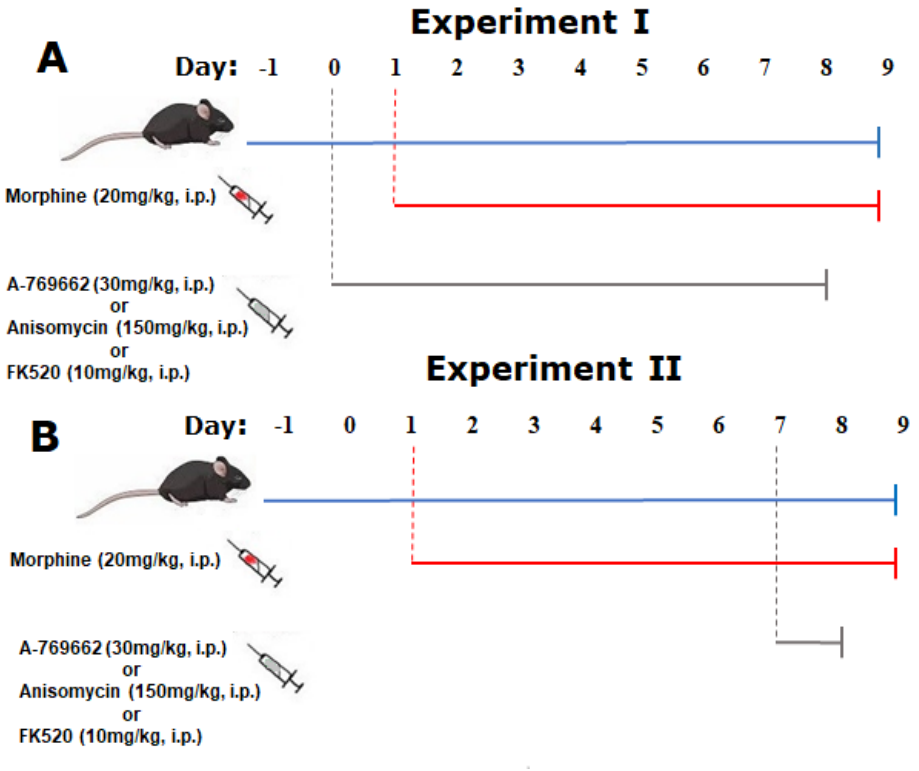


**Figure 5.1: A schematic illustration of experiment I and II. (A; experiment I)** Represents chronic administration of A-769662 (30 mg/kg, i.p.) or anisomycin (150 mg/kg, i.p.) or FK520 (10 mg/kg, i.p.) schedule where these drugs were administered 20 h before first morphine i.p. injection and then repeated subsequently for 8 consecutive days (once daily after morning morphine injection). **(B; experiment II)** Represents acute administration of A-769662 (30 mg/kg, i.p.) or anisomycin (150 mg/kg, i.p.) or FK520 (10 mg/kg, i.p.) schedule where these drugs were injected in morphine tolerant mice on day 7 (first injection) and day 8 (second injection) 20 h before morning morphine i.p. injection on day 8 and 9. The blue line represents pain assessment using tail-flick test. The red line represents morning and evening injections of morphine (20 mg/kg, i.p.). The grey line represents i.p. drugs administrations.

### 5.2.4.1 Design of experiment I

*Naïve* mice were divided into 5 different experimental groups as indicated in Table 5.2. Group 1 (n= 7) consisted mice receiving morphine (20 mg/kg, i.p.) twice daily at 12 h intervals (8 am/8 pm) for 9 consecutive days (day1-9 in Table 5.1), group 2 (n= 6) received saline under identical conditions and served as controls. To determine the influence of the three different treatment on morphine analgesic effects, mice were injected with A-769662 (30 mg/kg, i.p.) or anisomycin (150 mg/kg, i.p.) or FK520 (10 mg/kg, i.p.) 20 h prior the morning morphine (20 mg/kg, i.p.) on each testing day (day 0-8; group 3, n= 6-8). To determine the effect of the drug treatment without morphine, mice were injected i.p. with A-769662 (30 mg/kg, i.p.) or anisomycin (150 mg/kg, i.p.) or FK520 (10 mg/kg, i.p.) 20 h prior to the morning saline i.p. injection for 8 following days (group 4, n= 7). Finally, in group 5 (n= 6), mice were injected i.p. with an equivalent vehicle solution without A-769662, anisomycin and FK520 (controls).

**Table 5.2: Design of experiment I.**

| Group | Morphine 20mg/kg (i.p.) | Saline (i.p.) | Chronic administration | | | Vehicle (i.p.) | Number of mice in each group |
|---|---|---|---|---|---|---|---|
| | | | A-769662 30mg/kg (i.p.) | Anisomycin 150 mg/kg (i.p.) | FK520 10mg/kg (i.p.) | | |
| 1 | + | | | | | | 7 |
| 2 | | + | | | | | 6 |
| 3 | + | | | + | | | 6-8 |
| 4 | | + | | + | | | 7 |
| 5 | | | | | | + | 6 |

*Naïve* mice were randomly assigned into 5 groups and received different treatment: (1) i.p. morphine twice daily; morning injections were given between 9:00 am and 10:00 am, and evening injections were given between 8:00 pm and 9:00 pm; (2) controls; i.p. saline twice daily between 9:00 am and 10:00 am and between 8:00 pm and 9:00 pm; (3) morphine + Drug; morphine twice daily (as above) followed by i.p. A-769662 or anisomycin or FK520 (given between 1:00 pm and 2:00 pm) and then morphine evening injection;.(5) i.p. A-769662 or anisomycin or FK520 were given once daily (as above). (5) controls; i.p. vehicle once daily between 1:00 pm and 2:00 pm.

### 5.2.4.2 Design of experiment II

*Naïve* mice were divided into 5 different experimental groups as depicted in Table 5.3. A paradigm to induce morphine analgesic tolerance was identical as mentioned. Briefly, morphine at a dose of 20 mg/kg was administered i.p. every 12 h (group 1, n= 7). Control mice (group 2, n= 6) were injected with saline under identical conditions. Mice were rendered tolerant to morphine after 6 days of treatment. On day 7, mice were injected with A-769662 (30 mg/kg, i.p.) or anisomycin (150 mg/kg, i.p.) or FK520 (10 mg/kg, i.p.) (group 3, n= 8) 20 h prior to the morning morphine i.p. injection on day 8. The injection was repeated 20 h after (on day 8). For testing of the action of the different drugs without morphine control mice were injected i.p. with A-769662 (30 mg/kg, i.p.) or anisomycin (150 mg/kg, i.p.) or FK520 (10 mg/kg, i.p.) (group 4, n= 7) 20 h prior to the morning saline i.p. injection on day 8 and day 9. In group 5 (n= 6), mice were injected i.p. with equivalent vehicle solution without A-769662, anisomycin and FK520 (controls).

**Table 5.3: Design of experiment II.**

| Group | Morphine 20mg/kg (i.p.) | Saline (i.p.) | Acute administration | | | Vehicle (i.p.) | Number of animals |
|---|---|---|---|---|---|---|---|
| | | | A-769662 30mg/kg (i.p.) | Anisomycin 150 mg/kg (i.p.) | FK520 10mg/kg (i.p.) | | |
| 1 | + | | | | | | 7 |
| 2 | | + | | | | | 6 |
| 3 | + | | | + | | | 8 |
| 4 | | + | | + | | | 7 |
| 5 | | | | | | + | 6 |

*Naïve* mice were randomly assigned into 5 groups and received different treatment: (1) i.p. morphine twice daily; morning injections were given between 9:00 am and 10:00 am, and evening injections were given between 8:00 pm and 9:00 pm; (2) controls; i.p. saline twice daily between 9:00 am and 10:00 am and between 8:00 pm and 9:00 pm; (3) morphine + Drug; morphine twice daily (as above) followed by i.p. A-769662 or anisomycin or FK520 (given between 1:00 pm and 2:00 pm) on day 7 and day 8, and then morphine evening injection;.(5) i.p. A-769662 or anisomycin or FK520 were given once daily on day 7 and day 8. (5) controls; i.p. vehicle once daily between 1:00 pm and 2:00 pm.

### 5.2.5 Statistical data analysis

Data analysis and statistical comparisons were performed using GraphPadPrism, version 8.01 for Windows (GraphPad Software, CA, www.graphpad.com). Tail-flick latencies are expressed in seconds and presented as the means ± SEM. Each group included 6 to 8 mice. Comparisons between groups were performed using analysis of variance for ordinary measurements (two-way ANOVA) followed by Bonferroni's multiple comparison post-hoc test and Student's t-test was used when two groups were compared. A value of $P<0.05$ was considered statistically significant.

## 5.3 Results

### 5.3.1 Administration of A-769662 inhibited the development and maintenance of morphine-induced analgesic tolerance and restored the analgesic effect of morphine in *naïve* mice

As illustrated in Figure 5.2, administration of morphine (20 mg/kg, i.p.) to *naïve* mice produced a significant and potent analgesic effect on the first day of morphine treatment when compared to saline injected mice (day 1: 9.62 ± 0.11 s *vs.* 3.43 ± 0.21 s). For next 6 days of morphine administration (20 mg/kg, i.p.; twice daily), morphine consistently produced significant analgesia effect when compared to saline-injected mice, although the effect decreased gradually on the following days of chronic morphine administration. On day 8, morphine antinociceptive effect significantly decreased when compared to its efficacy on day 1 (day 8: 3.75 ± 0.17 s *vs.* day 1: 9.62 ± 0.11 s) and there was no significant difference in comparison to vehicle/saline treated mice (day 8: 3.45 ± 0.15 s *vs.* 3.60 ± 0.16 s, respectively) indicating for the development of morphine-induced analgesic tolerance.

Morphine-induced analgesic tolerance was observed until day 9 (treatment: $F_{(1,121)} = 908.20$, $P<0.0001$).

Interestingly, administration of A-769662 (30 mg/kg, i.p., once daily; Figure 5.2 A) for 9 consecutive days 20 h before morphine administration significantly prevented the development and maintenance of morphine analgesic tolerance as measured 30 min after morphine administration by tail-flick latency in *naïve* mice (Figure 5.2 A; treatment effect: $F_{(4,319)} = 768.70$, $P<0.0001$). Statistically significant difference in response to tail-flick stimulation between morphine-treated group and mice administered with A-769662 prior to morphine was observed on day 5 (day 5: 7.71 ± 0.21 s *vs.* 8.88 ± 0.08 s). This positive influence of A-769662 on the analgesic effect of morphine was continued over all tested days. Results were as follows: morphine + A-769662 *vs.* morphine, day 6: 7.87 ± 0.49 s *vs.* 5.90 ± 0.44 s; day 7: 7.58 ± 0.50 s *vs.* 4.97 ± 0.39 s; day 8: 7.61 ± 0.38 *vs.* 3.75 ± 0.17 s; day 9: 7.48 ± 0.52 s *vs.* 4.07 ± 0.13 s.

Control animals injected with vehicle or saline did not show any significant change in their response to tail-flick stimulation over all 9 days of testing (Figure 5.2 A). In addition, A-769662 (30 mg/kg, i.p., once daily) administered alone for 9 consecutive days had no significant effect on tail-flick latency when compared to the latency values of the vehicle/saline-treated group ($P>0.05$).

As illustrated in Figure 5.2 B, administration of two consecutive doses of A-769662 (30 mg/kg, i.p., once daily) on day 7 and then on day 8 of morphine (when tolerance to analgesic effect of morphine was established) resulted in fully restoring morphine's analgesic effectiveness. Specifically, after 6 days of morphine administration (20 mg/kg, i.p., twice daily every 12 h), mice were tolerant to morphine analgesia as measured by the tail-flick test (day 1: 9.20 ± 0.07 s *vs.* day 6: 5.15 ± 0.88 s). On day 7 of morphine treatment, a single injection of A-769662 (30 mg/kg, i.p.) 20 h prior to morphine injection on day 8 fully restored morphine analgesic efficiency (Figure 5.2 B). The tail-flick latencies measured 30 min after morphine injection on day 8, preceded by administration of A-769662, significantly increased when compared to morphine treated group (morphine + A-769662 *vs.* morphine, day 8: 7.28 ± 0.32 s *vs.* 4.97 ± 0.39 s, treatment effect: $t_{(13)}= 4.57$, $P<0.0005$). Additional systemic injection of A-769662 on day 8, prior to morphine injection on day 9, resulted in the maintenance of the analgesic effect of morphine (morphine + metformin *vs.* morphine, day 9: 7.14 ± 0.41 s *vs.* 3.06 ± 0.16 s, treatment effect $t_{(13)}= 8.737$, $P<0.0001$).

Acute administration of A-769662 alone had no antinociceptive properties as the tail-flick latency did not vary significantly from the respective values in mice chronically injected with saline/vehicle. Results were as follows: A-769662 *vs.* vehicle/saline, (day 8:3.48 ± 0.16 s *vs.* 3.60 ± 0.15 s/ 3.45 ± 0.15 s; day 9: 2.83 ± 0.31 s *vs.* 2.96 ± 0.10 s/3.01 ± 0.19 s).

### 5.3.2 Administration of anisomycin inhibited the development and maintenance of morphine-induced analgesic and restored the analgesic effect of morphine in *naïve* mice

As illustrated in Figure 5.3 A, administration of morphine (20 mg/kg, i.p.) to *naïve* mice produced a significant and potent analgesic effect on the first day of morphine treatment when compared to saline injected mice (day 1: 8.98 ± 0.01 s *vs.* 3.51 ± 0.21 s). For next 6 days of morphine administration (20 mg/kg, i.p.; twice daily), morphine consistently produced significant analgesia effect when compared to saline-injected mice, although the effect decreased gradually on the following days of chronic morphine administration. On day 8, morphine antinociceptive effect significantly decreased when compared to its efficacy on day 1 (day 8: 4.15 ± 0.22 s *vs.* day 1: 8.98 ± 0.01 s) and there was no significant difference in comparison to vehicle/saline treated mice (day 8: 3.53 ± 0.22 s *vs.* 3.53 ± 0.14 s, respectively) indicating for the development of morphine-induced analgesic tolerance (treatment: $F_{(1,110)}$ =995.10, P<0.0001)

Interestingly, administration of anisomycin (150 mg/kg, i.p., once daily) 20 h before morphine administration significantly prevented the development and maintenance of morphine analgesic tolerance as measured 30 min after morphine administration by tail-flick latency in *naïve* mice (Figure 5.3 A; treatment effect: $F_{(4,285)}$= 1238, P<0.0001). Statistically significant difference in response to tail-flick stimulation between morphine-treated group and mice administered with anisomycin prior to morphine was observed on day 5 of the experiment (day 5: 7.97 ± 0.31 s *vs.* 8.98 ± 0.01 s). This positive influence of anisomycin on the analgesic effect of morphine was continued over all tested days. Results were as follows: morphine + anisomycin *vs.* morphine, day 6: 6.01 ± 0.31s *vs.* 8.51 ± 0.22 s; day 7: 5.31 ± 0.25 s *vs.* 8.35 ± 0.17 s; day 8: 4.15 ± 0.22 *vs*. 7.22 ± 0.24 s.

Control animals injected with vehicle or saline did not show any significant change in their response to tail-flick stimulation over the days of testing (Figure 5.2 A). In addition, anisomycin (150 mg/kg, i.p., once daily) administered alone for 8 consecutive days had no significant effect on tail-flick latency when compared to the latency values of the vehicle/saline-treated group (P>0.05).

As illustrated in Figure 5.3 B, administration of two consecutive doses of anisomycin (150 mg/kg, i.p., once daily) on day 7 and then on day 8 of morphine treatment (when tolerance to analgesic effect of morphine was established) resulted in fully restoring morphine's analgesic effectiveness. Specifically, after 6 days of morphine administration (20 mg/kg, i.p., twice daily every 12 h), mice were tolerant to morphine analgesia as measured by the tail-flick test (day 1: 9.00 ± 0.01 s *vs.* day 6: 5.13 ± 0.70 s). On day 7 of morphine treatment, a single injection of anisomycin (150 mg/kg, i.p.) 20 h prior to morphine injection on day 8 fully restored morphine analgesic efficiency (Figure 5.3 B). The tail-flick latencies measured 30 min after morphine injection, on day 8 preceded by administration of anisomycin was significantly increased when compared with morphine treated group (morphine + anisomycin *vs.* morphine, 7.20 ± 0.29 s *vs.*4.15 ± 0.22 s treatment effect: $t_{(13)}$= 8.04, P<0.05). Additional systemic injection of anisomycin on day 8 prior to morphine injection on day 9 resulted in maintenance of the analgesic effect of morphine (morphine + anisomycin *vs.* morphine, 6.68 ± 0.38 s *vs.* 3.98 ± 0.18 s treatment effect: $t_{(13)}$= 6.11, P<0.05).

Acute administration of anisomycin (150 mg/kg, i.p., once daily) alone had no antinociceptive properties as the tail-flick latency did not vary significantly from the respective values in mice chronically injected with saline/vehicle. Results were as follows: anisomycin *vs.* vehicle/saline, day 8: 3.53 ± 0.21 s *vs.* 3.53 ± 0.14 s/ 3.75 ± 0.20 s; day 9: 3.55 ± 0.26 s *vs.* 3.75 ± 0.20 s/3.75 ± 0.20 s.

### 5.3.3 Administration of FK520 did not inhibit the development and maintenance of morphine-induced analgesic and did not restore the analgesic effect of morphine in *naïve* mice

As illustrated in Figure 5.4 A, administration of morphine (20 mg/kg, i.p.) to *naïve* mice produced a significant and potent analgesic effect on the first day of morphine treatment when compared to saline injected mice (day 1: 9.00 ± 0.04 s *vs.* 4.08 ± 0.21 s). For next 6 days of morphine administration (20 mg/kg, i.p.; twice daily), morphine consistently produced significant analgesia effect when compared to saline-injected mice, although the effect decreased gradually on the following days of chronic morphine administration. On day 8 morphine antinociceptive effect significantly decreased when compared to its efficacy on day 1 (day 8: 4.20 ± 0.63 s *vs.* day 1: 9.00 ± 0.04 s) and there was no significant difference in comparison to vehicle/saline treated mice (3.11 ± 0.19 s and 3.75 ± 0.18 s, respectively) indicating for the development of morphine-induced analgesic tolerance. Morphine-induced analgesic tolerance was observed on day 9 (treatment effect: $F_{(1,121)}$ = 875.00, P<0.0001).

Administration of FK520 (10 mg/kg, i.p., once daily) 20 h before morphine failed to prevent morphine induced analgesic tolerance as measured 30 min after morphine administration by tail-flick latency in *naïve* mice (Figure 5.4 A). A similar trend of morphine induced tolerance was observed in both morphine treated groups (morphine-injected mice and morphine + FK520-injected mice). Analysis revealed that, there was no difference in morphine antinociceptive effect in mice injected with morphine compared to mice injected with morphine preceded by FK520 (morphine *vs.* morphine + FK520, day1: 9.11 ± 0.07 s *vs*. 9.12 ± 0.04 s; day 2: 8.98 ± 0.01 s *vs.* 9.00 ± 0.06 s; day 3: 8.98 ± 0.01 s *vs.* 8.95 ± 0.06 s; day 4: 8.05 ± 0.19 s *vs.* 7.75 ± 0.41 s: day 5: 7.22 ± 0.12 s *vs.* 6.91 ± 0.26 s; day 6: 7.11 ± 0.19 s *vs.* 6.82 ± 0.14 s; day 7: 5.42 ± 0.17 s *vs*. 5.67 ± 0.22 s; day 8: 4.60 ± 0.36 *vs*. 4.35 ± 0.11 s; day 9: 3.90 ± 0.20 s *vs.* 3.87 ± 0.34 s, treatment: $F_{(1,43)}$= 0.32, P= 0.57, n.s).

The tail-flick latencies in morphine + FK520 group were statically different compared to saline-injected mice on days 1 to 7 (morphine + FK520 *vs*. saline, day1: 9.00 ± 0.06 s *vs*. 4.08 0.21 s; day 2: 9.00 ± 0.06 s *vs.* 3.75 ± 0.14 s; day 3: 8.95 ± 0.06 s *vs.* 3.78 ± 0.22 s; day 4: 7.75 ± 0.41 s *vs.* 3.61 ± 0.09 s: day 5: 6.91 ± 0.26 s *vs.* 3.53 ± 0.13 s; day 6 :6.82 ± 0.14 s *vs.* 3.55 ± 0.20 s; day 7: 5.67 ± 0.22 s *vs*. 3.93 ± 0.09 s, treatment effect: $F_{(4,319)}$=929.3, P<0.0001), whereas no significant difference was found on day 8 and day 9 (4.35 ± 0.11 s *vs*. 3.75 ± 0.18 s; 3.87 ± 0.34 s *vs*. 3.65 ± 0.16 s, respectively). In addition, administration of FK520 (10 mg/kg, i.p., once daily) alone for 8 consecutive days had no significant effect (P<0.05) on tail-flick latency when compared to the latency values of the vehicle/saline-treated animals.

As illustrated in Figure 5. 4 B, administration of two consecutive doses of FK520 (10 mg/kg, i.p., once daily) on day 7 and then on day 8 of morphine treatment (when tolerance to analgesic effect of morphine was established) failed to restoring morphine's analgesic effectiveness as it did not change the effect of morphine 30 min after its administration in morphine-tolerant mice. There was no significant difference in tail-flick latencies on days 8 and 9 compared with those for morphine alone (morphine + acute FK50 *vs*. morphine, day 8: 4.06 ± 0.22 s *vs.* 4.20 ± 0.63 s treatment: $t_{(13)}$= 1.28, P= 0.22 n.s; day 9: 3.70 ± 0.11 s *vs.* 3.90 ± 0.20 s, treatment: $t_{(13)}$= 0.87, P= 0.39 n.s).

Acute administration of FK50 (10 mg/kg, i.p., once daily) alone had no antinociceptive properties as the tail-flick latency did not vary significantly from the respective values in mice chronically injected with saline/vehicle. Results were as follows: FK520 *vs.*

vehicle/saline, (day 8: 3.33 ± 0.11 s *vs.* 3.11 ± 0.19 s/ 3.25 ± 0.23 s; day 9: 3.45 ± 0.15 s *vs.* 3.26 ± 0.06 s/3.35 ± 0.21 s).

### 5.3.4 A-769662 or anisomycin or FK520 in combination with morphine induced body weight loss in *naïve* mice

***A-769662***

As illustrated in Figure 5.5 A, repeated administration of morphine (20 mg/kg, i.p., twice daily) for 9 consecutive days induced gradually decrease in mouse body weight when compared with body weight values measured on day 0. The statistically significant difference in body weight was recorded on day 8 in *naïve* mice treated with morphine when compared with saline treated animals (treatment effect: $F_{(4,319)}$= 3.07, P<0.05). Administration of A-769662 (30 mg/kg, i.p., once daily) or vehicle did not affect the mouse body weight when compared with saline treated animals.

***Anisomycin***

As illustrated in Figure 5.5 B, repeated administration of morphine (20 mg/kg, i.p., twice daily) for 9 consecutive days induced gradually decrease in mouse body weight when compared with body weight values measured on day 0 (Figure 5.5 B). The statistically significant difference in body weight was recorded on day 7 in *naïve* mice co-treated with morphine and anisomycin (150 mg/kg, i.p., once daily) and anisomycin alone when compared with saline treated animals (treatment effect: $F_{(4,285)}$= 2.69, P<0.05). Administration of vehicle did not affect the mouse body weight when compared with saline treated animals.

***FK520***

As illustrated in Figure 5.5 C, repeated administration of morphine (20 mg/kg, i.p., twice daily) for 9 consecutive days induced gradually decrease in mouse body weight when compared with body weight values measured on day 0 (Figure 5.5 A). The statistically significant difference in body weight was recorded on day 7 in *naïve* mice treated with morphine when compared with saline treated animals (treatment effect: $F_{(4,319)}$= 3.94, P<0.0001). Administration of FK520 (10 mg/kg, i.p., once daily) or vehicle did not affect the mouse body weight when compared with saline treated animals.

It should be noted that despite of some effect of the treatment on the animal body weight, all animals subjected to experimental testing remain healthy and did not show any signs of

unexpected distress. However, in *naïve* mice co-treated with morphine and anisomycin or anisomycin alone showed signs related to high dose (body weight loss).

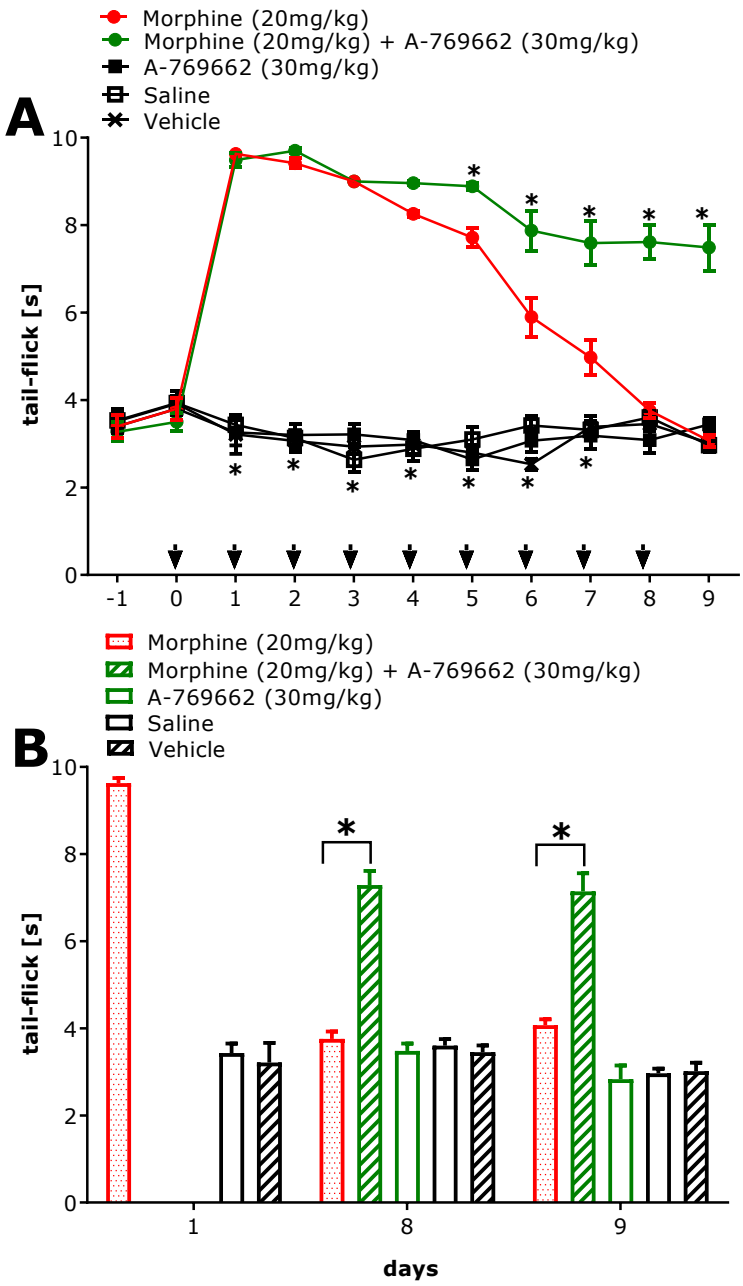


**Figure 5.2: A-769662 blocked the development and maintenance of morphine-induced tolerance (A) and restored the analgesic effect of morphine in morphine tolerant in *naïve* mice (B).** Tolerance was induced by morphine (20 mg/kg, i.p.) administered twice daily for 9 consecutive days. **(A)** Repeated systemic administration of A-769662 (30 mg/kg, i.p.) 20 h before first morphine injection and then subsequently for 8 days once daily after morning morphine injection blocked the development of morphine tolerance, as measured by tail-flick conducted daily 30 min after morning morphine. The data are presented as the mean responses ± SEM, n= 6-8 mice per group; *vs. morphine; *P<0.05 (two-way ANOVA followed by Bonferroni's test). The arrow indicates i.p. A-769662 administration. **(B)** On day 7 when morphine tolerance was developed a single systemic administration of A-769662 (30 mg/kg, i.p.) 20 h before next morning morphine injection restored the analgesic effect of morphine on day 8, as measured by tail-flick conducted 30 min after morning morphine. Another systemic injection of A-769662 (30 mg/kg, i.p.) 20 h before next morning morphine maintained the analgesic effect of morphine on day 9. The data are presented as the mean responses ± SEM, n= 8 mice per group; *vs. morphine; *P<0.05 (un-paired Student's t-test).

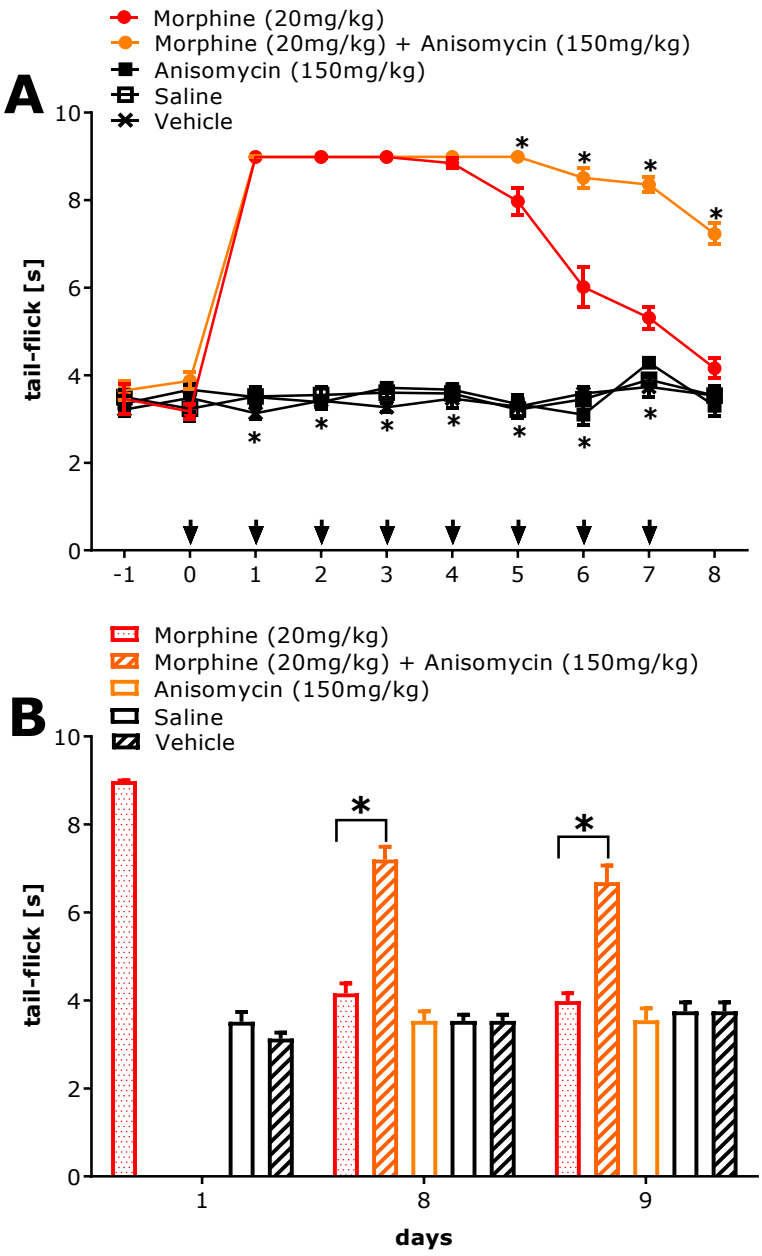


**Figure 5.3: Anisomycin blocked the development and maintenance of morphine-induced tolerance (A) and restored the analgesic effect of morphine in morphine tolerant in *naïve* mice (B).** Tolerance was induced by morphine (20 mg/kg, i.p.) administered twice daily for 9 consecutive days. **(A)** Repeated systemic administration of anisomycin (150 mg/kg, i.p.) 20 h before first morphine injection and then subsequently for 8 days once daily after morning morphine injection blocked the development of morphine tolerance, as measured by tail-flick conducted daily 30 min after morning morphine. The data are presented as the mean responses ± SEM, n= 6-8 mice per group; *vs. morphine; *P<0.05 (two-way ANOVA followed by Bonferroni's test). The arrow indicates i.p. anisomycin administration. **(B)** On day 7 when morphine tolerance was developed a single systemic administration of anisomycin (150 mg/kg, i.p.) 20 h before next morning morphine injection restored the analgesic effect of morphine on day 8, as measured by tail-flick conducted 30 min after morning morphine. Another systemic injection of anisomycin (150 mg/kg, i.p.) 20 h before next morning morphine maintained the analgesic effect of morphine on day 9. The data are presented as the mean responses ± SEM, n= 8 mice per group; *vs. morphine; *P<0.05 (un-paired Student's t-test).

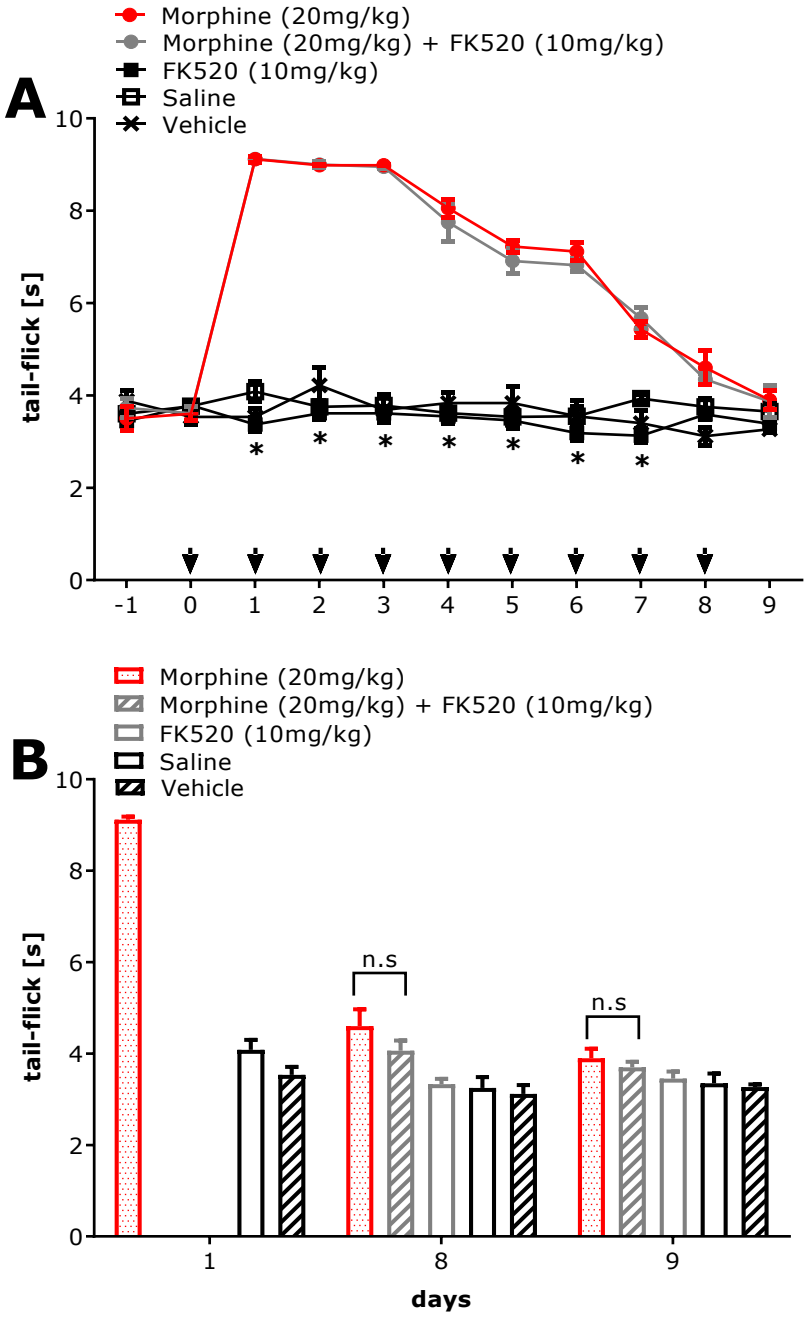


**Figure 5.4: FK520 blocked the development and maintenance of morphine-induced tolerance (A) and restored the analgesic effect of morphine in morphine tolerant in *naïve* mice (B).** Tolerance was induced by morphine (20 mg/kg, i.p.) administered twice daily for 9 consecutive days. **(A)** Repeated systemic administration of FK520 (10 mg/kg, i.p.) 20 h before first morphine injection and then subsequently for 8 days once daily after morning morphine injection had no effect on the development of morphine tolerance, as measured by tail-flick conducted daily 30 min after morning morphine. The data are presented as the mean responses ± SEM, n= 6-8 mice per group; *vs.* morphine; *P<0.05 (two-way ANOVA followed by Bonferroni's test). The arrow indicates i.p. FK520 administration. **(B)** On days 7 and 8 when morphine tolerance was developed a single systemic administration of FK520 (10 mg/kg, i.p.) 20 h before next morning morphine injection dose not restored the analgesic effect of morphine on days 8 and 9, as measured by tail-flick conducted 30 min after morning morphine. The data are presented as the mean responses ± SEM, n= 8 mice per group; *vs. morphine; *P<0.05 (un-paired Student's t-test).

