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**INSIGHTS INTO THE ROLE OF SPINAL mTOR IN THE
MODULATION OF INFLAMMATION AND NOCICEPTION**

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ABSTRACT

Pain, especially chronic pain, is a major clinical problem. Pain therapy has proven to be complicated and the results are often unsatisfactory. Despite many years of research, the number of available pain therapeutics remains relatively small. The available therapeutics are often not efficient enough and associated with adverse effects, which may be problematic when treating pain both in the short and long term. In order to develop new and improved pain therapeutics the identification of novel pharmaceutical drug targets is of crucial importance. This thesis investigates the protein mTOR as a new potential drug target to treat inflammatory pain. In addition, methodological approaches to obtain reliable in vitro spinal astrocyte cell cultures were studied.

To determine whether spinal mTOR plays a role in inflammatory pain processing, activation of spinal mTOR in rats was measured following the induction of a peripheral inflammation. An increased activation of the mTOR pathway was seen in spinal dorsal horn neurons following injection of carrageenan into the hindpaw. Inhibition of spinal mTOR with rapamycin resulted dose-dependently in decreased nociceptive behavior, further supporting a role for mTOR in inflammatory pain signaling.

In vitro model systems are important tools to study cellular signaling events. In order to obtain reliable cell cultures from rodent tissue with properties similar to human tissue cultures, the culture conditions are of vital importance. Using a variety of specific markers to identify astrocytes, the culture conditions were studied in astrocyte cultures generated from spinal cord samples taken from human tissue, as well as two genetically different rat substrains. Culturing rat astrocytes in medium optimized for astrocytes, results in lower contamination and cells with a more astrocyte-like phenotype. In addition, the choice of rat substrain can also affect the characteristics of the cultured astrocytes.

Microglia and astrocytes have received much attention in central pain processing research. Preclinical studies have demonstrated an important role for these cells in pain signaling. To examine the possible role of mTOR in astrocyte activation, two glia inhibitors, the methyl xanthines pentoxifylline and propentofylline, were used. Both substances inhibited astrocyte activation in vitro as demonstrated by reduced astrocyte proliferation and growth, and an altered morphology. Further, pentoxifylline and propentofylline counteracted TNF-induced mTOR activation in cultured astrocytes but did not affect EGF-induced mTOR activation. These data suggest that pentoxifylline and propentofylline act in an mTOR- and stimulus-dependent fashion to inhibit astrocyte activation.

In addition to targeting mTOR directly, pharmacological interference with factors associated with mTOR signaling, that can indirectly regulate activation of mTOR and other signaling pathways may prove useful as potential drug targets. One such factor that may be linked to mTOR regulation is caveolin-1, a protein that can act as a regulatory factor in several disease states. Although the importance of caveolin-1 in pain signaling is not yet clarified, links to TNF signaling indicated in the present thesis, suggest a possible role in inflammatory pain processing and further studies are required to determine if caveolin-1 is an important regulator of mTOR signaling. As a whole, this thesis provides insight into the role of mTOR in spinal pain processing. Further studies of the molecular function of mTOR in neurons and glial cells in the dorsal horn may provide a basis for the development of new therapeutics for the treatment of inflammatory pain.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid
MTT	3-/4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PDK1	3-phosphoinositide-dependent kinase 1
DAPI	4',6-diamidino-2-phenylindole
ATP	Adenosine triphosphate
Aldh1L1	Aldehyde dehydrogenase 1 member L1
ANOVA	Analysis of variance
AUC	Area under the curve
AM	Astrocyte medium
ATM	Ataxia telangiectasia mutated
BSA	Bovine serum albumin
JNK	c-Jun N-terminal kinase
CGRP	Calcitonin gene-related protein
CNS	Central nervous system
CSF	Cerebrospinal fluid
CD11b	Cluster of differentiation molecule 11b
CFA	Complete Freund's Adjuvant
Cnx-43	Connexin 43
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
DMSO	Dimethyl sulfoxide
DNA-PK	DNA-dependent protein kinase
DRG	Dorsal root ganglion
DMEM	Dulbecco's Modified Eagle Medium
ED1	Ectoderm dysplasia 1
ED2	Ectoderm dysplasia 2
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor 2

FKBP-12	FK506-binding protein 12
GABA	Gamma-butyric acid
GFAP	Glial fibrillary acidic protein
Glt-1	Glutamate transporter 1
Gs	Glutamine synthetase
IGF-1	Insulin-like growth factor 1
IL-10	Interleukin 10
IL-1 β	Interleukin 1 β
IL-6	Interleukin 6
i.t.	Intrathecal
iGluR	Ionotropic glutamate receptor
LPS	Lipopolysaccharide
LC	Locus coeruleus
LTP	Long-term potentiation
MHC	Major histocompatibility complex
mLST8	Mammalian lethal with Sec13 protein 8
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte attractant protein 1
NMDA	N-methyl-D-aspartate
NGF	Nerve growth factor
NeuN	Neuronal N
NO	Nitric oxide
NSAID	Non-steroidal inflammatory drug
S6K	P70S6 kinase
PFA	Paraformaldehyde
PTX	Pentoxifylline
PAG	Periaqueductal gray
PBS	Phosphate buffered saline
PIP3	Phosphatidylinositol 3,4,5 triphosphate
PI3K	Phospho-inositide-3-kinase

PDGF-CC	Platelet-derived growth factor-CC
DEPTOR	Pleckstrin (DEP)-domain containing mTOR-interacting protein
PRAS40	Proline-rich AKT substrate 40 kDa
PPF	Propentofylline
PGE ₂	Prostaglandin E ₂
Rheb	Ras homolog enriched in brain
RAPTOR	Regulatory-associated protein of mTOR
RVM	Rostral ventral medulla
S100b	S100 binding protein b
SSRI	Selective serotonin reuptake inhibitor
SEM	Standard error of the mean
TLR4	Toll-like receptor 4
TKI	TOR kinase inhibitor
TRPA1	Transient receptor potential cation channel member A1
TRPV1	Transient receptor potential vanilloid receptor 1
4E-BP	Translation initiation factor 4E-binding protein
TCA	Tricyclic antidepressant
TSC2	Tuberous sclerosis complex 2
TNF	Tumor necrosis factor

1 INTRODUCTION

The ability to feel pain is a vital function for the survival of an organism by providing information about an injury or potential injury and protecting the body from further damage in order to aid healing. The sensation of pain is subjective and comprises not only a sensory/discriminative component but also emotional and cognitive components. The concept of nociceptive afferent neurons was proposed by Sherrington in 1906 to describe the detection of a noxious stimulus or a potentially harmful event by a certain class of sensory receptors (nociceptors) (Sherrington, 1906). It is important to distinguish between the neural processes of encoding and processing noxious stimuli (nociception) and the complex sensory, psychological and emotional response to a noxious stimulus (pain). Since animals are not able to verbally express the psychological and emotional consequences of a noxious event, the most accurate term to describe what we study as pain in animals is nociception.

Pain, especially chronic pain, is an immense clinical problem and despite many years of research the number of available therapeutics to treat pain is still rather small. Furthermore, the available drugs are often not efficient enough and are also often associated with deleterious side effects which may be problematic both in the short- and long-term treatment of pain.

The dorsal horn of the spinal cord is an important relay station for pain transmission and modulation. In the dorsal horn, peripheral primary afferent fibers synapse onto central projection neurons to relay pain signals from the periphery to the thalamus and other supraspinal regions. During recent years, much attention has been given also to the role of non-neuronal cells such as astrocytes and microglia, in pain mechanisms in the dorsal horn (Milligan and Watkins, 2009). Animal experiments indicate that these cells are important in synaptic transmission both under normal conditions and under pathophysiological states. In pain processing, astrocytes and microglia are likely to contribute to increased pain by releasing signaling molecules, leading to a potentiation of the pain transmission (Milligan and Watkins, 2009). These signaling molecules include cytokines and chemokines, such as tumor necrosis factor (TNF), interleukin 1 β (IL-1 β) and monocyte chemoattractant protein-1 (MCP-1). Cytokines are signaling proteins released by immune cells and glia. Chemokines are a family of cytokines with

chemotactic properties. Upon release, cytokines and chemokines bind to their receptors, initiating intracellular signaling cascades, resulting most often in the facilitation of inflammation and nociception. However, some of the cytokines released by glia may also have suppressive effects on inflammation (Ren and Dubner, 2010).

Understanding the physiology and pathophysiology of pain is vital to the search for new drug targets for pharmacological treatment of pain. This thesis deals with the mechanisms of the intracellular protein kinase mammalian target of rapamycin (mTOR) in neuronal and glial cells in the dorsal horn of the spinal cord and its potential role as a pharmacological target to treat inflammatory pain.

1.1 PRIMARY AFFERENT NEURONS

Nociceptors are specialized primary afferent neurons with free nerve endings that respond to noxious stimuli. Two types of nerve fibers are involved in the conduction of pain signals from the periphery to the central nervous system (CNS). Myelinated A δ fibers signal sharp pain from heat, mechanical and chemical stimuli. Unmyelinated C-fiber afferents respond to intense heat, pain from sustained mechanical stimulation and chemical stimuli, resulting in a painful burning sensation (Meyer et al., 2006).

Apart from conveying pain signals to the CNS, nociceptive C-fibers also contribute to the local peripheral inflammation by releasing neuropeptides such as substance P and calcitonin gene-related peptide (CGRP). These peptides contribute to the inflammatory state by inducing vasodilation and plasma extravasation (neurogenic inflammation) (Meyer et al., 2006; Ren and Dubner, 2010).

1.2 CENTRAL PAIN PATHWAYS

In the dorsal horn of the spinal cord, the nociceptive primary afferent neurons form synapses with projection neurons, whose axons extend rostrally to convey information to the thalamus and other brain regions, and also with inhibitory or excitatory interneurons that in turn make contacts with other neurons within the spinal cord (Fields et al., 2006).

The major ascending pathways project from the dorsal horn to the thalamus (the spinothalamic tract) and the brainstem (spinobulbar and spinomesencephalic tracts),

including terminations in the parabrachial nucleus and the periaqueductal grey (PAG) (Dostrovsky and Craig, 2006).

Pain modulatory systems include local neuronal circuits in the dorsal horn and descending pathways projecting from the PAG, rostral ventral medulla (RVM) and locus coeruleus (LC) to the dorsal horn. The effects exerted by the descending pathways include both inhibition and facilitation of pain transmission in the dorsal horn. Stimulation of PAG, RVM or LC neurons may inhibit nociceptive signaling by activating noradrenergic and serotonergic projection neurons or indirectly by activating inhibitory interneurons in the dorsal horn (Dostrovsky and Craig, 2006).

1.3 ANATOMICAL ORGANIZATION OF THE DORSAL HORN OF THE SPINAL CORD

The dorsal horn is typically divided in six layers, termed laminae I-VI (Rexed, 1952). The majority of nociceptive primary afferents terminate in laminae I and II in the superficial dorsal horn. Lamina I contains a high density of myelinated A δ fiber terminals while many terminals of unmyelinated C-fiber projections are found in both laminae I and II (Light and Perl, 1979; Averill et al., 1995; Plenderleith et al., 1990). Some A δ afferents also terminate in laminae IV-V. Dorsal horn neurons implicated in nociceptive processing are divided into two classes, nociceptive-specific neurons predominantly present in the superficial dorsal horn and wide dynamic range neurons in the deep dorsal horn (laminae V-VI). Nociceptive-specific neurons are unresponsive to touch, have a relatively high mechanical threshold for activation and respond to painful stimuli (Christensen and Perl, 1970). In contrast, wide dynamic range neurons respond both to gentle innocuous and noxious stimuli (Mendell, 1966).

1.4 PATHOLOGIC PAIN

When pain persists for more than 3 - 6 months, the pain may be considered chronic (Merskey and Bogduk, 1994; Russo and Brose, 1998). In contrast to the protective role of acute pain, long lasting pain does not serve a useful purpose and is therefore pathological. Aspects of pathological pain may include reduced thresholds for neuronal activation, an increased response to a given stimuli and the occurrence of spontaneous activity in nociceptive fibers, corresponding to an increased sensitivity to normally non-

noxious stimuli (allodynia), noxious stimuli (hyperalgesia) and spontaneous pain (Julius and Basbaum, 2001).

Pain sensitization may occur both at the site of the injury (peripheral sensitization) and in the CNS (central sensitization). Peripheral sensitization is due to a local release of inflammatory mediators including TNF, IL-1 β , prostaglandin E₂ (PGE₂), nitric oxide (NO) and interleukin 10 (IL-10) from immune cells. These mediators may serve to facilitate or resolve the local inflammation (Julius and Basbaum, 2001). In addition, the primary afferents may undergo phenotypic changes, with altered expression of neuropeptides including substance P and CGRP and receptors such as the transient receptor potential vanilloid receptor 1 (TRPV1). TRPV1, for example, plays a crucial role in inflammation-induced heat hyperalgesia (Caterina et al., 2000). Following inflammation, the expression of TRPV1 in peripheral nerve fibers is increased and inflammatory mediators, such as bradykinin, sensitize TRPV1 and lead to reduced sensory thresholds and increased sensitivity of primary afferents (Carlton and Coggeshall, 2001; Nicholas et al., 1999; Tohda et al., 2001). An altered expression of sodium channels in dorsal root ganglia (DRGs) has also been observed following peripheral nerve damage, indicating a role for sodium channels in increased pain sensitivity (Devor, 2006; Dib-Hajj et al., 2010). In some clinical pain conditions, such as fibromyalgia, where there is no peripheral pathology, central sensitization and/or an imbalance in endogenous pain modulatory systems, appears to be the main cause of the experienced symptoms (Russell et al., 1994; Gracely et al., 2002; Cook et al., 2004).

Central sensitization has many underlying mechanisms. Wind-up and long-term potentiation (LTP) are two electrophysiological phenomena commonly associated with central sensitization. Wind-up is a form of plasticity that occurs in activated synapses, where the response of the dorsal horn neuron increases even though the repeated nociceptor stimulus does not change. Wind-up manifests only during the stimulus and ends with the termination of the stimulus (Zimmermann et al., 2001). LTP involves a long-lasting potentiation of the input from excitatory primary afferents to second order neurons in the dorsal horn (Latremoliere and Woolf, 2009). LTP may be induced by activation of N-Methyl-D-aspartate (NMDA) receptors and Ca²⁺-permeable ionotropic glutamate receptors (iGluRs). This activation leads to potentiation of NMDA and 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA) receptor

transmission through modulation of the channel properties of the NMDA and AMPA receptors, recruitment of NMDA and GluA1-containing AMPA receptors to the synapse as well as the removal of GluA2-containing AMPA receptors from the synapse (Larsson and Broman, 2011).

1.5 PHARMACOLOGICAL INHIBITION OF PAIN

The main groups of drugs used to treat pain are opiates, non-steroidal anti-inflammatory drugs (NSAIDs), paracetamol, antidepressant drugs and anticonvulsant drugs. For thousands of years opiates have been used for recreational and medicinal purposes to produce euphoria, analgesia, sleep and to prevent diarrhea. Opioid receptors are G-protein coupled receptors present both in the periphery and in the CNS. Upon activation, potassium flux is increased and voltage-dependent calcium channels are inhibited, decreasing intracellular Ca^{2+} and leading to decreased neurotransmitter release and reduced neuronal excitability. Morphine and other opiates are effective in most types of acute and chronic pain, although less effective in neuropathic pain (Dickenson and Kieffer, 2006).

Antipyretic analgesics such as NSAIDs and paracetamol are the most commonly used over-the-counter drugs to treat pain. NSAIDs are potent inhibitors of cyclooxygenase 1 and 2 (COX-1 and COX-2), leading to a reduced prostaglandin production and decreased pain sensitization and inflammation (McQuay and Moore, 2006). Paracetamol differs from NSAIDs by its weak anti-inflammatory effects and its lack of unwanted gastrointestinal effects. The mechanism of action of paracetamol is not fully understood, however, recent results demonstrate that the anti-nociceptive effect of paracetamol is mediated indirectly through TRPV1 receptors and directly via transient receptor potential cation channel, member A1 (TRPA1) (Mallet et al., 2010; Andersson et al., 2011).

Tricyclic antidepressants (TCAs) and antiepileptic drugs such as gabapentin and carbamazepine are commonly used to treat neuropathic pain. TCAs inhibit neuronal noradrenaline and serotonin reuptake, a mechanism that is likely to contribute to analgesia. Selective serotonin reuptake inhibitors (SSRIs) have less analgesic efficacy, suggesting noradrenaline to be the primary monoamine responsible for the analgesic effects of TCAs (Yaksh, 2006). Carbamazepine is chemically related to the TCAs and

inhibits noradrenaline uptake in addition to blocking sodium channels (Sang and Hayes, 2006).

Neuropathic pain is associated with excessive neuronal activity in pain pathways. Thus, inhibiting excitatory mechanisms may be an effective way of treating neuropathic pain. Several of the clinically available anticonvulsants to treat neuropathic pain bind to and inhibit voltage-activated calcium channels and sodium channels, thereby reducing neuronal firing and neurotransmitter release. Inhibition of sodium channels is also the mechanism of action of local anesthetics, which are also used to systemically treat neuropathic pain. In addition to the effects on ion channels, anticonvulsants may also increase the synthesis and release of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), resulting in a reduction in excitatory neuronal transmission (Taylor et al., 1998; Sang and Hayes, 2006).

Most current pain-relieving drugs exert their effects through extracellular mechanisms and by binding membrane bound receptors. However, much research is currently focusing on intracellular targets, such as molecular signaling cascades, in order to specifically inhibit key proteins regulating nociceptive processing. Developing kinase inhibitors that block abnormal flux through transduction pathways involved in pain signaling is one possible approach. An obvious potential problem with protein kinase inhibitors as therapeutic drugs, however, is poor selectivity. mTOR, for example, is present in a variety of cell types and is involved in many cellular processes (Zoncu et al., 2011). This raises concerns about using mTOR inhibitors as therapeutics for pain, due to the potential of unwanted side effects. In patients, long-term treatment with the mTOR inhibitor rapamycin as an immunosuppressant is known to be accompanied with side effects such as hypertriglyceridemia, diarrhea and impaired wound healing. However, most side effects are dose-dependent and can be managed or reversed with discontinuation of drug treatment (Jacot and Sherris, 2011). Presently, very little is known about possible differences in mTOR signaling in different cell types. Studies of the role of mammalian target of rapamycin complex 2 (mTORC2) in prostate cancer demonstrated that genetic ablation of mTORC2 only had effects on tumor development and not on normal prostate function (Guertin et al., 2009). Thus, the functional role of mTOR in pathology may be different from its physiological role and specifically

suppressing certain forms of mTOR in pathological conditions may not necessarily be associated with severe side effects.

Preclinical pain research using animal models is a main approach to find new targets for treatment of pain. Although many of the drugs developed based on animal research have turned out to be less effective in humans compared to animals, as in the case with substance P antagonists, animal studies continue to provide important information and a number of new potential targets derived from animal research are currently being evaluated clinically (Hill, 2001). Potential new classes of analgesics that are being tested include adenosine receptor ligands, TRPV1 antagonists, glutamate receptor antagonists, CGRP receptor antagonists and nerve growth factor (NGF) antibodies (Szallasi et al., 2007). It is well known that adenosine has antinociceptive properties in rodent models of pain. Several clinical trials have investigated the analgesic effects of adenosine receptor ligands in humans and currently, adenosine is being evaluated for treatment of neuropathic pain using intrathecal (i.t.) administration (Zylka, 2011). The TRPV1 agonist capsaicin has been used for years as a topical analgesic (inducing receptor desensitization). TRPV1 antagonists are also undergoing clinical trials in pain states such as chronic low back pain and dental pain (Szallasi et al., 2007).

Antagonists of the NMDA glutamate receptor have received new attention in the development of therapeutics for neuropathic pain. Several such compounds have entered or completed phase II clinical trials with positive results. NGF is a major mediator of both inflammatory and neuropathic pain (Hefti et al., 2006). Elevated levels of the neurotrophic factor NGF have been seen in inflammatory conditions such as arthritis and inflammation following injection of Complete Freund's Adjuvant (CFA) in both humans and animals (Aloe et al., 1992; Donnerer et al., 1992; Woolf et al., 1994). Blocking NGF in animal models of inflammatory pain prevents behavioral hyperalgesia, and the development of NGF antibodies to treat pain in humans is currently ongoing (McMahon et al., 1995; Lewin et al., 1994; Woolf et al., 1994; Woolf et al., 1997).

1.6 NEURON-GLIA COMMUNICATION

CNS glial cells include microglia, astrocytes and oligodendrocytes and constitute around 70% of the total cell population of the brain and spinal cord. Microglia,

astrocytes and oligodendrocytes are well known for their roles in host defense, synapse function and myelination (DeLeo et al., 2006). Microglia derive from monocytes and are commonly referred to as the resident macrophages of the CNS and express surface antigens such as Ectoderm Dysplasia 1 and 2 (ED1, 2), Major Histocompatibility Complex (MHC) and clusters of differentiation molecule 11b (CD11b). Microglia are usually the first cells to respond to a pathological stimulus within the CNS (DeLeo et al., 2006).

Astrocytes play an important role in synaptic transmission (Panatier et al., 2011). In addition to having a role in maintaining the extracellular milieu homeostasis and scavenging neuronal waste products, astrocytes actively contribute to synaptic communication. A number of studies show that astrocytes modulate synaptic transmission by releasing mediators called gliotransmitters, such as glutamate and adenosine triphosphate (ATP) (DeLeo et al., 2006). Astrocytes are also able to change their expression of voltage-gated channels and neurotransmitter receptors in response to increased synaptic signaling (DeLeo et al., 2006). Antibodies that bind to the cytoskeletal protein glial fibrillary acidic protein (GFAP) are a widely used specific marker for astrocytes (Eng et al., 1985). However, astrocytes are not a homogenous population of cells. Some astrocytes express GFAP, voltage-independent potassium currents, glutamate transporters and are coupled by gap junctions while others express low levels of GFAP and glutamate transporters, voltage-dependent sodium and calcium channels, AMPA receptors and are not coupled by gap junctions (Wallraff et al., 2004).

1.7 ASTROCYTES AND CYTOKINES

Peripheral inflammation or tissue injury results in activation of microglia and astrocytes in the dorsal horn (Gosselin et al., 2010). Activation of glia is associated with cell proliferation and morphological changes such as hypertrophy and increased arborization and ramification of processes. Other signs of glial activation consist of an increase of CD11b and GFAP (Milligan and Watkins, 2009). Glial activation also

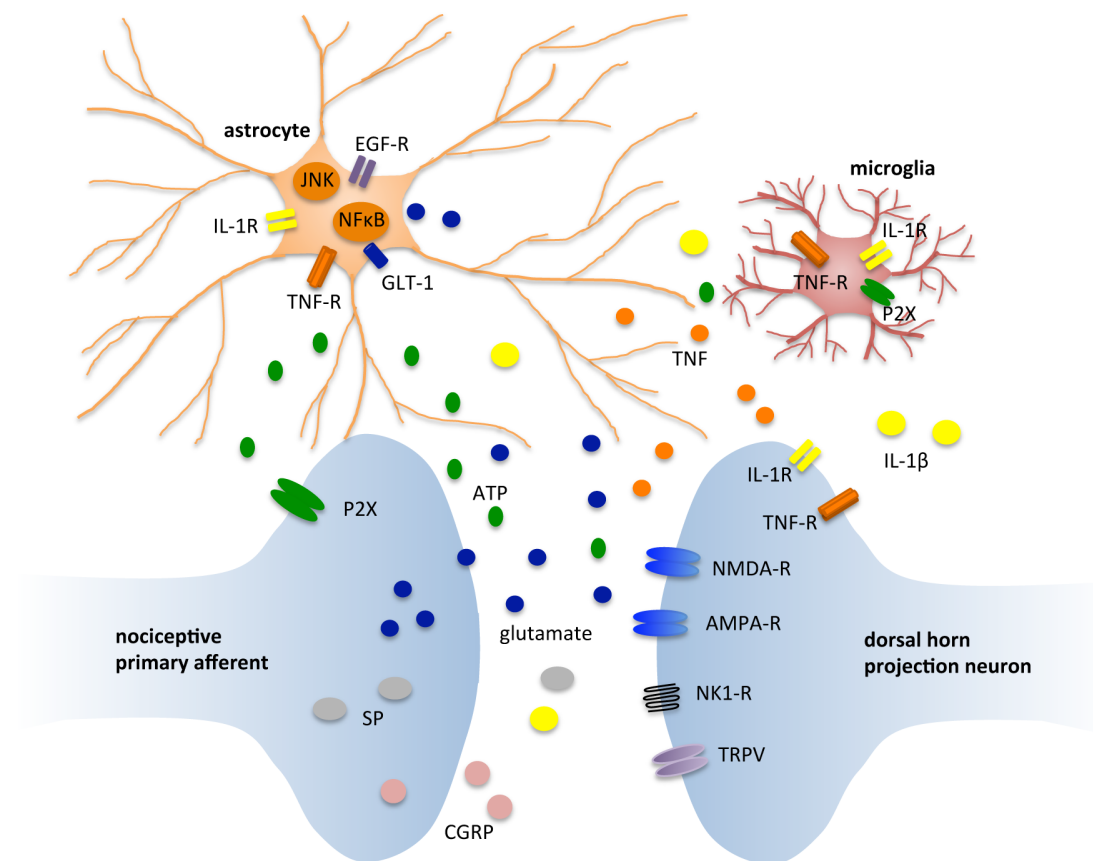


Fig. 1. Neuron-glia communication. Following a peripheral injury, primary afferent nerve terminals release neurotransmitters, such as substance P, CGRP and ATP as well as other mediators into the dorsal horn of the spinal cord leading to astrocyte and microglia activation and neuronal sensitization. Activated microglia and astrocytes release for example glutamate, ATP, IL-1 β and TNF, which bind to receptors on both neurons and glia, leading to an increase in synaptic transmission. The expression of GLT-1 on astrocytes is decreased on activated astrocytes, which impairs the glutamate uptake in the synaptic cleft further facilitating neuronal transmission. Cytokines such as TNF activate the JNK and NF κ B pathways in astrocytes, which increase NMDA and AMPA receptor activity and expression of inflammatory cytokines. CGRP, calcitonin gene-related protein; ATP, adenosine triphosphate; IL-1 β , interleukin 1 β ; TNF, tumor necrosis factor; GLT-1, glutamate transporter 1; JNK, c-Jun N-terminal kinase; NF κ B, nuclear factor kappa B; NMDA, N-methyl-D-aspartate; AMPA, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid.

includes release of cytokines and chemokines, such as IL-1 β , TNF and MCP-1. These factors are able to activate a number of intracellular signaling pathways, such as the intracellular mitogen-activated protein kinases (MAPKs) in both glial cells and neurons (Gosselin et al., 2010). The family of MAP kinases includes extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. The MAPKs are present in both glial cells and neurons and are important in a number of cellular processes. The MAPKs are also involved in nociceptive signaling in the dorsal horn. Activation of JNK, p38 and/or ERK leads to activation of a number of transcription factors, resulting in an

increased production of proinflammatory cytokines and chemokines, neurotrophic factors, prostaglandins and nitric oxide (Hameed et al., 2010).

1.8 mTOR

The pathway involving the serine threonine protein kinase mTOR is an important regulator of various cellular processes, such as cell metabolism, growth and proliferation, autophagy, ageing and synaptic plasticity (Zoncu et al., 2011; Laplante and Sabatini, 2009; Weichhart and Säemann, 2009). Dysregulation of mTOR may be involved in the etiology behind for example tumor formation, diabetes and neurodegenerative diseases (Laplante and Sabatini, 2009; Dazert and Hall, 2011).

The mTOR pathway is highly conserved from yeast to humans and is affected by a number of stimuli, including growth factors, energy status, amino acid levels and cellular stress (Weichhart and Säemann, 2009; Mendoza et al., 2011). The mTOR protein belongs to the phospho-inositide-3-kinase (PI3K) family, which includes enzymes such as ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK) (Fry, 2001). mTOR exists as two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The majority of the research on mTOR has been focused on mTORC1, primarily because of lack of pharmacological tools selective to mTORC2. Thus, the regulation of mTORC2 is less well understood. The main focus of this thesis is on mTORC1.

mTORC1 consists of the kinase mTOR, the scaffolding protein regulatory-associated protein of mTOR (RAPTOR), mammalian lethal with Sec13 protein 8 (mLST8), the proline-rich AKT substrate 40 kDa (PRAS40) and disheveled, egl-10, and pleckstrin (DEP)-domain containing mTOR-interacting protein (DEPTOR). The mTORC1 inhibitor rapamycin is a bacterial macrolide shown to have immunosuppressive and anti-proliferative properties (Wullschleger et al., 2006). Rapamycin forms a complex with FK506-binding protein 12 (FKBP-12) and inhibits the activity of mTORC1 by preventing FKBP-12 from binding to mTORC1. Rapamycin is largely selective for mTORC1, however, prolonged treatment with rapamycin in high doses may inhibit mTORC2 in some cell lines (Zoncu et al., 2011).

mTOR is activated by signals such as growth factors, insulin, amino acids and stress. The activation of, for example, a growth factor receptor leads to the activation of the kinase PI3K, which generates phosphatidylinositol 3,4,5 triphosphate (PIP₃). PIP₃ recruits the protein kinase AKT to the plasma membrane where it is activated by 3-phosphoinositide-dependent kinase 1 (PDK1) at thr-308. AKT phosphorylates the tuberous sclerosis complex 2 (TSC2), releasing the GTPase Ras homolog enriched in brain (Rheb), which directly activates mTORC1. Upon activation of mTORC1, p70S6 kinase (S6K) and translation initiation factor 4E-binding protein (4E-BP) is phosphorylated and activated. This in turn, promotes translation of cell growth and cell cycle proteins, such as c-Myc and cyclin D1 (Wullschleger et al., 2006). Activation of mTORC2 leads to activation and phosphorylation of AKT at ser-473, resulting in cellular events affecting cytoskeletal organization, metabolic regulation and survival (Wullschleger et al., 2006).