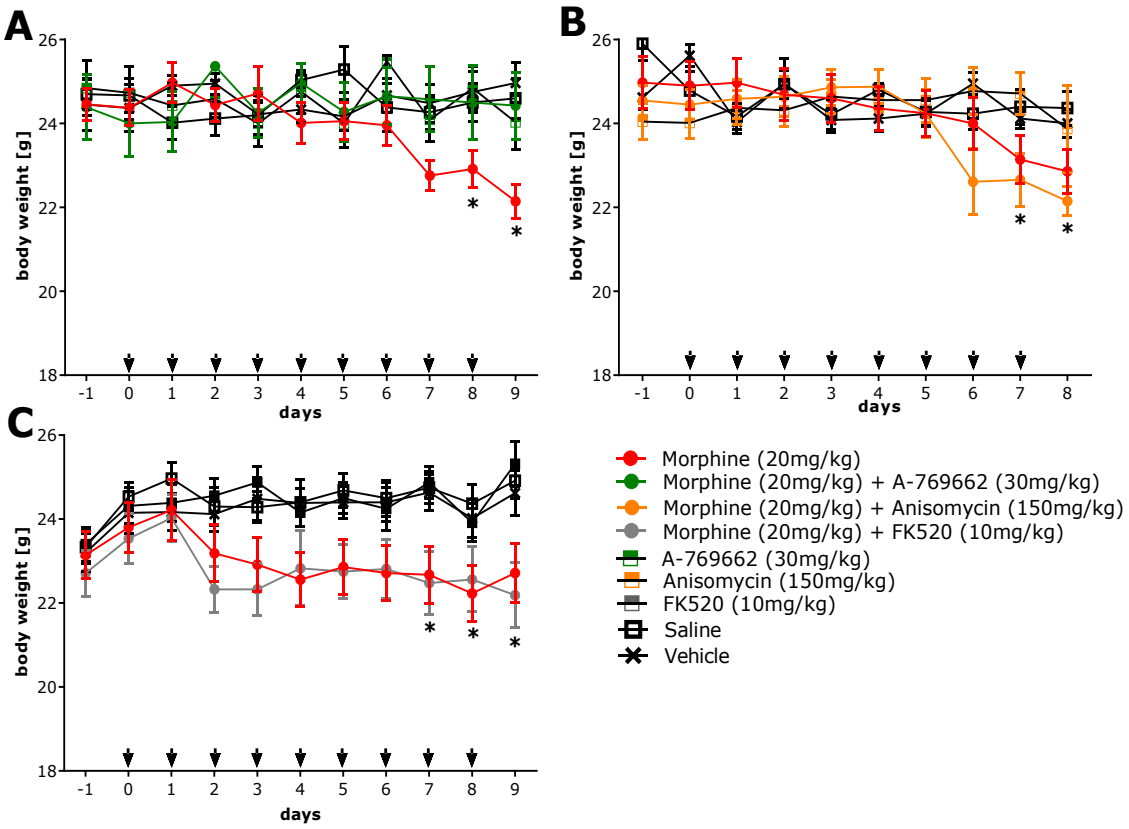


**Figure 5.5: Mean body weight changes of the *naïve* mice injected with (A) A-769662, (B) Anisomycin and (C) FK520.** Repeated administration of morphine (20 mg/kg, i.p.) twice daily for 9 consecutive days induced a gradual decrease in mouse body weight compared with saline/vehicle treated mice, the significant decrease was detected on day 7 or 8 of chronic morphine administration in the three experiment. **(A)** Repeated systemic administration of A-769662 (30 mg/kg, i.p.) 20 h before first morphine injection and then subsequently for 8 days once daily after morning morphine injection had no significant effect on mouse body weight ,as well in mice injected with A-769662 alone compared with vehicle/saline injected mice. **(B)** Repeated systemic administration of anisomycin (150 mg/kg, i.p.) 20 h before first morphine injection and then subsequently for 8 days once daily after morning morphine injection had significant effect on mouse body weight, as well in mice injected with anisomycin alone compared with vehicle/saline injected was observed on day 7. **(C)** Repeated systemic administration of FK520 (10 mg/kg, i.p.) 20 h before first morphine injection and then subsequently for 8 days once daily after morning morphine injection had no significant effect on mouse body weight ,as well in mice injected with FK520 alone compared with vehicle/saline injected mice. The data are presented as the mean responses ± SEM, n= 6-8 mice per group; *vs. saline; *P<0.05 (two-way ANOVA followed by Bonferroni's test). The arrow indicates i.p. A-769662 or anisomycin or FK520 administration.

## 5.4 Discussion

The principal findings in this chapter are as follows: (1) AMPK activation with A-769662 had a significant inhibitory impact on the development and maintenance of morphine tolerance, (2) administration of the protein synthesis inhibitor anisomycin, produced similar behavioural effects to those observed after metformin and CCI-779 treatment and identified as inhibition of the development and maintenance of antinociceptive tolerance to morphine, (3) administration of FK520, which binds to FKBP12 and does not inhibit mTORC1 activity, had no effect on the development and maintenance of morphine tolerance. Thus, the present data demonstrate that the reduction of morphine tolerance resulting from administration of A-769662, AMPK activator/mTORC1 inhibitor and anisomycin (a protein synthesis inhibitor) is similar to the effects produced by metformin and CCI-779 (Chapters 3). This may suggest that morphine tolerance appears to be mediated by the mTORC1 pathway and potentially involving regulation of protein synthesis.

***A-769662 blocked the development and maintenance of morphine-induced tolerance and restored morphine analgesia in naïve mice.***

The behavioural tests showed that mice developed antinociceptive tolerance to morphine. This was evident as the tail-flick latencies decreased in animals that repeatedly received morphine, since a comparable decrease was not seen in animals pre-treated with A-769662. These results suggested that AMPK activation by A-769662 is effective at attenuating morphine induced tolerance and restored morphine analgesia in tolerant mice.

A-769662 is well known as a direct activator of AMPK *in vitro* as well as *in vivo* research. Several studies have reported that AMPK activation is a requirement of A-769662 action, for example, the effect of A-769662 on glucose uptake under condition mimics ischemia in rat hearts (Timmermans et al., 2014), in regulating energy metabolism in the adipose tissue of mice (Wu et al., 2018) and regulation of lipid metabolism in the livers of mice (Foretz et al., 2018). Moreover, several cells assays and mutagenesis studies determined that A-769662 specifically bound to the β1 subunit, the regulatory site in the heterodimer structure of AMPK, resulted in allosteric activation of this kinase. Thus, using A-769662 in this current study was a valuable pharmacological target to study the physiological role of AMPK on morphine tolerance. Therefore, all the data generated from A-769662 administration to *naïve* mice is dependent on AMPK activation. This data confirms importance of AMPK activation induced by metformin administration and its effect on morphine-induced tolerance. While signalling mechanisms underlying metformin effects are not clear and often suggested to be

off target, the data related to the use of A-769662 may suggest that similar pathway potentially underlies behavioural effects produced by both metformin and A-769662.

The beneficial effect of AMPK activator on morphine tolerance has been addressed in pre-clinical studies. Based on the evidence that morphine tolerance resulted from activation of MAPK cascade (ERK, JNKs and p38/SAPKs) that led to changes in neuronal and glial mechanisms in the nervous system, it has been reported that AMPK activator blocked morphine tolerance mediated by microglial activation in *naïve* CD-1 mice (Han et al., 2014). They demonstrated that chronic injection of resveratrol at a dose of (10 mg/kg, .ip.) 15 min prior to morphine for 7 days attenuates morphine induced tolerance (Han et al., 2014). Furthermore, the same research group reported that intrathecal resveratrol suppressed spinal microglial activation *via* a reduction in p38/NF-kB signalling in the microglia cell in *naïve* CD-1 mice (Han et al., 2014). Likewise, Tsai and co-workers (2012) demonstrated an enhancement in the antinociceptive action of morphine measured by the tail-flick in a tolerant rats after intrathecal resveratrol administration, suggesting that AMPK activation regulate the of NMDAR expression in the spinal cord dorsal horn (Tsai et al., 2012). Moreover, it has been reported that systemic administration of metformin at a dose of (200 mg/kg) attenuated morphine tolerance in CD-1 mice as measured by the tail-flick test (Pan et al., 2016). The immunohistochemistry and immunoblotting revealed that metformin exerted its action *via* regulating the p38/MAPK pathway (Pan et al., 2016).

Importantly, the beneficial effect of AMPK as an inhibitor of the translation process in protein synthesis, was investigated in different research. It has been documented that targeting AMPK led to a reduction in the behavioural signs of several types of pathological pain and improved opioid efficacy. Specifically, it has been documented that administration of A-769662 or metformin enhanced the mechanical allodynia in SNI mice and suppressed protein synthesis in the trigeminal ganglia of mice (Melemedjian et al., 2011). Their immunoblotting showed a significant decrease in the phosphorylation level of 4EBP, mTOR and S6RP with metformin treatment, while A-769662 revealed a reduction in 4EBP, mTOR, S6RP, ERK, Akt and eLF4E (Melemedjian et al., 2011). Similarly, the immunoblotting of the ipsilateral side of the sciatic nerve obtained from rats exposed to SNL treated with metformin or A-769662, displayed a significant reduction in elF4G activity with greater levels of 4EBP and elF4E (Melemedjian et al., 2011). This suggested that AMPK activation suppressed the translation regulation process of protein synthesis in neuropathic pain. It should be pointed out that there was no difference in the mechanical threshold in SNI mice

treated with A-769662 or metformin, in their published paper. However, the mechanism of action to activate AMPK is different between A-769662 and metformin (increased the phosphorylation of the α-subunit at T172). This finding above is relatively interesting, as neuropathic pain had a similarity mechanism with morphine tolerance and both conditions displayed activation of the mTORC1 pathway.

Numerus pre-clinical studies in cancer research presented that inhibition of mTORC1 led to activation of AMPK *via* different activators. For example, administration of OSU-53 in thyroid cancer cells (Plews et al., 2015), GSK621 in leukaemia (Sujobert et al., 2014) and thalidezine acted as an activator of apoptosis in cancer cells (Law et al., 2017).

***Anisomycin blocked the development of morphine induced tolerance and restored morphine analgesia in naïve mice.***

The beneficial effect of blocking protein synthesis by anisomycin on improving morphine analgesia was potent in both chronic and acute administration.

It has been recognised that tolerance to morphine is associated with up regulation of several kinases, such as PKCγ and CamKIIα in the dorsal horn which are involved in spinal plasticity (Xu et al., 2014). Generally, the induction and maintenance of spinal plasticity in neurons requires protein synthesis. Protein synthesis underlying the activity of spinal plasticity is critically regulated at the level of translation. Additionally, mTORC1 is acknowledged as a master regulator of protein synthesis in the cells. It regulates the initiation step and the translational rate of the protein synthesis process, which in turn controls neuroplasticity (Jobim et al., 2012). Importantly, phosphorylated mTORC1 and its downstream effectors: S6RP, p70S6K and 4EBP1 are involved in the translation apparatus. Numerous studies have shown a greater expression level of the initiation mRNA translation marker in dorsal horn neurons after chronic morphine administration (Xu et al., 2014). Therefore, targeting the mTORC1 signalling pathway is extremely important in regulating synaptic plasticity that is implicated in opioid efficacy in neurons.

The observation from the behavioural test (tail-flick) and the immunoblotting obtained from the previous chapters in *naïve* animals support the involvement of the mTORC1 pathway in morphine tolerance. These results were in agreement with Xu et al. (2014). Thus, it a natural next step to confirm the role of the inhibition of protein synthesis on morphine efficacy by anisomycin, a specific protein inhibitor. The antibiotic anisomycin is well known for

inhibiting mRNA translation (Kardalinou et al., 1994), as it exerts a strong effect on different initiation factor resulting in inhibiting protein synthesis. Anisomycin activates the p38 MAPK pathway upon the activation of p38 MAPK phosphorylates several protein kinases and transcription factors that induces macrophage apoptosis, which known to be associated with substantial protein inhibition in mammalian cells accompanied by phosphorylation of the α subunit of initiation factor eIF2 and the caspase-dependent cleavage of initiation factors eIF4G, eIF4B, eIF2a where protein synthesis is down-regulated. (Kim et al., 1998; Clemens et al., 2000). The caspases play an important role in protein synthesis down-regulation and exert their effects on translation (Clemens et al., 2000).

As expected, the *naïve* mice pre-treatment with anisomycin showed greater tail-flick latencies compared with morphine treated animals over the 9 days of the experiment indicating that the plastic changes in the spinal cord cause the synthesis of new proteins that enhanced synaptic plasticity, which in turn counteract morphine analgesia leading to the development and maintenance of morphine tolerance. Thereby, inhibition of protein synthesis modulates morphine efficacy by blocking the development of tolerance in *naïve* mice.

Interestingly, acute administration of anisomycin inhibiting protein synthesis resulted in reversal of morphine-induced tolerance and maintained morphine analgesia in tolerant mice. This signifies that a single or repeated administration of the protein inhibitor can interfere with the intracellular process that initiates protein translation, which inhibited or blocked the synthesis of the new kinases that induced neuroplasticity that contributed to the mechanisms of morphine tolerance. It has been documented that single or multiple anisomycin administration for mice at a dose of (50-75 mg/kg, i.p.) resulted in impairment of contextual fear conditioning (Lattal et al., 2004), demonstrating that single administration of anisomycin is sufficient to cause impairment in memory consolidation, as multiple administration inhibits protein synthesis (Lattal et al., 2004).

Other interesting results observed in this current study are that mice treated with chronic anisomycin before morphine showed a greater but (not significant) response to the tail-flick test on day 8 compared with tail latencies measured after acute administration of anisomycin in tolerant mice. Thus, it could be that repeated anisomycin treatment led to protein inhibition as well as depletion of proteins with short half-lives. However, in acute administration, anisomycin blocked the synthesis of new proteins and anisomycin had no

effect on the existing proteins that had been previously synthesized due to chronic morphine administration implicated in the anticipative tolerance to morphine. This interesting result was observed with metformin, CCI-779 and A-769662 administration in to the *naïve* mice. However, the average lifetime of proteins in the mice brain is in the region of 9 days (Price et al., 2010). Therefore, the difference in tail-flick latencies in the various approaches (chronic and acute) is small or even minor.

***FK520 had no effect on the development and maintenance of morphine induced tolerance and did not restore morphine analgesia in naïve mice***

As expected, administration of ascomycin had no effect on morphine analgesia as this drug did not block morphine-induced tolerance and moreover, it did not reverse morphine tolerance in *naïve* mice. This indicates that metformin and CCI-779 had an absolute specificity of action on mTORC1 signalling and mTORC1 inhibition is critical in regulating morphine analgesic efficacy.

Interestingly, both CCI-779 and FK520 drugs rely on binding to the FKBP family, specifically the FK506-pinding-protien 12 (FKBP12) to exert their immunosuppressant effect in mammalian cells. Subsequently, this binding enabled the drug (CCI-779 or ascomycin) to form a complex with FKBP12 (Arndt et al., 1999). Consequently, this complex interacts with different mechanistic targets to induce its immunosuppressant action. Furthermore, it should be mentioned that the CCI-779-FKBP12 complex targets mTOR inhibiting its activity, while FK520- FKBP12 complex interacts with calcineurin and does not inhibit mTOR (Arndt et al., 1999). Thus, FK50 was used in this current study as a negative control due to the difference in the mechanism of action between the mTORC1 inhibitors and FK520. In line with the data presented here, it has been documented that intrathecal co-administration of rapamycin, but not FK520 with morphine blocked the development of morphine-induced tolerance and hyperalgesia and markedly reversed the reduction in pain thresholds in animal models of morphine-induced tolerance and hyperalgesia (Xu et al., 2014). This observation suggest that mTORC1 play a vital role in morphine-induced tolerance and hyperalgesia and inhibition this pathway improved the analgesic effect of morphine especially when prolong therapy of opioid is required.

Taken together, the findings observed in this chapter support the possible role of the mTORC1 pathway in the development and maintenance of morphine tolerance in *naïve* mice. Therefore, to summarize the results obtained from *naïve* mice regarding the tail-

flick latencies obtained from previous studies presented in chapter 3 as well as presented here, figure 5.6 and 5.7 illustrates the differences in responses to morphine-induced tolerance in *naïve* mice pre-treated with metformin, CCI-779, A-769662, anisomycin and FK502 20 h prior morphine at two different treatment approaches (chronic and acute).

*Note:* the raw data for the different vehicle treated animals were not different, and results obtained for all vehicle treatments were pooled for statistics and graphs.

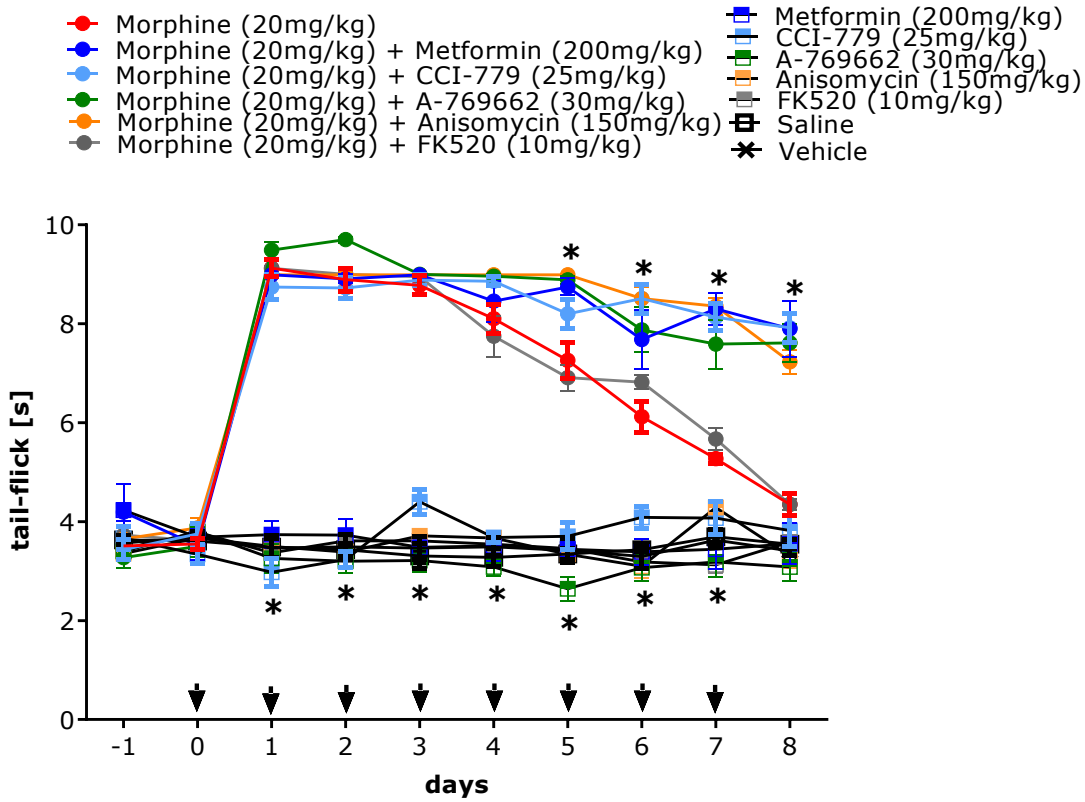


**Figure 5.6: Metformin, CCI-779, A-769662 and anisomycin but not FK520 blocked the development and maintenance of morphine-induced analgesic tolerance in *naïve* mice.** Tolerance was induced by morphine (20 mg/kg, i.p.) administered twice daily for 9 consecutive days. Repeated systemic administration of metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) or A-769662 (30 mg/kg, i.p.) or anisomycin (150 mg/kg, i.p.) 20 h before first morphine injection and then subsequently for 8 days once daily after morning morphine injection blocked the development of morphine induced tolerance, as measured by tail-flick test conducted daily 30 min after morning morphine administration. However, Repeated systemic administration of FK520 (10 mg/kg, i.p.) 20 h before first morphine injection and then subsequently for 8 days once daily after morning morphine injection did not produce morphine antinociception, indicating for the development of morphine-induced analgesic tolerance. The data are presented as the mean responses ± SEM, n= 6-24; **vs.* morphine; *P<0.05 (two-way ANOVA followed by Bonferroni's test). Arrows indicate metformin/CCI-779/ A-769662 / anisomycin /FK520 injections.

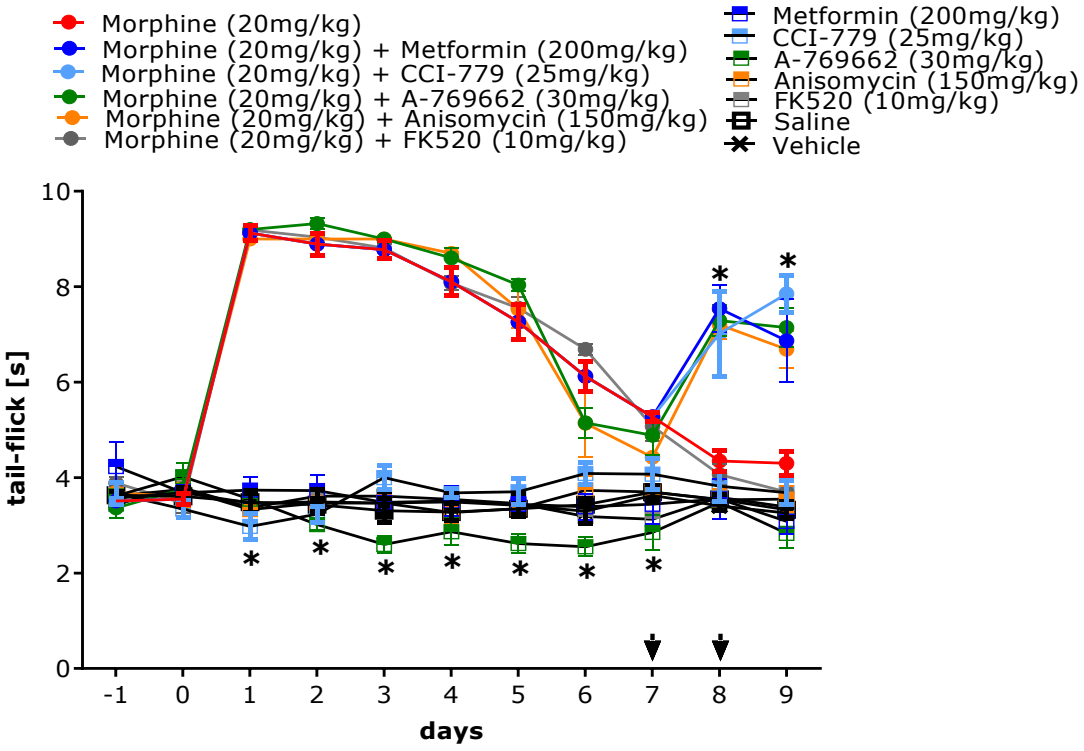


**Figure 5.7: Metformin, CCI-779, A-769662 and anisomycin but not FK520 restored the analgesic effect of morphine in morphine tolerant *naïve* mice.** Tolerance was induced by morphine (20 mg/kg, i.p.) administered twice daily for 9 consecutive days. On day 7 when morphine tolerance was developed a single systemic injection of metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) or A-769662 (30 mg/kg, i.p.) or anisomycin (150mg/kg, i.p.) 20 h before next morning morphine injection restored the analgesic effect of morphine on day 8, as measured by tail-flick conducted 30 min after morning morphine. Additional another systemic injection of metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) or A-769662 (30 mg/kg, i.p.) or anisomycin (150 mg/kg, i.p.) 20 h before next morning morphine maintained the analgesic effect of morphine on day 9. However administration of FK50 (10 mg/kg, i.p.) on day 7 and 8 20 h before morphine injection failed to restored morphine analgesia. The data are presented as the mean responses ± SEM, n= 5-24; **vs.* morphine;*P<0.05 (two-way ANOVA followed by Bonferroni's test). Arrows indicate metformin/CCI-779/ A-769662 / anisomycin /FK520 injections.

Taken together, these identical statistical findings in *naïve* mice pre-treated with mTORC1 inhibitors (metformin, CCI-779) or A-769662 or anisomycin, proved the hypothesis of this thesis that inhibition of the mTORC1 pathway blocks the establishment and maintenance of morphine-induced tolerance. Therefore, this valuable data emphasised the importance of mTORC1 as a novel and tractable target for the improvement of opioid analgesia especially when opioids are used in high doses for prolonged period. In addition, the data obtained here support the idea that metformin, a widely clinically available anti-diabetic drug that inhibits mTORC1 pathway through activation of AMPK, may offer a novel and clinically viable avenue for enhancing morphine analgesic efficacy in chronic pain. Thus, in the following chapter (Chapter 6), this finding in relation to the neuropathic pain model induced by spared nerve injury of the sciatic nerve (SNI model) to mimic condition of chronic pain that is closer to the clinics was validated. Therefore, the data may create rational strategies to tackle opiate

tolerance in the frame of the opiate rotation protocol, where metformin, a relatively safe and accessible medication, would be use with morphine administration when long-term opioid treatment is required and necessary.

# Chapter 6. The effect of the mammalian target of rapamycin complex 1 (mTORC1) inhibitors on the analgesic effect of morphine in neuropathic mice

## 6.1 Introduction

The diagnosis and pharmacological therapies for neuropathic pain has received a considerable amount attention in literature, including a revision of its definition. The initial definition used by the International Association for the Study of Pain (IASP) described neuropathic pain as "*pain initiated or caused by a primary lesion, dysfunction, or transitory perturbation of the peripheral or central nervous system*" (Merskey, 1994). In 2008, this definition was revised by Treede and colleagues to "*pain caused by a lesion or disease of the somatosensory system*" and this definition has now been widely accepted (Treede et al., 2008). Neuropathic pain can be divided into central (e.g., spinal cord injury) and peripheral (e.g., pain associated to diabetes) (Watson and Sandroni, 2016). Neuropathic pain is varied in its severity and impact it can have on a patient's quality of life as well as how it responds to treatment (Attal and Bouhassira, 2015; Allison et al., 2016). Furthermore, neuropathic pain can be very difficult to treat to the satisfaction of patients and clinicians due to the variability of the response of the medication to pain patient, versus the response of pain patient to medication (Wong et al., 2018). Analgesic medication, such as prescribed drugs like opioids or over the counter medication such as paracetamol, do not provide effective analgesic relief for every patient with neuropathic pain (Gatti et al., 2009), which emphasises the importance for a new approach to provide patients with other options to manage their neuropathic pain. Nevertheless, opioids are among the most effective and commonly used analgesics in clinical practice (He et al., 2009; Smith, 2012). Most professional societies recommend opioids as the second or third line treatment (Finnerup et al., 2015). However, it is currently recommended that strong opioids (particularly oxycodone and morphine) should be kept in reserve as a third-line agent because of the potential negative side effects to users, such as tolerance and addiction (Finnerup et al., 2015).

Generally, chronic pain treatment requires prolong morphine administration and this is regularly hindered by the need for dose escalation due to the development of morphine analgesic tolerance (He et al., 2009). This increases in morphine concentration not only puts an individual at a higher risk of severe side effects like respiratory depression but also increases the probability to be opioid addicted (Morgan and Christie, 2011).

This is particularly important in patients suffering with neuropathic pain who are less responsive to opioids, and therefore they require much higher dose of the drug (Jadad et al., 1992; Hanks and Forbes, 1997; Smith and Meek, 2011).

Over the last several years, a growing body of literature has indicated that hyperactive central states resulting from nervous system injury lead to neuronal plastic changes within the CNS (e.g., neuroplasticity of the spinal cord) that play a significant role in hyperalgesia associated with nerve injury or inflammation (Mao et al., 1995). Moreover, the mechanisms that may underlie the neuropathic pain may simultaneously contribute to the development of tolerance to the analgesic effects of morphine, resulting in a state of neural hyperexcitation (Smith, 2012). Importantly, the site of action contribute to hyperalgesia associated with injured nerve and morphine tolerance has also been shown to be in the superficial laminae, specifically laminae I and II of the dorsal horn in spinal cord (Smith and Meek, 2011). Thus, the complexity and the relationship between hyperalgesia as a result of nerve injury and opioid-induced tolerance, calls for a more comprehensive look into some of the clinical issues associated with pain management. In the previous chapter, it was demonstrated that chronic metformin/CCI-779 co-administration with morphine blocked the development and maintenance of morphine tolerance. This observation, in line with other, suggests that mTORC1 may serve as a target to improve opioid therapeutic effectiveness. Since mechanisms regulating opioid analgesic efficacy seem to share commonalities with mechanisms underlying chronic pain (Mao et al., 1995), it was hypothesized that inhibition of the mTORC1 pathway by metformin and/or CCI-779 may result in blockade of the establishment and maintenance of morphine-induced tolerance in neuropathic pain. It was also hypothesized that this strategy may restore analgesic effectiveness of morphine in neuropathic mice.

In this chapter, a model of neuropathic pain induced by spread nerve injury (SNI) in mice was employed. This model changes how mice respond to mechanical and thermal nociceptive stimuli and, in this aspect, it mimics the symptoms of mechanical and thermal hypersensitivity in patients suffering from neuropathic pain (Niederberger et al., 2008).

## 6.2 Material and methods

### 6.2.1 Subject

Adult male C57BL/6J mice (8 weeks of age, Charles River, UK) weighting 25-30 g at the beginning of the study were used. Mice were housed 4 per polyethylene cage on a sawdust bedding under standard conditions (12 h light/dark cycle, lights on from 8:00 am) with food and water available *ad libitum*. Experimental protocols were approved by the AWERB Committee at Durham and Newcastle University and were consistent with the guidelines provided by the UK Animals (Scientific Procedures) Act 1986. For more details see Chapter 2.

### 6.2.2 SNI surgery

The spared nerve injury model involves partial nerve injury as described previously by Decosterd and Woolf, 2000 (for more details see Chapter 2). Briefly, it consist of a tight ligation and cut of the common peroneal and tibial branches of the sciatic nerve on the left side, with preservation of the sural nerve, resulting in consistent and reproducible pain hypersensitivity in the territory of the spared sural nerve.

### 6.2.3 Preparation and administration of drugs

***Morphine***

To induce analgesic tolerance, morphine (morphine sulphate salt pentahydrate; Sigma-Aldrich, UK) was prepared in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK) immediately prior to injections. Mice were weighted and then injected intraperitoneally (i.p.) with morphine (40 mg per kg body weight) or equivalent vehicle (saline) solution without morphine, as a control group, twice daily for 10 consecutive days in a volume of 4 ml per kg body weight.

***Metformin***

For systemic (i.p.) administration, metformin (metformin hydrochloride; Cat. No. 2864; Tocris Bioscience, UK) was prepared in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK) immediately prior injections. Mice were weighed and then injected i.p. with metformin (200 mg per kg body weight) or equivalent vehicle (saline) solution without metformin as a control group. Metformin/vehicle was administered i.p. 20 h before i.p. first injection of morphine was given and then i.p. administration of metformin/vehicle was continued for 9 consecutive days. The timing and concentration of metformin injections were based on

previously published research using metformin (Melemedjian et al., 2011; Obara et al., 2015).

***CCI-779***

For systemic (i.p.) administration, CCI-779 (temsirolimus; Cat. No. T-8040; LC Laboratories, USA) was prepared in pure ethanol as a stock solution at 60mg/mL on the day of experiment and diluted to 2.5 mg/mL in 0.15M NaCl, 5% polyethylene glycol 400, 5% Tween 20 (Ravikumar et al., 2004; Obara et al., 2015) immediately before injection. Mice were weighed and then injected intraperitoneally (i.p.) with a 1% v/w solution of CCI-779 (25 mg per kg body weight) or equivalent vehicle (0.15M NaCl, 5% polyethylene glycol 400, 5% Tween 20) solution without CCI-779 as a control group. CCI-779/vehicle was injected 20 h before first i.p. injection of morphine was given and then i.p. administration of CCI-779/vehicle was continued for 9 consecutive days. The timing and concentration of CCI-779 injections were based on previously published research using CCI-779 (Obara et al., 2011; Obara et al., 2015).

### 6.2.4 Nociceptive threshold in SNI mice

*Tail flick test* (Analgesia Meter, Ugo Basile, Italy) was carried out in SNI mice as described previously in Chapter 2. In brief, tail-flick latency to noxious heat was determined by applying a heat stimulus to the dorsal tail surface approximately 2 cm from the tip of the tail. The cut off time for the tail-flick latency was set to 9 s. SNI mice were tested for their baseline latencies before the injury of the sciatic nerve on day -1 and on day 0 (prior the first morphine injection; see Table 6.1). Then, each morning, mice were tested for analgesia 30 min after i.p. morphine administration. Mice were also monitored daily for any signs of heat-induced skin lesions resulting from repeated tail-flick testing. No animal developed injuries that may affect their nociceptive threshold to tail-flick stimulation.

*Mechanical hypersensitivity* was assessed as described in details in Chapter 2. In brief, mechanical hypersensitivity in mice with SNI was measured by using a series of calibrated nylon von Frey filaments (Stoelting, Wood Dale, IL, USA) ranging from 0.04 to 2g. The von Frey filaments were applied, through the mesh floor, in an ascending order to the lateral part surface of the injured hind paw until it bent. Mice were tested for baseline before SNI surgery on day -1 and then 40 min post morphine injection on days 1-10.

*Cold hypersensitivity* was assessed as described in Chapter 2. In brief, a drop of acetone was applied on the hind paw ipsilateral to the injury approximately 10 min after the von Frey test. Mice were tested for baseline before SNI surgery on day -1 and then 50 min post morphine injection on days 1-10.

### 6.2.5 Experimental design

To establish the role of mTORC1 inhibition on morphine-induced tolerance in SNI mice two experiment were conducted:

*Experiment I*: On day 0 (Table 6.1, Figure 6.1) SNI mice were injected i.p. with metformin or CCI-779 20 h prior i.p. administration of morphine and the injections were repeated for 8 consecutive days in order to assess the role of mTORC1 in the development and maintenance of morphine tolerance.

*Experiment II*: On day 8 (Table 6.1, Figure 6.1) SNI morphine tolerant mice were injected i.p. with metformin or CCI-779 20 h prior i.p. administration of morphine to determine whether a single injection of mTORC1 inhibitors influenced and restored the morphine analgesic effect in morphine tolerant mice. The i.p. injection of mTORC1 inhibitors was also repeated on day 9 to determine whether any following injection of mTORC1 inhibitors potentiated the effect resulting from the first injection.

Table 6.1 and Figure 6.1 below summarizes the experimental design of both experiments.

**Table 6.1: A summary of experiments I and II illustrating schedule of chronic and acute administration of both mTORC1 inhibitors in SNI mice.**

| Days | Experiment I | | Experiment II | |
|---|---|---|---|---|
| | Chronic metformin or CCI-779 | | Acute metformin or CCI-779 | |
| | Morning | Evening | Morning | Evening |
| - 4 (marked as -1 in graphs) | BT → SNI | | BT → SNI | |
| 0 | BT → D | | BT | |
| 1 | BT → M → BT → D | M | BT → M → BT | M |
| 2 | M → BT → D | M | M → BT | M |
| 3 | M → BT → D | M | M → BT | M |
| 4 | M → BT → D | M | M → BT | M |
| 5 | M → BT → D | M | M → BT | M |
| 6 | M → BT → D | M | M → BT | M |
| 7 | M → BT → D | M | M → BT | M |
| 8 | M → BT → D | M | M → BT → D | M |
| 9 | M → BT → D | M | M → BT → D | M |
| 10 | M → BT | Tissue collection | M → BT | Tissue collection |

**TF**: tail-flick test
**D**: drug; metformin (200mg/kg, i.p.) or CCI-779 (25mg/kg, i.p.)
**M**: morphine (20mg/kg, i.p.)
**Chronic administration**: metformin (200mg/kg, i.p.) or CCI-779 (25mg/kg, i.p.) injected 20 h before first morning morphine i.p. injection; then repeated for 8 consecutive days
**Acute administration**: metformin (200mg/kg, i.p.) or CCI-779 (25mg/kg, i.p.) injected on day 8 20 h before morning morphine i.p. injection on day 9. Also, another systemic injection of metformin (200mg/kg, i.p.) or CCI-779 (25mg/kg, i.p.) injected on day 9 20 h before morning morphine i.p. injection on day10. After completion of chronic and acute administrations mice were sacrificed for tissue collection (spinal cord and sciatic nerve) to determine changes in the activity mTORC1 pathway.