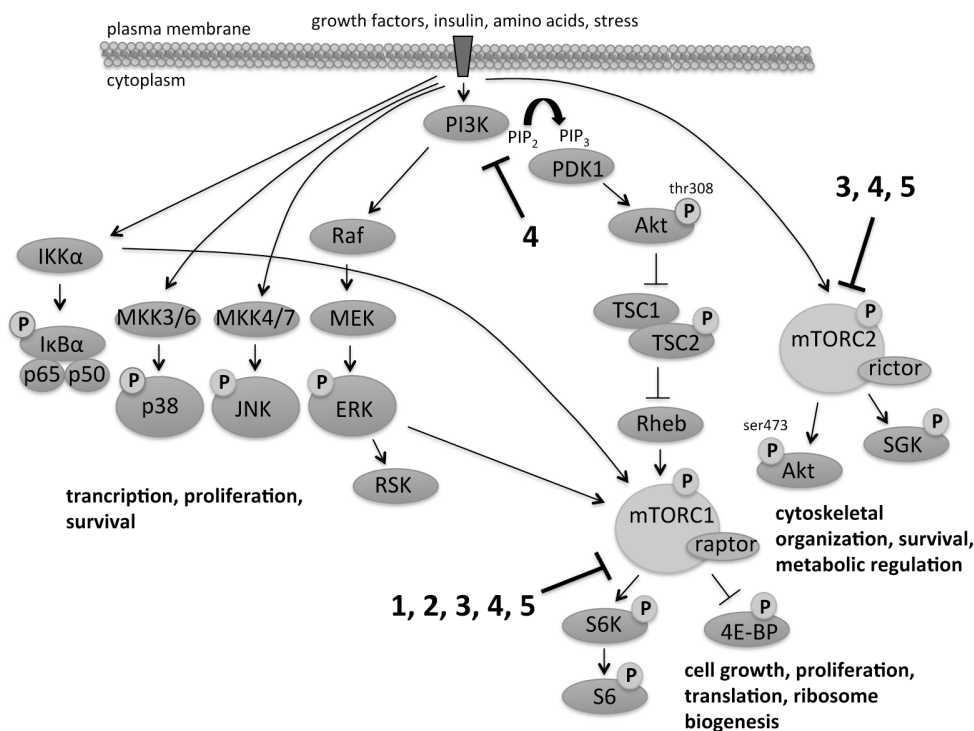


Fig. 2. The mTOR signaling pathway. Mammalian target of rapamycin (mTOR) is activated by stimuli such as growth factors, insulin, amino acids and stress. These stimuli lead to activation of PI3K and PDK1, which phosphorylate and activates AKT at thr-308. Activated AKT phosphorylates and inactivates TSC2, which together with TSC1 negatively regulates Rheb. Phosphorylation of TSC2 enables Rheb to bind mTORC1, leading to activation of downstream targets such as S6K, S6 and 4E-BP. The stimulating signal also activates mTORC2, phosphorylating its downstream targets AKT (ser-473) and SGK. Activation of mTORC1 and mTORC2 signaling pathways promotes cellular processes such as translation, ribosome biogenesis, transcription and actin organization. Available mTOR inhibitors are rapamycin (1), rapalogs (2), ATP-competitive inhibitors (3), PI3/mTOR inhibitors (4) and siRNA directed towards raptor and rictor (5). Arrows represent activation, whereas bars represent inhibition.

1.8.1 mTOR in pathology

As a major regulator of cell growth, proliferation and mobility, mTOR is commonly activated in cancer tumor cells. Constitutive activation of the mTOR pathway can lead to pathology and contribute to tumor growth (Zask, 2011). During recent years, a number of patents for mTOR inhibitors have been granted and several compounds are now in clinical trials for various cancer treatments. Rapamycin and its analogs, the rapalogs, are clinically important drugs used in patients subjected to organ transplantation, coronary disease and recently for the treatment of kidney cancer (Zask, 2011). However, the results from clinical trials with rapalogs have been less successful than expected. The limited effects seen in studies utilizing rapalogs may be because only mTORC1 is inhibited, leaving mTORC2 largely unregulated, and feedback signaling, where inhibition of mTORC1 leads to increased activation of other protein kinases, such as AKT and ERK (Carracedo et al., 2008). Recently, second generation mTOR inhibitors, so called TOR kinase inhibitors (TKIs), have entered clinical trials. These compounds inhibit mTORC1 and mTORC2 activity by directly acting on the ATP binding site on mTOR in an FKBP-12-independent fashion (Carew et al., 2011). Inhibitors targeting both PI3K and mTOR signaling pathways have also been evaluated in clinical trials and both TKIs and PI3K/mTOR inhibitors have proven more effective as antitumor therapeutics than rapalogs. Since several signaling pathways are commonly activated in cancer, these substances may have therapeutic advantages to the more selective rapalogs (Carew et al., 2011; Zask, 2011).

	Compound/class of compound	Target	Reference
1	Rapamycin	mTORC1	Price et al., 1992 Kuo et al., 1992
2	Rapalogs (e.g. everolimus, and temsirolimus)	mTORC1	Huang et al., 2003 Bayle et al., 2006
3	ATP-competitive mTOR inhibitors (e.g. Torin 1 and Torin 2)	mTORC1/2	Thoreen et al., 2009 Liu et al., 2012
4	Dual PI3K/mTOR inhibitors (e.g. NVP-BEZ235 and PI-103)	PI3K/mTORC1/2	Roper et al., 2011
5	siRNA: Rictor Raptor	mTORC1 mTORC2	Sarbassov et al., 2004 Jacinto et al., 2004

Table 1. mTOR inhibitors. Current approaches to inhibit mTOR signaling.

In recent years, studies have strongly indicated that mTOR is involved in pain processing. I.t. administered rapamycin inhibits formalin-induced hypersensitivity and intraplantar injection of rapamycin decreases mechanical allodynia induced by nerve injury or capsaicin (Price et al., 2007; Jiménez-Díaz et al., 2008). mTOR has been reported to be expressed and constitutively phosphorylated in myelinated A fibers in the skin (Jiménez-Díaz et al., 2008). However, if and how spinal mTOR regulates various forms of pain remains largely unexplained. In this thesis, the role of spinal mTOR in inflammation-induced pain is investigated.

2 AIMS

- I. To investigate the possible role of mTOR in inflammatory nociception
- II. To evaluate spinal astrocyte cultures as an in vitro model system to study molecular mechanisms involved in spinal sensitization
- III. To study molecular mechanisms of mTOR possibly contributing to spinal sensitization
- IV. To identify new potential regulators of inflammatory pain

3 MATERIALS AND METHODS

For a more detailed description of the materials and methods used, please see the Materials and Methods sections in each individual paper (I-III).

IN VIVO METHODOLOGY

3.1 LABORATORY ANIMALS (PAPER I, II AND III)

Adult male Holzman and Sprague-Dawley rats weighing 200-250 g were used (Harlan Industries, Indianapolis, IN, USA; Scanbur BK, Sollentuna, Sweden; Harlan, Netherlands; Charles River, Germany). The rats were housed in groups of 4-5 (Karolinska Institutet) or single-housed (University of California, San Diego). The rats were kept in rooms with a temperature of 20-22°C and a relative humidity of 40-65% on a 12-h light/dark cycle. Food and water were provided ad libitum. To minimize stress in connection with the experiments the animals were acclimatized for 5 days after their arrival to the animal department and handled once daily for one week prior to the initiation of experiments. All experiments were approved by and performed in accordance with the guidelines of the Ethical Committee of Northern Stockholm and the Institutional Animal Care Committee of the University of California, San Diego.

3.2 INDUCTION OF INFLAMMATORY PAIN AND PHARMACOLOGICAL TREATMENT (PAPER I)

To permit bolus i.t. injections, chronic lumbar i.t. injection catheters (PE-5) were implanted i.t. under isoflurane anesthesia with the caudal end at level T12, at the rostral margin of the lumbar enlargement. The catheters were externalized at the base of the skull as previously described (Yaksh and Rudy, 1976). The rostral end of each cannula was heat fixed to PE-10 tubing that was externalized to allow for injections. Five days of recovery was required prior to initiation of injections and behavioral testing. The rats were monitored daily and removed from the study if any signs of neurological dysfunctions were noted, if there was a weight loss greater than 10% over 5 days or if the cannula became occluded.

Rapamycin was delivered via i.t. catheter in 10 µl vehicle (0.5% ethanol in saline) followed by 10 µl saline flush. To induce local inflammation, 2% carrageenan was injected subcutaneously into the plantar side of the hindpaw under brief isoflurane anesthesia. Carrageenan was injected 10 minutes after rapamycin delivery. Nociceptive testing was initiated 1 hour after drug administration.

3.3 BEHAVIORAL TESTING OF NOCICEPTION (PAPER I)

3.3.1 Mechanical allodynia

To assess tactile allodynia, the rats were placed in individual plexiglas compartments with wire mesh bottoms. Following a 30-minute acclimation period, mechanical allodynia was tested using von Frey filaments and the Dixon up-down method as previously described (Chaplan et al., 1994). Briefly, calibrated filaments (Stoelting, Wood Dale, IL, USA) with buckling forces between 0.41 and 15.2 g were applied perpendicular to the mid-paw plantar surface until the filament was slightly bent and held there for 4-6 s. A positive response was noted if the paw was briskly withdrawn. Von Frey testing was performed prior to and 60, 120, 150, 180 and 240 minutes after injection of carrageenan.

3.3.2 Thermal hyperalgesia

Heat-evoked paw withdrawal response was assessed using a device similar to that described by Hargreaves and colleagues (Hargreaves et al., 1988). The apparatus consists of a glass surface (maintained at 25 °C) on which the rats are placed individually in plexiglas cubicles. The thermal nociceptive stimulus originates from a focused projection bulb positioned below the glass surface. A timer is actuated by the light source and latency is defined as the time required for the paw to show a brisk withdrawal as detected by phosphodiode motion sensors that stops the timer and terminates the stimulus. Tests were performed prior to and 90, 120, 150, 180, 240 and 360 minutes after injection of carrageenan.

IN VITRO METHODOLOGY

3.4 ESTABLISHMENT OF SPINAL ASTROCYTE CULTURES (PAPER II, III AND PRELIMINARY DATA)

Male Sprague-Dawley rats from Charles River (Germany) or Harlan (Netherlands) were used to generate spinal cell cultures. The animals were deeply anesthetized with isoflurane, decapitated and the spinal cords removed by hydroextrusion using cold saline. The spinal cords were homogenized in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS; Sigma), 50 U/ml penicillin, 50 µg/ml streptomycin and 1 mM sodium pyruvate (complete DMEM). The cells were cultured in complete DMEM at 37 °C in a humidified incubator with 5% CO₂ until approximately 90% confluency was reached. In paper II, the astrocytes were cultured in DMEM supplemented with 10% FBS purchased from Gibco (16000-044), Sigma (F7524), Hyclone (SH30079.03) or Astrocyte Medium (AM; ScienCell). AM was supplemented with 2% FBS (ScienCell), 50 U/ml penicillin, 50 µg/ml streptomycin and Astrocyte Growth Supplement (ScienCell) to provide a final concentration of 10 µg/ml bovine serum albumin (BSA), 10 µg/ml apo-transferrin, 5 µg/ml insulin, 2 ng/ml fibroblast growth factor 2 (FGF-2), 2 ng/ml insulin-like growth factor 1 (IGF-1), 1 µg/ml hydrocortisone and 20 nM progesterone. To remove microglia, the cells were shaken and the medium replaced with complete DMEM. As astrocytes in vitro are cultured in growth medium containing serum, the astrocytes are normally in an activated state. In order to return the astrocytes to a non-activated state, the cells were cultured in DMEM containing only 0.1% serum for 24 - 48 hours or serum-free DMEM for 48 hours prior to the initiation of experiments.

3.5 PHARMACOLOGICAL TREATMENT (PAPER I, II, III AND PRELIMINARY DATA)

In paper I, PC12 cells were pre-treated with 100 nM rapamycin for 30 minutes followed by stimulation with 20% FBS for 20 minutes. In paper II, spinal astrocytes were stimulated with 150 ng/ml platelet-derived growth factor-CC (PDGF-CC) for 4 hours. In paper III and Preliminary data, spinal astrocytes were pre-treated with 1 mM pentoxifylline, propentofylline or phosphate buffered saline (PBS) for 30 minutes, followed by stimulation with 50 ng/ml TNF or 200 ng/ml epidermal growth factor

(EGF) for 15 minutes. In other experiments, SH-SY5Y cells were stimulated with 50 ng/ml TNF for 15 minutes – 24 hours.

3.6 CELL LINES (PAPER I AND II)

The rat PC12 neuronal cell line and the human neuroblastoma SH-SY5Y cell line were purchased from ATCC, Manassas, VA, USA. Neurons were cultured in complete DMEM and serum starved with serum free DMEM for 24 hours prior to initiation of experiments.

Human spinal astrocytes were purchased from ScienCell (Carlsbad, CA, USA). The astrocytes were cultured in AM, supplemented with 2% FBS (ScienCell). Prior to the initiation of experiments the astrocytes were cultured in DMEM containing 0.1% AM or serum free DMEM for 48 hours.

3.7 WESTERN BLOT (PAPER I, II, III AND PRELIMINARY DATA)

Proteins extracted from lysed spinal astrocytes were subjected to NuPAGE 4-12% agarose gel electrophoresis (Invitrogen) followed by electrophoretic transfer to nitrocellulose membranes (Osmonics Inc., Minnetonka, MN, USA). After blocking non-specific binding sites with non-fat milk the membranes were incubated with primary antibodies overnight, followed by incubation with secondary antibodies. The primary antibodies p-S6K, p-S6, p-4E-BP1, p-JNK, p-ERK, p-AKT (ser-473), p-AKT (thr-308), cleaved caspase-3, I κ B α , caveolin-1, total S6K, total S6, total 4E-BP1, total JNK, total ERK, total AKT and GAPDH were all purchased from Cell Signaling Technology. β -actin was purchased from Sigma. The specific proteins were detected with chemiluminescent reagents (Supersignal, Pierce Biotechnology Inc., Rockford, IL, USA) and then stripped of antibodies and re-probed with subsequent primary antibodies as needed. Immunopositive bands were quantified using Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Data were normalized to β -actin or GAPDH.

3.8 QUANTITATIVE REAL-TIME PCR (PAPER I AND II)

mRNA from spinal cord or cultured spinal astrocytes was isolated with RNA Stat (Tel-Test, Friendswood, TX, USA) or Trizol (Invitrogen). Quantitative real-time PCR was

performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) to determine relative mRNA levels, using the GeneAmp 7000 Sequence Detection system (Applied Biosystems). Pre-developed specific primers were used to detect the genes of interest (Applied Biosystems). C6 cells stimulated with 20% serum for 2 hours were used as standard curve samples and used to calculate the cDNA concentration equivalents in the test samples. The data obtained were normalized to HPRT1 or GAPDH gene expression.

3.9 TUNEL ASSAY (PAPER II)

The effect of basal medium (DMEM) on cell survival was assessed by Tunel staining. Spinal astrocytes were cultured in AM followed by 48 hours of serum starvation. The cells were then fixed with 4% paraformaldehyde (PFA) and Tunel staining was performed using the Click-IT Tunel Alexa flour imaging assay (Invitrogen). The nuclei were visualized by incubation with Hoechst 33258 (Sigma). Images were acquired with a Cell observer microscope (Zeiss) and analyzed with CellProfiler (Carpenter et al., 2006). The amount of apoptotic cells was expressed as percent of Tunel positive cells.

3.10 CELL GROWTH ASSAYS (PAPER III)

3.10.1 MTT

Growth and viability of the astrocytes were measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) (5 mg/ml). The astrocytes were seeded at approximately 3,000 cells/well and treated with various concentrations of pentoxifylline or propentofylline (10 – 1,000 μ M) once daily for 1 - 6 days. The cells were incubated with 50 μ g MTT per well for 4 hours, followed by the addition of dimethyl sulfoxide (DMSO) to solubilize the formazan crystals. The absorbance of the purple formazan was measured at 570 nm. All samples were done in triplicate.

3.10.2 Cell count

To assess astrocyte proliferation, the astrocytes were seeded at approximately 40,000 cells/well and treated with pentoxifylline or propentofylline (1 mM) for 1 – 6 days. To count the cells, the astrocytes were trypsinized and resuspended in trypan blue. The viable cells were counted in a light microscope. All samples were done in triplicate.

3.11 TRANSFECTION (PRELIMINARY DATA)

Human SH-SY5Y neuroblastoma cells (ATCC, Manassas, VA, USA) were used for transfection using siRNA. Lipofectamine 2000 (Invitrogen) was used as a transfection agent and AMAXA Nucleofector (Lonza) was used for transfection by electroporation. SH-SY5Y cells were incubated with 50 nM caveolin-1 siRNA or negative siRNA (scrambled siRNA of similar length) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a control together with Lipofectamine in DMEM for approximately 6 hours. The growth medium was then replaced with complete DMEM. After 72 hours the cells were harvested and prepared for western blot analysis.

For transfection via electroporation, the cells were resuspended in the Nucleofector solution, 50 nM cav-1 siRNA or negative siRNA was added and the cells were then processed in the AMAXA Nucleofector apparatus. After the addition of complete DMEM, the cells were cultured in 37°C for approximately 48 hours, then cultured in serum free medium for 24 h, followed by harvest and western blot analysis.

3.12 HISTOLOGY AND CONFOCAL IMAGING (PAPER I, II AND III)

3.12.1 Immunohistochemistry

Animals were perfused and spinal cords harvested 4 hours after intraplantar carrageenan injection. The animals were deeply anesthetized with isoflurane and perfused intracardially with saline followed by 4% PFA. The lumbar spinal cords were removed and post-fixed in 4% PFA for 6 hours. Spinal cords were then cryoprotected in 20% sucrose for 48 hours and 30% sucrose for 24 hours. The lumbar segments L4-6 were frozen and cut into 14 or 30 μ m thick sections. The sections were permeabilized with Triton-X and non-specific binding sites were blocked with non-fat milk. Following incubation with the primary antibodies directed against p-S6 (ser-235/236), GFAP, OX42, Neuronal N (NeuN) and subsequent secondary antibody incubation, the sections were dried and mounted on glass slides using cover slip mounting medium containing DAPI. Confocal images were acquired using a confocal microscopy system (Zeiss, LSM 710) operated by LSM ZEN 2008 software.

3.12.2 Immunocytochemistry

Spinal astrocytes were cultured on cover slips treated with Poly-L-lysine and laminin. In paper II, the astrocytes were cultured in AM, Sigma, Gibco or Hyclone media. In paper III, the astrocytes were treated with 1 mM pentoxifylline, 1 mM propentofylline or 100 nM rapamycin once daily for 6 days. The cells were fixed with 4% PFA and permeabilized with Triton-X. Non-specific binding sites were blocked with non-fat milk and the cells were incubated with primary antibodies followed by secondary antibodies and mounted on glass slides using a cover slip mounting medium containing 4',6-diamidino-2-phenylindole (DAPI). Microglia contamination was quantified by counting cells positive for Iba1 and DAPI labeling. The counting was performed by a blinded experimenter using a ImageJ software (NIH). Confocal images were acquired using a confocal microscopy system (Zeiss, LSM 710) operated by LSM ZEN 2008 software. Images for quantitative measurements were collected with a Nikon Eclipse TE300 camera. Three images per coverslip were collected and analyzed. The results were presented as an average of the three images.

3.13 STATISTICAL ANALYSES

The statistical software GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA, USA) was used for all statistical analyses.

3.13.1 Nociceptive behavior

Tactile allodynia data are presented as withdrawal thresholds and area under the curve (AUC). Thermal hyperalgesia data are presented as thermal withdrawal latency and AUC. AUC represents the area under the time-effect curve after stimulation and defines the magnitude of carrageenan-induced sensitization. The percent reduction of baseline response latency is plotted against time and the resulting parameter is percent change per minute. Increasing values of AUC indicate increasing hyperalgesia. Data were analyzed by one-way analysis of variance (ANOVA) followed by Neuman-Keul's multiple comparison test. A p-value of <0.05 was considered statistically significant.

3.13.2 Western blot

Western blot membranes were imaged and the pixel intensities were normalized and expressed as mean \pm standard error of the mean (SEM). In paper I, drug treated samples

are compared to saline treated samples and presented as percent of control. In paper III and Preliminary data, PBS and drug treated samples are compared to TNF or EGF treated samples and presented as the percent of TNF or EGF treated samples. siRNA treated samples were compared to untreated or PBS treated samples. Data were analyzed by one-way ANOVA followed by Neuman-Keul's multiple comparison test. A p-value of <0.05 was considered statistically significant.

3.13.3 Quantitative real-time PCR

Data are expressed as relative units \pm SEM (paper I) or change in % from control \pm SEM (paper II). In paper II, changes in mRNA expression in test samples are compared to AM samples and presented as % change from AM \pm SEM. Data were analyzed by one-way ANOVA followed by Neuman-Keul's (paper I) or Bonferroni's (paper II) posthoc test. A p-value of <0.05 was considered statistically significant.

3.13.4 Immunocytochemistry

Data are expressed as % \pm SEM. One-way ANOVA followed by Bonferroni's posthoc test were used to analyze the data. A p-value of <0.05 was considered statistically significant.

4 METHODOLOGICAL CONSIDERATIONS

4.1 ANIMAL MODELS OF PAIN

There are a number animal models developed to mimic pain in humans. Models of inflammatory pain include those utilizing carrageenan, CFA, capsaicin and formalin, as well as various models based on evoked immune responses to endogenous collagen injection. Models of neuropathic pain include, for example, partial nerve damage or complete transection of a peripheral nerve.

There is a growing discussion regarding the validity and reliability of the current animal pain models. Considerable debate is ongoing concerning the duration of the pain and if a model should be considered chronic and if the methods of testing nociceptive behavior reflect actual symptoms of pain. In humans, pain is generally considered chronic when it persists more than six months (Russo and Brose, 1998). In animals, the time line generally is much shorter and more variable. In the carrageenan model of inflammatory pain, used in the present study, carrageenan is injected into the hindpaw of the animal and gives rise to a local inflammation characterized by swelling, redness and pain behavior. The symptoms of paw edema and increased pain behavior last for more than four days (Iadarola et al., 1988).

The issue of clinical relevance and translation from animal research to human conditions is one of constant debate. A major obstacle in research using animal models to mimic human disorders is communication between the subject and the experimenter. In pain research using humans as subjects, an important part of testing the pain is to ask questions to the subject. However, in animal research this is not possible. Instead, the nociceptive tests have to rely on the interpretation of the behaviors of animals in terms of nociception or pain.

To study pain mechanisms at a spinal level, drugs are commonly administered i.t., using i.t. catheters or via lumbar injections. A method of administering drugs by i.t. catheters in rats was developed by Yaksh and Rudy in 1976 and consists of surgically implanting a catheter starting at the base of the skull and terminating at the T12 level. The catheter may also be placed through the L5/6 interspace (Størkson et al., 1996).

The i.t. catheter method of drug delivery is a widely used method for spinal drug delivery but has been criticized for being more invasive than lumbar injections, where the animal is anesthetized for a shorter period and does not have a foreign object permanently implanted and possibly causing mechanical trauma and/or inflammation (DeLeo et al., 1997). Studies of the effects of i.t. catheterization have shown that post implantation, the expression of GFAP and OX42 in the spinal cord is increased (DeLeo et al., 1997). However, in the study by DeLeo and colleagues, a PE-10 catheter was used, which is thicker than the PE-5 catheter that was used in our study. We have also observed that the expression of GFAP and OX42 in the spinal cord following catheterization using PE-5 catheters is not increased (unpublished data). Following the surgical implantation of the catheter, the animals were monitored and removed from the study if any neurological dysfunctions were noted, if there was more than 10% weight loss over 5 days or if the catheter became occluded. Fewer than 5% of the animals were excluded from the study.

Although the use of i.t. catheters has been criticized, there are advantages in using this method. It allows for a continuous and repeated delivery of drugs and minimizes the risk of delivery of the drug in an unwanted location.

4.2 IN VITRO SYSTEMS

Cell cultures are often used as systems to study cellular mechanisms. These systems can be manipulated in a more controlled fashion compared to in vivo models and are suitable for studying a particular signaling pathway or a particular cell type. However, the controlled environment and lack of other cell types may result in a poor resemblance of the in vivo situation. These matters should be taken into consideration when interpreting data obtained from experiments on cell cultures.

Primary cell cultures are established from fresh tissue. Subsequent passages of cells are referred to as secondary cultures. Cell lines are established cell cultures that will proliferate indefinitely. Using primary cell cultures may be a way to mimic in vivo models in a more accurate way compared to cell lines, which are often of a tumor origin. However, primary cells have been shown to be more difficult to genetically manipulate with, for example, RNA silencing, compared to cells from a cell line.

In paper II, primary rat and human spinal astrocyte cultures were used and compared. However, the rat cultures were established from adult rats while the human astrocytes were of fetal origin. This issue may lead to differences in the outcome of the results and should be taken into consideration when interpreting the data.

5 RESULTS AND DISCUSSION

5.1 ROLE OF SPINAL mTOR IN INFLAMMATORY HYPERSENSITIVITY (PAPER I)

In paper I we investigated the intracellular mechanisms of mTOR in the spinal regulation of a peripherally induced injury using the carrageenan model of inflammatory pain.

To study carrageenan-induced activation of the mTOR pathway in the spinal cord, we measured the activation of factors both upstream and downstream of mTORC1 following injection of carrageenan. Carrageenan (4%) was injected into the plantar side of the hindpaw, resulting in a local inflammation of the paw, including increased swelling and redness. Studies have demonstrated phosphorylation of mTORC1 itself as a less reliable sign of activation than factors downstream of mTOR (Sabatini, 2006). Therefore, the downstream proteins S6K, S6 and 4E-BP1 are commonly used as readouts for mTORC1 activation. Previous reports have shown that the GTPase Rheb increases mTORC1 activation (Bai et al., 2007). In order to further assess activation of the mTOR pathway, we measured Rheb gene expression in the lumbar spinal cord following carrageenan injection. Measurements of activated proteins were made prior to, and at 1, 2, 4, 8, 12, 18 and 24 hours after carrageenan treatment.

Carrageenan injection resulted in activation of the mTOR pathway in the spinal cord. The carrageenan treated animals displayed an increase in phosphorylation and activation of S6K (Fig. 3), S6 and 4E-BP1 in the lumbar spinal cord 4 hours after induction of inflammation compared to naïve animals, indicating mTOR activation. A significant activation of 4E-BP1 was seen also at earlier time points, starting at 1 hour. Increased Rheb gene expression was seen at the 2-hour time point, providing further support for an activated mTOR pathway, and suggesting that carrageenan induces Rheb in the spinal cord, possibly leading to activation of mTOR.

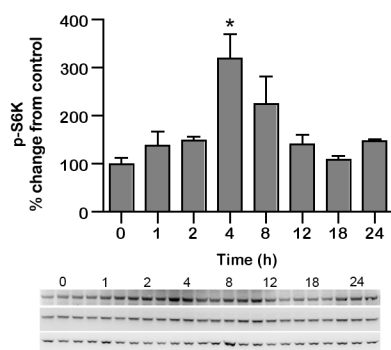


Fig. 3. A peripheral inflammation activates the mTOR pathway in the spinal cord. The histogram displays % change of p-S6K compared to naïve control ($t=0$ h) in the spinal cord at different time points ranging from 0-24 h following carrageenan-induced inflammation. A representative western blot of p-S6K is shown below the bar graph. Data are reported as mean \pm SEM, $n=4$. * denotes $p<0.05$ compared to naïve control.