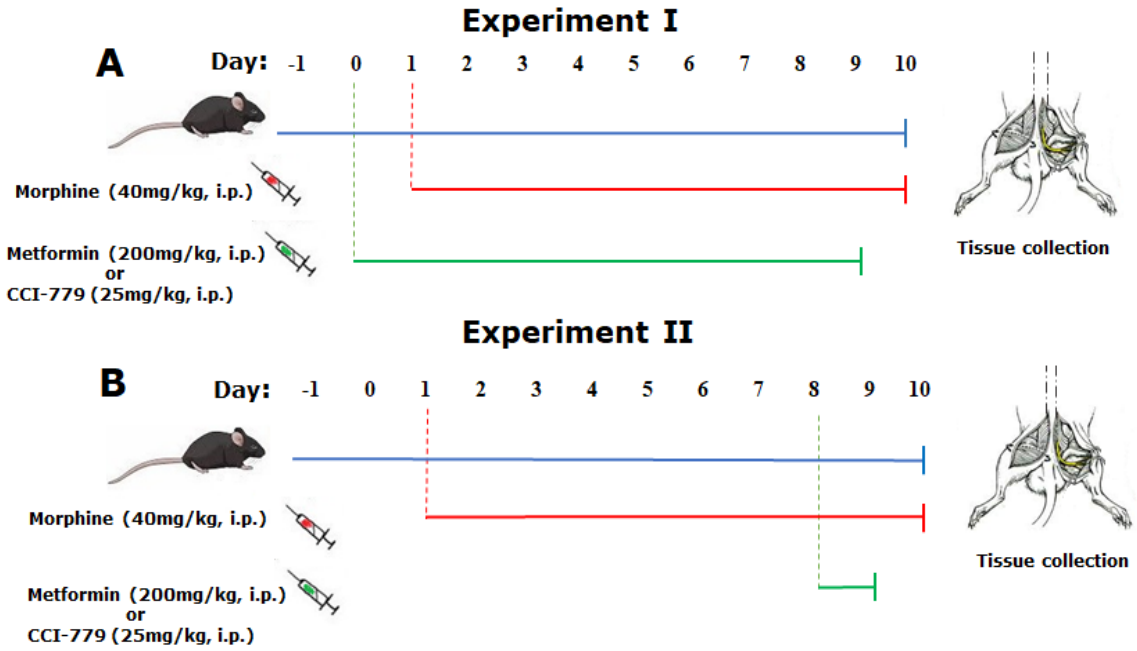


**Figure 6.1: A schematic illustration of experiment I and II. (A; experiment I)** Represents the chronic administration of metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) schedule where these drugs were 20 h before first morphine i.p. injection and then repeated subsequently for 9 consecutive days (once daily after morning morphine injection). **(B; experiment II)** Represents the acute administration of metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) schedule where these drugs were injected in morphine tolerant mice on day 9 (first injection) and day 10 (second injection) 20 h before morning morphine i.p. injection on day 9 and day 10.. The blue line represents pain assessment using tail-flick test. The red line represents morning and evening injections of morphine (20 mg/kg, i.p.). The green line represents i.p. mTORC1 inhibitors administrations. Final step involved tissue collection for biochemical analysis.

### 6.2.5.1 Design of experiment I

SNI mice were divided into into 5 different groups as indicated in Table 6.1. Group 1 (n= 6) consisted mice receiving morphine (40 mg/kg, i.p.) twice daily at 12 h intervals (8 am/8 pm) for 10 consecutive days (day 1-10 in Table 6.1), group 2 (n= 6) received saline under identical conditions and served as controls. To determine the influence of mTORC1 inhibitors on morphine analgesic effects, SNI mice were injected with metformin (200 mg/kg, i.p.) or CCI-779 (25mg/kg, i.p.) 20 h prior the morning morphine (40 mg/kg, i.p.) on each testing day (day 0-9; group 3, n= 5). To determine the effect of mTORC1 inhibitors without morphine, SNI mice were injected i.p. with metformin (200 mg/kg, i.p) or CCI-779 (25 mg/kg, i.p.) 20 hr prior to the morning saline i.p. injection for 9 following days (group 4, n= 6-7). In group 5 (n= 6), mice were injected i.p. with an equivalent vehicle solution without mTORC1 inhibitors (controls).

**Table 6.1: Design of experiment I.**

| Group | Morphine (40 mg/kg, i.p.) | Saline (i.p.) | Chronic administration of mTORC1 inhibitors | | Vehicle (i.p.) | Number of animals |
|---|---|---|---|---|---|---|
| | | | Metformin (200 mg/kg, i.p.) | CCI-779 (25 mg/kg, i.p.) | | |
| 1 | + | | | | | 6 |
| 2 | | + | | | | 6 |
| 3 | + | | + | | | 5 |
| 4 | | + | + | | | 6 |
| 5 | | | | | + | 6 |

The SNI mice were randomly assigned in to 5 groups and received different treatment: (1) i.p. morphine twice daily; morning injections were given between 9:00 am and 10:00 am, and evening injections were given between 8:00 pm and 9:00 pm; (2) controls; i.p. saline twice daily between 9:00 am and 10:00 am and between 8:00 pm and 9:00 pm; (3) morphine + mTORC1 inhibitors; morphine twice daily (as above) followed by i.p. mTORC1 inhibitors(given between 1:00 pm and 2:00 pm) and then morphine evening injection;.(5) i.p. mTORC1 inhibitors were given once daily (as above).

### 6.2.5.2 Design of experiment II

SNI mice were divided into 5 different experimental groups as depicted in Table 6.2. A paradigm to induce morphine analgesic tolerance was identical as mentioned. Briefly, morphine at a dose of 20 mg/kg was administered i.p. every 12 h (group 1, n = 6). Control mice (group 2, n= 6) were injected with saline under identical conditions. SNI mice were rendered tolerant to morphine after 7 days of treatment. On day 8, mice were injected with metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) (group 3, n= 5-6) 20 h prior to the morning morphine i.p. injection on day 9. The injection was repeated 20 h after (on day 9). For testing of the action of mTORC1 inhibitors without morphine control mice were injected i.p. with metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) (group 4, n= 6-7) 20 h prior to the morning saline i.p. injection on day 9 and day 10. In group 5 (n= 6), mice were injected i.p. with equivalent vehicle solution without mTORC1 inhibitors.

**Table 6.2: Design of experiment II.**

| Group | Morphine (40mg/kg, i.p.) | Saline (i.p.) | Acute administration of mTORC1 inhibitors | | Vehicle (i.p.) | Number of animals |
|---|---|---|---|---|---|---|
| | | | Metformin (200mg/kg, i.p.) | CCI-779 (25mg/kg, i.p.) | | |
| 1 | + | | | | | 6 |
| 2 | | + | | | | 6 |
| 3 | + | | + | | | 5-6 |
| 4 | | + | + | | | 6-7 |
| 5 | | | | | + | 6 |

The SNI mice were randomly assigned in to 5 groups and received different treatment: (1) i.p. morphine twice daily; morning injections were given between 9:00 am and 10:00 am, and evening injections were given between 8:00 pm and 9:00 pm; (2) controls; i.p. saline twice daily between 9:00 am and 10:00 am and between 8:00 pm and 9:00 pm; (3) morphine + mTORC1 inhibitors; morphine twice daily (as above)followed by i.p. mTORC1 inhibitors (given between 1:00 pm and 2:00 pm) on day 8 and day 9, and then morphine evening injection; (5) i.p. mTORC1 inhibitors were given once daily on day 8 and day 9.

### 6.2.6 Statistical data analysis

Data analysis and statistical comparisons were performed using GraphPadPrism, version 8.01 for Windows (GraphPad Software, CA, www.graphpad.com). Behavioural results are presented in the graphs as mean ± SEM. Each group included 5 to 7 mice. Comparisons between groups were performed using analysis of variance for ordinary measurements (two-way ANOVA) followed by Bonferroni's multiple comparison post-hoc test and Student's t-test, used when two groups were compared. A value of $P<0.05$ was considered statistically significant.

## 6.3 Results

### 6.3.1 The mammalian target of rapamycin complex 1 (mTORC1) inhibitors blocked the development and maintenance of morphine tolerance in neuropathic mice

The first dose of morphine (40 mg/kg, i.p.) was administered 4 days after SNI surgery (Table 6.1, Figure 6.1). Mice subjected to SNI were treated with higher dose of morphine when compared to *naïve* animals as it has been documented that morphine loses some of its effectiveness under neuropathic pain conditions and therefore the dose of morphine for this study was selected from literature referring to similar experimental design (Osikowicz et al., 2008).

As illustrated in Figure 6.2, the first dose of morphine (40 mg/kg, i.p.) induced a strong analgesic effect when compared to saline injected mice as measured with the tail-flick (day 1: 8.77 ± 0.13 s, *vs*. 3.00 ± 0.50 s), von Frey (day 1: 0.93 ± 0.19 g *vs*. 0.07 ± 0.02 g) and acetone (day 1: 7.20 ± 1.57 s *vs.* 11.15 ± 1.15 s) tests. However, repeated morphine administration (40 mg/kg, i.p., twice daily) resulted in a gradual decrease in morphine analgesic effectiveness. This was observed in the von Frey (Figure 6.2 B) and acetone (Figure 6.2 C) tests on day 2 and 3. On day 4, statistical analysis revealed that there was no significant difference between morphine treated mice and saline injected animals in withdrawal response to von Frey (day 4: 0.24 ± 0.07 g *vs*. 0.06 ± 0.01 g; treatment effect: $F_{(1,192)}= 30.35$, $P <0.05$) and acetone (day 4: 7.31 ± 0.85 *vs.* 12.04 ±1.54 s; treatment effect: $F_{(1,176)}= 28.63$, $P<0.05$) stimulations. Interestingly, in the tail-flick test, morphine (40 mg/kg, i.p., twice daily) produced significant antinociception when compared to saline treated animals throughout the whole 10 days of behavioural testing (Figure 6.2 A). While there was a lack of a significant drop in morphine analgesic efficacy, morphine effect gradually decreased when compared to its efficacy on the first day of administration (day 1: 8.25 ± 0.28 s *vs*. day 10: 4.66 ± 0.52s: treatment effect: $F_{(1,170)}= 466.40$, $P<0.0001$).

#### 6.3.1.1 Metformin

As illustrated in Figure 6.3, pre-treatment with metformin (200 mg/kg, i.p., once daily) 20 h before morphine (40 mg/kg, i.p., twice daily) administration significantly prevented the development and maintenance of morphine analgesic tolerance as measured after morphine administration by the tail-flick, von Frey and acetone tests in SNI mice (Figure 6.3). Metformin produced a significant increase in morphine analgesic effect when compared to morphine alone, and this effect was observed for the first time on day 4 in the von Frey test (day 4: 0.82 ± 0.17 g *vs*. 0.24 ± 0.06 g; Figure 6.3 B). This analgesic effect was then recorded on every subsequent day of experimental testing (treatment effect: $F_{(3,231)}$= 61.30, P<0.0001). Statistically significant difference in response to the acetone test was observed for the first time on day 7 (day 7: 6.97 ± 1.55 s *vs*. 10.97 ± 0.90 s; Figure 6.3 C) and then, similarly to the von Frey testing results, this analgesic effect was observed until day 10 (treatment effect: $F_{(3,211)}$= 32.04, P<0.0001). As mentioned above, administration of morphine consistently produced a significant analgesic effect in tail-flick latency over the 10 days of the experiment when compared with the saline treated animals but the morphine effect in mice pre-treated with metformin displayed a significantly higher latency in comparison with a group treated with morphine alone (treatment effect: $F_{(3,231)}$= 307.30, P<0.0001). This stronger analgesic effect in the morphine group pre-treated with metformin was observed for the first time on day 5 (day 5: 8.83 ± 0.13 *vs* 6.32 s ± 0.89 s) and was recorded on until day 10 of behavioural testing (Figure 6.3 A).

SNI mice injected with metformin (200 mg/kg, i.p., once daily) alone for 10 days showed no significant effect of the drug in the tail-flick, von Frey or acetone behavioural testing when compared to the latency values of the saline-treated animals (Figure 6.3, P>0.05).

#### 6.3.1.2 CCI-779

As illustrated in Figure 6.4, pre-treatement with CCI-779 (25 mg/kg, i.p., once daily) 20 h before morphine (40 mg/kg, i.p., twice daily) administration significantly prevented the development and maintenance of morphine analgesic tolerance as measured after morphine administration by the tail-flick, von Frey and acetone tests in SNI mice (Figure 6.4). CCI-779 produced a significant increase in morphine analgesic effect when compared to morphine alone and this effect was observed for the first time on day 3 in the von Frey test (day 3: 1.15 ± 0.32 g *vs.* 0.54 ± 0.12 g; Figure 6.3 B). This analgesic effect resulting from co-administration of CCI-779 and morphine was also recorded on day 7, 8, 9 and 10 (day 7: 0.94 ± 0.28 g *vs*. 0.39 ± 0.06 g; day 8: 0.65 ± 0.14 *vs*. 0.17 ± 0.06 g; day 9: 0.69 ± 0.12 g *vs*. 0.15 ± 0.02 g; day 10: 0.60 ± 0.10 g *vs.* 0.10 ± 0.02 g; treatment effect: $F_{(4,291)}$= 49.44,

P<0.0001). Statistically significant difference in response to cold stimuli between morphine-treated group and mice administered with CCI-779 prior to morphine was observed for the first time on day 8 of the experiment (day 8: 14.56 ± 3.37 s *vs*. 6.92 ±1.45 s; Figure 6.3 C) and the was recorded until day 10 (treatment effect: $F_{(4,266)}$= 53.72, P<0.0001).

As mentioned above, morphine consistently produced significant analgesic effect in the tail-flick test over 9 days of behavioural testing however the morphine effect in mice pre-treated with CCI-779 displayed a significantly higher tail-flick latency in comparison with a group treated with morphine alone. This was observed for the first time on day 6 (day 6: 8.80 ± 0.20 s *vs.* 7.37 s ± 0.38 s) and continued until day 10 (treatment effect: $F_{(4,341)}$= 430.90, P<0.0001).

SNI mice injected with CCI-779 (25 mg/kg, i.p., once daily) alone for 10 consecutive days showed no significant effect in the tail-flick, von Frey and acetone tests when compared to the withdrawal latency values of the saline-treated animals (Figure 6.4, P>0.05).

### 6.3.2 The mammalian target of rapamycin complex 1 (mTORC1) inhibitors restored the analgesic effect of morphine in neuropathic mice

Administration of two subsequent doses of metformin (200 mg/kg, i.p., once daily) or CCI-779 (25 mg/kg, i.p., once daily) on days 8 and then on day 9, when tolerance to the analgesic effect of morphine was developed, resulted in fully restoring morphine's analgesic effectiveness as observed in all three behavioural tests (Figure 6.5).

#### 6.3.2.1 Metformin

As illustrated in Figure 6.5 A, the tail-flick latencies measured 30 min after morphine injection on day 9 (40 mg/kg, i.p., twice daily), preceded by administration of metformin on day 8 (200 mg/kg, i.p.), were statistically significant compared with withdrawal latencies recorded in animals treated with morphine alone (day 9: 7.77 ± 0.90 s *vs.* 5.78 ± 0.58 s, $t_{(10)}$= 2.24, P= 0.04). Also, statistical analysis detected a significant difference in mechanical and cold hypersensitivity when compared with withdrawal latency values in animals treated with morphine alone (von Frey test: morphine + metformin *vs.* morphine, day 9: 0.53 0.19 g ± *vs.* 0.09 ± 0.01 g, $t_{(10)}$= 2.23, P= 0.04 and acetone test: morphine + metformin *vs.* morphine, day 9: 8.55 ± 0.80 s *vs.* 15.51 ± 0.91 s, $t_{(10)}$= 5.46, P= 0.0003; Figure 6.5 B and C).

Additional systemic injection of metformin (200 mg/kg, i.p.) on day 9, prior to morphine injection on day 10 (40 mg/kg, i.p., twice daily), resulted in maintenance of the analgesic effect of morphine. Results were as follow: tail-flick test: morphine + metformin *vs.* morphine, day 10: 8.25 ± 0.32 s *vs.* 5.55 ± 0.52 s, $t_{(10)}$= 4.43, P= 0.0013, von Frey test: morphine + metformin *vs.* morphine, day 10: 0.82 ± 0.20 g ± *vs.* 0.13 ± 0.02 g, $t_{(10)}$= 3.25, P= 0.0087and acetone test: morphine + metformin *vs.* morphine, day 10: 6.23 ± 0.47 s *vs.* 13.97 ± 1.47 s, $t_{(10)}$= 4.58, P= 0.0013; Figure 6.5). The behavioural testing values recorded from mice receiving metformin alone did not differ significantly from saline injected animals on any tested days (Figure 6.5, P>0.05).

**6.3.2.2 CCI-779**

As illustrated in Figure 6.6, administration of two consecutive doses of CCI-779 (25 mg/kg, i.p., once daily) on day 8 and then on day 9 (when tolerance to analgesic effect of morphine was established) resulted in fully restoring morphine's analgesic effectiveness observed in the three behavioural tests.

The tail-flick latencies measured 30 min after morphine injection (40 mg/kg, i.p., twice daily)on day 9, preceded by administration of CCI-779 (25 mg/kg, i.p), were statistically significant compared with latencies in animals treated with morphine alone (day 9: 7.77 ± 0.90 s *vs.* 5.78 ± 0.58 s, $t_{(9)}$= 2.44, P= 0.04; Figure 6.6 A). Also, statistical analysis detected a significant difference in mechanical and cold hypersensitivity when compared with withdrawal latency values in animals treated with morphine alone (von Frey test: morphine + CCI-779 *vs.* morphine, day 9: 0.58 0.06 g ± *vs.* 0.15 ± 0.02 g, $t_{(9)}$= 6.38, P< 0.0001and acetone test: morphine + metformin *vs.* morphine, day 9: 5.61 ± 0.80 s *vs.* 14.56 ± 0.71 s, $t_{(9)}$= 8.83, P<0.0001; Figure 6.6 B and C).

Additional systemic injection of CCI-779 (25 mg/kg, i.p., once daily) on day 9, prior to morphine injection (40 mg/kg, i.p., twice daily) on day 10, resulted in maintenance of the analgesic effect of morphine (Figure 6.6). Results were as follow: tail-flick test: morphine + CCI-779 *vs.* morphine, day 10: 8.20 ± 0.58 s *vs.* 4.66 ± 0.52 s, $t_{(9)}$= 4.50, P= 0.0015,von Frey test: morphine + metformin *vs.* morphine, day 10: 0.62 ± 0.12 g ± *vs.* 0.10 ± 0.01 g, $t_{(9)}$= 4.74, P= 0.0011 and acetone test: morphine + metformin *vs.* morphine, day 10: 8.02 ± 1.15 s *vs.* 13.54 ± 1.37 s, $t_{(9)}$= 3.00, P= 0.015; Figure 6.6 ). The withdrawal latency values did not differ significantly in mice receiving CCI-779 (25 mg/kg, i.p.) alone from saline injected animals on any tested days (Figure 6.6, P>0.05).

### 6.3.3 The mammalian target of rapamycin complex 1 (mTORC1) inhibitors in combination with morphine induced body weight loss in SNI mice

Repeated administration of morphine (40 mg/kg, i.p., twice daily) for 10 consecutive days induced gradually decrease in mouse body weight when compared with body weight values measured on day 0 (Figure 6.7). The statistically significant difference in body weight was recorded on day 9 in SNI mice co-treated with morphine and metformin (200 mg/kg, i.p., once daily) or CCI-779 (25 mg/kg, i.p., once daily) when compared with saline treated animals (treatment effect for metformin + morphine: $F_{(3,294)}= 14.25$, $P<0.0001$; treatment effect for CCI-779 + morphine: $F_{(4,291)}= 25.40$, $P<0.0001$). Administration of metformin (200 mg/kg, i.p., once daily), CCI-779 (25 mg/kg, i.p., once daily) or vehicle alone did not affect the mouse body weight when compared with saline treated animals.

It should be noted that despite some effect of the treatment on the animal body weight, all animals subjected to experimental testing remained healthy and did not show any signs of unexpected distress.

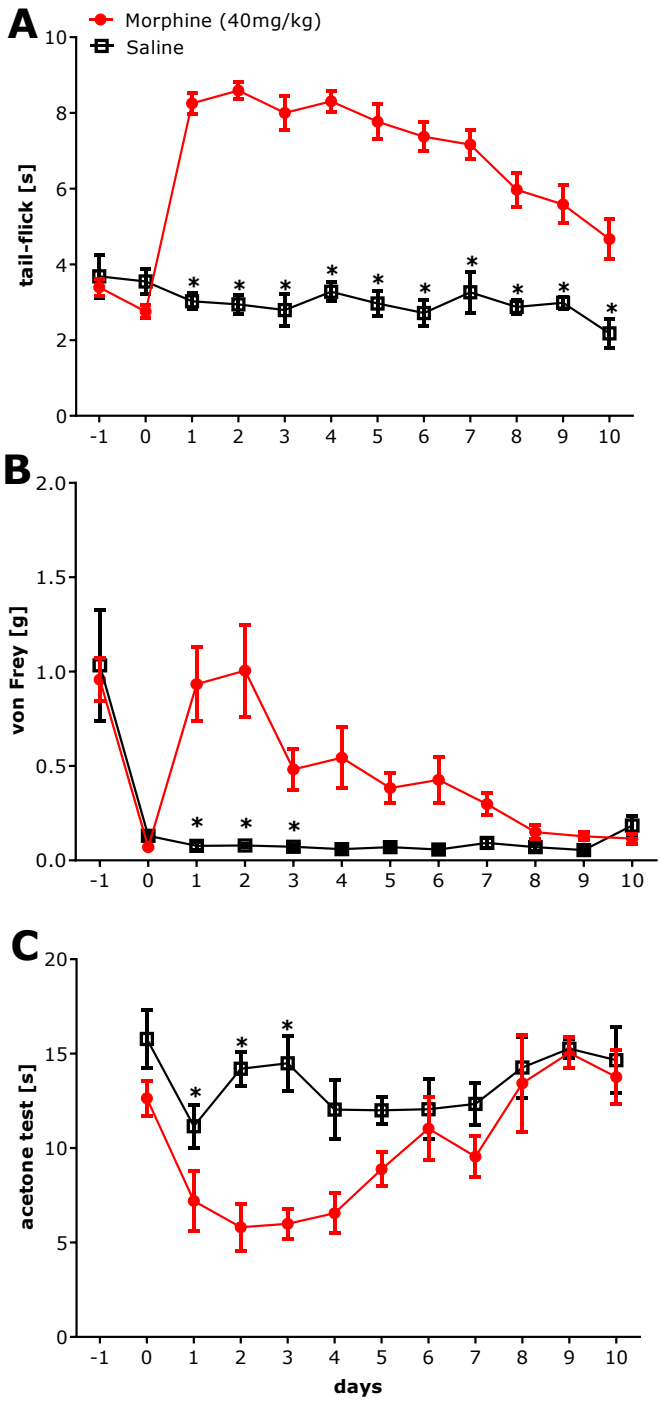


**Figure.6.2: Repeated administration of morphine resulted in analgesic tolerance in SNI mice.** Administration of morphine (40 mg/kg, i.p., twice daily) produced a strong analgesic effect on day 1 followed by a gradual decrease of the analgesic response to morphine treatment as measured by the tail-flick test. On day 4 mice developed a clear tolerance to mechanical **(B)** and cold **(C)** stimuli as indicated by a lack of significant difference when compared to saline treated animals in von Frey **(B)** and acetone **(C)** tests. SNI mice showed a gradual decrease in morphine-induced analgesia in tail-flick test **(A)** when compared to saline control. The data are presented as mean ± SEM, n= 6-12; *vs.* morphine; *P<0.05 (two-way ANOVA followed by Bonferroni's test).

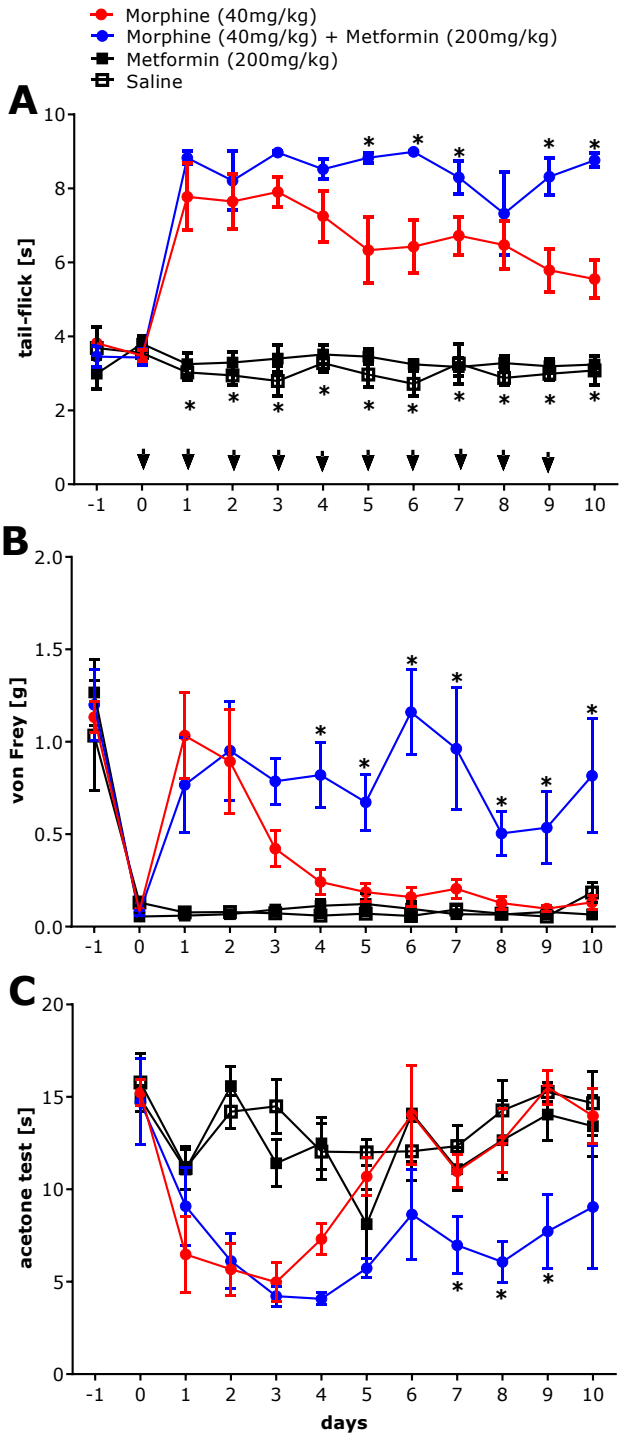


**Figure 6.3: Metformin blocked the development and maintenance of morphine-induced analgesic tolerance in SNI mice.** Tolerance was induced by morphine (40 mg/kg, i.p., twice daily) administered for 10 consecutive days. Repeated systemic administration of metformin (200 mg/kg, i.p.) 20 h before first morphine injection and then subsequently for 9 days (once daily after morning morphine injection) blocked the development and maintenance of morphine-induced tolerance as measured by the tail-flick **(A),** von Frey **(B)** and acetone **(C)** test conducted daily 30-50 min after morning morphine. The data are presented as mean ± SEM, n= 5-6 mice per group; *_vs_. morphine; *P<0.05 (two-way ANOVA followed by Bonferroni's test). Arrows indicate metformin injections.

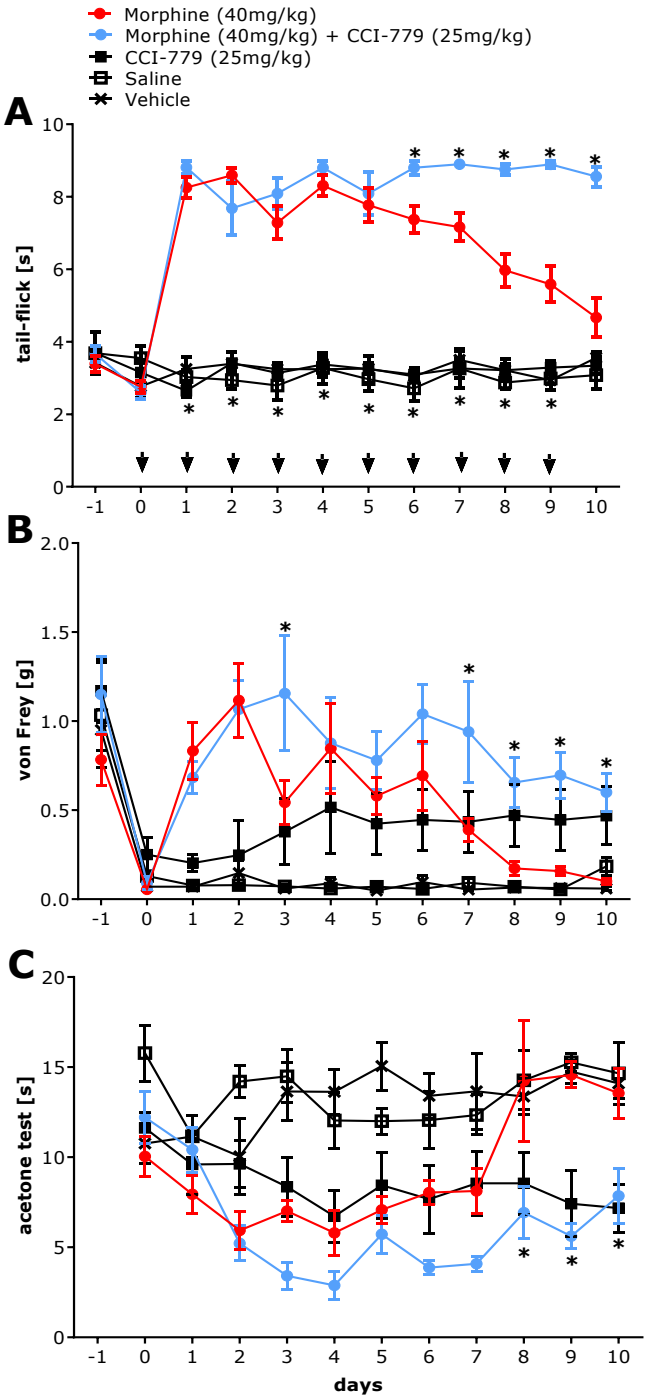


**Figure 6.4: CCI-779 blocked the development and maintenance of morphine-induced analgesic tolerance in SNI mice.** Metformin blocked the development and maintenance of morphine-induced analgesic tolerance in SNI mice. Tolerance was induced by morphine (40 mg/kg, i.p., twice daily) administered for 10 consecutive days. Repeated systemic administration of CCI-779 (25mg/kg, i.p.) 20 h before first morphine injection and then subsequently for 9 days (once daily after morning morphine injection) blocked the development and maintenance of morphine-induced tolerance as measured by the tail-flick **(A)**, von Frey **(B)** and acetone **(C)** test conducted daily 30-50 min after morning morphine. The data are presented as mean ± SEM, n= 6-7 mice per group; *_vs_. morphine; *P<0.05 (two-way ANOVA followed by Bonferroni's test). Arrows indicate CCI-779 injections.

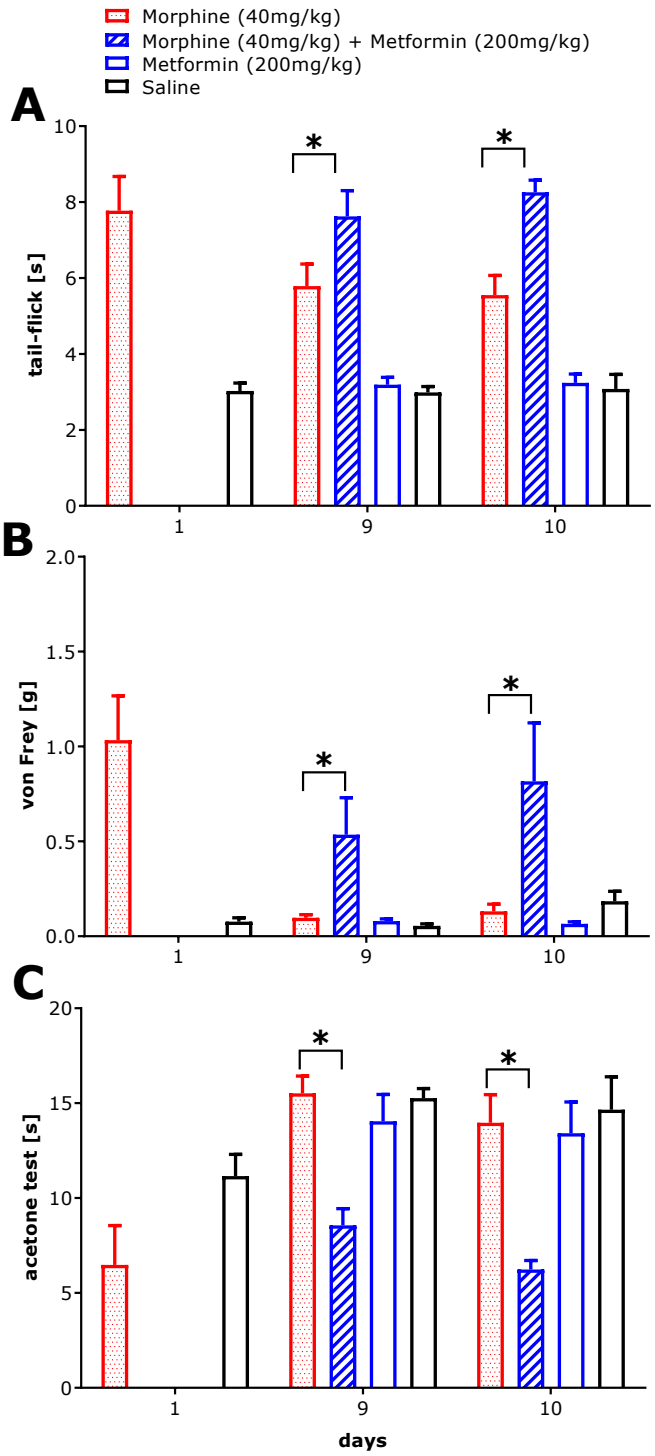


**Figure 6.5: Metformin restored the analgesic effect of morphine in morphine tolerant SNI mice.** Tolerance was induced by morphine (40 mg/kg, i.p., twice daily) administered for 10 consecutive days. On day 8, when morphine tolerance was developed, a single systemic administration of metformin (200 mg/kg, i.p.) 20 h before next morning morphine injection restored the analgesic effect of morphine on day 9, as measured by the tail-flick **(A)**, von Frey **(B)** and acetone **(C)** tests conducted 30-50 min after morning morphine. Additional systemic injection of metformin (200 mg/kg, i.p.) on day 9 20 h before next morning morphine maintained the analgesic effect of morphine on day 10. The data are presented as mean ± SEM, n= 5-6 mice per group; *vs*. morphine; *P<0.05 (un-paired Student's t-test).