To assess whether spinal inhibition of mTOR affects nociceptive behavior, we administered the mTOR inhibitor rapamycin (0.1, 1 or 3 μ g) i.t. 15 minutes prior to carrageenan injection. Nociceptive behavior was tested prior to, and at 90, 120, 150, 180 and 240 minutes after carrageenan injection. Calculation of the hyperalgesic index (AUC) showed that both thermal and mechanical hypersensitivity were significantly decreased following rapamycin treatment compared to vehicle and saline treated animals (Fig. 4A, B). This indicates that spinal mTOR regulates inflammation-induced spinal sensitization. The reduction of nociceptive thresholds correlate with the increase in 4E-BP1 phosphorylation, which was observed from 1 h to 4 hours after carrageenan injection. This suggests that 4E-BP1 regulates the initial mTOR-mediated hypersensitivity while S6K and S6, which displayed increased phosphorylation at the 4-hour time point, drive the hypersensitivity at later time points.

To determine the cellular location of activated mTOR in the spinal cord following a peripheral inflammation, we used immunohistochemistry and compared the tissue sections from animals with and without carrageenan treatment. P-S6 immunolabeling confirmed western blot data, displaying an increased phosphorylation of S6 in the ipsilateral superficial and deeper laminae 4 hours after carrageenan injection. Double labeling showed that p-S6 was localized in neurons but not in astrocytes or microglia. Rheb immunolabeling was seen in dorsal horn neurons, further supporting mTOR activation in neurons and not in glia.

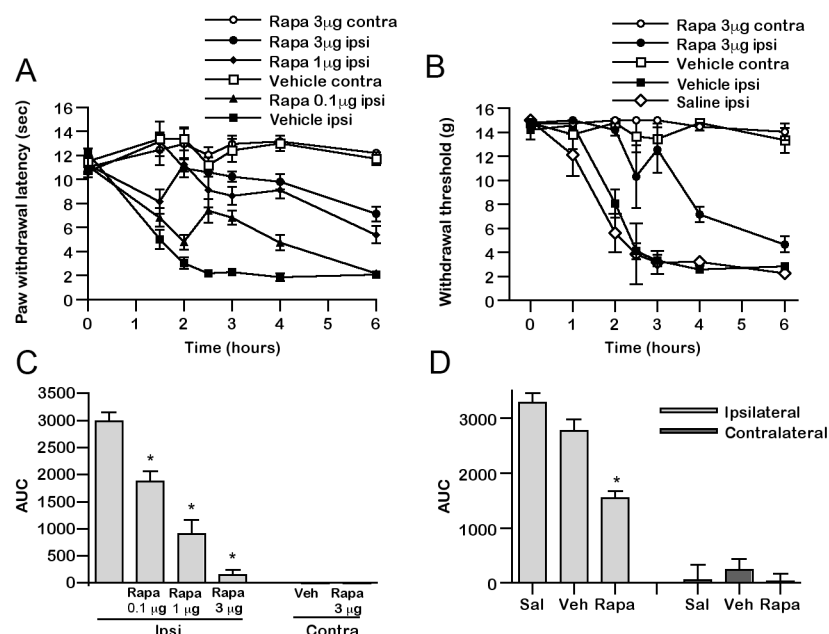


Fig. 4. Spinal mTOR inhibition attenuates carrageenan-induced pain behavior. Graphs display ipsilateral and contralateral (A) paw withdrawal latency reported in seconds (sec) and (B) tactile thresholds reported in grams (g) before ($t=0$) and after injection of carrageenan to the paw and i.t. injection of vehicle or rapamycin. The histograms display area under the curve (AUC, 0-240 min) for (C) tactile allodynia and (D) thermal hyperalgesia for each group shown in (A) and (B). Data are reported as mean \pm SEM, $n=6-7$. * denotes $p<0.05$ compared to vehicle control.

Our results demonstrate that carrageenan activates mTOR in dorsal horn neurons and not in microglia or astrocytes. This was later confirmed by Xu and colleagues (Xu et al., 2011), who showed activated mTOR in neurons in the superficial laminae following carrageenan injection. However, Xu and colleagues also demonstrated mTOR activation in astrocytes and microglia. These results are in contrast to our results, as we did not see any activation of the mTOR pathway in glial cells in the dorsal horn of the spinal cord.

Glial activation in animal models of inflammatory pain is less studied than in neuropathic pain models. In models of neuropathic pain, a clear upregulation of both astrocyte and microglial markers is seen (Garrison et al., 1991; Tanga et al., 2004). However, activation of glia is less clear in inflammatory models. Although biochemical changes can be seen in activated glial cells, the morphological signs of activation are less obvious (Schreiber et al., 2008; Eijkelkamp et al., 2010). While astrocyte and microglia activation is strong in models of neuropathic pain, only moderate activation is commonly seen in models of inflammatory pain. An upregulation of GFAP is seen after

carrageenan injection in the knee (Gabriel et al., 2009) and a moderate activation of microglia and astrocytes is seen after injection of the inflammatory agents zymosan or formalin (Sweitzer et al., 1999). A clear astrocyte activation has been reported in a model of bone cancer pain, which includes a strong inflammatory component (Honore et al., 2000; Yao et al., 2011). Spinal treatment with glia inhibitors such as minocycline, pentoxifylline or fluorocitrate decrease inflammation-induced pain behavior, which also suggests an inflammation-induced glia activation in the spinal cord (Hua et al., 2005; Saito et al., 2010; Gao and Ji, 2010).

There are several confounding factors that have to be considered when interpreting and comparing results of studies on glial activation, including differences due to the choice of animal model and the timeframe used for the experiments. Spinal astrocyte activation has also been demonstrated to change depending on the housing situation of the laboratory animals. Housing of the animals socially (several animals per cage) in an enriched environment decreases astrocyte activation and mechanical allodynia induced by carrageenan injection in the knee compared to single-housed animals without an enriched environment (Gabriel et al., 2009).

The moderate glial activation induced by carrageenan may explain the activation of mTOR found in neurons and not in glia. There may be a possibility for mTOR activation in glial cells in models of a more pronounced glial activation. In a study by Codeluppi and colleagues, mTOR activation was seen in astrocytes following spinal cord injury (Codeluppi et al., 2009). Géranton and colleagues demonstrated constitutive mTOR activity in glia 7 days following spared nerve injury, although no increase in mTOR activity in glial cells was seen compared to control animals (Géranton et al., 2009). These results indicate that mTOR may be activated in glial cells during certain pathological conditions. Unpublished data from our laboratory show increased activation of mTOR in dorsal horn neurons and not in glial cells following spinal nerve ligation on day 3 compared to control animals. These disparate findings highlight the complexity of the role of mTOR in pathology.

5.2 CHARACTERIZATION OF SPINAL ASTROCYTE CULTURES (PAPER II)

Primary cell cultures are commonly used as tools to study cellular mechanisms. It is therefore important to evaluate the cultures as in vitro systems in order to increase reproducibility and to produce more valid results. In paper II, we investigated what the optimal conditions for cultured rat spinal astrocytes should be to acquire characteristics that are most similar to cultured human spinal astrocytes. We characterized and compared spinal astrocytes in vitro originating from human spinal cord tissue and rat spinal cord tissue from two different rat substrains, cultured in media supplemented with four different types of sera.

The purity of the culture used is of essential importance for the reliability of the results obtained. Microglia is a common contaminator in astrocyte cultures and may lead to poor results. Therefore, the level of microglia contamination was determined in cultures established from Harlan Sprague Dawley and Charles River Sprague Dawley rats using Iba-1 immunolabeling, and Cd11b and Il-1 β mRNA expression. Charles River astrocyte cultures were contaminated with microglia to a higher extent compared to Harlan astrocyte cultures. Harlan cells cultured in AM displayed the lowest levels of microglia contamination compared to Hyclone-, Sigma- or Gibco-supplemented media. AM is supplemented with 1 μ g/ml hydrocortisone, which inhibits microglia proliferation and may be the reason for the lower levels of microglia in the astrocytes cultured in AM (Gaunter et al., 1992). In addition, AM contains lower serum levels (2%) compared to Hyclone, Sigma or Gibco media, which are supplemented with 10% serum. The lower serum concentration may also add to the reduced microglia growth in the AM cultured astrocytes. This is in accordance with a previous study demonstrating similar results using primary neuronal cultures (Fischer et al., 1992).

To investigate whether culture medium alters the phenotype of cultured rat astrocytes, gene expression of commonly used astrocyte markers were monitored, including glutamine synthetase (Gs), connexin-43 (Cnx-43), GFAP, S100 binding protein β (S100 β), glutamate transporter-1 (Glt-1) and aldehyde dehydrogenase 1 member L1 (Aldh1L1). Harlan and Charles River rat astrocytes were compared, cultured in AM, Sigma-, Gibco- or Hyclone-supplemented media. Charles River rat astrocytes displayed higher levels of the astrocyte markers Gs, Cnx-43 and Glt-1 when cultured in AM

compared to the other media (Fig. 5A). Harlan rat astrocytes expressed similar levels of astrocyte markers, suggesting that culturing rat astrocytes in AM results in cells with a more astrocyte-like phenotype.

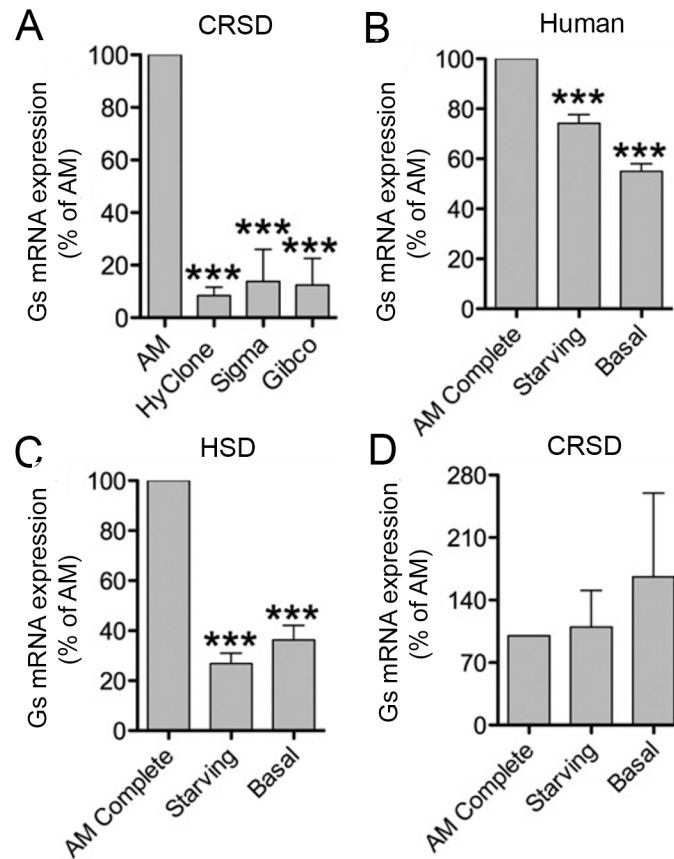


Fig. 5. Effects of culture medium and serum starvation on human and rat astrocytes in culture. (A) Charles River rat astrocytes (CRSD) cultured in Astrocyte Medium (AM) express higher mRNA levels of the astrocyte marker glutamine synthetase (Gs) compared to rat astrocytes cultured in Hyclone, Sigma or Gibco media. (B) Human and (C) Harlan rat astrocytes (HSD) but not (D) Charles River rat astrocytes decrease the mRNA levels of Gs in response to 48-hour serum starvation (Starvation) and complete serum withdrawal (Basal) compared to complete AM. Data are reported as mean \pm SEM, $n=3$. *** represents $P<0.001$ compared to AM levels.

Our data show that culturing astrocytes in AM results in astrocytes with higher levels of Gs, Cnx-43 and Glt-1. Based on these findings, we investigated the effects of serum starvation on rat and human astrocytes after culturing the cells in AM. Serum starvation of Harlan and Charles River rat astrocytes resulted in a decrease in Gs (Fig. 5C, D) and GFAP levels (Harlan) and an increase in Glt-1 levels (Charles River). These changes are consistent with a more quiescent astrocyte phenotype following serum starvation. Thus, the effects of serum withdrawal appear to result in a more resting state of the astrocytes from both substrains.

The human astrocytes responded to serum starvation with decreased levels of Gs (Fig. B) and GFAP compared to complete AM, similar to the effects of Harlan rat astrocytes (Fig. 5C). This suggests that Harlan rat astrocytes cultured in AM respond in a more similar fashion to human astrocytes than Charles River rat astrocytes.

The Harlan astrocytes appear to have similar mRNA expression patterns as the human astrocytes. Thus, to further examine the characteristics of these cells, the protein expression of the astrocyte markers were investigated using immunocytochemistry. Both human and Harlan astrocytes expressed GFAP, nestin, GLT-1, S100 β and Aldh1L1.

Taken together, these results indicate that spinal astrocytes originating from human and Harlan rats have similar gene and protein expression patterns when cultured in AM compared to media supplemented with sera from Hyclone, Sigma or Gibco. In addition, microglia contamination is significantly reduced in astrocyte cultures grown in AM compared to astrocytes cultured in Hyclone-, Sigma- or Gibco-supplemented media, suggesting that culturing cells in AM leads to the optimal conditions for producing human and rat astrocytes with comparable characteristics.

5.3 THE mTOR PATHWAY AS A POTENTIAL TARGET FOR INHIBITING INFLAMMATORY PAIN SIGNALING IN THE DORSAL HORN (PAPER III AND PRELIMINARY DATA)

Identifying new targets to develop new pharmaceutical drugs to treat pain is an important step in the search for more effective pain drugs. In these studies, we investigated the role of mTOR in inflammatory cell signaling in both astrocytes and neuron-like cells to further provide insight into mTOR signaling.

5.3.1 Pentoxifylline and propentofylline inhibit TNF-induced mTOR activation in cultured spinal astrocytes (Paper III)

Release of cytokines and activation of astrocytes and microglia in the dorsal horn of the spinal cord are likely to be important for central sensitization following nerve injury and peripheral inflammation. By inhibiting glial activation in the spinal cord with, for example, methyl xanthines, nociception caused by inflammation can be reduced (Vale et al., 2004; Dorazil-Dudzic et al., 2004). Thus, glial inhibition may be a potential

approach to treat pain. In paper III, we investigated the effects of the methyl xanthines pentoxifylline and propentofylline on astrocyte activation and TNF-induced mTOR activation in cultured spinal astrocytes.