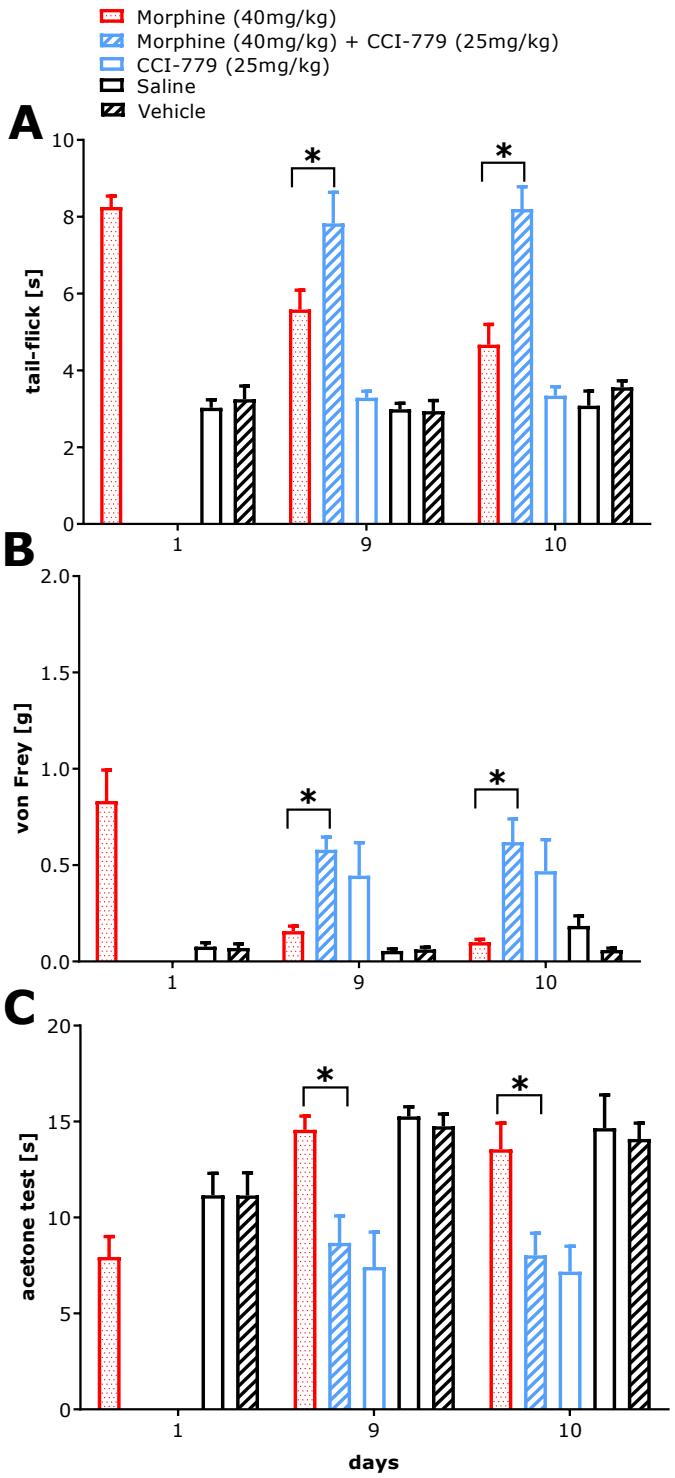


**Figure 6.6: CCI-779 restored the analgesic effect of morphine in morphine tolerant SNI mice.** Tolerance was induced by morphine (40 mg/kg, i.p., twice daily) administered for 10 consecutive days. On day 8, when morphine tolerance was developed, a single systemic administration of CCI-779(25 mg/kg, i.p.) 20 h before next morning morphine injection restored the analgesic effect of morphine on day 9, as measured by the tail-flick (A), von Frey (B) and acetone (C) tests conducted 30-60 min after morning morphine. Additional systemic injection of CCI-779 (25 mg/kg, i.p.) on day 9 20 h before next morning morphine maintained the analgesic effect of morphine on day 10. The data are presented as mean ± SEM, n= 6-7 mice per group; *$vs.$ morphine; *P<0.05 (un-paired Student's t-test).

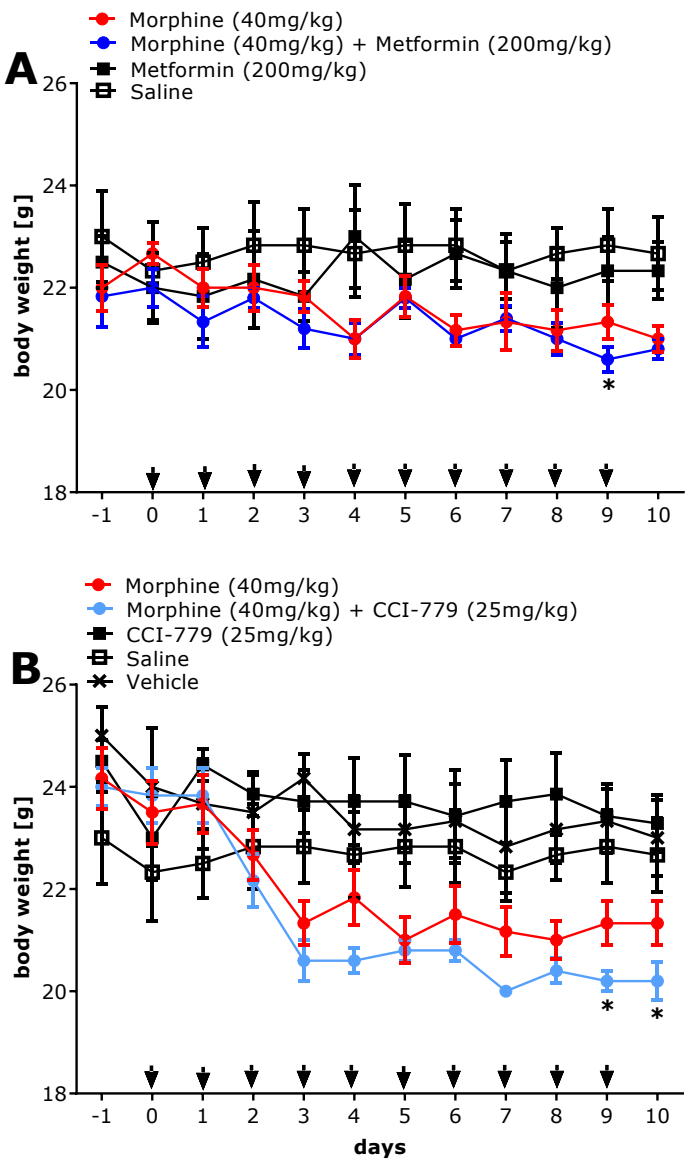


**Figure 6.7: Metformin (A) and CCI-779 (B) in combination with morphine induced body weight loss in SNI mice**. Repeated administration of morphine (40 mg/kg, i.p., twice daily) for 10 consecutive days induced a gradual decrease in body weight when compared with saline treated mice. The co-administration of morphine with metformin (200 mg/kg, i.p., A) or CCI-779 (25 mg/kg., i.p., B) induced significant body loss on days 9 and 10. The data are presented as mean ± SEM, n= 5-12 mice per group; *vs. saline; *P<0.05 (two-way ANOVA followed by Bonferroni's test). Arrows indicate metformin/CCI-779 injections.

## 6.4 Discussion

In this chapter, it was demonstrated that systemic administration of mTORC1 inhibitors, metformin and CCI-779, inhibited the development and maintenance of antinociceptive tolerance to morphine in neuropathic mice. This was evident in the sustained responses to morphine in the presence of metformin or CCI-779 for 10 days, compared to morphine tolerant animals not being exposed to mTORC1 inhibitors. This observation may have an important clinical implication as treatment of neuropathic pain with available analgesics is challenging. Even morphine, the most potent analgesic drug, is not effective in the control of neuropathic pain. Here, the use of two drugs that are registered for clinical use opens up the possibility for an immediate conversion to the clinic.

The fact that mTORC1 inhibitors blocked the development and maintenance of morphine-induced tolerance in neuropathic pain has been also reported in the literature by others. Specifically Xu et al. (2015), revealed that intrathecal co-infusion of rapamycin attenuated morphine-induced tolerance in rats exposed to SNL surgery. In their neuropathic pain model, opioid tolerance was induced on day 8 post SNL *via* continuous intrathecal infusion of morphine (Xu et al. 2015).

In line with the data presented here, co-infusion of rapamycin prevented morphine-induced tolerance and hyperalgesia in both SNL and sham rats (Xu et al., 2015). They showed that co-infusion of rapamycin blocked the reductions in the morphine's maximal possible analgesic effects and paw ipsilateral withdrawal thresholds to mechanical stimuli (von Frey) in both SNL and sham rats (Xu et al., 2015) thus, they found that the development of morphine induced tolerance and hyperalgesia was at a similar time point (day 3) in injured and non-injured rats (Xu et al., 2015) hence showing that SNL did not change the initiation development of morphine induced tolerance and hyperalgesia. This is in contrast to observations presented here as morphine- induced tolerance occurred faster when compared to *naïve* mice (Chapter 3)

It is well documented that opioids lack analgesic efficacy after nerve damage that facilitates or accelerates the development of morphine tolerance (Arner and Meyerson, 1988). Several research studies addressed the interaction of morphine tolerance and neuropathic pain in different animal pain models, as they investigated opioid tolerance and pain-related behaviour after surgery resulting in neuropathy and compared with sham surgery. Reports suggested that peripheral nerve injury facilitated the development of morphine tolerance

(Yaksh and Harty, 1988; Mao et al., 1995; Ossipov et al., 1995; Christensen et al., 2000; Fundytus et al., 2001), whereas others suggested that nociceptive stimulation did not alter or accelerate morphine tolerance development (Yu et al., 1997; Xu et al. 2015). It is worth mentioning that the comparison between numerous published studies is so complicated due to the variety in morphine treatment regimens, pain models and the behavioural measures used in each study.

To support the data presented here, Osikowicz, (2008) showed that morphine-induced tolerance developed faster in animals subjected to neuropathy pain and current studies were design based on their work. Moreover, results demonstrated in this chapter, in line with the findings from different literature that revealed that peripheral nerve injury enhanced the earlier development of antinociceptive tolerance to morphine. For example, Raghavendra et al. (2002), reported the rapid development of antinociceptive tolerance in tail-flick and paw-pressure tests to morphine injection on day 3 in nerve-injured rats compared with sham rats. Moreover, it has been stated that morphine tolerance developed faster in von Frey test in allodynic animals than in the tail-flick test (Yu et al., 1997). They revealed that the effect of intrathecal morphine injection on mechanical allodynia was maintained for 2 days with significant reduction on day 3 (Yu et al., 1997). The different pain models used in each research may have a fundamentally different sensitivity to opioids in addition to the pathophysiology. In this presented work, rapid tolerance was observed in relation to morphine effects in mechanical and cold tests in SNI mice (unlike development antinociceptive tolerance in tail-flick test). These results denoted that within the same model of neuropathic pain (SNI), pathophysiological mechanisms mediating the abnormal reactions to noxious stimuli are different, although there was no sham group to compare the time course of morphine tolerance development in von Frey and acetone tests, as the *naïve* mice in Chapter 3 were tested by tail-flick test only *naïve* animals do not respond to von Frey and acetone tests.

The basis for the deficiency of opioid efficacy in neuropathic conditions is not clear. Numerous studies addressed the association between opioid tolerance and neuropathic pain as both have similar mechanisms that result in diminishing the antinociceptive properties of opioids. Nerve injury has been shown to have a profound impact on the reorganisation of the nociceptive circuits within the CNS, including significant changes in microglia morphology (Wen et al., 2011) and the expression of gene changes (Kononenko et al., 2018) that may account for the low efficacy of opioid drugs. Moreover, both neuropathic pain and tolerance

combined with the release of excitatory amino acids, such as glutamate acting at NMDA receptors in the dorsal horn of the spinal cord subsequently lead to the activation of PKC. It has been demonstrated that inhibition of PKC decreases morphine tolerance and reduced neuropathic pain (Mao et al., 1992; Mayer et al., 1995). Likewise, it has been reported that the production of nitric oxide contributed to neuropathic pain as well as morphine tolerance (Mayer et al., 1999). Importantly, the development of morphine tolerance may involve adaptive changes in neuronal circuits and μ-opioid receptor, like internalization and desensitization. These adaptive changes may be related to protein translation process (Costa-Mattioli et al., 2009). In line with this observation, it was demonstrated that chronic morphine administration is reported to increased protein synthesis in the spinal dorsal horn (Xu et al., 2014). Additionally, Xu and colleagues (2015) revealed the beneficial effect of mTORC1 inhibition on morphine analgesia in neuropathic rats as already described above. Interestingly, in this present study, a systemic chronic administration of CCI-779 significantly blocks the development of morphine-induced analgesic tolerance in mice treated daily with morphine and acute administration restore the morphine analgesia in tolerant mice. These findings support the previous observations in healthy/*naïve* mice (Chapter 3), where potentially repeated morphine could elevate mTORC1 activity and inhibit this pathway, reducing the induction and maintenance of morphine tolerance (Xu et al., 2014). Remarkably, chronic administration of CCI-779 produced antinociception to mechanical and cold stimuli in SNI mice. This is not surprising as the antinociceptive effects of CCI-779 have been documented in several experimental models of neuropathic and inflammatory pain. It has been reported that repeated administration of CCI-779 resulted in a reduction in mechanical allodynia in the lateral part of the hind paw in SNI mice (Obara et al., 2011). Moreover, it has been stated that the efficacy of CCI-779 is increased after direct nerve damage (Asante et al., 2010). Thus, repeated administration of CCI-779 had no effect on neuronal responses in the absence of nerve injury as the data presented in Chapter 3 demonstrates but the antinociceptive effect of CCI-779 was observed after SNI.

The data presented here is the first evidence to demonstrate the beneficial effect of metformin on morphine induced tolerance in neuropathic mice, as repeated administration of metformin blocked the development of morphine tolerance, most probably *via* inhibition of the mTORC1 pathway.

Antinociceptive effect produced by metformin is quite well documented in the literature. Indeed, it has been postulated that metformin alleviated neuropathic allodynia in a mouse

model of SNI (Melemedjian et al., 2011). Similarly, it has been established that metformin alleviated pain amplification by attenuating hyperexcitability in sensory neurons as it significantly reduced mechanical hyperalgesia, heat hyperalgesia and cold allodynia in an animal model of painful diabetic neuropathy induced by streptozotocin (Ma et al., 2015). Also, in the mouse model of chemotherapy-induced peripheral neuropathy, metformin improved mechanical allodynia resulting from cisplatin induced sensory deficits (Mao et al., 2014). Also, recent study has demonstrated that metformin at a dose of 50 mg/kg reduced sensitivity to mechanical and thermal allodynia in an animal model of spinal cord injury (SCI) (Afshari et al., 2018), demonstrating that metformin showed neuroprotective and anti-inflammatory effects, resulting in attenuating neuropathic pain and hyperalgesia after nerve damage (Afshari et al., 2018). Furthermore, it has been reported recently that metformin treatment for 7 days reversed mechanical and cold hypersensitivity in male but not in female ICR mice (Inyang et al., 2019). Here the authors, showed that metformin has a persistent effect on the mechanical withdrawal threshold 32 days after metformin treatment was terminated (Inyang et al., 2019). This antinociceptive effects of metformin are proposed to be associated with inhibition of the translation machinery occurring after nerve injury involving mTORC1 inhibition. In fact Melemedjian et al., (2011) assessed the biochemical changes in translation machinery occurring after nerve injury induced by SNL in the sciatic nerve and dorsal root ganglion (DRG) in rats supporting the idea that targeting the mTORC1 pathway results in reversing mechanical allodynia which occurs in response to different models of peripheral nerve injury in mice and rats (Melemedjian et al., 2011; Ma et al., 2015; Afshari et al., 2018; Inyang et al., 2019). However, data presented in this study revealed that mice treated with metformin alone, experienced no beneficial analgesic effect on the injured paw, as there were no differences in the mechanical withdrawal threshold compared to saline treated mice, but a weak or slight effect but not significant in response to cold stimuli on the first day of metformin treatment. This discrepancy is rather puzzling and requires further investigation. However, the fact that metformin blocked morphine tolerance and restored analgesic effect of morphine, in the present study suggest that these behavioural effects were due to inhibition of mTORC1. This could be supported by fact that this current study employed a dose of metformin that in a series of behavioural studies showed inhibition of this pathway *via* activation of AMPK (Melemedjian et al., 2011; Inyang et al., 2019). Moreover, behavioural effects produced by metformin seemed to follow the same pattern of changes that has been observed for CCI-779 that produced the effects *via* inhibition of mTORC1.

A few reports have evaluated the effects of mTORC1 inhibitors on a mouse's body weight in neuropathic conditions. One of the interesting results observed in this study was that none of the inhibitors had an effect on the mouse's body weight. It has been documented that long term metformin administration reduced body weight gain in high fat diet diabetic C57BL/6J mice and had no effect on mice fed with a standard diet (Matsui et al., 2010), showing that metformin improves glucose intolerance and reduces adipose mass (Matsui et al., 2010). Moreover, Eskens et al. (2013), stated that 14 days of metformin treatment in drinking water at a concentration of 0.33 mg/ml had no effect on C57BL/6J mice compared with diabetic mice, while in the animal model of diabetic neuropathy, the rat's body weight was reduced significantly. Furthermore, it has been presented that metformin at a dose of 50 mg/kg resulted in significant body weight loss in rats subjected to spinal cord injury (Afshari et al., 2018). In fact, administration of morphine induced weight loss and this was reported in different studies with different experimental designs. For example, it has been documented that acute or chronic morphine administration induced weight loss in rats (Lilius et al., 2009). Similarly, Hook and co-workers have shown that a single intrathecal administration of morphine produced weight loss in rats (Hook et al., 2009). Also, it has been documented that in morphine self-administration mice exposed to spinal cord injury showed a significant body weight loss in comparison to sham animals, and the weight loss was increased by morphine administration (Woller et al., 2014). These experiments as well as the present study showed weight loss occurring as a result of morphine administration. It has been suggested that weight loss can result from solely to withdrawal syndrome or alterations in food and fluid consumption (Nogueiras et al., 2012).

In conclusion, this chapter has verified the importance of using mTORC1 inhibitors (metformin and CCI-779) with morphine in order to acquire the optimal antinociceptive effect with reduced tolerance to morphine in neuropathic pain. Also, it supported the idea that metformin, a widely clinically available anti-diabetic drug, may play clinically important role in the regulation of opioid analgesic efficacy in chronic pain. In the following chapter, (Chapter 7) it was hypothesized that inhibition of the mTORC1 pathway by metformin and/or CCI-779 may result in the improvement of opioid analgesic efficacy in neuropathic pain to identify the ability of both metformin and CCI-779 to improve morphine efficacy in neuropathic pain.

# Chapter 7. The mammalian target of rapamycin complex 1 (mTORC1) inhibitors potentiated the analgesic effect of morphine in neuropathic mice

## 7.1 Introduction

Pain resulting from peripheral nerve injury is one of the most critical health problems (Menorca et al., 2013). Currently available therapeutic medicines (e.g., morphine, gabapentin) reduce but do not eliminate characteristic symptoms of neuropathic pain, such as allodynia (see definition in Chapter 1). Moreover, to date, no medication has shown long-term efficacy and tolerability for neuropathic pain conditions. Also morphine and other classic opioids, while effective in the treatment of acute pain, are not long-term effective analgesics in relieving neuropathic pain (Gilron et al., 2005). It has been suggested that neuropathic pain may be attenuated by higher doses of opioids in comparison with those useful in acute pain control, but this is accompanied by an aggravation of unwanted side effects such as sedation, dizziness, nausea, vomiting, constipation, physical dependence, tolerance and respiratory depression (Ricardo Buenaventura et al., 2008). Clinical studies in humans and research in animals have suggested that neuropathic pain might respond poorly to opioid treatment under chronic administration of this type of drugs (Decosterd et al., 2004; Gilron et al., 2005). The use of opioids for long period may results in the development of tolerance to its analgesic effect. Moreover, the similarity in the cellular mechanism that drive both tolerance and neuropathic pain decrease opioids beneficial effects and accelerate the development of tolerance (Porreca et al., 1998). Thus neuropathic pain does not always respond well to morphine or other opioids. There is a pressing need for the identification of new therapeutic strategies to improve management of neuropathic pain and consequently efficacy of opioid-based treatments that would be beneficial to clinical practice.

The mTORC1 pathway is well established as a regulator of pain sensitivity (Xu et al., 2014). There is also increasing evidence supporting mTORC1's role in the regulation of opioid efficacy, particularly in chronic pain when prolonged treatment is required (Xu et al., 2014). Results presented in Chapter 3 (*naïve* mice) and Chapter 6 (neuropathic mice) also emphasized the importance of the mTORC1 pathway in the regulation of morphine-induced tolerance, indicating for mTORC1 as a potential target for the improvement of morphine-driven therapeutic intervention in chronic pain. While neuropathic pain and tolerance share some common pathological mechanisms (Mayer et al., 1999), it is important to determine the extent to which alterations in mTORC1 activity within nociceptive pathways underlie

the analgesic responsiveness to morphine in neuropathic pain and also how these changes correspond to the loss of morphine efficacy in neuropathic pain. Therefore, it was hypothesized that inhibition of the mTORC1 pathway by metformin and/or CCI-779 may result in the improvement of opioid analgesic efficacy in neuropathic pain that may be observed behaviourally by potentiation of morphine analgesic effect leading to effective reduction of mechanical hypersensitivity in mice with neuropathic pain. This strategy could be potentially clinically important as higher doses of opioids are required to achieve pain relief (Osikowicz et al., 2008) and this approach would allow for decreasing morphine dosing and therefore would allow for longer treatment.

In addition, it was also hypothesized that pharmacological inhibition of mTORC1 in peripheral nerve fibers expressing activated mTOR (P-mTOR) would potentiate morphine analgesia after metformin/CCI-779 peripheral intraplantar (i.pl.) application in neuropathic pain. Outcomes of this experiment, together with previous work that revealed opioid analgesia through peripheral opioid receptors in neuropathic pain (e.g., Obara et al., 2009 and Stein et al., 2009) could be therapeutically important since peripheral use of opioids has been proven to minimize the risk of side effects (Stein et al., 2009).

## 7.2 Material and Methods

### 7.2.1 Subject

Adult male C57BL/6J mice (8 weeks of age, Charles River, UK) weighting 25-30 g at the beginning of the study were used. Mice were housed 4 per polyethylene cage on a sawdust bedding under standard conditions (12 h light/dark cycle, lights on from 8:00 am) with food and water available *ad libitum*. Experimental protocols were approved by the AWERB Committee at Durham and Newcastle University and were consistent with the guidelines provided by the UK Animals (Scientific Procedures) Act 1986. For more details see Chapter 2.

### 7.2.2 SNI surgery

The spared nerve injury (SNI) model involves partial nerve injury as described previously by Decosterd and Woolf, 2000 (for more details see Chapter 2). Briefly, it consist of a tight ligation and cut of the common peroneal and tibial branches of the sciatic nerve on the left side, with preservation of the sural nerve, resulting in consistent and reproducible pain hypersensitivity in the territory of the spared sural nerve.

### 7.2.3 Preparation and administration of drugs

***Morphine***

To determine a dose response curve for morphine-induced analgesic effect, morphine (morphine sulphate salt pentahydrate; Sigma-Aldrich, UK) was prepared in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK) immediately prior to injections. Mice were weighted and then injected (i.p.) with morphine (3, 10, 20 mg per kg body) or equivalent vehicle solution (saline) without morphine as a control group in a volume of 4 ml per kg body weight.

***Metformin***

*Systemic (i.p.) administration:* For systemic (i.p.) administration, metformin (metformin hydrochloride; Cat. No. 2864; Tocris Bioscience, United Kingdom) was prepared in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK) immediately before injections. Mice were weighed and then injected i.p. with metformin (200 mg per kg body weight) or equivalent vehicle (saline) solution without metformin as a control group. Metformin/vehicle was administered i.p. once 20 hours before i.p. injection of morphine (3, 10, 20 mg per kg body). The timing and concentration of metformin injections were based on previously published research using metformin (Obara et al., 2011; Obara et al., 2015; Melemedjian et al., 2013).

*Intraplantar (i.pl.) administration:* For intraplantar (i.pl.) administration, metformin (metformin hydrochloride; Cat. No. 2864; Tocris Bioscience, United Kingdom) was given into SNI-injured hind paw at a dose of 100 nmol (50 µl of 100 mM solution) prepared immediately before injections in a sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK). Intraplantar injections were given over 30 s in a volume of 50 µl. Control animals received 50 µl of equivalent vehicle solution (saline) without metformin. Metformin/vehicle was administered i.pl. once 20 h before i.p. injection of morphine (3, 10, 20 mg per kg body).

***CCI-779***

*Systemic (i.p.) administration:* For systemic (i.p.) administration, CCI-779 (temsirolimus; Cat. No. T-8040; LC Laboratories, USA) was prepared in pure ethanol as a stock solution at 60mg/mL on the day of experiment and diluted to 2.5 mg/mL in 0.15M NaCl, 5% polyethylene glycol 400, 5% Tween 20 (Ravikumar et al., 2004) immediately before injection. Mice were weighed and then injected intraperitoneally (i.p.) with a 1% v/w solution of CCI-779 (25 mg per kg body weight) or equivalent vehicle (0.15M NaCl, 5% polyethylene glycol 400, 5% Tween 20) solution without CCI-779 as a control group. CCI-779/vehicle was injected 20 hours before i.p. injection of morphine (3, 10, 20 mg per kg

body). The timing and concentration of CCI-779 injections were based on previously published research using CCI-779 (Obara et al., 2011; Obara et al., 2015).

*Intraplantar (i.pl.) administration:* For intraplantar (i.pl.) administration, CCI-779 (temsirolimus; Cat. No. T-8040; LC Laboratories, USA) was given into SNI-injured hind paw at a dose of 12.5 nmol (i.e., 50 µl of 250 µM solution) and was prepared immediately before injections in a vehicle solution containing 0.15M NaCl, 5% polyethylene glycol 400, 5% Tween 20 and 20% ethanol (Ravikumar et al., 2004). Intraplantar injections were given over 30 s in a volume of 50 µl. Control animals received 50 µl of equivalent vehicle (0.15M NaCl, 5% polyethylene glycol 400, 5% Tween 20) solution without CCI-779. CCI-779/vehicle was injected i.pl. 20 h before i.p. injection of morphine (3, 10, 20 mg per kg body). The timing and concentration of CCI-779 injections were based on previously published research using CCI-779 (Obara et al., 2011).

### 7.2.4 Nociceptive threshold in SNI mice

In all experiments, mice were habituated to a plexiglas behaviour chamber under ambient light for 2-3 days before the beginning of the experiment. The experimenter remained blind to the treatment during the testing procedure.

*Mechanical hypersensitivity* was assessed as described in Chapter 2. In brief, mechanical hypersensitivity in mice with SNI was measured by using a series of calibrated nylon von Frey filaments (Stoelting, Wood Dale, IL, USA) ranging from 0.04 to 2g. The von Frey filaments were applied, through the mesh floor, in an ascending order to the lateral surface of the injured hind paw until it bent. Mice were tested for baseline before and after SNI surgery (day -1 and 4), and then 30 min post morphine injection on day 5 and 7.

*Cold hypersensitivity* was assessed as described in Chapter 2. In brief, a drop of acetone was applied on the lateral part of the hind paw ipsilateral to the injury approximately 10 min after the von Frey test. Mice were tested for baseline before and after SNI surgery (day -1 and 4), and then 40 min post morphine injection on day 5 and 7.

### 7.2.5 Experimental design

To identify the extent to which mTORC1 inhibition improves morphine analgesic efficacy in mice subjected to neuropathic pain two separate experiments were conducted:

*Experiment I:* SNI mice (n= 64) were given systemic i.p. single dose of mTORC1 inhibitors (metformin or CCI-779) or equivalent vehicle solution 20 h before i.p. morphine administration.

*Experiment II:* SNI mice (n= 64) were given peripheral i.pl. single dose of mTORC1 inhibitor (metformin or CCI-779) or equivalent vehicle solution 20 h before i.p. morphine administration.

#### 7.2.5.1 Design of experiment I

SNI mice were divided into 8 different groups as indicated in Table 7.1. On day 4 after SNI, metformin (200 mg/kg, i.p.) or equivalent vehicle solutions without the inhibitor was administered 20 h before morphine i.p. injections (3, 10, 20 mg/kg). On day 5, a single i.p. morphine injection (3, 10, 20 mg/kg) or saline was given to SNI mice. Pain threshold assessments were taken on the ipsilateral hindlimb before metformin and morphine administrations and then 30 min after morphine i.p. injection. A short break from injections and testing (2-3 days) was allowed for washout of the drugs, before the experiment was repeated with the other mTORC1 inhibitor CCI-779 (25mg/kg, i.p.).

On day 6 after SNI, CCI-779 (25 mg/kg, i.p.) or equivalent vehicle solutions without the inhibitor was administered 20 h before morphine i.p. injections (3, 10, 20 mg/kg). On day 7, a single i.p. morphine injection (3, 10, 20 mg/kg) or saline was given to SNI mice. Pain threshold assessments were taken on the ipsilateral hindlimb before CCI-779 and morphine administrations and then 30 min after morphine i.p. injection.

The whole experiment was repeated with a new group of SNI-injured mice. However, the order of the mTORC1 inhibitors was reversed and therefore CCI-779 was given first and then metformin was administered after the washout period. The experiment I was repeated a total of four times - twice with metformin and twice with CCI-779.

**Table 7.1 Design of experiment I.**

| Group | Morphine doses | | | Saline (i.p.) | 20 h prior morphine-treatment | | Number of animals |
|---|---|---|---|---|---|---|---|
| | (3mg/kg) (i.p.) | (10mg/kg) (i.p.) | (20mg/kg) (i.p.) | | mTORC1 inhibitors (i.p.) | Vehicle (i.p.) | |
| 1 | + | | | | | + | 6 |
| 2 | | + | | | | + | 6 |
| 3 | | | + | | | + | 6 |
| 4 | | | | + | | + | 6 |
| 5 | + | | | | + | | 10 |
| 6 | | + | | | + | | 10 |
| 7 | | | + | | + | | 10 |
| 8 | | | | + | + | | 10 |

SNI mice were randomly assigned in to 8 groups and received different treatment: (1),(2) and (3) mice were pre-treated with vehicle given between 1:00 pm and 2:00 pm, then 20 h after mice received one injections of one of three doses of morphine (3, 10, 20 mg/kg); (4) control, mice were pre-treated with vehicle given between 1:00 pm and 2:00 pm, then 20 h after mice received one injection of saline; (5), (6) and (7) mice were pre-treated with mTORC1 inhibitors given between 1:00 pm and 2:00 pm, then 20 h after mice received one injections of one of three doses of morphine (3, 10, 20 mg/kg); (8) mice were pre-treated with mTORC1 inhibitors given between 1:00 pm and 2:00 pm, then 20 h after mice received one injections of saline.

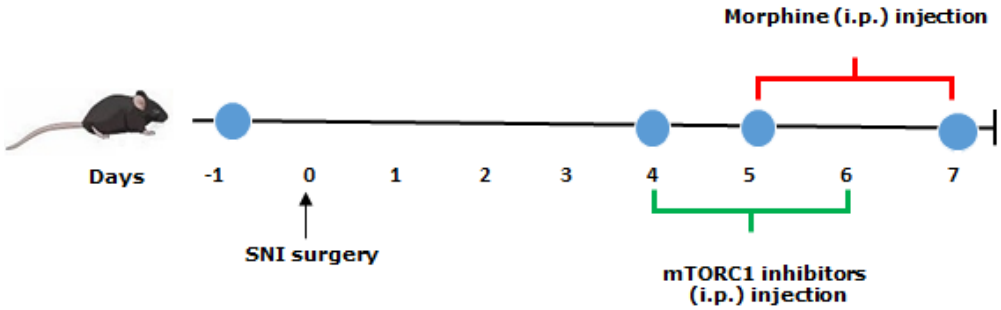


**Figure 7.1: A schematic diagram summering a timeline of the experiment I**. Baseline for mechanical and cold stimulation thresholds was taken one day (day -1) before the induction of neuropathic pain by spread nerve injury SNI (day 0). Following recovery, another post-surgery baseline was taken on day 4. Then, the mTORC1 inhibitors metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) or equivalent vehicle solutions without the inhibitor, were administered on day 4 and 6. On days 5-7, another set of tests were taken, one before any morphine injections (3, 10, 20 mg mg/kg, i.p.) and another 30 min following morphine injection.The blue dots represent behavioral test measured in response to mechanical (von Frey filaments) and cold (acetone test) stimuli. The red double brace represents a single injection of morphine at one of three doses (3, 10 and 20 mg/kg, i.p.) on day 5 and 7. The green double brace represents metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) injection on day 4 and 6 once 20 h before morphine doses.

### 7.2.5.2 Design of experiment II

SNI mice were divided into 8 different groups as indicated in Table 7.2. On day 4 after SNI, metformin (100 nmol /50 µl, i.pl) or equivalent vehicle solutions without the inhibitor was administered 20 h before morphine i.p. injections (3, 10, 20 mg/kg). On day 5, a single i.p. morphine injection (3, 10, 20 mg/kg) or saline was given to SNI mice. Pain threshold

assessments were taken on the ipsilateral and contralateral hindlimb before metformin and morphine administrations and then 30 min after morphine i.p. injection. A short break from injections and testing (2-3 days) was allowed for washout of the drugs, before the experiment was repeated with the other mTORC1 inhibitor CCI-779 (12.5 nmol /50 µl, i.pl). On day 6 after SNI, CCI-779 (12.5 nmol /50 µl, i.pl.) or equivalent vehicle solutions without the inhibitor was administered 20 h before morphine i.p. injections (3, 10, 20 mg/kg).

On day 7, a single i.p. morphine injection (3, 10, 20 mg/kg) or saline was given to SNI mice. Pain threshold assessments were taken on the ipsilateral and contralateral hindlimb before CCI-779 and morphine administrations and then 30 min after morphine i.p. injection.

The whole experiment was repeated with a new group of SNI-injured mice. However, the order of the mTORC1 inhibitors was reversed; CCI-779 was given first and then metformin was administered after the washout period. The experiment II was repeated a total of four times - twice with metformin and twice with CCI-779.

**Table 7.2 Design of experiment II.**

| Group | Morphine doses | | | Saline (i.p.) | 20 h prior morphine-treatment | | Number of animals |
|---|---|---|---|---|---|---|---|
| | (3mg/kg) (i.p.) | (10mg/kg) (i.p.) | (20mg/kg) (i.p.) | | mTORC1 inhibitors (i.pl.) | Vehicle (i.pl.) | |
| 1 | + | | | | | + | 6 |
| 2 | | + | | | | + | 6 |
| 3 | | | + | | | + | 6 |
| 4 | | | | + | | + | 6 |
| 5 | + | | | | + | | 10 |
| 6 | | + | | | + | | 10 |
| 7 | | | + | | + | | 10 |
| 8 | | | | + | + | | 10 |

SNI mice were randomly assigned in to 8 groups and received different treatment: (1), (2) and (3) mice were pre-treated with vehicle given between 1:00 pm and 2:00 pm, then 20 h after mice received one injections of three dose of morphine (3, 10, 20 mg/kg); (4) control, mice were pre-treated with vehicle given between 1:00 pm and 2:00 pm, then 2 h after mice received one injection of saline;(5), (6) and (7) mice were pre-treated local mTORC1 inhibitors given between 1:00 pm and 2:00 pm, then 2 h after mice received one injections of three dose of morphine (3, 10, 20 mg/kg); (8) mice were pre-treated with local mTORC1 inhibitors given between 1:00 pm and 2:00 pm, then 20 h after mice received one injections of saline.

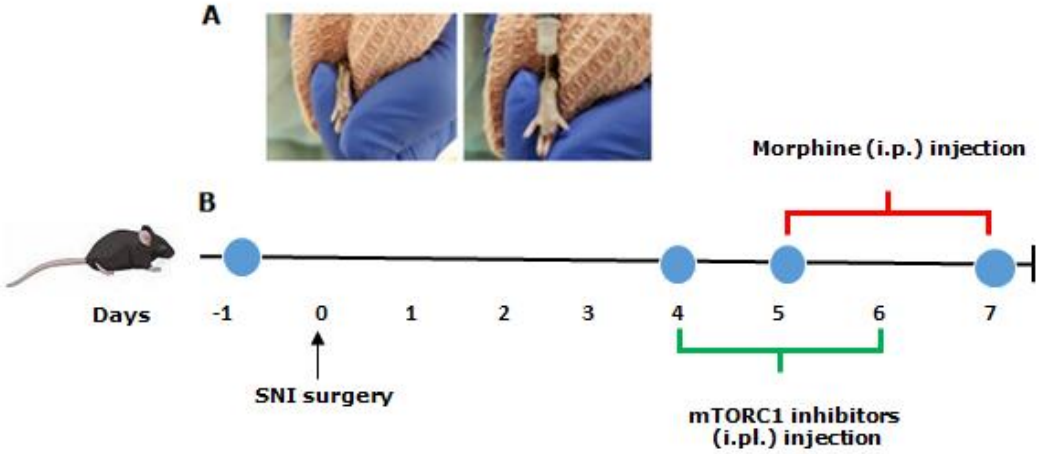


**Figure 7.2: A schematic diagram summering a timeline of the experiment II. (A)** Mouse was restrained in a cloth and received intraplantar injections (i.p over 30 s in a volume of 50 µl into the ipsilateral paw. **(B)** Baseline for mechanical and cold stimulation threshold was taken one day (day -1) before the induction of neuropathic pain by spread nerve injury SNI (day 0). Following recovery, another post-surgery baseline was taken on day 4. Then, the mTORC1 inhibitors metformin (100 nmol /50 µl, i.pl.) or CCI-779 (12.5 nmol/50 µl, i.pl.) or equivalent vehicle solutions without the inhibitor, were administered on day 4 and 6. On days 5-7, another set of tests were taken, one before any morphine injections (3, 10, 20 mg mg/kg, i.p.) and another 30 min following morphine injection. The blue dots represent behavioral test measured in response to mechanical (von Frey filaments) and cold (acetone test) stimuli. The red double brace represents a single injection of morphine at one of three doses (3, 10 and 20 mg/kg, i.p.) on day 5 and 7. The green double brace represents metformin (100 nmol /50 µl, i.pl.) or CCI-779 (12.5 nmol/50 µl, i.pl.) injection on day 4 and 6 once 20 h before morphine doses.

### 7.2.6 Statistical data analysis

Data analysis and statistical comparisons were performed by GraphPad Prism, version 8.01 for Windows, (GraphPad Software, CA, www.graphpad.com). Behavioral results are presented in the graphs as mean ± SEM. Each group included 6-10 mice. Statistical analysis for behavioral results was performed by two-way analysis of variance (two-way ANOVA) followed by Bonferroni's multiple comparison post-hoc tests and Student's t test was used when two groups were compared. A value of $P<0.05$ was considered to be statistically significant.

## 7.3 Results

### 7.3.1 Systemic (i.p.) administration of the mammalian target of rapamycin complex I (mTORC1) inhibitors potentiated the analgesic effect of morphine in neuropathic mice

As illustrated in Figure 7.3, all mice that were subjected to SNI surgery developed mechanical (Figure 7.3 A) and cold (Figure 7.3 B) hypersensitivity in the paw ipsilateral to the injury (left paw). Specifically, the mechanical withdrawal threshold in response to von Frey filament decreased significantly compared with the basal value before the SNI surgery (0.06 ± 0.01 g *vs.* 1.65 ± 0.06 g, $t_{(36)}= 35.66$, $P<0.0001$; Figure 7.3 A). Also, SNI mice

showed an enhanced response in acetone test, observed as a longer duration of response to cold stimulus, when compared to the sensitivity prior to the SNI surgery (9.90 ± 0.30 s *vs*. 1.98 ± 0.28 s, $t_{(36)}$= 24.07, P<0.0001; Figure 7.3 B). As illustrated in Figure 7.4, morphine produced dose response relationships observed in both vehicle and mTORC1 inhibitors pre-treated groups in both mechanical (von Frey; Figure 7.4 A, B) and cold (acetone; Figure 7.4 C, D) behavioral tests. Interestingly, a single systemic injection of metformin (200 mg/kg, i.p.; Figure 7.4 A, C) or CCI-779 (25 mg/kg, i.p.; Figure 7.4 B, D) 20 h prior to one of three tested doses of morphine (3, 10 and 20 mg/kg, i.p.) resulted in potentiation of morphine analgesia in von Frey (Figure 7.4 A; Figure 7.4 B) and acetone (Figure 7.4 C; Figure 7.4 D) tests. Post-test analysis revealed statistically significant difference in response to von Frey test at higher doses of morphine (10 and 20mg/kg, i.p.). Results were as follows: morphine 20 mg/kg + vehicle pre-treatment *vs.* morphine 20 mg/kg + metformin pre-treatment: 0.57 ± 0.15 g *vs.* 0.98 ± 0.17 g, $F_{(1,55)}$= 8.60, P= 0.004, Figure 7.4 A, morphine 10 mg/kg and 20 mg/kg + vehicle pre-treatment *vs*. morphine 10 mg/kg and 20 mg/kg + CCI-779 pre-treatment: 0.14 ± 0.01 g and 0.31 ± 0.09 g *vs.* 0.85 ± 0.17 g and 0.81 ± 0.19 g, $F_{(1,56)}$= 15.87, P= 0.0002, Figure 7.4 B). However, the withdrawal duration thresholds was higher in metformin or CCI-779 pre-treated groups compared with vehicle pre-treatment animals.

In the cold hypersensitivity test, paw-withdrawal latency in response to acetone and measured after morphine (3, 10 and 20 mg/kg, i.p.) administration was decreased in mice pre-treated with mTORC1 inhibitors in comparison with vehicle pre-treated mice indicating for potentiation of morphine-induced analgesia in the presence of mTORC1 inhibition (Figure 7.4). Specifically, systemic administration of metformin (200 mg/kg, i.p., Figure 7.4 C) and CCI-779 (25mg/kg, i.p., Figure 7.4 D) potentiated the morphine analgesic effect in response to cold (acetone test) stimuli ($F_{(1,56)}$= 13.95, P= 0.0004 and $F_{(1,56)}$= 11.47, P= 0.0013, respectively). Post-test analysis revealed statistically significant difference in response to acetone test at the highest dose of morphine (20mg/kg, i.p.). Results were as follows: morphine 20 mg + vehicle pre-treatment *vs.* morphine 20 mg + metformin pre-treatment (6.94 ± 0.56 s *vs.* 4.06 ± 0.59 s,), morphine 20 mg + vehicle pre-treatment *vs*. morphine 20 mg + CCI-779 pre-treatment (7.11 ± 1.02 s *vs.* 4.25 ± 0.72 s). In addition, statistical analysis revealed a significant interaction between morphine doses and mTORC1 inhibitors in von Frey but not in cold hypersensitivity test (morphine x metformin: $F_{(3,56)}$= 18.23, P< 0.0001; morphine x CCI-779: $F_{(3,56)}$= 14.32, P< 0.0001 ).

Neuropathic mice injected with metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) alone showed no significant effect in von Frey or acetone tests when compared to the values of the vehicle/saline-treated animals (Table 7.3).

The observed potentiation of the analgesic effects of morphine seems to be more pronounced in von Frey than in acetone test (Figure 7.4).

### 7.3.2 Peripheral (i.pl.) administration of the mammalian target of rapamycin complex 1 (mTORC1) inhibitors potentiated the analgesic effect of morphine in neuropathic mice

#### 7.3.2.1 Ipsilateral paw

Mice were subjected to SNI surgery and developed mechanical (Figure 7.3 C) and cold (Figure 7.3 D) hypersensitivity in the paw ipsilateral to the injury. Specifically, the mechanical withdrawal threshold in response to von Frey filaments significantly decreased when compared with the basal value before the SNI surgery (0.048 ± 0.01 g *vs*. 1.54 ± 0.04 g, $t_{(63)}$= 40.39, P<0.0001, Figure 7.3C). Also, the SNI mice showed an enhanced response in acetone test observed as a longer duration of response to the cold stimulus, when compared to the sensitivity prior to the SNI surgery (12.96 ± 0.66 s *vs*. 3.03 ± 0.14 s, $t_{(63)}$= 25.85, P<0.0001, Figure 7.3 D).

As illustrated in Figure 7.5, local peripheral i.p. injection of mTORC1 inhibitors directly to the plantar surface of the injured hind paw 20 h before morphine resulted in significant increase in paw withdrawal threshold to mechanical stimuli observed 30 min after morphine injection. Specifically, local administration of metformin (100 nmol/50 µl, i.pl., Figure 7.5 A) and CCI-779 (12.5 nmol/50 µl, i.pl., Figure 7.5 B) potentiated the morphine analgesic effect in von Frey test ($F_{(1,56)}$= 77.44, P<0.0001 and $F_{(1,56)}$= 73.37, P<0.0001, respectively). Post-test analysis revealed statistically significant difference in response to von Frey test at the two higher dose of morphine (10 mg/kg and 20mg/kg, i.p.). Results were as follows: (morphine 10 mg/kg + vehicle pre-treatment *vs.* morphine 10 mg/kg + metformin pre-treatment: 0.2 ± 0.04 g *vs.* 0.86 ± 0.09 g; morphine 20 mg/kg + vehicle pre-treatment *vs.* morphine 20 mg/kg + metformin pre-treatment: 0.39 ± 0.02 g *vs*. 1.36 ± 0.10 g; morphine 10 mg/kg + vehicle pre-treatment *vs.* morphine 10 mg/kg + CCI-779 pre-treatment: 0.25 ± 0.04 g *vs.* 0.87 ± 0.08 g; morphine 20 mg/kg + vehicle pre-treatment *vs.* morphine 20 mg/kg + CCI-779 pre-treatment: 0.34 ± 0.04 g *vs.* 1.38 ± 0.13 g).

In cold hypersensitivity test, statistical analysis showed a significant decrease in paw withdrawal latency to cold stimulus as measured in the acetone test in all groups pre-treated with mTORC1 inhibitors when compared with vehicle pre-treatment (Figure 7.5). Here, both metformin (100 nmol /50 µl, i.pl., Figure 7.5 C) and CCI-779 (12.5 nmol/50 µl, i.pl., Figure 7.5 D) potentiated the morphine analgesic effect in acetone test ($F_{(1,56)}$= 70.79, P<0.0001 and $F_{(1,56)}$= 41.14, P<0.0001, respectively). Post-test analysis revealed statistically significant difference in response to acetone test at all tested dose of morphine (3, 10 and 20mg/kg, i.p.). Interestingly, administration of metformin (100 nmol /50 µl, i.pl.) or CCI-779 (12.5 nmol/50 µl, i.pl.) in the absence of morphine induced significant (P<0.05) increase in the paw withdrawal response to cold, but not mechanical stimulus in comparison with values after the SNI surgery. Results were as follows: after SNI *vs.* metformin pre-treatment (10.53 ± 0.56 s *vs*. 7.77 ± 0.43 s, $t_{(9)}$= 0.001); after SNI *vs.* CCI-779 pre-treatment (11.81 ± 0.91 s *vs*. 8.80 ± 0.63 s, $t_{(9)}$= 0.01; Table 7.4).

#### 7.3.2.2 Contralateral paw

As only, the left side of the sciatic nerve was injured, the paw contralateral to the side of SNI injury served as a *naïve* control and received no treatment. Interestingly however, the statistical analysis revealed that there were small but statistically significant differences in the withdrawal responses of the control paw between pre-SNI and post-SNI as measured by the von Frey test (1.55 ± 0.03 g *vs*. 1.27 ± 0.02 g, $t_{(63)}$= 5.34, P<0.0001) and acetone test (1.95 ± 0.13 *vs.* 3.38 ± 0.08 s, $t_{(63)}$= 5.36, P= 0.0017) and presented in Figure 7.3 (E and F). This result is likely to be a false positive error resulting from very small error bars (von Frey test: mean differences = 0.26; acetone test: mean difference = 0.78) that may be an indicative of a Type I error (Table 7.5).

As depicted in Figure 7.6, no significant differences were detected in withdrawal thresholds to mechanical stimulation or in paw withdrawal latency to acetone stimulus in the contralateral paw after local i.pl. injection of mTORC1 inhibitors in the plantar surface of the injured hind paw 20 h before morphine (3, 10 and 20mg/kg, i.p.), metformin treatment effect in von Frey test: $F_{(1,56)}$= 0.001 , P= 0.97, Figure 7.6 A; treatment effect in acetone test: $F_{(1,56)}$= 0.33 , P= 0.56, Figure 7.5 C and CCI-779 treatment effect in von Frey test: $F_{(1,56)}$= 0.07, P= 0.77, Figure 7.5 B; treatment effect in acetone test: $F_{(1,56)}$= 0.16, P= 0.68, Figure 7.5 D.

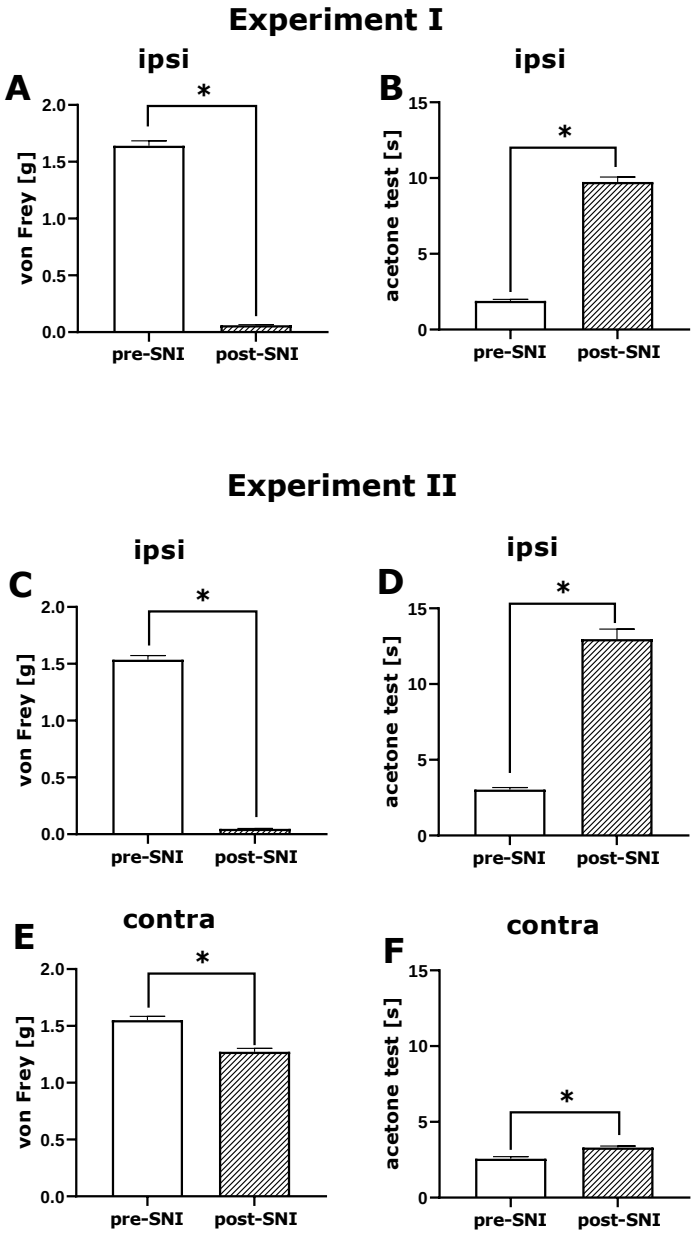


**Figure 7.3: Spared nerve injury (SNI) produced significant change in response to mechanical (A, C, E) and cold (B, D, F) stimuli in mice.** The von Frey and acetone tests were used to measure the mechanical paw withdrawal threshold and cold withdrawal latency in pre-SNI and post-SNI mice. **Experiment I**: On day 4 after SNI surgery, mice showed an enhanced response to mechanical **(A)** and cold **(B)** stimulation in the lateral part of the injured hind paw compared with their values prior the surgery. **Experiment II**: On day 4 after SNI surgery, mice showed an enhanced response to mechanical **(C)** and cold **(D)** stimulation in the lateral part of the injured hind paw (ipsi) compared with their values prior the surgery. The non-injured hind paw (contra) showed a weak but significant response to mechanical **(E)** and cold **(F)** stimulation compared with their values prior the surgery. Data are presented as mean ± SEM, n= 64 mice per group, *P<0.05 (paired Student's t-test).

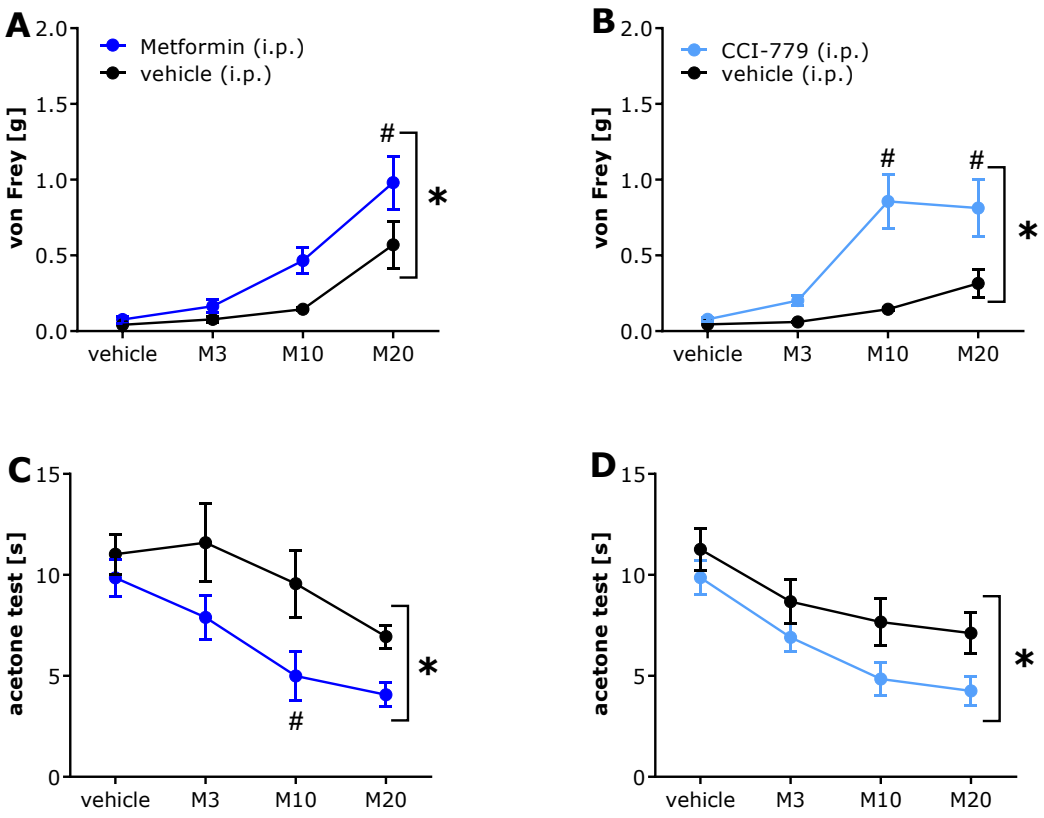


**Figure 7.4: Systemic administration of metformin (A, C) and CCI-779 (B, D) potentiated the analgesic effect of morphine in neuropathic mice.** A single systemic administration of metformin (200 mg/kg, i.p.) and CCI-779 (25 mg/kg, i.p.) 20 h before morphine (M, 3, 10, 20 mg/kg, i.p.) potentiated the analgesic effect of morphine on day 5-7 after the spared nerve injury (SNI), as measured by von Frey **(A, B)** and acetone tests **(C, D)** 30-40 min after morphine injection. Metformin or CCI-779 were injected i.p. once 20 h before one morphine dose. The control group received vehicle according to the same experimental schedule. The data are presented as the mean responses ± SEM, n= 6-10 mice per group. */# $P<0.05$ (two- way ANOVA, followed by Bonferroni's test).

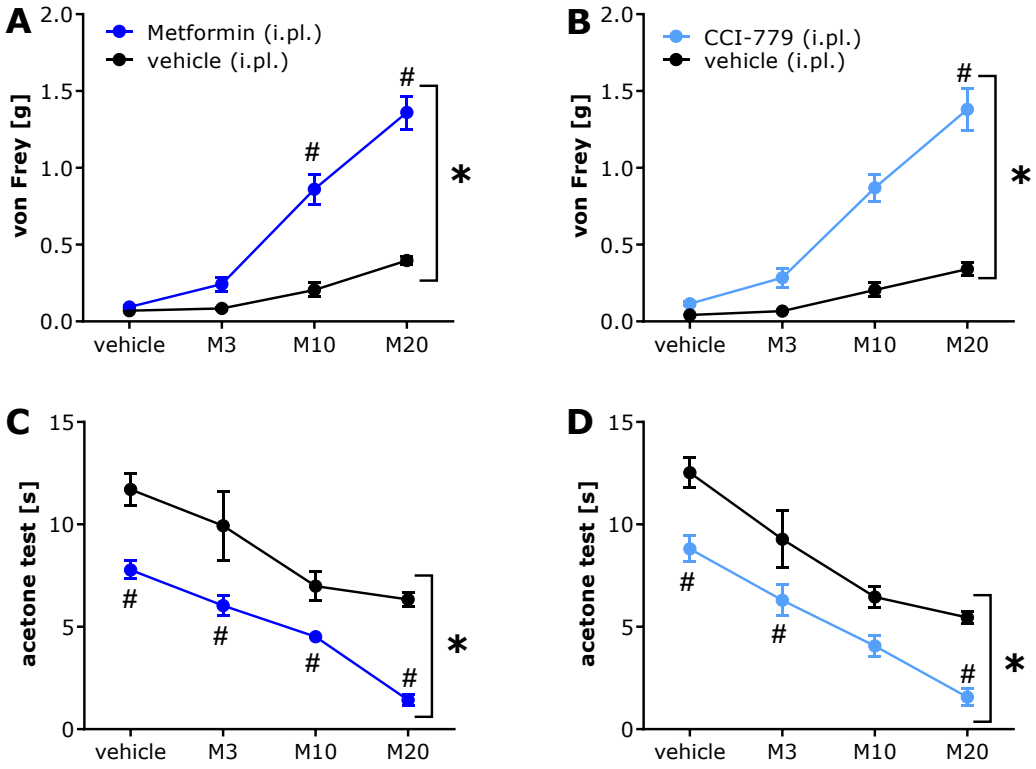


**Figure 7.5: Local administration of metformin (A, C) and CCI-779 (B, D) potentiated the analgesic effect of morphine in neuropathic mice.** A single local administration of metformin (100 nmol /50 µl, i.pl.) or CCI-779 (12.5 nmol/50 µl, i.pl.) 20 h before morphine (M, 3, 10, 20 mg/kg, i.p.) potentiated the analgesic effect of morphine on day 5-7 after the spared nerve injury (SNI), as measured by von Frey **(A, B)** and acetone test **(C, D)** 30-50 min after morphine injection. Metformin or CCI-779 were injected i.pl. once 20 h before one morphine dose. The control group received vehicle according to the same experimental schedule. The data are presented as the mean responses ± SEM, n= 6-10 mice per group. */# *vs*. vehicle; */# $P<0.05$ (two-way ANOVA, followed by Bonferroni's test).

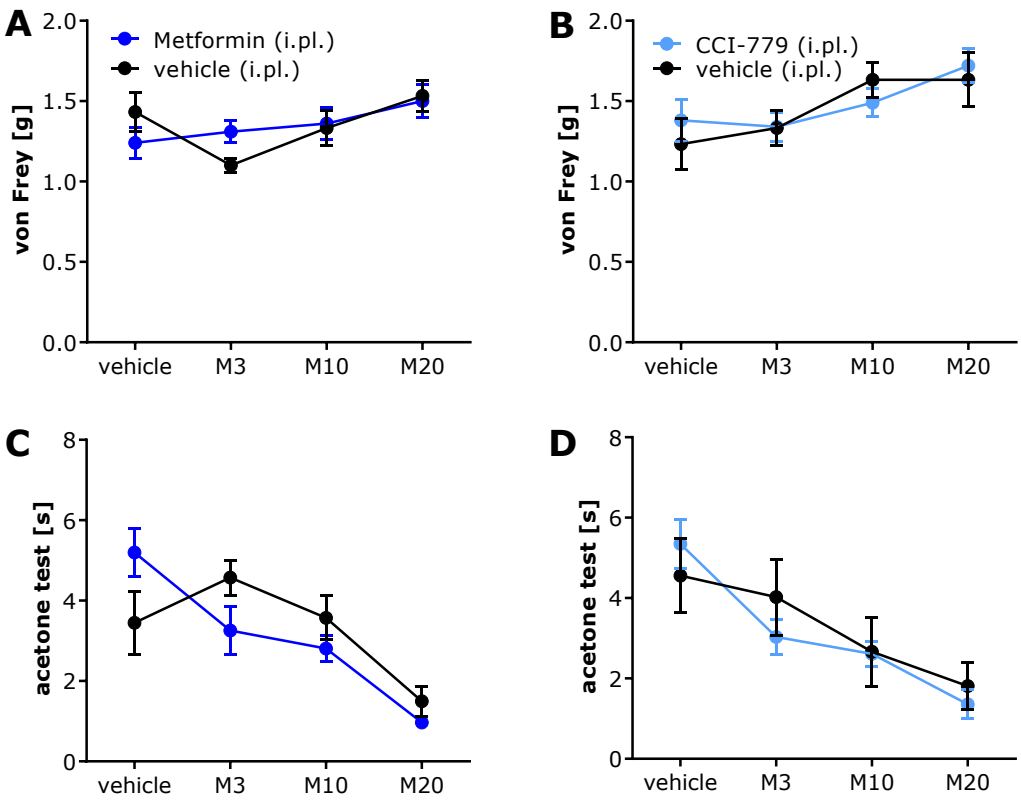


**Figure 7.6: Local administration of metformin (A, C) and CCI-779 (B, D) in injured hind paw had no effect on the contralateral hind paw in neuropathic mice.** A single local administration of metformin (100 nmol /50 µl, i.pl.) or CCI-779 (12.5 nmol/50 µl, i.pl.) 20 h before morphine (M, 3, 10, 20 mg/kg, i.p.) in the injured hind paw did not change the analgesic effect of morphine on day 5-7 after the spared nerve injury (SNI), as measured by von Frey **(A, B)** and acetone test **(C, D)** 30-40 min after morphine injection. Metformin or CCI-779 were injected i.pl. once 20 h before one morphine dose. The control group received vehicle according to the same experimental schedule. The data are presented as the mean responses ± SEM, n= 6-10 mice per group. */# *vs*. vehicle; */# P<0.05 (two-way ANOVA, followed by Bonferroni's test).

**Table 7.3: A summary of behavioral responses measured on the ipsilateral hind paw in response to mechanical (von Frey test) and cold (acetone test) stimuli in mice subjected to neuropathic pain induced by speared nerve injury (SNI) and treated with morphine and/or the mammalian target of rapamycin complex 1 (mTORC1) inhibitors (metformin or CCI-779).**

| **von Frey** | **Metformin (200 mg/kg, i.p.)** | | | **CCI-779 (25 mg/kg, i.p.)** | | |
|---|---|---|---|---|---|---|
| | pre-SNI | post-SNI | 30 min | pre-SNI | post-SNI | 30 min |
| saline | 1.68±0.16 | 0.04±0.01 | 0.04±0.01 | 1.68±0.16 | 0.05±0.01 | 0.04±0.01 |
| morphine 3mg | 2.00±0.01 | 0.04±0.01 | 0.07±0.02 | 2.00±0.01 | 0.04±0.01 | 0.06±0.01 |
| morphine 10mg | 1.45±0.10 | 0.05±0.01 | 0.14±0.02 | 1.45±0.10 | 0.04±0.01 | 0.14±0.01 |
| morphine 20mg | 1.65±0.12 | 0.06±0.02 | 0.57±0.15 | 1.65±0.12 | 0.05±0.01 | 0.31±0.09 |
| morphine 3mg + mTORC1 inhibitor | 1.70±0.08 | 0.08±0.01 | 0.16±0.04 | 1.70±0.08 | 0.05±0.01 | 0.20±0.03 |
| morphine 10mg + mTORC1 inhibitor | 1.55±0.13 | 0.05±0.01 | 0.46±0.08 | 1.55±0.13 | 0.05±0.01 | 0.85±0.17 |
| morphine 20mg +mTORC1 inhibitor | 1.50±0.10 | 0.06±0.01 | 0.98±0.17 | 1.50±0.10 | 0.06±0.01 | 0.81±0.19 |
| mTORC1 inhibitor | 1.68±0.12 | 0.07±0.02 | 0.07±0.02 | 1.68±0.12 | 0.10±0.03 | 0.07±0.01 |
| F | $F_{(2,14)}$= 122.00, P<0.0001 | | | $F_{(2,14)}$= 103.50, P<0.0001 | | |

| **acetone test** | **Metformin (200 mg/kg, i.p.)** | | | **CCI-779 (25 mg/kg, i.p.)** | | |
|---|---|---|---|---|---|---|
| | pre-SNI | post-SNI | 30 min | pre-SNI | post-SNI | 30 min |
| saline | 2.26±0.35 | 8.68±1.93 | 11.02±0.99 | 2.26±0.35 | 7.71±0.23 | 11.26±1.03 |
| morphine 3mg | 1.83±0.13 | 8.92±0.72 | 11.59±1.92 | 1.83±0.13 | 10.69±0.92 | 8.67±1.08 |
| morphine 10mg | 3.07±0.34 | 8.55±0.62 | 9.56±1.66 | 3.07±0.34 | 11.30±1.79 | 7.66±1.16 |
| morphine 20mg | 1.71±0.28 | 7.11±0.74 | 6.94±0.56 | 1.71±0.28 | 8.21±0.41 | 7.11±1.02 |
| morphine 3mg + mTORC1 inhibitor | 1.84±0.11 | 7.69±1.50 | 7.89±1.07 | 1.84±0.11 | 9.92±0.60 | 6.90±0.70 |
| morphine 10mg + mTORC1 inhibitor | 1.50±0.16 | 7.88±1.19 | 4.99±1.22 | 1.50±0.16 | 10.36±0.98 | 4.84±0.82 |
| morphine 20mg +mTORC1 inhibitor | 1.95±0.32 | 9.06±1.35 | 4.06±0.59 | 1.95±0.32 | 10.10±0.67 | 4.25±0.72 |
| mTORC1 inhibitor | 1.48±0.23 | 9.37±0.80 | 9.85±0.93 | 1.48±0.23 | 9.21±0.80 | 9.86±0.84 |
| F | $F_{(2,14)}$= 47.47, P<0.0001 | | | $F_{(2,14)}$ = 45.68, P<0.0001 | | |

Withdrawal threshold to mechanical stimulus was assessed with von Frey filaments (g), while withdrawal latency to cold stimulus was measured by the acetone test (s). Measurements were taken before induction of neuropathic pain as a baseline pain threshold (pre-SNI), and then after the SNI (post-SNI) and 30 min following of morphine injection with (3, 10, 20 mg/kg, i.p). Data are presented as mean ± SEM, n= 6-10 mice per group. Comparison of the groups with repeated measure one-way ANOVA followed by Bonferroni's test.

**Table 7.4: A summary of behavioural responses measured on the ipsilateral hind paw in response to mechanical (von Frey test) and cold (acetone test) stimuli in mice subjected to neuropathic pain induced by speared nerve injury (SNI) and treated with morphine and/or the mammalian target of rapamycin complex 1 (mTORC1) inhibitors (metformin or CCI-779).**

| **von Frey** | **Metformin (100 nmol, i.pl.)** | | | **CCI-779 (12.5 nmol, i.pl.)** | | |
|---|---|---|---|---|---|---|
| | pre-SNI | post-SNI | 30 min | pre-SNI | post-SNI | 30 min |
| saline | 1.50±0.13 | 0.04±0.01 | 0.04±0.01 | 1.50±0.13 | 0.04±0.01 | 0.04±0.01 |
| morphine 3mg | 1.59±0.11 | 0.06±0.02 | 0.08±0.01 | 1.59±0.11 | 0.05±0.01 | 0.06±0.01 |
| morphine 10mg | 1.52±0.10 | 0.05±0.01 | 0.20±0.04 | 1.52±0.10 | 0.05±0.01 | 0.25±0.04 |
| morphine 20mg | 1.60±0.15 | 0.04±0.01 | 0.39±0.02 | 1.60±0.15 | 0.04±0.01 | 0.34±0.04 |
| morphine 3mg + mTORC1 inhibitor | 1.61±0.10 | 0.05±0.01 | 0.24±0.04 | 1.61±0.10 | 0.05±0.01 | 0.28±0.06 |
| morphine 10mg + mTORC1 inhibitor | 1.44±0.07 | 0.05±0.01 | 0.86±0.09 | 1.44±0.07 | 0.04±0.01 | 0.87±0.08 |
| morphine 20mg +mTORC1 inhibitor | 1.46±0.11 | 0.06±0.02 | 1.36±0.10 | 1.46±0.11 | 0.04±0.01 | 1.38±0.13 |
| mTORC1 inhibitor | 1.58±0.08 | 0.04±0.01 | 0.09±0.01 | 1.58±0.08 | 0.04±0.01 | 0.11±0.01 |
| F | $F_{(2,14)}$= 60.25, P<0.0001 | | | $F_{(2,14)}$ = 58.83, P<0.0001 | | |

| **acetone test** | **Metformin (100 nmol, i.pl.)** | | | **CCI-779 (12.5 nmol, i.pl.)** | | |
|---|---|---|---|---|---|---|
| | pre-SNI | post-SNI | 30 min | pre-SNI | post-SNI | 30 min |
| saline | 2.99±0.48 | 12.20±1.26 | 11.70±0.78 | 2.99±0.48 | 14.36±0.94 | 12.52±0.74 |
| morphine 3mg | 3.15±0.69 | 12.14±0.51 | 9.93±1.60 | 3.15±0.69 | 16.26±0.95 | 9.27±1.38 |
| morphine 10mg | 3.42±0.56 | 12.47±0.56 | 6.98±0.70 | 3.42±0.56 | 13.94±0.89 | 6.45±0.51 |
| morphine 20mg | 3.77±0.50 | 11.51±0.59 | 6.33±0.33 | 3.77±0.50 | 13.43±0.89 | 5.45±0.30 |
| morphine 3mg + mTORC1 inhibitor | 2.80±0.53 | 12.53±0.53 | 6.03±0.48 | 2.80±0.53 | 11.16±0.53 | 6.29±0.76 |
| morphine 10mg + mTORC1 inhibitor | 2.71±0.53 | 12.57±0.54 | 4.25±0.23 | 2.71±0.53 | 11.80±0.76 | 4.06±0.53 |
| morphine 20mg +mTORC1 inhibitor | 2.76±0.36 | 11.48±0.45 | 1.42±0.27 | 2.76±0.36 | 10.46±1.10 | 1.56±0.42 |
| mTORC1 inhibitor | 2.69±0.41 | 10.53±0.56 | 7.77±0.43 | 2.69±0.41 | 11.81±0.91 | 8.80±0.63 |
| F | $F_{(2,14)}$= 47.28, P<0.0001 | | | $F_{(2,14)}$ =52.49, P<0.0001 | | |

Withdrawal threshold to mechanical stimulus was assessed with von Frey filaments (g), while withdrawal latency to cold stimulus was measured by the acetone test (s). Measurements were taken before induction of neuropathic pain as a baseline pain threshold (pre-SNI), and then after the SNI (post-SNI) and 30 min following of morphine injection with (3, 10, 20 mg/kg, i.p). Data are presented as mean ± SEM, n= 6-10 mice per group. Comparison of the groups with repeated measure one-way ANOVA followed followed by Bonferroni's test.

**Table 7.5: A summary of behavioural responses measured on the contralateral hind paw in response to mechanical (von Frey test) and cold (acetone test) stimuli in mice subjected to neuropathic pain induced by speared nerve injury (SNI) and treated with morphine and/or the mammalian target of rapamycin complex 1 (mTORC1) inhibitors (metformin or CCI-779).**

| **von Frey** | **Metformin (100 nmol, i.pl.)** | | | **CCI-779 (12.5 nmol, i.pl.)** | | |
|---|---|---|---|---|---|---|
| | pre-SNI | post-SNI | 30 min | pre-SNI | post-SNI | 30 min |
| saline | 1.51±0.17 | 1.50±0.13 | 1.43±0.12 | 1.51±0.17 | 1.23±0.06 | 1.23±0.15 |
| morphine 3mg | 1.75±0.15 | 1.45±0.11 | 1.40±0.18 | 1.75±0.15 | 1.40±0.10 | 1.33±0.11 |
| morphine 10mg | 1.55±0.09 | 1.33±0.06 | 1.33±0.11 | 1.55±0.09 | 1.30±0.06 | 1.63±0.10 |
| morphine 20mg | 1.45±0.10 | 1.36±0.17 | 1.53±0.09 | 1.45±0.10 | 1.30±0.16 | 1.63±0.16 |
| morphine 3mg + mTORC1 inhibitor | 1.46±0.10 | 1.29±0.09 | 1.31±0.06 | 1.46±0.10 | 1.28±0.09 | 1.34±0.08 |
| morphine 10mg + mTORC1 inhibitor | 1.58±0.11 | 1.20±0.13 | 1.36±0.09 | 1.58±0.11 | 1.28±0.09 | 1.34±0.08 |
| morphine 20mg +mTORC1 inhibitor | 1.52±0.11 | 1.18±0.04 | 1.50±0.10 | 1.52±0.11 | 1.28±0.07 | 1.72±0.07 |
| mTORC1 inhibitor | 1.52±0.08 | 1.46±0.13 | 1.24±0.09 | 1.52±0.08 | 1.35±10 | 1.38±0.12 |
| F | $F_{(2,14)}$= 8.70, P = 0.0035 | | | $F_{(2,,14)}$ = 9.70, P = 0.002 | | |

| **acetone test** | **Metformin (100 nmol, i.pl.)** | | | **CCI-779 (12.5 nmol, i.pl.)** | | |
|---|---|---|---|---|---|---|
| | pre-SNI | post-SNI | 30 min | pre-SNI | post-SNI | 30 min |
| saline | 2.65±0.34 | 2.87±0.48 | 3.44 ± 0.77 | 2.65±0.34 | 3.42±0.28 | 3.00±0.67 |
| morphine 3mg | 2.16±0.53 | 3.06±0.40 | 3.90±0.30 | 2.16±0.53 | 3.71±0.36 | 2.88±0.50 |
| morphine 10mg | 2.12±0.26 | 3.54±0.40 | 3.02±0.33 | 2.12±0.26 | 3.77±1.14 | 2.06±0.51 |
| morphine 20mg | 2.55±0.46 | 3.25±0.58 | 1.49±0.37 | 2.55±0.46 | 3.85±0.60 | 1.81±0.58 |
| morphine 3mg + mTORC1 inhibitor | 2.25±0.49 | 3.66±0.37 | 2.99±0.46 | 2.25±0.49 | 3.76±0.61 | 3.03±0.43 |
| morphine 10mg + mTORC1 inhibitor | 3.31±0.44 | 3.47±0.17 | 2.81±0.32 | 3.31±0.44 | 3.21±0.36 | 2.61±0.308 |
| morphine 20mg +mTORC1 inhibitor | 2.66±0.54 | 3.22±0.41 | 2.19±0.16 | 2.66±0.54 | 3.00±0.42 | 1.35±0.36 |
| mTORC1 inhibitor | 2.76±0.48 | 2.72±0.19 | 2.48±0.33 | 2.76±0.48 | 3.25±1.08 | 3.23±0.39 |
| F | $F_{(2,,14)}$= 2.42, P= 0.124 | | | $F_{(2,14)=}$ 5.49, P= 0.017 | | |

Withdrawal threshold to mechanical stimulus was assessed with von Frey filaments (g), while withdrawal latency to cold stimulus was measured by the acetone test (s). Measurements were taken before induction of neuropathic pain as a baseline pain threshold (pre-SNI), and then after the SNI (post-SNI) and 30 min following of morphine injection with (3, 10, 20 mg/kg, i.p). Data are presented as mean ± SEM, n= 6-10 mice per group. Comparison of the groups with repeated measure one-way ANOVA followed by Bonferroni's test.