As described above, astrocyte activation is characterized by proliferation and morphological changes. Hence, we determined the effects of pentoxifylline and propentofylline on astrocyte proliferation and morphology. To measure proliferation of spinal astrocytes in culture, we used the MTT and cell count assays. Spinal astrocytes were cultured according to the protocol above (see Materials and Methods). Briefly, the cells were cultured in medium containing 10% serum, treated with various concentrations (0.1 μ M - 1 mM) of pentoxifylline or propentofylline once daily for 1 to 6 days and proliferation was measured daily. Both pentoxifylline and propentofylline inhibited astrocyte proliferation (Fig. 6A, B, C, D). This finding is compatible with the hypothesis that mTOR regulates astrocyte proliferation.

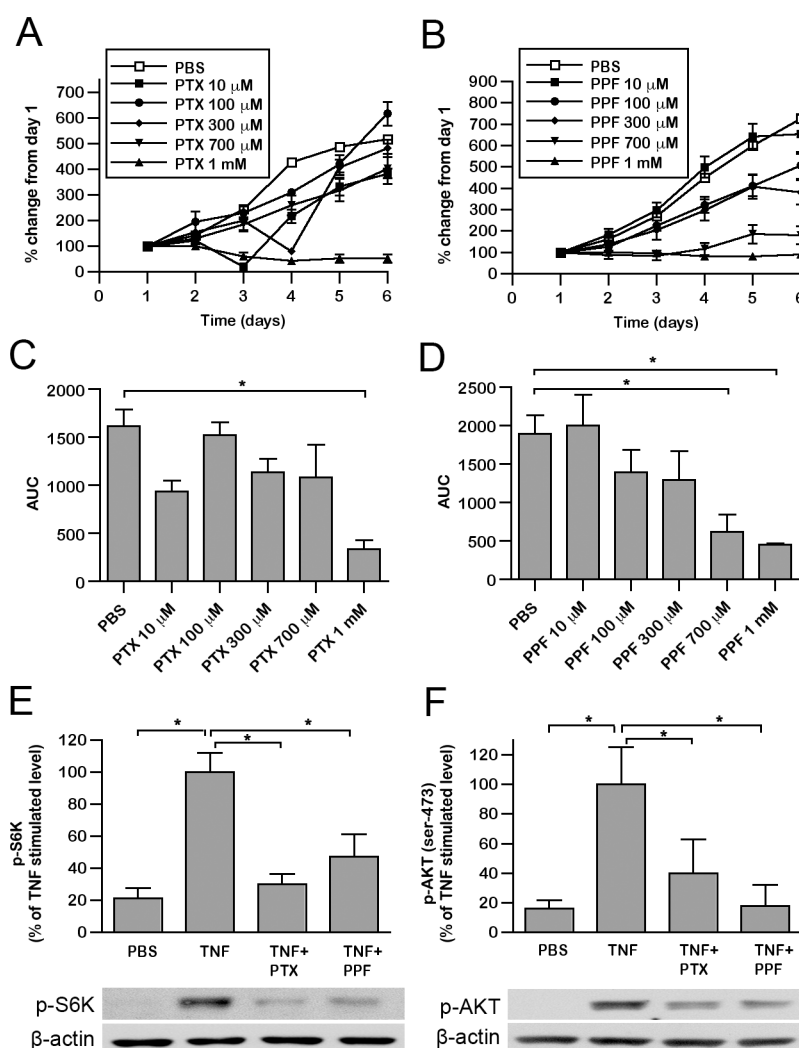


Fig. 6. Pentoxifylline and propentofylline inhibit astrocyte proliferation and TNF-induced mTOR activation. The graphs (A) and (B) display astrocyte growth after treatment with PBS or increasing concentrations of (A) pentoxifylline (PTX) or (B) propentofylline (PPF). The histograms (C) and (D) display area under the curve (AUC) for inhibition of astrocyte growth by (C) pentoxifylline and (D) propentofylline shown in (A) and (B). The bar graphs (E) and (F) display the levels of (E) p-S6K and (F) p-AKT (ser-473) following treatment with PBS, TNF only or TNF following pre-treatment with pentoxifylline (PTX) or propentofylline (PPF). Levels are expressed as % of TNF stimulated level. Representative western blots of p-S6K and p-AKT (ser-473) are shown below each histogram. Data are presented as mean \pm SEM. n = 5-6. * denotes $p < 0.05$ compared to control.

A previous study has shown that propentofylline changes the morphology of astrocytes, turning them from an active state to a more quiescent phenotype (Tawfik et al., 2006). Our results show that pentoxifylline also produces this effect (Fig. 7A, B, C). Interestingly, the mTORC1 inhibitor rapamycin does not change morphology of the astrocytes, suggesting that this effect is regulated by mTORC2 rather than mTORC1 (Fig. 7A, D).

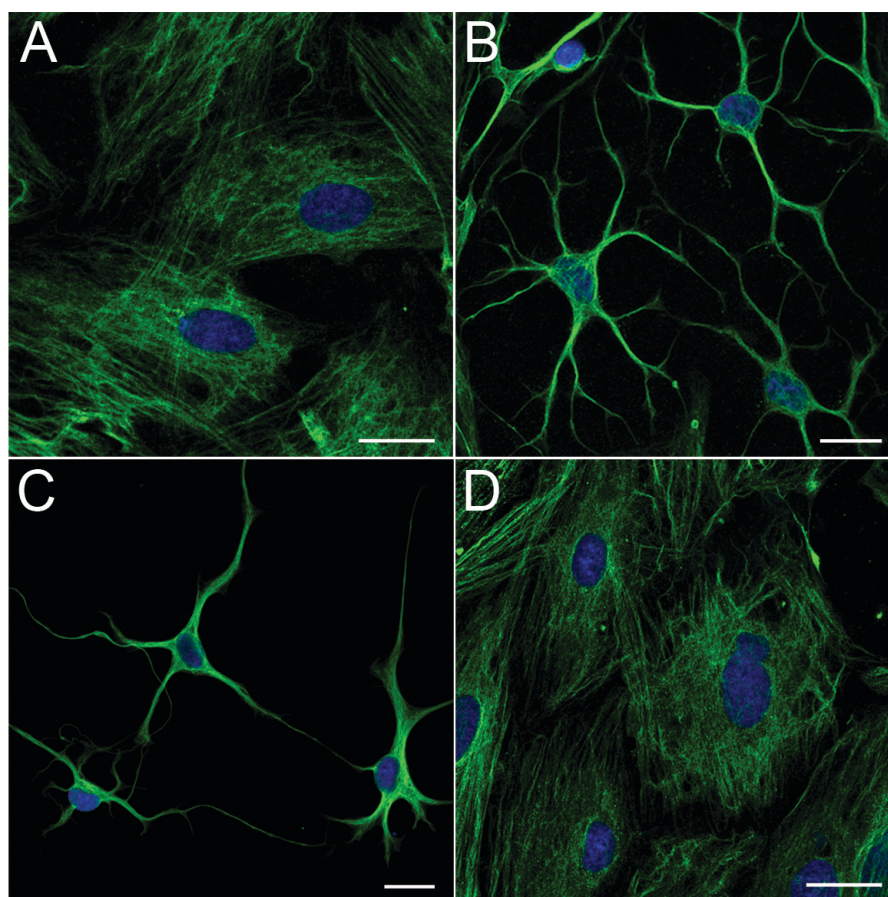


Fig. 7. Pentoxifylline and propentofylline but not rapamycin change the morphology of spinal astrocytes. Confocal microscopy images depicting vimentin immunoreactivity in spinal astrocytes cultured in medium containing 10% FBS following treatment of (A) PBS, (B) pentoxifylline, (C) propentofylline or (D) rapamycin. Scale bar represents 20 μ m.

To determine TNF-induced mTOR activation, the spinal astrocytes were cultured in serum-starved medium for 24 hours prior to the experiment, followed by pre-treatment with pentoxifylline or propentofylline for 30 minutes and stimulation with TNF for 15 minutes. TNF stimulation caused a significant increase in p-S6K, indicating activation of mTORC1 (Fig. 6E).

TNF also activated AKT (ser-473), commonly used as an indicator for mTORC2 activation (Fig. 6F). Pre-treatment with pentoxifylline or propentofylline led to a reduction of TNF-induced activation of both p-S6K and p-AKT (ser-473) (Fig. 6E, F). Thus, our data suggest that TNF activates both mTORC1 and mTORC2, and pentoxifylline and propentofylline can inhibit both pathways.

TNF is a known activator of MAPKs and the nuclear factor kappa B (NF κ B) pathway. Therefore, we investigated the effects of pentoxifylline and propentofylline on ERK, JNK and NF κ B activation. Stimulation with TNF produced a significant activation of ERK, JNK as well as the NF κ B pathway activation as shown by degradation of I κ B α . Pentoxifylline significantly inhibited TNF-induced JNK activation. Propentofylline treatment led to a similar, though not statistically significant effect. Neither pentoxifylline nor propentofylline counteracted the TNF-induced activation of ERK and NF κ B. These results indicate that pentoxifylline and propentofylline inhibit TNF-induced activation of JNK in spinal astrocytes, while the NF κ B pathway on the other hand, seems to be activated by TNF without any modulatory influences by pentoxifylline and propentofylline.

To further investigate whether mTOR activation is regulated by JNK, spinal astrocytes were pre-treated with a common JNK inhibitor, SP600125, prior to TNF stimulation. The mTOR pathway was activated by TNF, as previously shown. As with pentoxifylline and propentofylline, SP600125 significantly counteracted the TNF-induced increase of p-S6K, suggesting that TNF-induced mTORC1 activation in spinal astrocytes may be regulated by JNK.

To summarize, our results suggest that pentoxifylline and propentofylline inhibit astrocyte activation and TNF-induced mTOR activation. In addition, JNK inhibition also leads to reduced mTOR activation.

5.3.2 mTOR in cultured spinal astrocytes is activated by EGF but is not inhibited by pentoxifylline and propentofylline (Preliminary data)

EGF has been shown to increase astrocyte proliferation and to increase GFAP expression by activating the EGF receptor (EGF-R) (Liu and Neufeld, 2004). The EGF-R is absent in mature astrocytes in the normal mammalian CNS (Gomez-Pinilla et al., 1988). However following CNS injury, EGF is released by microglia and the EGF-R is expressed on reactive astrocytes (Nieto-Sampedro et al., 1988). In response to ligand binding, the EGF-R dimerizes and activates an intrinsic tyrosine kinase generating a complex that results in activation of intracellular signaling pathways such as PI3K/AKT, ERK and p38 (Zhang and Neufeld, 2007).

A previous study reported that EGF activates mTOR in spinal astrocytes in vitro and by inhibiting mTOR astrocyte proliferation is reduced (Codeluppi et al., 2009). Here, we investigated if EGF-induced mTOR activation could be inhibited by pentoxifylline and propentofylline.

Spinal astrocytes were cultured according to the protocol above (see Materials and Methods). As in Paper III, the astrocytes were cultured in serum-starved DMEM for 24 hours prior to initiation of the experiments. Astrocytes were pre-treated with 1 mM pentoxifylline or 1 mM propentofylline for 30 minutes, followed by stimulation with 200 ng/ml EGF for 15 minutes and then subjected to western blot. The membranes were incubated with primary antibodies against p-S6K, total S6K, p-AKT (ser-473), p-AKT (thr-308), total AKT, p-JNK, total JNK, p-ERK, total ERK or β -actin.

Western blot analysis demonstrated that EGF stimulation activated mTORC1, as seen by significantly increased levels of S6K phosphorylation, confirming previous results (Fig. 8A) (Codeluppi et al., 2009). EGF also significantly activated AKT (thr-308) compared to PBS treatment (Fig. 8B). AKT (thr-308) is upstream of mTORC1 and is part of the PI3K-AKT-mTORC1 pathway, thus confirming an activation of the mTORC1 pathway. In addition to activating mTORC1, EGF also significantly increased p-AKT (ser-473), indicating mTORC2 activation (Fig. 8C).

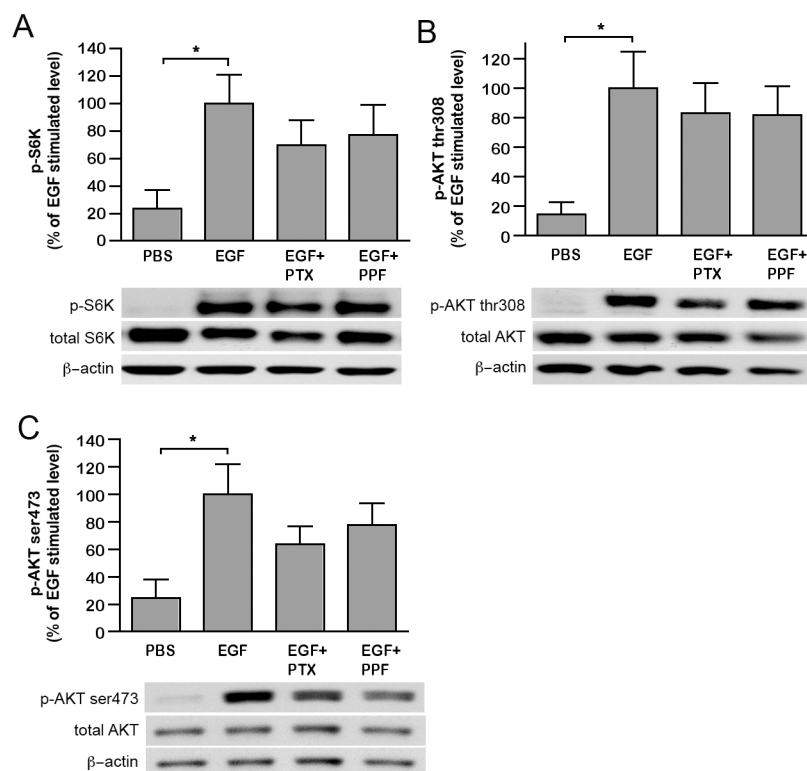


Fig. 8. Pentoxifylline and propentofylline do not inhibit EGF-induced mTOR or AKT activation in spinal astrocytes. The bar graphs display the levels of (A) p-S6K, (B) p-AKT (thr-308) and (C) p-AKT (ser-473) following treatment with PBS, EGF only or EGF following pretreatment with pentoxifylline (PTX) or propentofylline (PPF). The levels of phosphorylated proteins are expressed as % of EGF stimulated levels. Representative western blots of p-S6K, p-AKT (thr-308) and p-AKT (ser-473) are shown below each bar graph. Data are presented as mean \pm SEM, $n = 5-6$. * denotes $p < 0.05$ compared to the EGF treated group.

EGF significantly activated the MAPKs JNK and ERK (Fig. 9A, B). Treatment with pentoxifylline or propentofylline did not significantly inhibit activation of either of these proteins, though there was a tendency towards inhibitory effects (Fig. 9A, B).