## 7.4 Discussion

In this chapter, it was demonstrated that both systemic (i.p.) and peripheral (i.pl.) administration of mTORC1 inhibitors tested 20 h after a single exposure to morphine improved the analgesic efficacy of morphine observed by significant inhibition of mechanical and cold hypersensitivity in mice subjected to neuropathic pain. Specifically, SNI-induced mechanical and cold hypersensitivity in mice was alleviated in animals pre-treated with metformin and/or CCI-779 before morphine administration. In contrast, animals treated with morphine alone only showed a slight reduction in mechanical and cold hypersensitivity. Thus, these observations emphasised that mTORC1 inhibition with either metformin or CCI-779 progressively potentiated the analgesic efficacy of morphine and highlighted a possible therapeutic potential for mTORC1 inhibitors and opioids for the treatment of neuropathic pain.

Morphine is the most effective available analgesic medicine to treat moderate to severe acute pain. However, pain linked to peripheral neuropathy does not always respond well to morphine (Porreca et al., 1998). It is well documented that damaged nerves lead to incomplete morphine efficacy and therefore, require a greater concentration of morphine in turn leading to increased risk of undesirable side-effects.

The similarities in cellular mechanism between morphine induced tolerance and neuropathic pain is often associated with decreased morphine clinical utility (McQuay, 2002; Porreca et al., 1998). The mechanisms that lead to lower effectiveness of morphine in neuropathic pain are not fully understood (Przewlocki and Przewlocka, 2001; Przewlocki and Przewlocka, 2005). Porreca et al. (1998), showed that a reduction in morphine analgesic potency which may be due to the degeneration of primary afferent neurons following a nerve injury resulted in a decreased number of presynaptic opioid receptors (Porreca et al., 1998). Further studies proposed that the involvement of the N-methyl-D-aspartate (NMDA) receptor appears to be a vital link to opioid receptor desensitisation, as the N-methyl-D-aspartate (NMDA) receptor cascade appears to be a common and essential link between morphine tolerance and induced pain sensitivity (Mayer et al., 1999; Ballantyne, 2003). Recently, it has been documented that alternation in protein translation in the nervous system appears to trigger opioid tolerance and hyperalgesia and therefore protein translation may regulate opioid efficacy in pain (Xu et al., 2014; Zhang et al., 2019).

In this current study, mice were exposed to morphine injection for the first time 5-7 days. This first exposure to morphine injection showed a slight enhancement in the mechanical withdrawal threshold and withdrawal duration in response to noxious stimuli compared with

saline injected mice. It should be pointed out that the analgesia achieved from morphine injection was not sufficient to enhance the response to mechanical or cold stimuli in SNI mice that would indicate for analgesic effect produced by morphine. Several studies recognised that morphine is relatively ineffective in rodent neuropathic pain models compared to its effect on inflammatory pain (Sandner-Kiesling et al., 2001; Decosterd et al., 2004; Rashid et al., 2004).

Interestingly, studies in both humans and animals showed that the combination of morphine with other drugs can result in more effective in pain control than single agents in treating neuropathic pain. For example, in randomised, double-blind study, Gilron et al. (2005), found that gabapentin significantly enhances the analgesic effect of morphine (Gilron et al., 2005). Similarly, Deng et al. (2017), argue that systemic administration of vitamin B complex potentiates morphine antinociception suggesting that vitamin B complex inhibited upregulation of Iba-1 expression and decreased p38 MAPK phosphorylation which in turn improved morphine efficacy (Deng et al., 2017). Moreover, a recent study reported that pre-treatment with a single dose of histamine H3 (E-162) and H4 (TR-7) receptor antagonists improved the response to morphine in mice subjected to the CCI pain model (Popiolek-Barczyk et al., 2018). The authors explained that blocking of both histamine receptors significantly potentiated the morphine antinociceptive effect as measured by mechanical (von Frey test) and thermal stimuli (Cold plate test), demonstrating that histamine receptors H3 and H4 are implicated in nociceptive transmission under neuropathic pain conditions (Popiolek-Barczyk et al., 2018). Likewise, it has been reported that pre-emptive treatment with glial inhibitors (minocycline) in rats exposed to CCI surgery improved morphine efficacy in response to tactile allodynia (von Frey test) and thermal hyperalgesia (cold plate test) (Mika et al., 2007). Furthermore, it was discovered that administration of non-selective inhibitors of nitric oxide synthase (L-NAME, TRIM and 1400W) improved morphine analgesia in mechanical allodynia (von Frey test) and thermal hyperalgesia (cold plate test) in CCI exposed rats (Makuch et al., 2013).

All these observations indicate that neuropathic pain leads to neuroplasticity changes within a multiple system which could be the direct cause of the modulation of nociceptive function in peripheral and central sensitisation in requirement of a higher morphine dose to achieve pain relief (de Conno et al., 1991; White et al., 2007; Oliveira Júnior et al., 2016). All these changes result from the modulation of protein translation which appears to be responsible for the mechanism that contributes to neuropathic pain and reduces the analgesic effect of morphine. Several pathological processes in the nervous system including neuropathic pain

are regulated *via* activation of mTORC1 and its downstream effectors that promote protein synthesis implicated in neuroplasticity in the nervous system (Swiech et al., 2008). Therefore, targeting mTORC1 appears important in relation to improving neuropathic pain and morphine analgesia. In line with this hypothesis, the inhibition of the mTORC1 pathway with metformin or CCI-779 potentiated morphine analgesia in SNI mice as demonstrated in this chapter.

Significantly, active mTORC1 was shown to be involved in pain hypersensitivity related to chronic pain, although it was not involved in acute pain (Géranton et al., 2009). It has been documented that an intrathecal administration of rapamycin attenuated neuropathic pain (He et al., 2016; Wang et al., 2016). Additionally, it has been documented that peripheral intraplantar administration of rapamycin had no beneficial effect on acute pain induced by local capsaicin injection into the rat's hind paw (Jiménez-Díaz et al., 2008). They showed that this administration route for rapamycin did not alter the withdrawal latency to heat (Hargreaves test), mechanical withdrawal threshold (von Frey test) and withdrawal response duration (pinprick test) in *naïve* rats (Jiménez-Díaz et al., 2008). This observation suggested that inhibition of mTORC1 had no effect on acute pain possibly due to a small number of fibres that contain the important biochemical elements that mediates local mRNA translation regulated by mTORC1 (Jiménez-Díaz et al., 2008). Therefore, in this current study, the SNI model was employed, as it has been reported that higher levels of mTORC1 have been detected in neuropathic pain conditions.

The nociceptive pain resulted from SNI surgery linked to changes in the sensitivity of nociceptor peripheral terminals (Decosterd and Woolf, 2000), as activation of primary afferent neuron causes suprathreshold firing in Aδ-mechanoreceptor (myelinated fibres) and C fibres (unmyelinated fibres) (Amir and Devor., 1999) resulting in enhanced pain sensitivity in the ipsilateral side of the mouse's hind paw leading to mechanical and cold allodynia, as presented in the results section.

Evidence generated from immunohistochemical staining demonstrated that local translation in primary afferent fibres mediated by mTORC1 is restricted to A-fibres. Thus, A-fibres supported the local protein synthesis machinery, for two reasons: mTORC1 does express in its fibres and the ribosomal particles usually present in axons near to the neurilemma cell (Schwann cell) that are around A-fibres to form the myelin sheath (Jiménez-Díaz et al., 2008; Hendriks et al., 2008). Therefore, local protein synthesis in primary afferent fibres is thought to contribute to synaptic plasticity following tissue injury as well as the regeneration of the damaged nerve.

It should be pointed out that nerve injury enhanced the excitability of dorsal horn neurons which generated a second level of signal amplification (central sensitisation). The central sensitisation resulted from complex changes in the excitability of neurons amplified the signals of A-fibres arising from the tissue surrounding the site of injury. For example, the experimental animals showed an increase in the pain thresholds in the tissue around the peripheral injury in response to mechanical test. Thus, several studies demonstrated that blocking the local translation machinery *via* mTORC1 inhibitors in A-fibres nociceptors regulates pain hypersensitivity.

It has been documented that local injection of rapamycin 4 h before a capsaicin injection (neuropeptide releasing agent that induced central sensitisation) blocked secondary but not primary hyperalgesia (Jiménez-Díaz et al., 2008). They reported that when rapamycin was injected away (in the lateral side hind paw) from the capsaicin injection site (centre of plantar area in the hind paw), rats showed enhancement in mechanical withdrawal threshold (von Frey test) and withdrawal duration (pinprick test), indicating that rapamycin had a beneficial effect on secondary hyperalgesia (Jiménez-Díaz et al., 2008). Furthermore, they stated that rapamycin had no effect on withdrawal latency (Hargraves test) when rapamycin and capsaicin were injected into the centre of the plantar of the rat's hind paw, signifying that rapamycin did not alter primary hyperalgesia (Jiménez-Díaz et al., 2008).

In 2009, Géranton and colleagues revealed that intrathecal administration of rapamycin 4 h before capsaicin injection in the centre area of the rat's paw, significantly enhanced the pain threshold measured by von Frey and pinprick tests (Géranton et al., 2009). Similarly, it has been reported that 6 h pre-treatment of systemic CCI-779 to C57BL/6J mice injected with capsaicin into the ankle enhanced the mechanical withdrawal threshold (von Frey test) compared with the vehicle treated group (Obara et al., 2011), indicating that inhibition of mTORC1 blocked secondary mechanical hyperalgesia (Obara et al., 2011). In contrast, CCI-779 failed to improve the mechanical withdrawal threshold (von Frey test) in C57BL/6J mice subjected to skin incision (postsurgical model), demonstrating that the inhibition of mTORC1 had no effect on primary mechanical hyperalgesia (Obara et al., 2011). Moreover, Seal and colleagues observed a change in the sensation in response to mechanical withdrawal threshold (von Frey test) in a mice model of post-surgical mediated by C-fibres (Seal et al., 2009).

All these findings support that mTORC1 mediate the local protein synthesis in A-fibres that reflects central sensitivity associated with the development and maintenance of chronic pain. Importantly, opioid receptors, synthesised in DRG neurons are expressed in the cell bodies

of sensory neurons and are transported to their central terminals in the superficial dorsal horn as well as the peripheral terminals.

Immunohistochemical studies have demonstrated that μ-opioid receptor expressed in both Aδ- and C-fibres (Besse et al., 1990; Abbadie et al., 2001). However, a recent study has shown that in mice, μ-opioid receptor expression is almost exclusively concentrated in unmyelinated fibres and a few at the dorsal root ganglion (Scherrer et al., 2009). As mentioned previously, after peripheral nerve injury, the number of μ-opioid receptors in primary afferent neurons decreased. This dynamic change in μ-opioid receptor expression is likely to have functional consequences on neuropathic pain-associated behaviours and opioid analgesia. Thus, it has been demonstrated that peripheral activation of the μ-opioid receptor provides an alternative strategy for neuropathic pain therapy with minimal central nervous system side-effects (Obara et al., 2004; Obara et al., 2007; Obara et al., 2009). However, the mechanisms by which μ-opioid receptor mRNA is down-regulated in injured DRG neurons after peripheral nerve injury are not completely understood. Nevertheless, a recent study suggested that the transcriptional repressor neuron-restrictive silence factor (NRSF), binds to the neuron-restrictive silencer element within the μ-opioid receptor gene and might be involved in nerve injury-induced downregulation of μ-opioid receptor mRNA in the DRG (Uchida et al., 2010).

In this current study, the local administration (i.pl.) of mTORC1 inhibitors demonstrated a higher morphine analgesia comparing the systemic injection of both inhibitors. It could be that the higher concentration of both inhibitors resulted in the inhibition of mTORC1-mediated local translation that might be involved in the downregulation of the μ-opioid receptor as well as regulating the function of voltage-gated channels that maintain the sensitivity of A-fibres, thus reducing spontaneous and ectopic firing from the nerve. In line with this finding, it has been documented that local administration (i.pl.) of rapamycin in to the injured hind paw enhanced the mechanical response to the pinprick test (A-fibre nociceptors stimuli) in SNI rats (Jiménez-Díaz et al., 2008). Similarly, it reported (i.pl.) administration of CCI-779 attenuated mechanical and cold hypersensitivity in the SNI mice (Obara et al., 2011), which suggested that inhibition of mTORC1 with rapamycin or CCI-779 locally decreased the mechanical hypersensitivity in neuropathic pain by reducing the sensitivity of A-fibres (Jiménez-Díaz et al., 2008; Obara et al., 2011).

The finding obtained by this study throws new light on the beneficial effect of mTORC1 inhibitors on opioid analgesia in neuropathic pain, as mTORC1-inhibition may compensate for the loss of morphine analgesia *via* translational changes and nociceptor regulation.

Moreover, the local injection of metformin or CCI-779 suggested that the involvement of peripheral mechanisms targeting mTORC1 led to the loss of fibre sensitivity, which in turn improved the analgesic effect. Likewise, local use of drugs has been proven to reduce the risk of side-effects, therefore offering a new strategy for prolonged opioid treatment with fewer side-effects. As a result of these studies, it appears that inhibiting mTORC1 activity during opioid administration has additive analgesic benefits. However, the influence of both mTORC1 inhibitors in the motivational and the reinforcing effects of morphine must be addressed. In the following chapter (Chapter 8), conditioned place preference (CPP) paradigm, which is a standard preclinical behavioural model was used to study the rewarding and aversive effects of drugs.

# Chapter 8. The effect of the mammalian target of rapamycin complex 1 (mTORC1) inhibitors on morphine-induced conditioned place preference (CPP) in *naïve* mice

## 8.1 Introduction

Repeated exposure to opioids like morphine and other abused drugs leads to an enduring neuroadaptations within a neural network containing dopaminergic, GABAergic and glutamatergic projections innervating different areas in the brain, such as the ventral tegmental area (VTA), nucleus accumbens (NAC), prefrontal cortex, dorsal striatum, amygdala and hippocampus (Nestler, 2005; Hyman et al., 2006; Bailey et al., 2012). From a molecular perspective, morphine-induced neuroplasticity consist of changes in protein expression that can regulate the important properties of neuron physiology such as transcriptional activation, mRNA translation or mRNA/protein stability and degradation (Ammon-Treiber and Höllt, 2005; Luo et al., 2011; Cai et al., 2016).

mTORC1 is a major regulator of protein synthesis and cell growth, which integrates different environmental signals implicated to cell survival; presence of growth factors, nutrient availability, cellular energy levels and hypoxic or genotoxic stress (Tang et al., 2002). Based on the ability of mTORC1 to modulate translation and its presence at post-synaptic sites (Tang et al., 2002), multiple studies have demonstrated the involvement of mTORC1 in memory formation in the hippocampus and suppression between glutamate and dopamine neurons in the ventral tegmental area (Mameli et al., 2007; Slipczuk et al., 2009; Li et al., 2010). Importantly, these areas or synapses are notably remodelled by morphine and other drugs of abuse (Niehaus et al., 2010). While there are limited number of studies on the effects of mTORC1 inhibitors upon behaviour, it is reported that rapamycin treatment exerts antidepressant-like activity in mice (Cleary et al., 2008) and blocks the sensitisation of a methamphetamine-induced conditioned place preference in rats (Narita et al., 2005). Besides, rapamycin significantly reduces cue-induced drug craving in abstinent human heroin addicts (Shi et al., 2009). Together, these data suggest that mTORC1 inhibitors may well serve as a potential pharmacotherapeutic for treating motivational and emotional dysfunction associated with reward system and the mTORC1 signalling pathway may be related to addiction processes in both humans and laboratory animals.

The present study tested the hypothesis that mTORC1 mediates the development and/or expression of morphine-induced reward by assessing the effects of metformin and CCI-779 pre-treatment within a conditioned place-preference (CPP) paradigm that also permitted measurement of sensitization of morphine-induced locomotor activation. The CPP paradigm is a standard pre-clinical conditioning model used to study drug reward mechanisms in rodents. Briefly, the task involves the association of an environment (a specific compartment, compromising visual and tactile stimuli) with a drug treatment (morphine and mTORC1inhibitors). A rewarding effect of the drug treatment is indicated when the mouse spends more time in the drug-paired compartment than in the vehicle paired compartment. The CPP procedure in this present study examined a wide range of doses used in various rodent CPP studies. Table 8.1 summarizes findings with morphine across a range of doses that were shown to condition a significant place preference.

**Table 8.1: Summery of several studies across a range of doses of morphine induced conditioned place preference.**

| Animal | CPP apparatus | Dose of morphine producing CPP | References |
|---|---|---|---|
| Male C57BL/6J and DBA/2J mice | Two square-base compartments | 2.5, 5 or 10 mg/kg, i.p. | *Cunningham et al., 1992* |
| Females Sprague–Dawley rats | Three-chamber design | 2.5, or 5 mg/kg, i.p. | *Campbell et al., 2000* |
| Male NMRI mice | Three-chamber design | 2.5and 10 mg/kg, i.p. | *Zarrindast et al., 2002* |
| Male OF1 strain mice | Three-chamber design | 5, 10, 20 and 40 mg/kg, i.p. | *Do Couto et al., 2003* |
| Female NMRI mice | Two square-base compartments | 5 and 10 mg/kg, s.c. | *Zarrindast et al., 2003* |
| Male C57BL/6J mice | Two square-base compartments | 0.32, 1, 3.2, or 10 mg/kg, i.p. | *Koek et al., 2016* |

A summary of studies displacing with models of morphine induced conditioned place preference in *naïve* rodents. This table was a basic to determine the experiment design and dose selection in the current study.

## 8.2 Material and method

### 8.2.1 Subject

Adult male C57BL/6J mice (8 weeks of age, Charles River, UK) weighing 25-30 g at the beginning of the study were used. Mice were housed in groups of 4 per polyethylene cage containing sawdust bedding according to standard welfare conditions (12 h light/dark cycle, lights on from 8:00 am) with food and water available ad libitum. Experimental protocols were approved by the AWERB Committee at Durham and Newcastle University and were consistent with the guidelines provided by the UK Animals (Scientific Procedures) Act 1986. For more details see Chapter 2.

### 8.2.2 Preparation and administration of drugs

***Morphine***

To determine a dose response curve for morphine-induced CPP (morphine sulphate salt pentahydrate; Sigma-Aldrich, UK) was dissolved in saline (0.9% NaCl; Fresenius Kabi Ltd., UK) immediately prior to injecion. Mice were weighed and injected intraperitoneally (i.p.) with morphine (0.3, 1, 3, 10 or 20 mg/kg body weight) or equivalent vehicle solution (saline) without morphine as a control group in a volume of 4 ml per kg body weight. The timing and concentration of morphine injections were based on previously published research (Koek, 2016).

***Metformin***

For systemic (i.p.) administration, metformin (metformin hydrochloride; Cat. No. 2864; Tocris Bioscience, UK) was prepared in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK) immediately prior injections. Mice were weighed and then injected i.p. with metformin (200 mg per kg body weight) or equivalent vehicle (saline) solution without metformin as a control group. Metformin/vehicle was administered i.p. once 20 h before i.p. injection of morphine was given. The timing and concentration of metformin injections were based on previously published literature on metformin (Melemedjian et al., 2011; Obara et al., 2015).

***CCI-779***

For systemic (i.p.) administration, CCI-779 (temsirolimus; Cat. No. T-8040; LC Laboratories, USA) was prepared in pure ethanol as a stock solution at 60mg/mL on the day of experiment and diluted to 2.5 mg/mL in 0.15M NaCl, 5% polyethylene glycol 400, 5% Tween 20 (Ravikumar et al., 2004: Obara et al., 2015) immediately before injection. Mice were weighed and then injected intraperitoneally (i.p.) with a 1% v/w solution of CCI-779 (25 mg per kg body weight) or equivalent vehicle (0.15M NaCl, 5% polyethylene glycol 400, 5% Tween 20) solution without CCI-779 as a control group. CCI-779/vehicle was injected 20 h before i.p. injection of morphine was given. The timing and concentration of CCI-779 injections were based on previously published research using CCI-779 (Obara et al., 2011; Obara et al., 2015).

### 8.2.3 CPP Apparatus

All CPP experiments were carried out using six identical CPP boxes purchased from a commercial supplier (CPP Apparatus; Model CPP-3013AT Med Associates, St Albans, VT, USA). Each box consisted of three compartments: two equally sized large conditioning

compartments (interior dimensions; 12.7 cm L × 16.8 cm W× 12.7 cm H) separated by a smaller, neutral compartment (12.7 cm L ×7.2 cm W × 12.7 cm H). The two conditioning compartments had different visual and tactile cues: one conditioning chamber had black walls and a stainless steel grid floor (Grid floor; product # ENV-3013BR, Med Associates, St Albans, VT, USA) and the other conditioning chamber had white walls and a floor of stainless mesh (Mesh floor; product # ENV-3013WM, Med Associates, St Albans, VT, USA). The middle compartment had grey walls and a floor made from solid plastic, an automatically controlled guillotine doors separated each conditioning compartment from the centre compartment that opened simultaneously to both the black and white compartments. The CPP apparatus was equipped with three individual light bulbs on the top door of each compartment. The intensity of ambient illumination was adjusted to provide the same intensity of light (maximum level; 100% light intensity). Automated data collection was accomplished through infra-red photo-beam detectors (sixteen infra-red photo-beam detectors were located 2.45 cm above the chamber floor) which identified the location of the mouse within the three compartments. The photo-beams were connected to a computer interface, and MED-PC IV behavioural software (MED Associates, Inc.), where an appropriate program was selected for each phase of the experiment.

As mentioned in Chapter 2, place conditioning involved three phases: a preconditioning baseline preference test (habituation), eight place conditioning trials, and a final place preference test conducted after varying periods of morphine abstinence. During habituation and testing phase, the infrared beam detectors were used to detect the time the mouse spent by (s) in the three compartments. During the conditioning phase, the infrared beam detectors measured the general ambulatory activity of the mouse in each of the conditioning compartments.

All experiments were performed in a sound attenuated and dimly lit room. Between tests, the compartments were cleaned with colourless, odourless and tasteless disinfectant (EndoSan; PCS No.97819, Clinipath Ltd, Hull, UK).

### 8.2.4 Experimental design

To establish the extent to which mTORC1 inhibitors modified the acquisition of morphine-induced CPP in *naïve* mice, two separate experiments were performed. The first experiment tested the effects of 20 h pre-treatment with metformin before each morphine conditioning

session, whereas the second experiment tested the effects of a 20 h pre-treatment with CCI-779 before each morphine conditioning session.

### 8.2.4.1 Design of experiment I

As depicted in Table 8.1, 135 experimentally *naïve* mice were randomly assigned into two separate pre-treatment groups: group 1, which included 6 subgroups received a 20 h pre-treatment with metformin (200 mg/kg, i.p.) before each conditioning day, while group 2, also containing 6 subgroups received an equivalent vehicle solution without the mTORC1 inhibitors. Both groups were conditioned with four morphine and four vehicles (0.9% saline, 4ml/kg volume of injection) sessions. The experiment examined five graded doses of morphine (0.3, 1, 3, 10 and 20mg/kg, i.p.) or vehicle. By way of summary, Figure 8.1 represents the timeline and a schematic diagram of the experimental design.

**Table 8.1: Design of experiment 1.**

| Group | Subgroup | Morphine doses | | | | | Saline (i.p.) | 20 h prior conditioning day | | Number of mice in each group |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.3 mg/kg (i.p.) | 1 mg/kg (i.p.) | 3 mg/kg (i.p.) | 10 mg/kg (i.p.) | 20 mg/kg (i.p.) | | metformin 200 mg/kg (i.p.) | Vehicle (i.p.) | |
| A | 1 | + | | | | | | + | | 8 |
| | 2 | | + | | | | | + | | 16 |
| | 3 | | | + | | | | + | | 7 |
| | 4 | | | | + | | | + | | 8 |
| | 5 | | | | | + | | + | | 8 |
| | 6 | | | | | | + | + | | 8 |
| B | 1 | + | | | | | | | + | 8 |
| | 2 | | + | | | | | | + | 16 |
| | 3 | | | + | | | | | + | 16 |
| | 4 | | | | + | | | | + | 16 |
| | 5 | | | | | + | | | + | 16 |
| | 6 | | | | | | + | | + | 8 |

*Naïve* mice were randomly assigned into two groups and received different treatment: in group A (1, 2, 3, 4 and 5) mice pre-treated with metformin in the afternoons (given between 1:00 pm and 2:00 pm), then 20 h after mice received one injections of particular dose of morphine (0.3, 1, 3, 10, 20 mg/kg);(6) control, mice pre-treated with metformin in the afternoons, then 20 h after mice received one injection of saline. In group B (1, 2, 3, 4 and 5) mice pre-treated with vehicle in the afternoons, then 20 h after mice received one injections of particular dose of morphine (0.3, 1, 3, 10, 20 mg/kg); (6) mice were pre-treated with vehicle in the afternoons (given between 1:00 pm and 2:00 pm), then 20 h after mice received one injection of saline.

### 8.2.4.2 Design of experiment II

As depicted in Table 8.2, 111 *naïve* mice were randomly assigned into two separate pre-treatment groups: group 1, which included 6 subgroups received a 20 h pre-treatment with CCI-779 (25 mg/kg, i.p.) before each conditioning day, while group 2, also the 6 subgroups received an equivalent vehicle solution without the inhibitors. Both groups were conditioned with four morphine and four vehicles (0.9% saline) sessions in a volume of 4 ml/kg. The

experiment examined five graded doses of morphine (0.3, 1, 3, 10 and 20mg/kg, i.p.) or vehicle. By way of summary, Figure 8.1 represents the timeline and a schematic diagram of the experimental design.

**Table 8.2: Design of experiment II.**

| Group | Subgroup | Morphine doses | | | | | Saline (i.p.) | 20 h prior conditioning day | | Number of mice in each group |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.3 mg/kg (i.p.) | 1 mg/kg (i.p.) | 3 mg/kg (i.p.) | 10 mg/kg (i.p.) | 20 mg/kg (i.p.) | | CCI-779 25 mg/kg (i.p.) | Vehicle (i.p.) | |
| **A** | 1 | + | | | | | | + | | 8 |
| | 2 | | + | | | | | + | | 16 |
| | 3 | | | + | | | | + | | 8 |
| | 4 | | | | + | | | + | | 8 |
| | 5 | | | | | + | | + | | 8 |
| | 6 | | | | | | + | + | | 8 |
| **B** | 1 | + | | | | | | | + | 8 |
| | 2 | | + | | | | | | + | 16 |
| | 3 | | | + | | | | | + | 8 |
| | 4 | | | | + | | | | + | 8 |
| | 5 | | | | | + | | | + | 7 |
| | 6 | | | | | | + | | + | 8 |

*Naïve* mice were randomly assigned into two groups and received different treatment: in group A (1, 2, 3, 4 and 5) mice pre-treated with CCI-779 in the afternoons (given between 1:00 pm and 2:00 pm), then 20 h after mice received one injections of particular dose of morphine (0.3, 1, 3, 10, 20 mg/kg);(6) control, mice pre-treated with CCI-779 in the afternoons, then 20 h after mice received one injection of saline. In group B (1, 2, 3, 4 and 5) mice pre-treated with vehicle in the afternoons, then 20 h after mice received one injections of particular dose of morphine (0.3, 1, 3, 10, 20 mg/kg); (6) mice were pre-treated with vehicle in the afternoons (given between 1:00 pm and 2:00 pm), then 20 h after mice received one injection of saline.

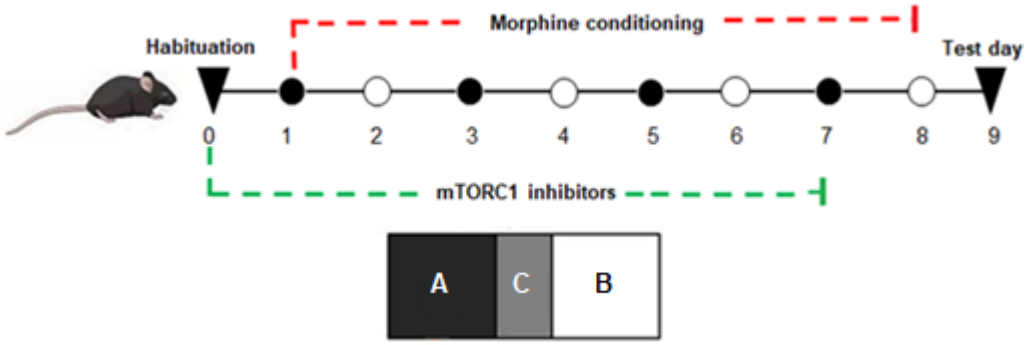


**Figure 8.1: A schematic diagram summering the experimental timeline of the CPP test.** All experiments took place in a dimly lit room. Habituation phase (Day-0), mice were placed in the center chamber (box C) with the guillotine doors raised and allowed to freely roam the apparatus for 15 min. The time spent in each chamber was recorded. Following habituation, mTORC1 inhibitors: metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.), was administered 20 h before each conditioning day with (0.3, 1, 3, 10 and 20 mg/kg). Morphine CPP conditioning sessions (Days 1-8), consisted of alternating sessions of morphine and saline injections with the guillotine doors in place and mouse confined in the conditioned chamber for 45 min. Place preference test (Day-9), mice were placed in the center chamber (box C) with the guillotine doors raised and allowed to freely roam the apparatus for 15 min. The time spent in each chamber was recorded. The red dashed line represents morphine CPP conditioning sessions. The green dashed line represents metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) administration.

### 8.2.5 Statistical data analysis

All analyses and statistical comparisons were performed using GraphPad Prism, version 8.01 for Windows, (GraphPad Software, San Diego, CA), except repeated measures ANOVA, which were conducted using IBM SPSS Statistics V23.0 software. If a significant F-value was obtained, post hoc analyses (Bonferroni's multiple comparison tests) were performed. Also, Student's t-tests were used when date between two groups were compared. A value of $P<0.05$ was considered to be statistically significant, P values are expressed in relation to comparisons to the vehicle pre-treated condition. Behavioural results are presented in the graphs as mean ± SEM.

Morphine-induced CPP, measured as the percentage time spent on the morphine-paired compartment, compared with the saline-paired (i.e., the percentage time spent excludes the time spent in the neutral unpaired central chamber) and was analysed by a two-way ANOVA with morphine dose (0-20 mg/kg) and (mTORC inhibitor) as between-subjects factors.

The percentage time spent in the drug-paired compartment was calculated using the formula:

$$(\%\ \text{time in drug-paired} = \frac{\text{time (s)in drug}-\text{paired}}{\text{time (s) in drug}-\text{paired} + \text{time (s) in vehicle}-\text{paired}} \text{ x } 100)$$

The results of locomotor activity during the conditioning trials were expressed as the percentage of activity in the drug-paired compartment, and was analysed using a two-way ANOVA with repeated measures.

The percentage of activity in the drug-paired compartment was calculated using the formula:

$$(\%\ \text{locomotor activity} = \frac{\text{activity in drug-paired compartment}}{\text{activity in vehicle-paired compartment}} \times 100)$$

Body weight during the conditioning trials was analysed using a two-way ANOVA with repeated measures.

## 8.3 Result

### 8.3.1 Habituation phase (initial preference)

During the habituation phase (900 s), the mice were allowed to explore the novel compartments within the CPP box. The mice spent equal amounts of time in the black and white compartments as shown in Figure 8.2. In general, statistical analyses revealed that mice did not show an unconditioned bias for either of the two compartments (315.62 ± 5.99 *vs.* 323.82 ± 7.20, respectively), which supported the unbiased method ($t_{(245)}$= 1.33, P= 0.18 n.s). Since there was no statistical significant difference between times spent in the two compartments, a non-biased fully counterbalanced design was employed throughout the CPP study.

### 8.3.2 The effect of mTORC1 inhibitors on morphine-induced motivational behaviour in *naïve* mice

#### 8.3.2.1 Metformin

***The effect of metformin on morphine-induced place preference***

Following four conditioning sessions with morphine and vehicle, the preference test allowed mice a free choice between the drug-paired and vehicle-paired compartment, where the absolute time spent in each compartment is presented in Figure 8.3, A. The statistical analysis regarding all groups injected with increasing doses of morphine yielded a significant main effect of morphine treatment ($F_{(1,123)}$= 35.1, P<0.0001). Bonferroni's multiple comparison tests revealed that doses of 1-20 mg/kg of morphine induced significant CPPs in comparison with the saline-treated control group. However, groups conditioned with saline or morphine in 0.3 mg/kg dose failed to produce significant CPP in mice.

Further analysis revealed no significant difference in morphine-induced CPPs across the various doses tested (1,3,10 and 20 mg/kg) in the total time spent either in the drug-paired or the vehicle-paired compartments (morphine dose: $F_{(3,63)}$= 1.68 , P= 0.18 n.s. ; morphine dose: $F_{(3,63)}$= 1.17, P= 0.33, n.s. respectively). Similarly, statistical analysis revealed no significant effect of morphine-induced CPP dose on the percentage measure of time spent in the drug-paired or in the vehicle-paired compartments on the test day by the mice (morphine dose: $F_{(3,63)}$= 1.22, P= 0.31 n.s.; morphine dose: $F_{(3,63)}$= 1.17, P= 0.33 n.s respectively). Both of these observations indicated that there was no difference between morphine doses (1, 3, 10 and 20 mg/kg) in the magnitude of the CPPs.

Pre-treatment with metformin prior to the eight conditioning sessions failed to modify the rewarding effects of morphine. Figure 8.3, B showed lack of effect of metformin pre-treatment on the development of morphine-induced CPPs. Statistical analysis of the absolute time and the calculated percentage time spent in the morphine-paired compartment failed to reveal an overall significant effect of metformin pre-treatment (treatment effect: $F_{(1,123)}$= 0.04, P= 0.84, n.s.; treatment effect: $F_{(1,123)}$= 0.11, P= 0.73, n.s, respectively). Pre-treatment of mice with metformin did not shift the morphine dose-response curve as shown in Figure 8.4. also, there was no significant interaction between metformin pre-treatment and morphine dose (interaction: $F_{(5,123)}$= 0.15, P= 0.97 n.s).

***The effect of metformin on morphine-induced locomotor activity***

Locomotor activity was measured over the eight morphine conditioning sessions, Figure 8.5 illustrates the locomotor activity of mice during the four conditioning trails from all groups of mice conditioned with different doses of morphine (0.3, 1, 3, 10, 20 mg/kg; i.p.) in the presence and absence of metformin pre-treatment (200 mg/kg; i.p.). Statistical analysis indicated that morphine increased locomotor activity (trial: $F_{(3,369)}$= 3.42, P<0.05). Interestingly a post hoc test confirmed a significant stimulatory effect of morphine at higher doses, indicating that morphine produced a dose-dependent increases over repeated conditioning sessions, effects were more prominent with doses of 10 and 20 mg/kg morphine while responses to 0.3, 1 and 3mg/kg morphine were not significant.