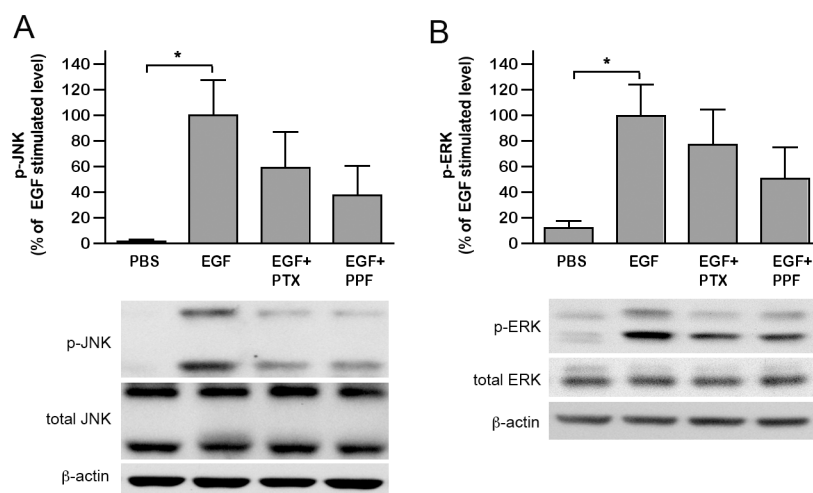


Fig. 9. Pentoxifylline and propentofylline do not inhibit EGF-induced JNK and ERK activation in spinal astrocytes. The bar graphs display the levels of (A) p-JNK and (B) p-ERK following treatment with PBS, EGF only or EGF following pretreatment with pentoxifylline (PTX) or propentofylline (PPF). The levels of phosphorylated proteins are expressed as % of EGF stimulated levels. Representative western blots of p-JNK and p-ERK are shown below each bar graph. Data are presented as mean \pm SEM, n = 5-6. * denotes $p < 0.05$ compared to the EGF treated group.

Proliferation is a hallmark of astrocyte activation and is driven by cytokines and growth factors that are released from surrounding microglia. It has previously been shown that EGF drives astrocyte proliferation mediated by mTOR (Codeluppi et al., 2009). Here we confirm that mTORC1 is activated by EGF in spinal astrocytes. However, in contrast to TNF-induced mTOR activation, treatment with pentoxifylline or propentofylline does not inhibit EGF-induced mTOR activation. EGF activated pathways similar to TNF, shown by activation of JNK, ERK and AKT, but pentoxifylline and propentofylline did not inhibit the activation of these factors when stimulated by EGF. One explanation for these results could be that pentoxifylline and propentofylline act upstream in the mTOR pathways, possibly on a receptor level, interacting with the TNF receptor.

5.3.3 Limited evidence found to support a role for caveolin-1 in the regulation of TNF-induced mTOR activation in a neuron-like cell line (Preliminary data)

Caveolin-1 is a membrane protein that is the primary component of caveolae. Caveolae are vesicular invaginations of the plasma membrane, where a variety of receptors, including the TNF receptor, are localized (D'Alessio et al., 2005). Caveolin-1 has also been found to act outside caveolae as a “molecular break” by directly interacting with, and thereby inhibiting, the kinase activity of many signaling molecules that are induced by growth factors, for example EGF (Couet et al., 1997). Caveolin-1 has been linked to, for example, inflammation and cancer, where it has been shown to regulate cell proliferation, cytokine production and NF κ B activation, (Williams and Lisanti, 2005; Wang et al., 2006; Ohnuma et al., 2004). Caveolin-1, which is expressed in most cells, including neurons, is induced by TNF in endothelial cells and forms a complex with the TNF receptor upon TNF stimulation (Feng et al., 2001; Head and Insel, 2007; Wang et al., 2008). Silencing of caveolin-1 blocks TNF-induced PGE₂ production and COX-2 expression, two factors associated with increased pain (Wang et al., 2008). Also, increased gene expression of caveolin-1 in DRGs has been seen in an experimental

model of peripheral inflammation (subcutaneous injection of CFA) (Rashid-Doubell, 2010). As mTOR has been shown to mediate inflammatory pain, we hypothesized that caveolin-1 may regulate mTOR activation induced by TNF.

Human SH-SY5Y neuroblastoma cells were cultured according to the protocol above (see Materials and Methods). Caveolin-1 was knocked down with siRNA using Lipofectamine 2000 as the transfection agent or using the AMAXA Nucleofector transfection system. Following transfection, the cells were stimulated with TNF for 5 minutes – 1 hour and then processed and subjected to western blot. The membranes were incubated with primary antibodies against p-S6K, p-JNK, caveolin-1 or GAPDH.

Previous studies have shown that TNF increases expression of caveolin-1 in endothelial cells (Wang et al., 2008). To investigate whether TNF increases caveolin-1 also in SH-SY5Y cells, cells were incubated with TNF for 1 - 24 hours. TNF stimulation did increase caveolin-1 expression at 24 hours in these cells, indicating a regulatory role for caveolin-1 in TNF signaling (Fig. 10A).

To determine if TNF activates the mTOR pathway in SH-SY5Y cells and to determine the time point for maximum activation, we stimulated the cells with TNF for 5, 15, 30, 45 and 60 minutes. The results showed that TNF activates the mTOR pathway in SH-SY5Y cells, as shown by an increase in p-S6K in TNF stimulated samples compared to control samples (Fig. 10B). Further, the maximum increase of p-S6K occurred at 15 minutes of TNF stimulation (Fig. 10B). This duration of incubation was used in the following experiments to activate mTORC1.

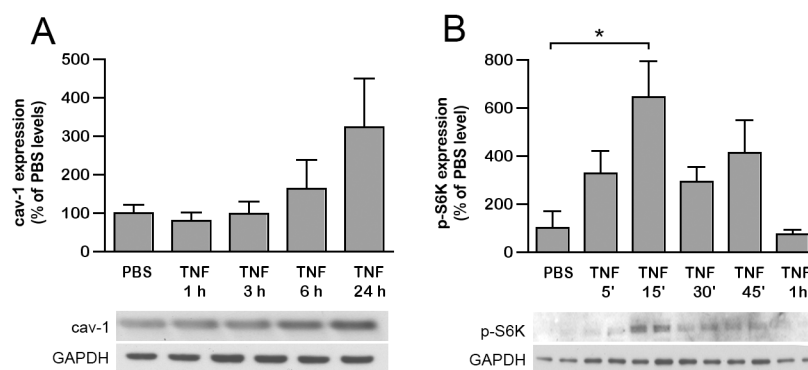


Fig. 10. TNF increases caveolin-1 expression and activates mTORC1 in SH-SY5Y cells. The bar graphs display the levels of (A) caveolin-1 (cav-1) and (B) p-S6K following treatment with (A) PBS or TNF for 1, 3, 6 or 24 hours or (B) PBS or TNF for 5, 15, 30, 45 or 60 minutes. The data are expressed as % of PBS treated levels. Representative western blots of caveolin-1 and p-S6K are shown below each bar graph. Data are presented as mean \pm SEM, $n = 2-4$.

To investigate the role of caveolin-1 in mTORC1 activation, we used siRNA as a way to silence caveolin-1 in SH-SY5Y cells. Transfection with caveolin-1 siRNA using Lipofectamine or the AMAXA Nucleofector reduced caveolin-1 expression in SH-SY5Y cells compared to control siRNA transfection (Fig. 11A). TNF stimulation for 15 minutes activated the mTOR pathway but caveolin-1 silencing did not inhibit mTORC1 activation compared to control samples (Fig. 11B).

As caveolin-1 has been implicated in MAPK signaling, we also investigated the effects of caveolin-1 silencing on TNF-induced JNK activation in SH-SY5Y cells (Zschocke et al., 2005; Wang et al., 2006; Head et al., 2011). TNF stimulation for 15 minutes activated the JNK pathway in SH-SY5Y cells. However, caveolin-1 silencing did not inhibit this activation (Fig. 11C). Expression of caveolin-1 after TNF stimulation for 5 min – 1 h was not changed (Fig. 11D).

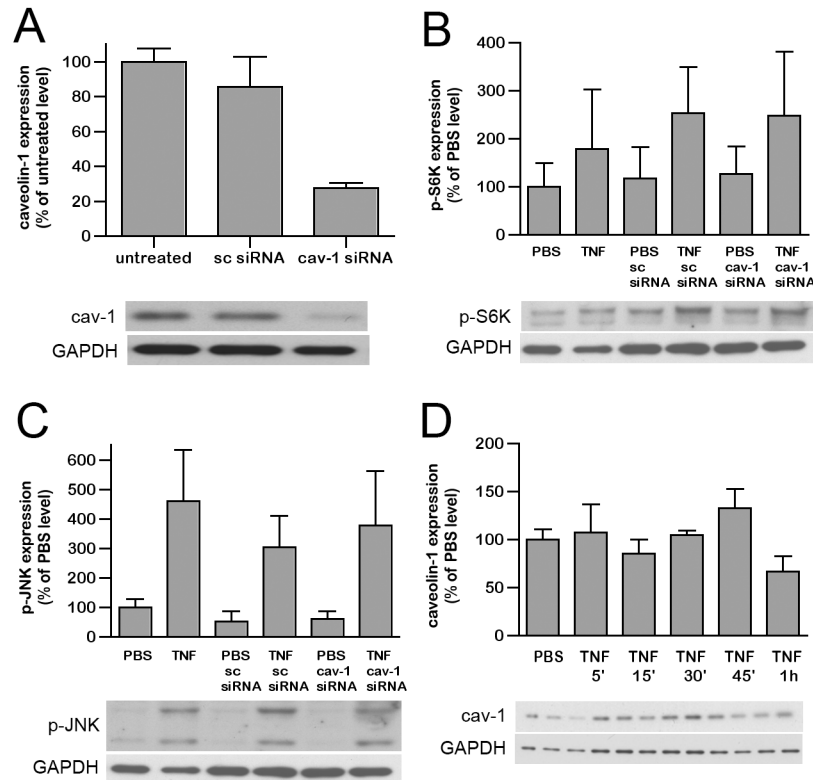


Fig. 11. Caveolin-1 RNA knock-down does not affect TNF-induced mTOR or JNK activation and 1 hour TNF stimulation does not change caveolin-1 expression. The bar graph (A) displays the levels of caveolin-1 (cav-1) following no treatment, scrambled siRNA (sc siRNA) or caveolin-1 siRNA (cav-1 siRNA). The bar graphs (B) and (C) display (B) p-S6K and (C) p-JNK levels following treatment with PBS only, TNF only, PBS or TNF with scrambled siRNA or PBS or TNF with caveolin-1 siRNA. The bar graph (D) displays the levels of caveolin-1 following treatment with PBS or TNF for 5, 15, 30, 45 and 60 minutes. Data are expressed as % of PBS treated levels. Representative western blots of caveolin-1, p-S6K and p-JNK are shown below each bar graph. Data are presented as mean \pm SEM, $n = 2-4$.

Caveolin-1 is a protein that has recently received attention as a potential therapeutic target in several diseases, mainly due to its role in mediating cellular signaling. Studies have shown that it has a role in inflammation and TNF signaling (D'Alessio et al., 2005; Feng et al., 2001). Previous studies have shown that TNF induces caveolin-1 expression in endothelial cells and our results indicate that TNF induces caveolin-1 also in SH-SY5Y cells.

As with mTOR, caveolin-1 appears to have opposing functions as a regulator of inflammation. Caveolin-1 seems to have a pro-inflammatory role through facilitation of TNF signaling in endothelial cells, and an anti-inflammatory role in macrophages, where caveolin-1 interacts with the toll-like receptor 4 (TLR4) upon lipopolysaccharide (LPS) stimulation, thereby reducing TNF and interleukin 6 (IL-6) production (Wang et

al., 2009). Knock down of caveolin-1 in endothelial cells inhibits TNF-induced PGE₂ and COX-2 expression, while in lung and colon tissue, caveolin-1 binds to COX-2 and increases COX-2 degradation, indicating both pro- and anti-inflammatory actions of caveolin-1 (Chen et al., 2010). Genetic deletion of caveolin-1 has a protective role against atherosclerosis and irritable bowel disease, suggesting a pro-inflammatory function of caveolin-1 whereas other data show that caveolin-1 is able to inhibit NFκB activation induced by LPS in lung tissue, demonstrating caveolin-1 as anti-inflammatory, further supporting opposing functions of caveolin-1 in the inflammatory process (Garreau et al., 2006; Frank et al., 2008; Chidlow et al., 2009). These data highlight the importance and complexity of caveolin-1 in inflammatory cell signaling.

Caveolin-1 has a regulatory function in autophagy, which is also controlled by mTOR (Martinez-Outschoorn et al., 2011; Jung et al., 2010). Furthermore, caveolin-1 regulates activation of the PI3K/AKT pathway in mouse embryonic stem cells, which is part of the mTOR pathway and caveolin-1 suppresses the mTOR-regulated protein cyclin D1 in a fibroblast cell line (Hulit et al., 2000; Park and Han, 2009). Thus, there may be a link between mTOR and caveolin-1. Silencing of caveolin-1 in SH-SY5Y cells did not, however, affect mTOR activation induced by TNF. Caveolin-1 has been implicated in MAPK signaling in various cell types (Zschocke et al., 2005; Wang et al., 2006; Head et al., 2011). However, TNF-induced JNK activation in SH-SY5Y cells was not changed by caveolin-1 silencing. This suggests that caveolin-1 may not be an important regulator in mTOR or JNK signaling induced by TNF. However, because silencing protein expression with siRNA does not yield a 100% loss of protein, the remaining caveolin-1 protein may be sufficient to maintain cellular signaling. Also, mTOR and JNK signaling events are transient, and our results demonstrate that a longer stimulation with TNF (24 hours) produced an increase in caveolin-1 expression. Caveolin-1 may be involved in regulating these pathways at other time points that were not measured in this study. It would be interesting to investigate targets further downstream of mTOR and JNK, in order to look at cellular events occurring at later time points. It is also important to consider that the effects of caveolin-1 on intracellular signaling are both stimulus- and cell type-specific and it is possible that knocking down caveolin-1 in another cell type may have an effect on mTOR signaling.

6 GENERAL DISCUSSION

The results of this thesis support a role for mTOR in nociceptive signaling in the spinal cord. Inhibition of mTOR in the spinal cord of rats by i.t. administration of rapamycin, prior to induction of a peripheral inflammation, decreases pain-related behavior, indicating that mTOR is important for inflammatory pain signaling in the dorsal horn. Previously, local inhibition of mTOR at the site of inflammation has been reported to result in reduced pain behavior, indicating a peripheral role of mTOR (Jiménez-Díaz et al., 2008). Thus, the anti-nociceptive effect of rapamycin following systemic administration is likely to be the result of a combination of peripheral and systemic mechanisms (Orhan et al., 2010; Obara et al., 2011).

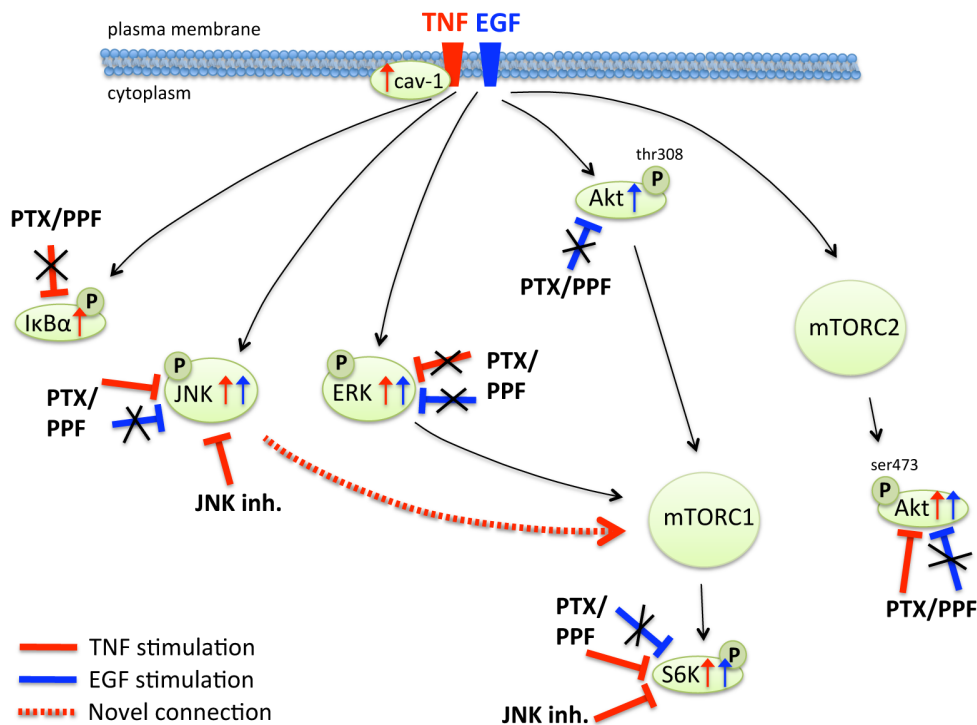


Fig. 12. Summary of findings. Both TNF and EGF activate the JNK, ERK, mTORC1 and mTORC2 pathways in spinal astrocytes. TNF also activates the NFκB pathway. Pentoxifylline and propentofylline inhibit TNF-induced JNK, mTORC1 and mTORC2 activation but not ERK or NFκB activation. Pentoxifylline and propentofylline do not inhibit EGF-induced activation of JNK, ERK, mTORC1 or mTORC2. A novel connection between JNK and mTORC1 has been demonstrated. Caveolin-1 expression is increased after TNF stimulation.

Our results together with other studies of mTOR in pain have clearly shown a role for mTOR in pain processing. Several studies have focused on investigating mTOR in neuropathic or formalin-induced pain but our results indicate that mTOR is also

involved in the regulation of inflammatory pain and these data have recently been corroborated by another study (Price et al., 2007, Jiménez-Díaz et al., 2008; Géranton et al., 2009; Asante et al., 2009, 2010; Xu et al., 2011). The exact role of mTOR in pain signaling is, however, far from clear. mTOR may be expressed in both A and C-fibers as well as in glial cells, and activation of mTOR seems to occur in different cell types depending on the pain model used (Jiménez-Díaz et al., 2008; Géranton et al., 2009; Asante et al., 2009). We show mTOR activation in dorsal horn neurons after a peripheral carrageenan injection and no upregulation of mTOR in glial cells. The study by Xu and colleagues demonstrates activation of mTOR in astrocytes and microglia after carrageenan injection, however, these results may be due to the poor specificity of the antibodies used in the study (Xu et al., 2011). In the spared nerve injury model, mTOR seems to be activated in astrocytes, although no increase in mTOR activation was seen 7 days after nerve injury (Géranton et al., 2009). In contrast, unpublished data from our laboratory show increased activity of mTOR in dorsal horn neurons and not in glia 3 days following spinal nerve ligation. These differing results may be explained by different models of neuropathic pain and different time points being investigated, but may also indicate a complicated role of mTOR in pain signaling.

It is still largely unknown if there are cell type-dependent differences in mTOR signaling. Although differences in the effects of mTOR inhibition have been reported between various cell types, the mechanisms are not clear. mTOR is expressed constitutively in most cell types and can be induced after tissue damage (Jiménez-Díaz et al., 2008; Géranton et al., 2009; Xu et al., 2010; Xu et al., 2011). The function of mTOR is complex, as it is involved in many cellular processes in both an inhibitory and facilitatory roles. Studies on innate immune cells demonstrate mTOR regulating both pro- and anti-inflammatory processes, suggesting that mTOR may have different, even opposing functions depending on cell type (Weichhart and Säemann, 2008). Seemingly opposing effects have also been reported for PI3K, a kinase upstream of mTOR, which regulates multiple signaling pathways depending on the cell type and organism (Weichhart and Säemann, 2008). It is also plausible that there are compensatory mechanisms that are activated after prolonged inhibition of mTOR. For example, such phenomena occur after long-term pain treatment with opioids, where compensatory mechanisms are activated, opposing the original pain modulating effects (Ahlbeck, 2011). Indeed, some of the side effects observed with long-term treatment of rapamycin

following organ transplantations are of a pro-inflammatory nature (Weichhart and Säemann, 2008).

A substantial part of the current preclinical pain research focuses on intracellular targets in order to modulate pain signaling. It is exciting to note that some of the preclinical drug candidates have advanced to clinical trials. Recently, a phase II clinical trial was completed for a p38 MAPK inhibitor. The inhibitor was tested for treatment of nerve injury-induced neuropathic pain and was found to be well tolerated and to reduce pain compared to placebo (ClinicalTrials.gov, NCT00390845). However, pain is a complex process and is comprised of several signaling pathways in different types of cells that all contribute to nociception. To target only one pathway may not be sufficient to treat pain. MAPK and mTOR signaling pathways have overlapping functions and involve phosphorylation of many common substrates, promoting cell survival and proliferation. In addition, there is extensive cross-talk between the pathways, regulating each other both positively and negatively, leading to a potential risk of resistance or reduced effects of therapeutics targeting only one pathway (Mendoza et al., 2011). Therefore, using a combination of drugs that each selectively targets a pathway may be a possible alternative. In the treatment of cancer and traumatic brain injury a combination of mTOR and ERK or AKT inhibitors has been reported to be more effective than mTOR inhibition alone (Proud et al., 2010; Park et al., 2011). Using a combination of drugs may hypothetically also reduce the doses needed for treatment, which may decrease potential side effects. Inhibitors that are able to inhibit not only mTORC1 but also mTORC2 have also recently become available, which may lead to an improved outcome in the treatment of pain.

Another issue concerning cross-talk between signaling pathways is the impact of oscillations in intracellular pathway activation (Kholodenko, 2000). In vitro studies have shown that activation of a common substrate by the MAPK and mTOR pathways may occur at different time frames, depending on regulatory loops (Yamnik and Holz, 2010). A signaling pathway may oscillate in activation with another pathway, which may be a way for the system to continuously respond to a sustained stimulus (Mendoza et al., 2011). To target more than one signaling pathway may be an option to increase the effectiveness of treatment.

Since the first studies of microglia and astrocyte activation were published, a large number of studies have shown glial activation in animal models of pain (Garrison et al., 1991; Coyle et al., 1998; Honore et al., 2000; Tsuda et al., 2005; Ji and Suter, 2007). However, to date there is little evidence of a similar activation of microglia and astrocytes in humans. To determine glial activation in humans is associated with methodological difficulties. A study from 2009 used post mortem tissue of a patient with complex regional pain syndrome, where spinal activation of both microglia and astrocytes was seen (Del Valle et al., 2009). Other studies have shown an increase in S100 β , a protein released by astrocytes, in cerebrospinal fluid (CSF) in patients with lumbar disc herniation and in serum of children with headaches (Brisby et al., 1999; Papandreou et al., 2005). These findings support the hypothesis that glial activation is important in pain signaling in human subjects as well as in animal models. However, more studies are required to determine the role of a glial activation in humans in pain conditions.

In 2009, the first clinical trial of a drug for pain treatment with glia as a target was conducted (ClinicalTrials.gov, NCT00813826). The efficacy of propentofylline was evaluated in patients with post-herpetic neuralgia. However, the results were disappointing as propentofylline failed to show efficacy. This may suggest that glial activation does not play a major role in neuropathic pain in humans. However, although glial activation in rodents has been shown in several models of neuropathic pain, it has not been shown in post-herpetic neuralgia (Watkins et al., 2012). Further, glia inhibitors may still be efficient in other types of pain states, such as inflammatory pain.

The failure of many clinical trials has sparked discussions regarding the possibility to translate preclinical animal data to clinical patients. A recent study reports differences between human and rodent cellular responses to an inflammatory stimulus and to propentofylline as an explanation for the failure of the clinical trial using propentofylline (Landry et al., 2011). The authors suggest more comparative studies of human cellular responses to rodent cells are warranted. In paper II, we demonstrate substrain and species differences between cultured astrocytes and also differences in expression patterns of astrocyte proteins depending on the type of culture medium used. In studies comparing human and rodent cells, it is important to consider the origin and the treatment of the cells. Cultured human cells deriving from the CNS, such as

microglia and astrocytes, are in many cases derived from fetal tissue while rodent cultures are often generated from adult brain or spinal cord tissue. Also, the process of acquiring human tissue and generating cultures is often more time consuming than generating rodent cultures and for obvious ethical reasons human cells are not available in the same way as rodent cells. Because of this, the human and rodent cells are in many cases not generated in identical ways. Despite these difficulties in achieving comparable cell cultures from humans and rodents, more research on the differences between human and rodent cells in culture is needed, as it may be a valuable way of explaining differences between results from animal studies and clinical trials.

The development of pharmacological pain therapeutics has proven to be a difficult and complex challenge and current options are often ineffective or poorly tolerated. Therefore, it is important to explore new approaches to treat pain, including investigating different cell types and signaling pathways that can mediate nociception. Although further studies are required, mTOR shows promise as a potential molecular target for drugs designed to treat pain. In addition to targeting mTOR itself, pharmacological interference with factors associated with mTOR signaling, that can indirectly regulate activation of mTOR and other signaling pathways may prove useful. One such factor that may be linked to mTOR regulation is caveolin-1, a protein that can act as a regulating factor in several disease states. Although the importance of caveolin-1 in pain signaling is not yet clarified, the links to TNF signaling suggest a possible role in inflammatory pain processing and further studies are required to determine if caveolin-1 is an important regulator of mTOR signaling.

7 CONCLUSIONS

1. Our results provide evidence for a regulatory role of spinal mTOR in nociceptive signaling induced by a peripheral inflammation.
2. Differences in cell culture conditions including rat substrain and culture media can profoundly affect the cell biology of astrocytes in culture.
3. Pentoxifylline and propentofylline inhibit astrocyte activation, possibly through inhibition of mTOR. However, the inhibitory properties of these methyl xanthines on mTOR activation in astrocytes appear to be stimulus-dependent.
4. Preliminary data indicate a role for caveolin-1 in TNF signaling, suggesting a possible role of caveolin-1 in inflammatory pain signaling. Its function in mTOR signaling may be less important although further studies are warranted.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Förmågan att känna smärta är en av våra viktigaste kroppsfunktioner som skyddar oss från skador och hjälper till så att uppkomna skador läker snabbare. Långvarig smärta är dock ett stort problem för många människor. Trots många års forskning kring smärta är de behandlingsalternativ som finns tillgängliga idag ofta inte tillräckligt effektiva och många har dessutom allvarliga biverkningar. För att utveckla nya, mer effektiva läkemedel mot smärta är det viktigt att hitta nya verkningsmekanismer och mål ("targets") för farmaka att verka på. Denna avhandling handlar om proteinet mTOR i nervceller och gliaceller, framför allt astrocyter, som ett nytt sådant möjligt mål för farmakologisk behandling av inflammatorisk smärta. Avhandlingen innehåller även metodologiska studier som visar hur olika faktorer kan påverka egenskaperna hos astrocyter i kultur.

För att undersöka vilken roll mTOR spelar vid inflammatorisk smärta användes en inflammatorisk smärtmodell i råtta. Resultaten visade att inflammationen aktiverade mTOR i ryggmärgen och att smärtbeteendet hos djuren kunde minskas genom att hämma mTOR. Detta tyder på att mTOR är inblandat i mekanismerna bakom inflammatorisk smärta.

För att studera cellsignalering används ofta odlade celler i kultur. Dessa system kan lätt manipuleras eftersom de är känsliga för yttre faktorer som till exempel sammansättningen av det tillväxtmedium som cellerna växer i. Ett problem med att använda cellkulturer från djur är dock att det kan vara svårt att översätta resultaten till vad som händer i mänskliga celler. Det är därför viktigt att klargöra hur man på bästa sätt kan få cellkulturer från djur att i största möjliga mån likna cellkulturer från människa. Resultaten i denna avhandling visar att valen av tillväxtmedium och djurstam har stor betydelse för egenskaperna hos odlade astrocyter från råtta och hur stor likhet som kan uppnås mellan cellkulturer från råtta och människa.

Djurstudier har visat att gliaceller i ryggmärgens bakhorn aktiveras i samband med smärtsamma tillstånd. Genom att blockera denna aktivering kan man minska smärtbeteendet hos djuren. I avhandlingen påvisas att hämning av astrocytaktivering med två gliahämmare samtidigt blockerar aktiveringen av mTOR i astrocyterna. Denna hämning av mTOR verkar dock vara beroende av på vilket sätt astrocyterna aktiveras.

Upptäckten av nya mekanismer för att minska smärta är mycket viktig för att kunna framställa nya smärtiläkemedel. Resultaten i denna avhandling pekar på mTOR som en ny angreppspunkt för att kontrollera inflammatorisk smärta. I fortsatta studier bör, förutom mTOR, även proteiner som är sammankopplade med mTOR undersökas. Detta kan skapa nya förutsättningar för att utveckla nya, effektiva och säkra smärthämmande läkemedel.

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