The locomotor response to all the doses of morphine tested was greater than the saline-treated condition in mice. In addition, statistical analysis revealed a significant interaction between morphine dose and conditioning trial (trial x morphine dose: $F_{(15,369)}$= 2.93,

P<0.001) suggesting that certain doses of morphine were producing stimulatory effects on locomotor activity with repeated conditioning trials.

There were no significant differences in the levels of morphine-induced locomotor activity of mice treated with vehicle or metformin prior to the eight conditioning sessions (treatment effect: $F_{(3,369)}$= 2.92, P= 0.87 n.s). This suggested that metformin pre-treatment had no effect on modulating morphine-induced stimulatory effects on locomotor activity over the four morphine conditioning trials.

#### 8.3.2.2 CCI-779

***The effect of CCI-779 on morphine-induced place preference***

As illustrated in Figure 8.6 A, mice showed significant CPP with graded doses of morphine as evidenced from the increase in time spent in the drug paired compartment compared with time in the vehicle-paired compartment (treatment effect: $F_{(1,100)}$= 42.37, P<0.0001). Significant morphine-induced CPPs were observed with 1, 3, 10 and 20 mg/kg doses of morphine (P<0.05, Bonferroni's post-hoc test). Pre-treatent with CCI-779 failed to modify morphine-induced CPPs on the time spent measure (treatment effect: $F_{(1,98)}$= 13.62, P= 0.0004; Figure 8.6 B), with post-hoc tests confirming significant CPPs at (1mg/kg).

Similarly, no effects of CCI-779 were observed when the percentage time spent in the morphine-paired compartment was analysed (treatment effect: $F_{(1,99)}$= 1.37, P= 0.24, n.s; Figure 8.7 A) and in the absolut time (treatment effect: $F_{(1,99)}$= 0.90, P=0.34 n.s; Figure 8.7 B). Therefore, there was no obvious shift of the morphine dose response curve by CCI-779 pre-treatment. Moreover, further analysis revealed there was no significant interaction between CCI-779 pre-treatment and morphine dose (interaction: $F_{(5,99)}$= 0.94, P= 0.24, n.s).

***The effect of CCI-779 on morphine-induced locomotor activity***

Locomotor activity measured over the four morphine and vehicle conditioning trials is illustrated in Figure 8.8. Statical analysis revaled increased in locomotor activity over trials, as (trial: $F_{(3,291)}$= 13.43, P<0.0001). Both pre-treated groups showed dose-dependent increases to morphine on locomotor activity which increased over the conditioning trials (trail x morphine dose: $F_{(15,291)}$= 1.72, P<0.05). Further analysis revealed that, the locomotor stimulatory effect of morphine at doses of 10 and 20 mg/kg were significantly greater than the responses to 0.3, 1 or 3 mg/kg doses of morphine (P<0.05, Bonferroni's post-hoc test). An analysis of differences in morphine-induced locomotor activity between the two pre-treatment groups did not reveal any significant effect of CCI-779 pre-treatment upon the

locomotor activity across the 4 conditioning trials (treatment effect: $F_{(3,291)}$= 0.80, P= 0.97 n.s).

### 8.3.3 The mammalian target of rapamycin complex 1 (mTORC1) inhibitors and morphine induced body weight loss in *naïve* mice.

As illustrated in Figure 8.9, the body weight of mice from all the groups was unaffected by treatment. Statistical analysis revealed no significant effect of conditioning with morphine doses (0.3-20 mg/kg) on body weight over the 8 sessions compared with saline-treated conditioned animals (treatment effect: $F_{(5,1300)}$= 8.30, P>0.05). Also, no significant effect was observed on body weight in the metformin or CCI-779 pre-treated groups of mice (treatment effect: $F_{(6,567)}$= 15.78, P>0.05; treatment effect: $F_{(6,563)}$= 30.63, P>0.05, respectively) over the 8 days of conditioning with morphine.

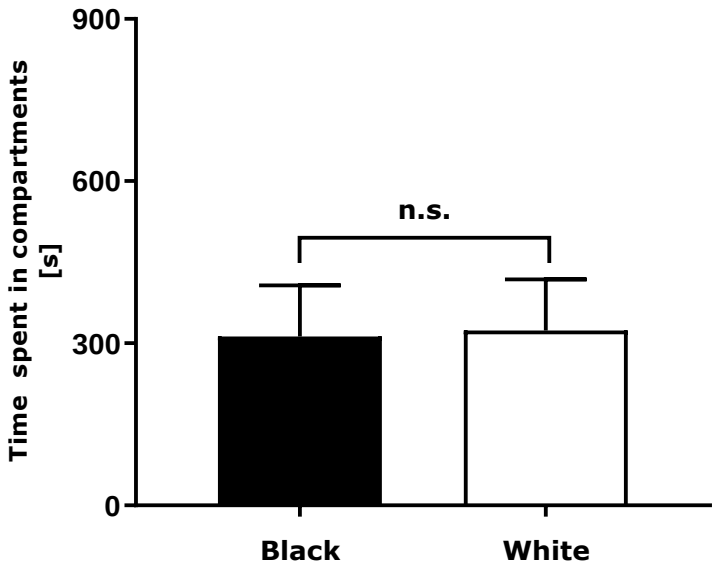


**Figure 8.2: Baseline preference in *naïve* mice**. Bar graphs displaying an average time in (sec) by *naïve* mice in the white or black compartment of the CPP apparatus during 900 sec of the initial preference test (habituation). Data are presented as mean ± SEM values, n= 246 mice per group, *P<0.05 (paired Student's t- test).

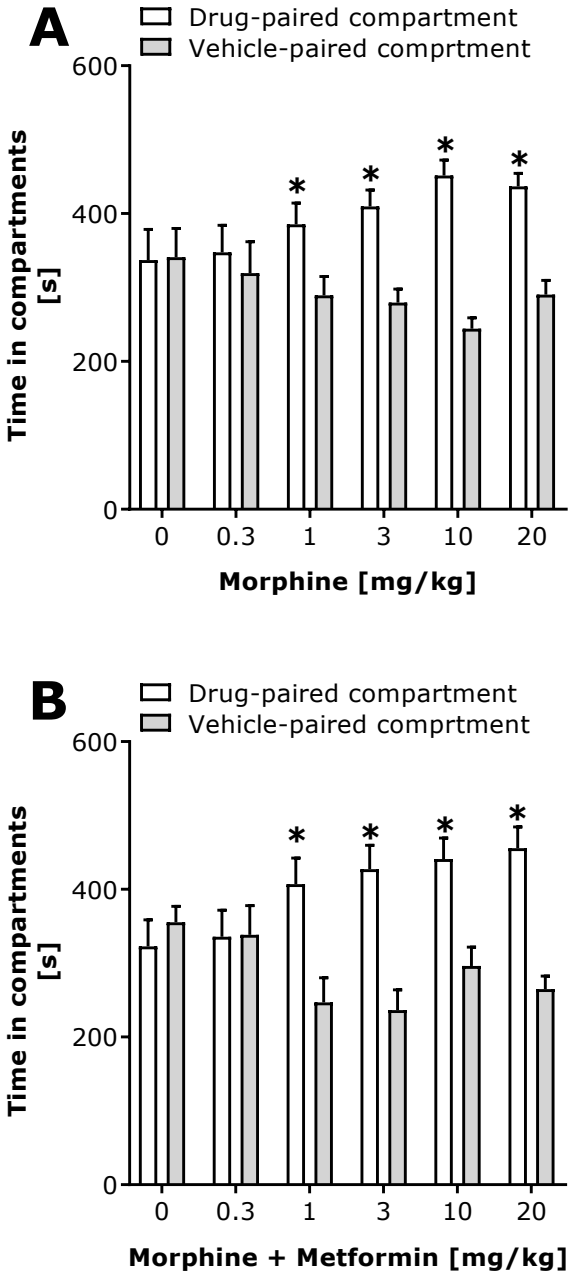


**Figure 8.3: Bar chart showing mean average time spent by *naïve* mice in the drug- and vehicle-paired compartments following place preference test. (A)** Effect of different doses on the development of morphine-induced CPP. Following conditioning with (0.3, 1, 3,10, 20 mg/kg; i.p), mice spent a greater amount of time in the morphine-paired chamber than in the saline-paired chamber.**(B)** Mice were conditioned with five doses of morphine (0.3, 1, 3,10, 20 mg/kg; i.p.) and pre-treated with metformin (200 mg/kg, i.p). Repeated systemic administration of metformin (200 mg/kg, i.p.) 20 h before first morphine/saline injection and then subsequently for 7 days once daily after morning morphine/saline injection (total of 4 conditioning sessions with morphine and 4 with saline) produced similar effect and did not modify morphine-induced conditioning. The data are presented as mean number of seconds ± SEM, n= 7-16 mice per group *P<0.05; indicates significant different from the saline-paired side effect (two-way ANOVA followed by Bonferroni's test t).

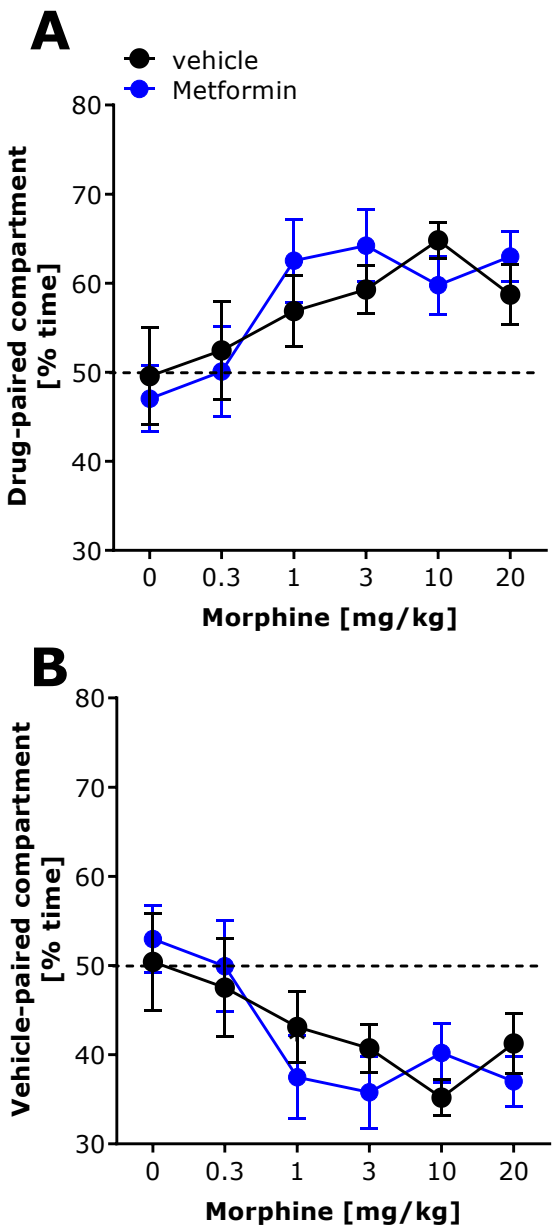


**Figure 8.4: Metformin did not affect the rewarding effects of morphine in *naïve* mice**. Summary of the % time spent between the morphine-paired **(A)** and saline-paired **(B)** compartment in the 15 min test for place-conditioning conducted following conditioning with 0.3, 1, 3, 10 and 20 mg/kg (i.p.) of morphine. Morphine produced dose-dependent increase in time spent in the morphine-paired compartment Repeated systemic administration of metformin (200 mg/kg, i.p.) 20 h before first morphine/saline injection and treated subsequently for 7 days once daily after morning morphine/saline injection (total of 4 conditioning sessions with morphine and 4 with saline) produced similar effect and did not potentiated morphine-induced conditioning. The data are presented as % time ± SEM, n= 7-16 mice per group (two-way ANOVA, followed by Bonferroni's test). Dotted line at 50% is used to represent positive CPP. The placed preference is considered to be achieved if the points are significantly different from the 50% baseline.

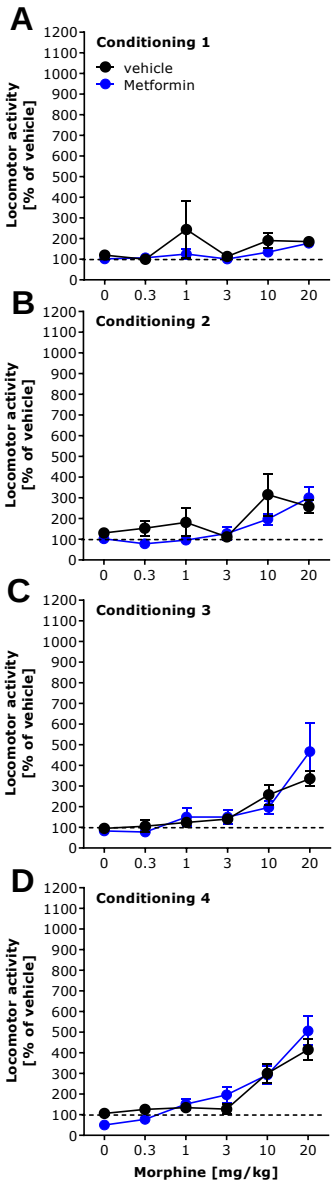


**Figure 8.5: Effect of metformin on morphine-induced locomotor activity during the four conditioning trials.** Locomotor activity after an i.p. injection of morphine or vehicle in *naïve* mice during the first morphine place conditioning trial (A) and during the subsequent three morphine conditioning trials (B, C and D) for doses 0.3-20mg/kg. Locomotor activity increased as increasing doses of morphine were tested but there was no effect of metformin pre-treatment on morphine-induced locomotor activity. Results with locomotor activity are shown as mean percentage of locomotor activity measured in the drug-paired compartment relative to the activity in the vehicle-paired compartment (± SEM), n= 7-16 mice per group (two-way ANOVA, followed by Bonferroni's test). The dotted line at 100% is used to represent the percentage of the stimulatory effect of morphine, where data points above indicate a stimulatory effect of morphine (locomotor activity in the drug-paired compartment is higher than in the vehicle-paired compartment) and data points below indicate a depressant effect (locomotor activity in the vehicle-paired compartment is higher than in the drug paired compartment).

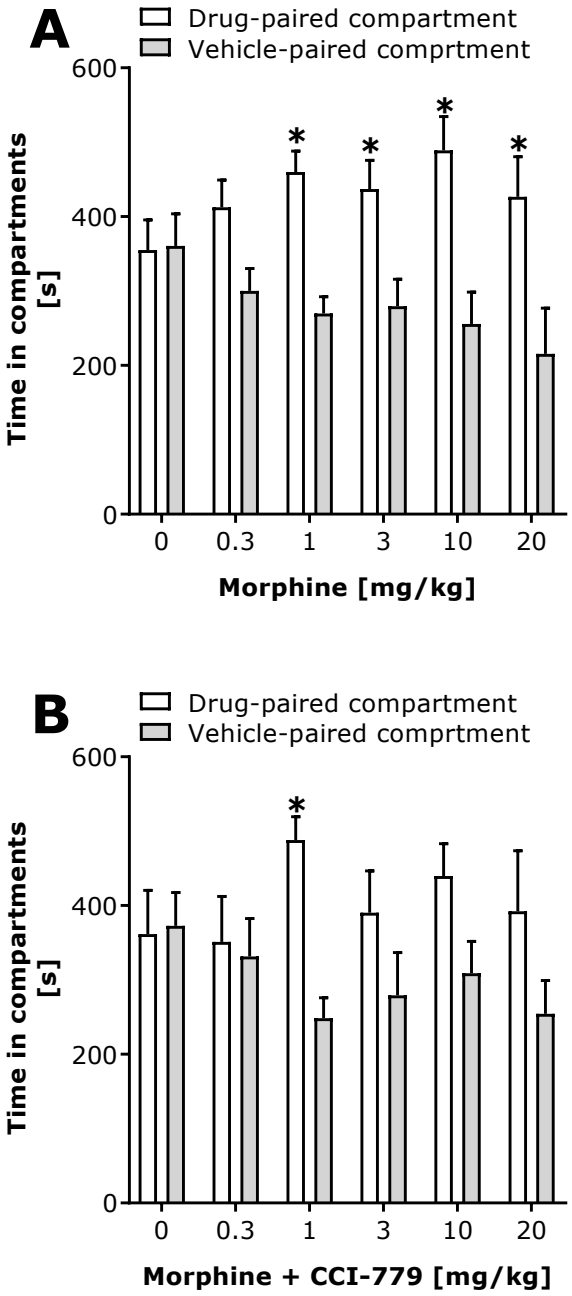


**Figure 8.6: Bar chart to show the mean average time spent by mice in the drug- and vehicle-paired compartments following conditioning with graded doses of morphine. (A)** Effect of different doses on the expression of morphine-induced CPP. Following conditioning with morphine (0.3, 1, 3,10, 20 mg/kg; i.p), mice spent more time in the morphine-paired chamber than in the saline-paired chamber.**(B)** Mice were conditioned with five doses of morphine (0.3, 1, 3,10, 20 mg/kg; i.p.) and pre-treated with CCI-779 (25 mg/kg, i.p). Repeated systemic administration of CCI-779 (25 mg/kg, i.p) 20 h before first morphine/saline injection and then subsequently for 7 days once daily after morning morphine/saline injection (total of 4 conditioning sessions with morphine and 4 with saline) produced similar effect and did not modify morphine-induced conditioning. The data are presented as mean number of seconds ± SEM, n= 7-16 mice per group. *P<0.05 indicates significant different from the saline-paired side effect (two-way ANOVA followed by Bonferroni's test).

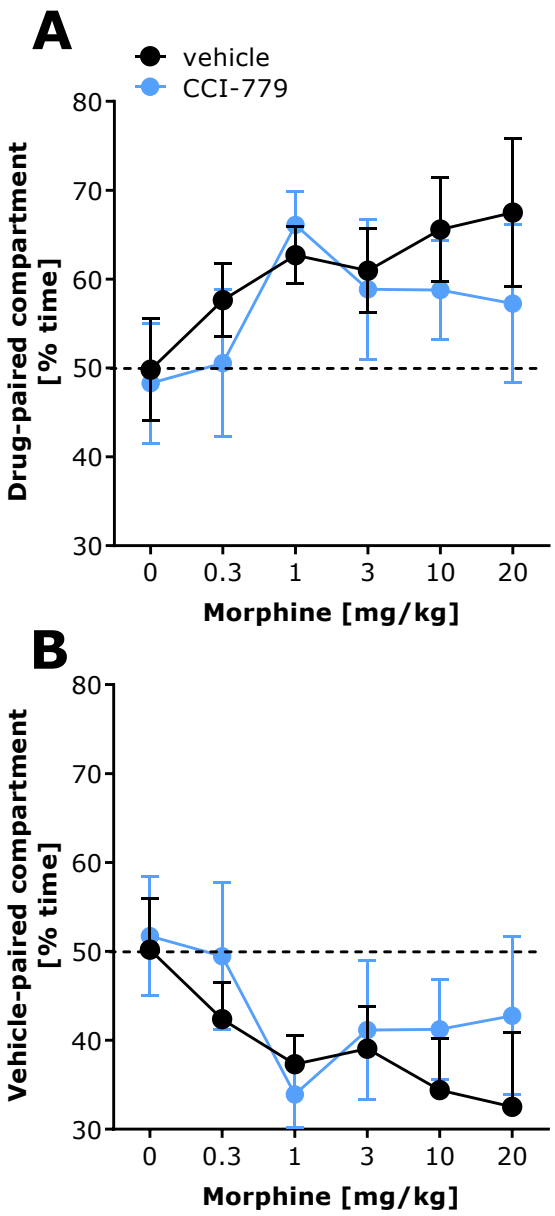


**Figure 8.7: CCI-779 did not modify the rewarding effects of morphine in mice**. Summary of the % time spent between the morphine-paired **(A)** and -unpaired **(B)** compartment in the 15 min test for place-conditioning conducted, following conditioning with 0.3, 1, 3, 10 and 20 mg/kg (i.p.) of morphine. Morphine produced dose-dependent increases in time spent in the morphine-paired compartment. Repeated systemic administration of CCI-779 (25 mg/kg, i.p.) 20 h before first morphine/saline injection and then subsequently for 7 days once daily after morning morphine/saline injection (total of 4 conditioning sessions with morphine and 4 sessions with saline) produced similar effect and did not potentiated morphine-induced conditioning. The data are presented as % time ± SEM, n= 7-16 mice per group (two-way ANOVA, followed by Bonferroni's test).

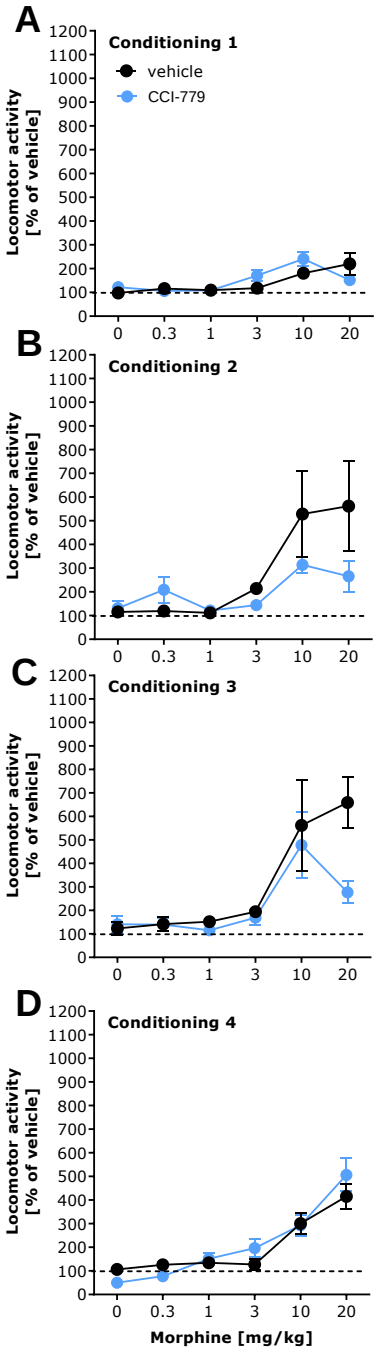


**Figure 8.8: Effect of CCI-779 on morphine-induced locomotor activity during the four conditioning trials.** Locomotor activity after an i.p. injection of morphine or vehicle in *naïve* mice during the first morphine place conditioning trial (A) and during the subsequent three morphine conditioning trials (B, C and D) for doses 0.3-20mg/kg. Locomotor activity increased as increasing doses of morphine were tested but there was no effect of cci-779 pre-treatment on morphine-induced locomotor activity. Results with locomotor activity are shown as mean percentage of locomotor activity measured in the drug-paired compartment relative to the activity in the vehicle-paired compartment (± SEM), n= 7-16 mice per group (two-way ANOVA, followed by Bonferroni's test). The dotted line at 100% is used to represent the percentage of the stimulatory effect of morphine, where data points above indicate a stimulatory effect of morphine (locomotor activity in the drug-paired compartment is higher than in the vehicle-paired compartment) and data points below indicate a depressant effect (locomotor activity in the vehicle-paired compartment is higher than in the drug paired compartment).

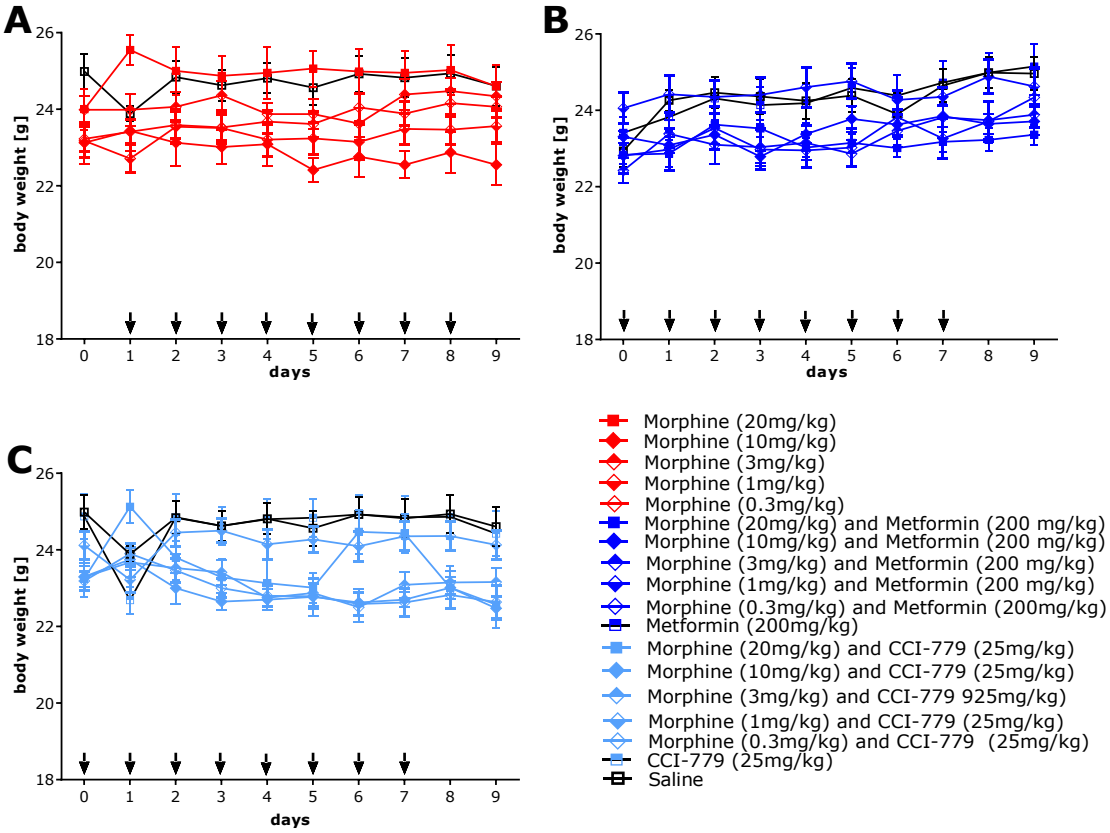


**Figure 8.9: Effect of conditioning with morphine (A) and pretreatment with the mTORC1 inhibitors, metformin (B) and CCI-779 (C) on mean body weight of mice.** Administration of morphine (0.03-20 mg/kg, i.p.) on alternate days or repeated administration mTORC1 inhibitors once daily for 8 consecutive had no effect on body weight compared with saline injected animals. The data are presented as the mean body weight ± SEM, n= 7-32 mice per group; **vs.* saline; *P<0.05 (two-way ANOVA, followed by Bonferroni's test). Arrows in graph (A) indicates conditioning with morphine, while arrows in graphs (B) and (C) indicates metformin/CCI-779 injection time.

## 8.4 Discussion

In this chapter, it was demonstrated that the systemic administration of mTORC1 inhibitors, metformin and CCI-779 had no effect on the motivational and reinforcing properties of morphine by demonstrating the influence of the 20 h pre-treatment of mTORC1 inhibitors on the acquisition of morphine-conditioned place-preference and the expression of morphine-induced locomotor sensitization in *naïve* mice. Thus, inhibition of the mTORC1 pathway may offer a novel approach towards the improvement of opioid therapy in humans, particularly when prolonged opioid efficacy is required.

During the habituation session, the mice were allowed to explore all three compartments of the CPP apparatus. Subsequently, as the mouse becomes familiar with the surrounding environment, it may choose to spend more time in one chamber, in preference to another. The tendency of the mouse to elect a particular chamber can be explained based on the ethological perspective that mice like dark places and tend to avoid bright places (Costall et al., 1989). In this study the natural affinity of mice to spend time in the chamber with the black walls is deterred by the addition of light. Consequently, this creates apparatus with an environment in which the mouse spends equal amounts of time in each chamber, as presented in the result section, since the three compartments (black, white and grey) comprised similar brightness, so that the influence of light was excluded. Moreover, data obtained from the habitation test showed that mice spent more time in the white or black compartments than the grey one. This behavioural observation can be explained by the fact that the lateral compartments (black, white) are bigger than the centre compartment (grey). This observation is in agreement with Orsini and colleagues, who reported that C57BL/6J mice spent less time in the central compartment (small), while the DBA mice spent equal time in all the compartments (Orsini et al., 2005), given that their experiment was conducted in CPP apparatus consisting of three compartments.

As expected, repeated pairing of morphine with the black or white chamber resulted in a robust place-preference when mice were tested in a drug-free state. This is consistent with other studies in the literature using opioids, such as morphine (Zarrindast et al., 2002; Orsini et al., 2005; Wang et al., 2010), heroin (Schlussman et al., 2008) and fentanyl (Finlay et al., 1988), as all tested drugs produced a reliable and robust place preference in the CPP paradigm. In this present study, morphine-induced CPP was examined across a range of doses that are typically used in rodent CPP studies, which resulted in consistent a morphine-induced CPPs over several experiments rewarding effect.

It should be pointed out that the half-life of morphine in mouse brain is roughly 1 h (Ishikawa et al., 1983). The interval between morphine and the subsequent session with saline in this presented study was 20 h. Thus, the washout period was sufficient to eliminate or to reduce morphine concentration between sessions leading to good, better or perfect morphine conditioning.

Importantly, pre-treatment with mTORC1 inhibitors metformin or CCI-779 in this study during repeated morphine exposure failed to affect the development of morphine-induced locomotion behaviour as measured during the conditioning phase and was insufficient to block morphine induced conditioned place-preference, when mice were tested in a drug-free state. Thus, mTORC1 inhibition had no effect on morphine reward. However, the data reported in the literature concerning the effects of the mTORC1 pathway with other rewarding stimuli in place conditioning studies are controversial. It has been documented that rapamycin pre-treatment at a dose of (10 mg/kg) 1 h prior to the conditioning session (8 days of alternating cocaine/saline) with cocaine had no effect on the acquisition of the CPP and locomotor activity in C57BL/6J mice (Bailey et al., 2012). Interestingly, the same research group determined that a single dose of rapamycin injected 1 h prior (post-conditioning test) on day 11 blocked the expression of cocaine CPP, suggesting that acute mTORC1 inhibitor may alter cocaine- induced conditioned place-preference in contrast to chronic administration. Additionally, it has been shown that rapamycin failed to block the development of methamphetamine-induced conditioned place preference in rats, as rapamycin was delivered *via* micro-injection directly into the nucleus accumbens at a dose of 0.025 pmol per rat, 24 h before methamphetamine administration for 5 days (4 days of alternating the methamphetamine/saline session) (Narita et al., 2005).

The interesting finding from this research was that rapamycin suppressed the development of sensitization to methamphetamine- induced CPP and based on the immunochemical data from the limbic forebrain regions confirmed that there was a high level of expression p70S6K following methamphetamine treatment (Narita et al., 2005). This suggests that the reward effect may contribute to synaptic plasticity and in turn, led to dopamine neurotransmission being accelerated in the mesolimbic system (Narita et al., 2005). Furthermore, several lines of evidence support the role of the mTORC1 pathway in addictive behaviours related to synaptic plasticity, as it was reported that a single rapamycin injection

attenuates CPP to alcohol in addition to reducing the expression of alcohol-induced locomotor sensitization in C57BL/6J mice and DBA/2J mice (Neasta et al., 2010).

In parallel, the same research group used western blot to measure activity of mTORC1. Their investigation found that acute exposure to alcohol in C57BL/6J mice and DBA/2J mice resulted in a significant increase in the phosphorylation level of p70S6K and 4EBP proteins (Neasta et al., 2010). This indicated that the mTORC1 signalling cascade contributed to the underlying mechanisms of alcohol-seeking behaviours (Neasta et al., 2010). Likewise, it has been identified that both mTOR complexes play an important role in opioid-induced structural plasticity (Mazei-Robison et al., 2011). They showed that chronic morphine decreased mTORC2 activity in the ventral tegmental area, whereas chronic morphine increases mTORC1 activity in the same brain region resulting in a change to the morphology of the neuron and subsequently, a reduction in dopamine output in the VTA (Mazei-Robison et al., 2011). This indicates that mTORC1 and mTORC2 are involved in morphine reward (Mazei-Robison et al., 2011). It should be mentioned that there is limited information regarding the role of mTORC2, mainly due to the lack of specific inhibitors with respect to this complex.

Targeting the mTORC1 signalling pathway may play a role in the acquisition of conditioned place-reference to morphine and other mechanisms involved in the rewarding properties. Thus, it has been reported that morphine conditioned preference is mediated by dopamine release and gabapentin has been shown to block the rewarding properties of morphine in hooded Lister rats (Andrews et al., 2001). They demonstrated that oral pre-treatment with gabapentin or pregabalin attenuated the development of morphine CPP and that pregabalin was capable of reversing place preference to morphine in hooded Lister rats. This implies that gabapentin or pregabalin had a beneficial effect in decreasing dopamine levels (Andrews et al., 2001), as the rewarding effects of opioids is linked to the increase in dopamine neurotransmission in the brain, specifically in the nucleus accumbens (Leone et al., 1991).

In the present study, both the chronically treated mice with mTORC1 inhibitors (metformin and CCI-779) failed to prevent the development of CPP to morphine. This finding was consistent with a previous report (Bailey et al., 2012). The same research group subsequently showed that a single administration of rapamycin blocked the development of cocaine-induced CPP. However, it should be noted that cocaine and morphine exert their rewarding effects *via* different neural substrates. Basically, cocaine blocks the reuptake of dopamine

and increases extracellular levels of dopamine in the nucleus accumbens creates the cells in (NAC) that receive signals from the (VTA) more sensitive to cocaine which enhances the reward effect, while morphine works by disinhibition of the tonic control exerted by GABA interneurons in the ventral tegmental area to increase dopamine levels in the nucleus accumbens (Goodman, 1996).

It might be argued that the failure of mTORC1 inhibitors (metformin, CCI-779) to block the development of morphine induced place-preference is due to a different underlying mechanisms of reward between cocaine and morphine or could also be related to pharmacokinetic factors. This signifies that higher metformin or CCI-779 concentrations, or greater mTORC1 inhibition may be required to interfere with morphine-induced CPP. However, Bailey and colleagues (2012) revealed that the phosphorylation level of p70S6K and S6 in the forebrain 24 h following repeated rapamycin treatment is significantly lower compared to vehicle-treated mice, suggesting that this reduction in the two markers of the mTORC1 pathway are not sufficient to produce the change on behaviour. Thus, factors other than metformin or CCI-779 pharmacokinetics or the mTORC1 signalling pathway may not play a crucial role in the acquisition of morphine CPP.

One more noteworthy result emerging from this study was that both of the mTORC1 inhibitors (metformin and CCI-779) had no effect on locomotor activity. This finding was consistent with further studies which addressed sensitivity to locomotor activity. For example, in an open field apparatus, metformin administration to C57BL/6J mice in drinking water at a dose of 2mg/ml administered for 4 weeks had no significant effect on locomotor activity, as measured by the distance travelled and rearing movements (Li et al., 2019). Additionally, in exercise wheel systems, the C57BL/6J mice treated with metformin had no effect on locomotor activity (Allard et al., 2016). Moreover, it has been documented that there was no significant difference in the distance travelled by C57BL/6J mice treated with metformin (50 or 300 mg/kg PO) for 7 days compared with vehicle-treated controls (Kim et al., 2013). There is a lack of data relating to the effect of repeated CCI-779 administration on the locomotor activity of animals. Nonetheless, it has been documented that administration of rapamycin to rats had no effect on the locomotor activity (Shih et al., 2012). Similarly, in an open field test, systemic rapamycin administration in C57BL/6J in the following dose range (5, 10, 20 and 50 mg/kg) for 4 days, had no effect on locomotor activity (Cleary et al., 2008).

Mice treated with morphine did not display changes in body weight compared with the observations reported in chapters 3 and 6. It could be due to the different experimental design, as in the present study, the mice were exposed to morphine once daily and on alternating days, while in the previous chapters, the mice were injected twice daily for 9-10 consecutive days. Conceivably, the effect of lower doses of morphine on body weight is negligible, as the higher doses had no effect.

In conclusion, the key finding of the present study was that metformin (200 mg/kg, i.p.) or CCI-779 (25 mg/kg, i.p.) in *naïve* mice failed to modify the development of morphine-induced CPP and had no effect on modifying morphine-induced locomotor activity. This suggests that the mTORC1 signalling pathway may not play an important role in the rewarding effects of morphine while this signalling pathway may be more prominent in mediating other effects of opioids, such as analgesia.

# Chapter 9. General discussion

## 9.1 Summary of findings

The mTORC1 pathway is well established as a regulator of pain sensitivity (e.g., Jiménez-Díaz, et al., 2008; Géranton et al., 2009; Asante et al., 2010; Obara et al., 2011; Kwon et al., 2017). This study is interested in defining the role of mTORC1 pathway in the regulation of morphine efficacy, especially in neuropathic pain when long-term use of opioid is required. Notably, in neuropathic pain, higher doses of morphine are required to achieve pain relief leading to the rapid development of morphine tolerance, which in turn restricts the clinical efficacy of opioids (Yaksh and Harty, 1988; Mao et al., 1995; Ossipov et al., 1995; Christensen et al., 2000; Fundytus et al., 2001). The data presented in this thesis demonstrate that targeting mTORC1 pathway *via* mTORC1 inhibitors improved opioid efficacy. This finding may have an interesting therapeutic implication specifically when long-term use of opioid is required. Chronic morphine administration, as mentioned previously, can be problematic due to the development of therapeutic desensitization that results in pharmacological tolerance to opioid analgesic effects. Thus, this thesis strongly suggests that upregulation of protein translation *via* mTORC1 mediated neuroplasticity is implicated in morphine efficacy. This finding is in agreement with other studies that reported that mTORC1 inhibition blocked the development and maintenance of morphine tolerance (Xu et al., 2014; Zhang et al., 2019). However, these studies demonstrated the beneficial effect of targeting mTORC1 following rapamycin administration, a therapy which, although it targets mTORC1 directly and selectively, is restricted due to its severe side-effects. Therefore, targeting AMPK, the upstream regulation of mTORC1 activity was investigated. This was achieved by using metformin, the first line in the treatment of type II diabetes with a good safety profile.

The originality of using metformin came from the finding that this drug blocked mTORC1 and inhibited the regulation of protein translation in the animal model of neuropathic pain (Melemedjian et al., 2011). Interestingly, metformin administration did not affect the rewarding and motivational properties of morphine, therefore supporting inhibition of the mTORC1 pathway as a safe strategy to improve morphine treatment that may not cause addiction or dependence, as addiction and dependence is associated with a serious global predicament that affects public health besides social and economic welfare (Martins et al., 2015).

### *9.1.1 Metformin blocked the development and maintenance of morphine tolerance in mice and potentiated the effects of morphine in amouse model of neuropathic pain*

Inhibition of the mTORC1 pathway by metformin blocked the establishment and maintenance of morphine-induced tolerance in *naïve* and neuropathic pain mice as well as potentiated morphine analgesia in neuropathic mice. Using behavioural *in vivo* methods, administration of morphine (twice daily, 20 mg/kg in *naïve* mice and 40 mg/kg in neuropathic mice, i.p.) produced a strong analgesic effect on the first day of administration, followed by a gradual decrease in the analgesic reaction to morphine over the following days of administration, indicating the development of analgesic tolerance. It is revealed that administration of mTORC1 inhibitor metformin (once daily, 200 mg/kg, i.p.), prior to morphine administration, strongly prevented the development and maintenance of morphine tolerance as measured 30-60 min after morphine administration by tail-flick latency (*naïve* and neuropathic mice), besides von Frey and acetone tests (neuropathic mice) that assessed symptoms directly associated with neuropathic pain. Moreover, administration of two subsequent doses of metformin on days7-9 when tolerance to the analgesic effect of morphine was achieved, resulted in morphine's analgesic effectiveness being fully restored. Parallel studies using the direct mTORC1 inhibitor CCI-779 (once daily, 25 mg/kg, i.p.) showed that these effects were attributed to the inhibition of mTORC1, while similar results were obtained in *naïve* and neuropathic mice.

Furthermore, behavioural studies in *naïve* mice using A-769662 (30mg/kg, i.p.), a direct AMPK activator/mTORC1 inhibitor, blocked the establishment and maintenance of morphine-induced tolerance similar to metformin and CCI-779. This observation, together with studies where the mTORC1-mediated mechanism was verified by using FK520 (10 mg/kg, i.p.), confirmed the involvement of the mTORC1 mechanism in the regulation of morphine-induced tolerance. Moreover, a known inhibitor of protein synthesis anisomycin (150 mg/kg, i.p.) was used to verify the involvement of the inhibition of translation in the regulation of morphine-induced tolerance by mTORC1 inhibitors. While this hypothesis was confirmed by using *in vivo* models, immunoblotting and immunohistochemistry approaches were also used to provide a basis to further explore the role of mTORC1 in the regulation of opioid systems. The immunoblotting showed that inhibition of mTORC1 activity in the spinal cord after metformin (once daily, 200 mg/kg, i.p.) and CCI-779 (once daily, 25 mg/kg, i.p.) treatment resulted in a decrease in the phosphorylation levels of two mTORC1 downstream targets, P-p70S6 kinase and P-S6 ribosomal protein. Additionally, antibodies were used to visualise the distribution and co-localization of mTORC1 effectors with NeuN

for neurons, GFAP for astrocytes and Iba-1 for microglia. It was established that chronic co-administration of metformin (once daily, 200 mg/kg, i.p.) and CCI-779 (once daily, 25 mg/kg, i.p.) inhibited P-S6RP immunoreactivity in dorsal horn neurones, while the majority of P-S6RP positive cells were identified in neurons. Thus, the inhibition of neuronal mTORC1 appear to be required to maintain morphine analgesia in *naïve* mice. Therefore, these observations emphasise the potential role of mTORC1 in opioid efficacy and suggested that targeting mTORC1 could modulate tolerance to opioid-like medications.

Importantly, systemic and local metformin administration potentiated morphine analgesia in neuropathic mice. Specifically, administration of metformin (once daily, 200 mg/kg, i.p.) or CCI-779 (once daily, 25 mg/kg, i.p.) 20 h prior to three graded doses of morphine (3, 10, 20 mg/kg, i.p.) administered on days 5-7 after SNI-induced nerve injury resulted in potentiation of morphine-induced analgesia. The injection of both mTORC1 inhibitors potentiated the morphine analgesic effect in response to mechanical (von Frey) and cold (acetone test) stimuli. Likewise, local administration of metformin (once daily, 100 nmol, i.pl.) or CCI-779 (once daily, 12.5 nmol, i.pl.) into the paw exhibiting symptoms of neuropathic pain potentiated morphine analgesia. This observation is particularly interesting, as peripheral use of drugs have been proven to reduce the risk of side-effects, thus offering a new strategy for prolonged opioid treatment with fewer side-effects.

To sum up, the behavioural studies in *naïve* as well as neuropathic mice support the idea that metformin, a common clinically available anti-diabetic drug, may play a clinically important role in the regulation of opioid analgesic efficacy, particularly when prolonged opioid treatment is required. Figure 9.1 illustrates mTORC1 signalling in normal/healthy, neuropathic pain and morphine-induced tolerance states together with the effect of different treatments used in this present study.

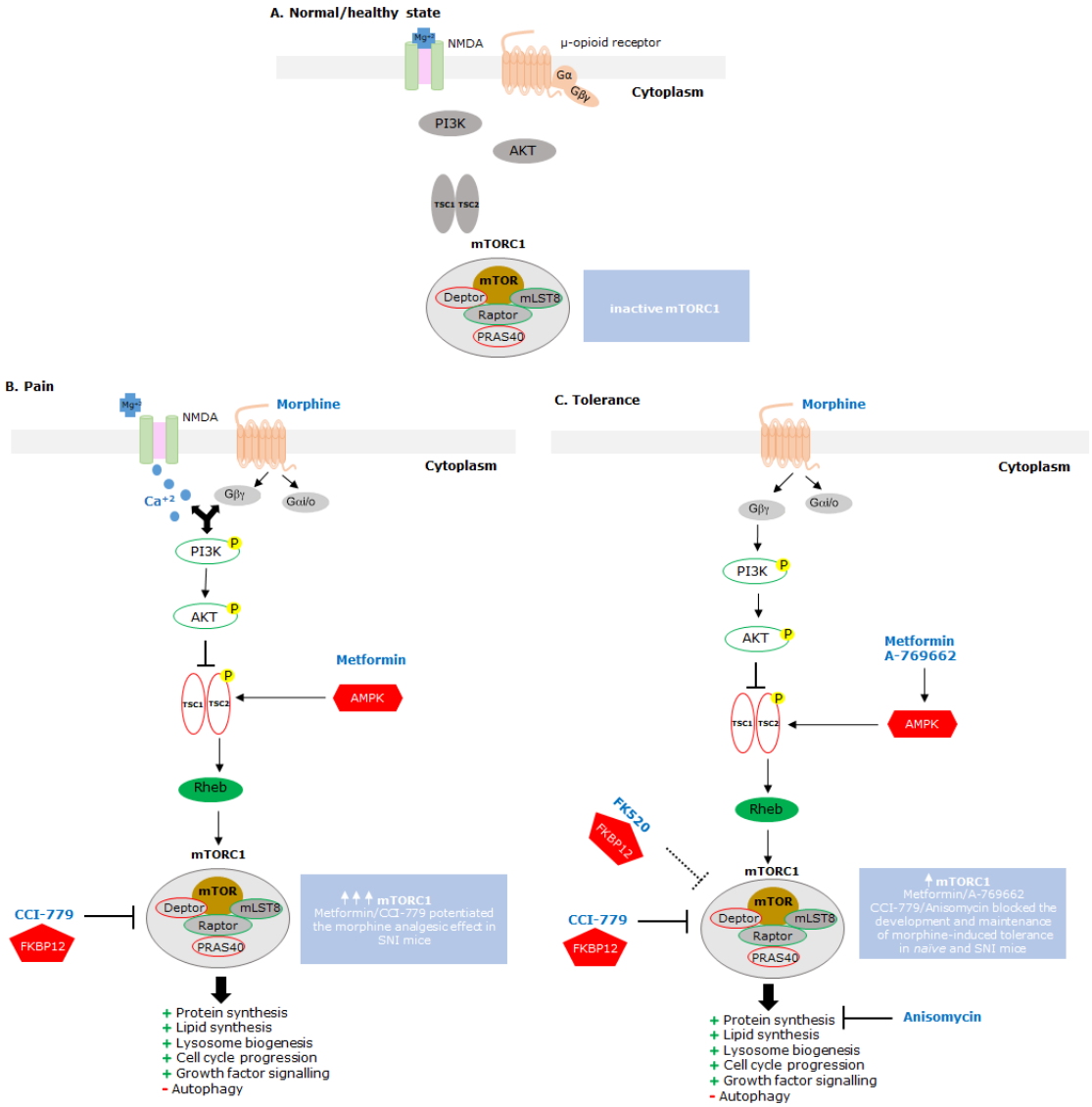


**Figure 9.1: mTORC1 signalling in normal/healthy, neuropathic pain and morphine-induced tolerance states. (A)** Under normal physiological conditions, mTORC1 acting *via* PI3K/Akt signalling is mediated by intrinsic factors (e.g., insulin and growth factors). The active mTORC1 regulates many fundamental cell processes such as cell growth, cell proliferation, cell survival, protein synthesis and autophagy. **(B)** It has been suggested that nerve injury leads to an increase in the spontaneous activity of the primary afferents that results in the release of glutamate, CGRP and substance P besides activation of glial cells and proinflammatory cytokines, for instance TNFα. These changes are suggested to increase mTORC1 activity resulting in activation of multiple cellular processes (e.g., protein synthesis) and subsequently in the development and maintenance of symptoms of neuropathic pain like hyperalgesia and allodynia. CCI-779 binds to cytosolic FKBP12 forming the CCI-779-FKBP12 complex that inhibits mTORC1 and potentiates morphine-induced analgesia in neuropathic mice. In addition, metformin activates AMPK leading to activation of TSC2 and enhancing the stability of the TSC1-TSC2 complex that also results in potentiation of morphine-induced analgesia in neuropathic mice. **(C)** The μ-opioid receptor is a GPCR which means that upon agonist binding (morphine) the GTP-bound Gα subunit dissociates from the Gβγ subunit. The Gβγ subunit then activates the PI3K/Akt/mTOR pathway due to prolonged exposure to morphine, which contributes to the development and maintenance of morphine tolerance both in *naïve* and neuropathic mice. In contrast, CCI-779 inhibits mTORC1 and blocks the development and maintenance of morphine-induced analgesic tolerance in *naïve* and SNI mice. In addition, metformin and A-769662 activate AMPK leading to inhibition of mTORC1 and the development and maintenance of morphine-induced analgesic tolerance in *naïve* and SNI mice. Administration of anisomycin, a protein synthesis inhibitor, also blocks the development and maintenance of morphine-induced analgesic tolerance in *naïve* mice. However, administration of FK520 which binds to FKBP12 but does not inhibit mTORC1 activity has no beneficial effect on the development and maintenance of morphine-induced analgesic tolerance in *naïve* mice.

### *9.1.2 Systemic metformin does not potentiate morphine-induced conditioned place preference in naïve mice*

The previous behavioural studies completed in this thesis revealed that inhibition of the mTORC1 pathway blocks morphine tolerance and improved the analgesic effect of morphine in *naïve* and neuropathic mice *via* metformin administration in a similar manner to the direct mTORC1inhibtor CCI-779, highlighting the functional role of metformin in pain regulation. Given the accumulated findings, where metformin acting *via* mTORC1 potentiated and restored morphine analgesia, it was recognised that it would be critical to determine the influence of mTORC1 inhibitors on the motivational and reinforcing effects of morphine. To determine the effect of combined metformin or CCI-779 with morphine treatment on motivational behaviour, the conditioned place preference (CPP) test was applied. Specifically, *naïve* mice were conditioned with morphine (0.3, 1, 3, 10 or 20 mg/kg, i.p.) using a full counterbalanced design, metformin (once daily, 200 mg/kg, i.p.) or CCI-779 (once daily, 25 mg/kg, i.p.) and were injected 20 h before morphine injection. Both of the mTORC1 inhibitors did not alter the morphine CPP acquisition as well as the sensitization of morphine-induced locomotor activation, indicating that inhibition of the mTORC1 pathway did not affect the rewarding and motivational properties of morphine. This observation strongly suggests that targeting mTORC1 *via* metformin may be a new therapeutic approach to improve morphine analgesia without the risk of morphine-induced addiction and dependence.

It should be pointed out that morphine produces its analgesic effect as well as reward effect by binding to μ-opioid receptors that are extensively distributed in the central and peripheral nervous system (Mansour et al., 1994; Dhawan et al., 1996). These receptors are expressed in many brain regions, including cortex, putamen, amygdala, hippocampus, hypothalamus, periaqueductal grey, thalamus, nucleus accumbens, insula and the ventral tegmental area Mansour et al., 1994; Dhawan et al., 1996; Mollereau and Mouledous, 2000; Walwyn et al., 2010). Additionally, mTORC1 is widely disrupted in the CNS including amygdala, medial prefrontal, hippocampus, auditory cortices and striatum and its role depends on the regions where it is stimulated, which leads to synaptic plasticity, neurogenesis and memory and learning deficits by stimulating protein synthesis (Mameli et al., 2007; Slipczuk et al., 2009; Li et al., 2010). Thus, several investigators addressed the role of the mTORC1 signalling pathway in several pathological conditions related to the function of CNS. However, the role of the mTORC1 signalling pathway on morphine induction and expression of CPP remains unclear as different brain areas may participate in the development of morphine CPP.

The acquisition of morphine or another drug of abuse requires long-lasting neuroadaptations in specific brain areas that leads to an activation of the reward system by way of the drug of abuse. Indeed, drugs which are used for recreational purposes, such as cocaine, tetrahydrocannabinol, morphine and alcohol produced an acute stimulation of mTORC1 signalling in the brain, particularly, in the mesolimbic system, while the induction of a long-lasting neuroadaptation necessitates further investigation (Wu et al., 2011; Puighermanal et al., 2013). It has been documented that 1 h but not 20 h following the single cocaine injection (15 mg/kg, i.p.), resulted in an increase in the phosphorylation level of P-S6RP in rat brains particularly, in NAc, cortex and VTA, though not in the hippocampus and cerebellum (Wu et al., 2011). Moreover, they found that rapamycin administration at a dose of 5 mg/kg (i.p.) for 4 consecutive days before cocaine blocked this enhancement of the P-S6RP level (Wu et al., 2011). Similarly, immunoblotting performed by Puighermanal and co-workers (2009) revealed that a single injection of tetrahydrocannabinol at a dose 10 mg/kg (i.p.) induced a rapid increase in p70S6K and eIF4G levels in the hippocampus of mice within 30 min and lasting for 2 h following drug administration. However, administration of rapamycin at a dose of 1mg/kg (i.p.) for 5 consecutive days before acute tetrahydrocannabinol injection decreased the level of p70S6K, but not the eIF4G level. In 2013, the same research group extended their observation of mTORC1 activity in other regains of the mouse brain. They showed that acute administration of tetrahydrocannabinol increased the level of P-p70S6K not only in the hippocampus but also in the amygdala, frontal cortex and the striatum (Puighermanal et al., 2013), suggesting that mTORC1 activation and other mechanisms might be behind the neuroadaptations in the brain (Puighermanal et al., 2019; Puighermanal et al., 2013). Additionally, it has been documented that acute administration of alcohol (2.5mg/kg, i.p.) resulted in increased levels of P-p70S6K and P-4EBP in the NAc of mice, as assessed at 30 min and 24 h post-alcohol administration (Neasta et al., 2010). Moreover, they showed that systemic administration of rapamycin inhibit the expression of alcohol-induced CPP (Neasta et al., 2010), indicating that activation of the mTORC1 signalling pathway involved in alcohol-related disorders (Neasta et al., 2010). Furthermore, morphine administration at a dose of 75 mg/kg through subcutaneous pellet for 5 consecutive days induced increases in P-p70S6K and p70S6K in rat VTA but not in the NAc was observed 24 h after the last morphine administration (Mazei-Robison, 2011). All these finding signifies the role of mTORC1 signalling pathway in the regulation of the mesolimbic system that is a dopaminergic pathway critical for rewarding processes induced by administration of abusive drugs.

In contrast, it has been reported that acute methamphetamine administration at a dose of 30mg/kg (i.p.) decreased the phosphorylation levels of Akt and mTOR in the mouse hippocampus 1 h after its administration and reduced the level of p70S6K 24 h after the administration (Gonçalves., 2012). In line with this observation, the immunoblotting done by Baily and co-workers (2012) showed that acute or chronic administration of cocaine (15mg/kg, i.p.) had no effect on P-p70S6K and p70S6K in mice NAc. Also, they showed that administration of rapamycin had no effect on cocaine acquisition of CPP but inhibited the expression of CPP (Bailey et al., 2012). In addition, it has been reported that methamphetamine administration at a dose of 2 mg/kg (i.p.) for 3 consecutive days did not change the level of P-p70S6K in comparison with the vehicle groups in the rat brain specifically in NAc (Narita et al., 2005). They also demonstrated that local administration of rapamycin into the NAc prior to methamphetamine injection did not change the acquisition of CPP (Narita et al., 2005) whereas morphine administration (7.5 mg/kg, i.p.) increased the phosphorylation of the Akt level in the hippocampus but not in the VTA and NAc of the rat brain and administration of rapamycin locally to the hippocampus 30 min before morphine blocked the acquisition of CPP (Cui et al., 2010). This suggests that activation of mTORC1 in the hippocampus plays an essential role in morphine-induced CPP. Taken together the finding from the behavioural and the biochemical studies, the functional role of inhibition of the mTORC1 pathway in changing animal behaviour in the CPP paradigm is controversial and requires further investigation particularly when long-term treatment of abusive drugs could be consider as therapeutic, e.g., opioids for long-term use in chronic pain.

### 9.2 Methodological considerations and limitations

***Drug doses/concentrations.*** The biguanide metformin is excreted unchanged in the urine, with a half-life of approximately 6-8 h. It might be argued that mice were injected with metformin 20 h before morphine thus, metformin pharmacokinetics may limit its beneficial effect. It has been documented that *in vitro* administration of metformin to H4IIE cells at a concentration 50 μM significantly activated AMPK 6 h following metformin treatment and AMPK continued to increase gradually up to 72 h (Hawley et al., 2002). Similarly, in the *in vivo* study, a significant reduction in neuropathic allodynia was observed on day 2 following systemic metformin treatment at a dose of 200mg/kg in SNI mice (Melemedjian et al., 2011) that is the same dose of metformin used in this current study. These findings therefore support the time course in relation to the activation of AMPK by administration of metformin, which in turn enhanced the analgesic effect of morphine.

***Benefits and limitations.*** Targeting the mTORC1 pathway *via* rapamycin and its rapalogues, such as CCI-779 for treating chronic pain is controversial in literature. On one hand, studies reported a beneficial effect of local or intrathecal treatment with rapamycin in reducing pain hypersensitivity in models of neuropathic and inflammatory pain (Jiménez-Díaz et al., 2008; Géranton et al., 2009; Asante et al., 2010; Melemedjian et al., 2010) as well as systemic treatment with CCI-779 in neuropathic pain model (Obara et al., 2011). Conversely, some clinical studies reported that long-term exposure to mTORC1 inhibitor, practically rapamycin and its rapalogues, has been linked to increased pain hypersensitivity in patients (Witzig et al., 2005; Molina et al., 2008; Massard et al., 2010) as well as in experimental animals (Zhang et al 2003; Melemedjian et al., 2013). It has been reported that systemic rapamycin treatment resulted in partial reversal of mechanical allodynia in mice exposed SNL and induced allodynia in sham mice (Melemedjian et al., 2013). Also, the same research group reported that administration of systemic rapamycin or CCI-779 induced allodynia in *naïve* mice (Melemedjian et al., 2013) suggesting that mTORC1 inhibition *via* rapamycin and its rapalogues mediated a feedback mechanism by activation of ERK thus, leading to hyperexcitability of sensory neuron that resulted in spontaneous pain and mechanical allodynia in animals (Melemedjian et al., 2013). Moreover, it has been documented that mTORC1 inhibition mediated feedback activation by Akt (Carracedo et al., 2008; Kinkade et al., 2008). In brief, the mechanism suggested in literature regarding the feedback loops is mediated by long-term mTORC1 inhibition as active mTORC1 stimulated protein synthesis *via* phosphorylate its downstream effectors p70S6K (p70S6K1 and p70S6K2) and 4EBP1 resulting in initiation of translation and elongation process leading to protein synthesis (Melemedjian et al., 2013). Moreover, active p70S6K1 reduced insulin receptor substrate (IRS-1) *via* phosphorylation on 636/639 residues resulting in inhibition of ERK and Akt signalling, but long-term inhibition of mTORC1 resulted in reduction in p70S6K activity and in turn enhanced both ERK and Akt signalling (Melemedjian et al., 2013). However, still the cellular mechanism underlying mTORC1 inhibition-induced pain is elusive and more *in vivo* rather than *in vitro* studies are required to clarify the impact of mTORC1-mediated negative feedback loop on pain control. Importantly, in this current study systemic administration of CCI-779 for 9 connective days in *naïve* mice and for 10 connective days in neuropathic mice did not increased pain hypersensitivity animals. Moreover, CCI-779 administration enhanced pain withdrawal threshold and withdrawal duration in neuropathic mice in agreement with previous observations by Obara et al. (2011). Interestingly, mTORC1 inhibition through AMPK activation and using metformin did not

induced pain hypersensitivity with long-term of mTORC1 inhibition (Melemedjian et al., 2013). The authors suggested that this effect resulted from that fact that active AMPK phosphorylated the inhibitory site in IRS-1 S789 and therefore prolong mTORC1 inhibition *via* metformin had no positive effect on ERK and Akt signalling. Moreover, it has been documented recently that metformin had long-lasting effect in decreasing the mechanical hypersensitivity observed up to 32 days after the end of treatment in SNI mice (Inyang et al., 2019).

***In vivo models - methodological issues.*** The C57BL/6J is the most widely used strain in animal research and approximately half of the cited articles used C57BL/6J strain (Bryant, 2011). In pain-related studies, C57BL/6J mice are widely used (Bryant, 2011). The C57BL/6J strain of mice naturally develop non-pigmented spots with different sizes on the distal part of their tails. It is therefore possible that the physiological or anatomical features of the tail could have an influence on the tail flick results, as it has been documented that the light spots in C57BL/6J may alter the response to the tail-flick test by recording a longer latency compared with fully pigmented mice (Wen et al., 2009). Moreover, the general guideline for the tail-flick test recommends that the light beam must be focused on an area 2 cm from the tip. Thus, by using this recommendation, the light beam could easily overlap with non-pigmented spots leading to differences in tail latencies between animals. This indicates that the pigment density and its location in the mouse tail is a subjective factor for the increase in pain-threshold (Wen et al., 2009). To avoid issues associated with tail pigmentation and response to pain stimuli, in this current study, mice were randomly assigned to different treated groups and the data analysis conducted and reported in this research was from an average of 5-12 mice per group. Each experimental design included control animals that were assigned randomly as well. Data analysis showed that no significant issue associated with tail pigmentation that may affect the experimental outcomes presented in this thesis was detected. Thus, all the data regarding the nociception level generated from the tail-flick assay in *naïve* and neuropathic mice can be considered as valuable, reliable and reproducible.

***In vitro models - methodological issues.*** It might be argued that in the biochemical experiments presented in this thesis, morphine pre-treatment with both mTORC1 inhibitors failed to induce a significant reduction on the protein levels of P- mTOR, mTOR, P-4EBP and 4EBP in the spinal cord. However, it was clearly showed that metformin and CCI-779 suppressed two major markers that reflected mTORC1 activity, P-p706SK and P-S6RP. The

lack of changes in the western blotting technique regarding P-mTORC1, mTORC1, P-4EBP and 4EBP in *naïve* mice as well in SNI mice, could be related to the timing when the tissue was collected, or it might necessitate a stronger immune component (antibodies) to give clear bands or the low concentration of the extracted proteins. In addition, by using immunohistochemistry technique, it could be important to determine colocalization of the μ-opioid receptor with mTORC1 downstream effectors in dorsal horn of *naïve* mice. It is however very challenging as antibodies available to detect μ-opioid receptor are not specific and verification of any signal related to μ-opioid receptor expression would require use of knockout animals.

### 9.3 Implications of current experimental results

The risk of opioid dependence concerning the management of acute and chronic pain is an issue that health care professionals, particularly doctors in hospitals, have recognised for a number of years. Opioids exhibit a considerable risk in contrast to other common classes of drugs due to their abusive properties. It is also worth noting that opioid dependence increases both hospital costs and length of stay for patients who are undergoing opioid-related adverse episodes, while also reducing patient satisfaction. Additionally, opioids can produce addiction, abuse, as well as the death of a person. Multimodal analgesia, a method that is dependent on non-opioid medication with the addition of opioids is required and may offer safer but more efficient way of pain control. Multimodal analgesia is acknowledged to be a viable alternative to opioid monotherapy. Several non-opioid analgesics have been noted to be extremely effective, as they are safer than opioids and exhibit fewer side-effects. As a result of these factors, multimodal analgesia is advocated as best practice by most authorities. The model is termed ‘multimodal’ for the reason that it necessitates the management of two or more drugs that act by different mechanisms used for providing analgesia. A further important point is that these drugs can be administered by the same route or by different routes. The combinations that are suggested for use consist of an opioid combined with a local anaesthetic or a drug from an alternative pharmacological class. Nevertheless, combined use of two opioids is rarely advocated as a multimodal treatment with respect to moderate to severe pain owing to the lack of knowledge concerning the possible advantages of such combinations. Moreover, concerns have been raised in relation to the possible side-effects, while there are misgivings in regards to the additional benefits.

Regarding individual patient, choice of treatment is dependent on the pain characteristics and the state of pain chronification. The more acute the pain, the greater the effect on the patient's life, and hence, the necessity for multimodal therapy. This thesis has provided what is thought to be the first evidence that metformin contributed to the inhibition of the development and maintenance of morphine-induced tolerance and potentiated morphine analgesia *via* targeting the mTORC1 pathway. Thus, the significance of mTORC1 as a novel and tractable target for the improvement of the effectiveness of opioid analgesic in chronic pain, supports the idea that metformin, a widely clinically available anti-diabetic drug, may offer a novel and clinically viable avenue for enhancing the efficacy of morphine analgesic, particularly in neuropathic pain.

### 9.4 Gender differences in relation to pain and the role of sex hormones

Pain response of different individuals can be influenced by a number of distinctive variables, which range from physiological, psychological and cultural to genetic and social factors (Holdcroft and Berkley, 2005; Greenspan et al., 2007). More recently, it has been reported that gender is a crucial issue in moderating the experience of pain. Research is indicating that males and females experience pain in a different way and furthermore, that they respond differentially to particular categories of analgesics (Paller et al., 2009). It is worth noting that both clinical and experimental studies report that females have lower pain tolerance and pain thresholds in contrast to males, as tested by various stimulus modalities (Barrett et al., 2002; Wiesenfeld-Hallin, 2005; Greenspan et al., 2007). Moreover, it has been reported that females experience pain that is more frequent, severe and longer lasting, which is frequently anatomically more disseminated than males with similar disease processes (Hurley and Adams, 2008).

It is recognised that there are many chronic pain states, for instance rheumatoid arthritis (RA), migraine, irritable bowel syndrome (IBS), temporomandibular disorder (TMD), as well as fibromyalgia, that are more commonly found in females (Holdcroft and Berkley, 2005; Greenspan et al., 2007). Nevertheless, certain long-standing conditions like cluster headaches and migraine without aura are more commonly found in males (Greenspan et al., 2007). Three imaging studies of the brain revealed gender differences in people concerning the intensity of cerebral activation and the spatial pattern in response to acute noxious stimuli (Derbyshire et al., 2002; Zubieta et al., 2002; Moulton et al., 2006). Another notable finding

is that response to analgesics is dependent on gender (Craft, 2003; Wiesenfeld-Hallin, 2005; Berkley et al., 2006). With respect to humans, women demonstrate a greater response to μ-opioid agonists in contrast to men (Cook et al., 2000; Craft, 2003), although in rodents, morphine has been observed to be more effective in males than in females (Berkley et al., 2006). It should be pointed out that sex hormones such as androgens, oestrogens and progestogens have been the focus of significant attention in studies into the mechanisms of gender differences in relation to pain. In females, the levels of plasma oestrogen differ throughout the menstrual cycle during the reproductive years (18-50) and on average are acknowledged to be 3–10 times greater than those found in men (Eldrup et al., 1987). It has been revealed that there is a relationship between pain and the menstrual cycle in relation to several chronic pain conditions. For instance, approximately 10% of female migraine sufferers have menstrual migraines, although headaches commonly occur at the beginning of menstruation (Martin and Lipton, 2008).

The female rat has a 4-5 day vaginal estrous cycle which is linked with changes in the hormone levels. The estrous cycle in rats is typically divided into three distinct stages; specifically, proestrous, estrous and diestrous (Krinke, 2000). In females which are experiencing the cycle, serum oestradiol concentration is low during the estrous stage, increases progressively during the diestrous stage, reaches its peak during the early proestrous stage and decreases during the late proestrous stage. Serum progesterone concentration attains its peak twice, once during the middle diestrous stage and subsequently during late proestrous stage (Butcher et al., 1974). Additionally, it has been documented that baseline pain sensitivity in rats reaches its peak during the proestrous and early estrous stages in contrast to the diestrous stage (Fillingim and Ness, 2000). Hind paw withdrawal latencies were noted to be considerably higher during the proestrous stage than during the estrous and diestrous stages in carrageenan-inflamed rats (Tall and Crisp, 2004).

In a TMD pain model, females in the proestrous stage were observed to exhibit fewer pain behaviours than those in the diestrous stages (Clemente et al., 2004). Moreover, modulation of opioid-mediated analgesia by means of the menstrual cycle has also been documented. Opioids are revealed to be less effective during the estrous stage compared to females tested in other stages (Craft et al., 2004). It has also been reported that the effectiveness of opioids differs between rats tested in early vs late proestrous (Stoffel et al., 2003). Research conducted by Inyang et al. (2019), using a model of neuropathic pain (SNI) showed that

metformin treatment reverses mechanical and cold hypersensitivity in male ICR mice, although that is not the case as regards females.

Overall, gender differences and the effect of the menstrual cycle indicate that the sex hormones of females may possibly affect behavioural responses to pain. However, the exact mechanisms by which sex hormones influence pain sensitivity, particularly in chronic pain conditions, has not yet been established. In light of these observations, it may be important to consider conduction of parallel studies using female mice that would confirm the effects resulting from mTORC1 inhibition on opioid efficacy observed in male mice. While such study may have significant values regarding the applicability of pre-clinical studies into clinical scenarios in humans, there should be a requirement of a thorough cost-benefit assessment regarding application of 3Rs principle (replacement, reduction and refinement in animal research).

## 9.5 Future studies

A considerable amount of evidence identified that mTORC1 has an essential role in opioid tolerance and chronic pain. This role was also recognised in my results obtained from several behavioural tests and molecular biology techniques. This thesis supports the valuable role of mTORC1 on morphine tolerance particularly in the neuropathic pain model and highlights the powerful effect of metformin as an indirect mTORC1 inhibitor to improve morphine analgesia. Thus, it will be interesting to continue some pre-clinical and clinical studies to extend and confirm the impact of these current observations.

### 9.5.1 Pre-clinical studies

***Study 1.*** Opioid tolerance occurs earlier in patients of a younger age, commonly develops during critical illness and results more frequently from prolonged intravenous infusions of short-acting opioids ( Colvin and Lambert, 2008; Anand et al., 2010). The current treatment options include: slowly tapering opioid doses, switching to longer-acting opioids, or specifically treating the symptoms of opioid withdrawal (Kral et al., 2015). Furthermore, a recent pre-clinical study presented that intensifying morphine treatment in adolescent rats causes long-lasting effects on the development of morphine tolerance and dependence during adulthood (Salmanzadeh et al., 2017). They established that the adolescent morphine treatment group significantly facilitates the development of tolerance to the analgesic effect of morphine and potentiate morphine withdrawal signs (Salmanzadeh et al., 2017).

The key finding from this thesis is that metformin attenuated morphine tolerance by blocking the mTORC1 signaling pathways in adult mice. Therefore, in the future, the efficacy of metformin co-administration with morphine in adolescent animals will be tested to address to what extent AMPK activators can improve opioid efficacy and prevent opioid tolerance during adulthood, where adolescence is characterised by neurobiological changes which remarkably affect behavioural manifestations in adulthood. Furthermore, adolescents are more likely to get engaged in risk-taking behaviours associated with the abuse of drugs (Wills et al., 1994).

***Study 2***. Physical dependence is a widely known consequence of morphine intake that is commonly associated with prolonged or repeated morphine administration. Additionally, withdrawal symptoms can be elicited even after a single injection prior to morphine exposure. In the CPP experiment, it was determined that mTORC1inhibtors did not affect the potentiation of morphine reward, nor did they antagonize or block morphine CPP acquisition in *naïve* (healthy) mice. Neuropathic pain however, has been shown to be associated with dysregulation of mTORC1 and its downstream effectors (Jiménez-Díaz, et al., 2008; Géranton et al., 2009; Asante et al., 2010; Obara et al., 2011; Kwon et al., 2017) an, interestingly, it has been documented that neuropathic pain increases the response to morphine reward (Kai et al., 2018). Thus, future studies should address the role of mTORC1 pathway targeted by mTORC1 inhibitors concerning morphine reward under neuropathic pain conditions. Such study would provide better understanding of the applicability of mTORC1 inhibition in the regulation of opioid efficacy in chronic pain and, importantly, would confirm this current observation indication for safety of combined mTORC1 inhibitors and morphine treatment for pain.

### 9.5.2 Clinical studies

Opioids are used in chronic sciatica (CS) along with anticonvulsants and antidepressants. However, while short-term treatment (9 weeks) has been shown to be efficacious (Khoromi et al., 2007), long-term use of opioids can be problematic due to the rapid development of profound analgesic tolerance (Dumas and Pollack, 2008). Based on the data from mice and presented here, it could be hypothesized that CS patients on metformin will show long-term (up to 7 months) responsiveness to oral morphine. Therefore, it may be worth to conduct an observational single arm clinical pilot experiment on a small group of patients with CS and

diabetes. These patients will be in receipt of metformin for the treatment of diabetes and will be clinical candidates for oral opioids for the treatment of CS related pain within routine clinical practice. During the pilot experiment, these patients will be subjected to oral morphine and will monitor their responsiveness to morphine for up to 8 months to determine if metformin, as a part of their co-treatment with morphine, will lead to efficacious pain control. This study may be planned as a pilot experiment and positive outcome of this study may lead to a possibility of double-blind randomized control trial that will prove if the effectiveness of morphine in these patients is due to co-treatment with metformin. Therefore, this pilot experiment may directly lead to the development of novel therapeutic strategy with immediate clinical benefit.

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