The role of protein kinase M zeta in chronic pain

Hibatulnaseer Nasir

Department of Experimental Medicine

McGill University, Montreal

April, 2016

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Abstract

Background

More than 20% of the world adult population suffers from chronic pain. Evidence shows that transition from acute to chronic pain depends on neuroplasticity in the spinal cord dorsal horn (SCDH). Although molecules responsible for the initiation of long-term memory in the SCDH have been identified, little is known about the maintenance of its long-term synaptic plasticity. PKMζ is a persistently active kinase that contributes uniquely to the maintenance of LTP and memory storage in the hippocampus. Interestingly, PKMζ is upregulated in SCDH after persistent nociception, and its inhibition using the myristoylated ζ-pseudosubstrate inhibitory peptide (ZIP) in the brain or spinal cord alleviates nociception. However, the role of ZIP and PKMζ action during persistent pain, and its exact regulation in the spinal cord are unknown. Here, we determine the role of PKMζ in models of persistent, long-lasting pain.

Results

Pharmacological inhibition of PKMζ in the rat had no effect on the reduced ipsilateral PWTs observed in rats with spared nerve injury (SNI), but transiently elevated contralateral PWTs, which were reduced several weeks after the injury. No change in reduced PWTs following hind paw inflammation induced by CFA was observed after i.t. ZIP or scrZIP treatment. ZIP reduced the late phase of nociception induced by 2% formalin, but only partially affected nociceptive responses to 3.5% formalin, and did not affect nociceptive responses to 5% formalin. ZIP pretreatment, and post-treatment prevented and reversed, respectively, i.c. capsaicin-induced remote allodynia, and ZIP post-treatment at either 24 h or 1 week persistently reduced i.m. acidic saline-induced remote allodynia, while no pain relieving effect was observed following i.t. scrZIP or full-length PKC inhibitor NPC15437, suggesting PKMζ may be responsible for the maintenance of remote allodynia. Moreover, ZIP prevented, and reversed long-lasting persistent mechanical allodynia in a

modified hyperalgesic priming model using either i.c. or i.pl. capsaicin. Importantly, we show for the first time that ZIP reversed long-lasting established allodynia following both priming and challenging i.c. capsaicin injections. Genetic deletion of *prkcz* supported the above pharmacological findings. Thus, $prkcz^{-/-}$, but not $prkcz^{+/-}$ or WT mice displayed attenuated responses in remote allodynia models, but not SNI-induced neuropathic pain. Importantly, these pain attenuation effects of *prkcz* KO were significantly more pronounced in male than in female mice.

We showed that i.t. injection of 10 nmol NMDA produced a significant decrease in PWTs for 24 - 48 h that was reversed by i.t. ZIP. Concordantly, a significant increase in PKMζ protein levels was observed 60 min post i.t. NMDA. Further, using pharmacological inhibition, we assessed the role of several PKMζ regulatory elements identified in hippocampal studies on spinal NMDA-induced hyperalgesia. Here, we found that spinal inhibition of PIN1 using juglone (5-hydroxynaphtho-quinone), which decreased PIN1 and increased PKMζ, but not full-length PKCζ, exacerbated i.t. NMDA-induced allodynia. Additionally, inhibition of PDK1 using GSK-2334470, which significantly reduced PKMζ phosphorylation, but not total PKMζ levels, prevented the development of NMDA-induced allodynia. Likewise, pretreatment with pep2m, an inhibitor of the interaction between *N*-ethylmaleimide-sensitive fusion protein (NSF) and the glutamate receptor subtype 2 (GluR2), prevented NMDA-induced allodynia. These data suggest PIN1, PDK1 and an NSF-GluR2 interaction are required for the establishment of PKMζ-dependent NMDA-induced hyperalgesia.

Significance

With its unique structure and function, PKMζ promises to be a key target for the treatment of chronic pain that depends on neuroplasticity. These studies have provided a detailed cellular and systems level analysis of the role of PKMζ in the maintenance of maladaptive neuroplasticity contributing to persistent pain. Our results illustrate a role for PKMζ in the maintenance of long-lasting persistent pain, particularly in remote allodynia models, where the contribution of central

sensitization can be separated from the role of peripheral inputs. Our results using varying concentrations of formalin highlight the notion that the analgesic effects of PKMζ inhibition may be masked at higher concentrations that produce increased peripheral inflammation. Further, our studies have contributed not only to an increased understanding of PKMζ regulation of the spinal cord, but also of its specific role in spinal nociception-induced neuroplasticity. Understanding spinal neuroplasticity is essential for drug development benefiting patients that currently cannot be treated optimally with state-of-the-art analgesics.

Résumé

Plus de 20% de la population mondiale adulte souffre de douleur chronique. Les évidences prouvent que la transition de la douleur aiguë à la douleur chronique dépend de la neuroplasticité dans la corne dorsale de la moelle épinière (CDME). Bien que les molécules responsables de l'initiation de la mémoire à long terme dans la CDME ont été identifiées, peu est connu sur le maintien de sa plasticité synaptique à long terme. La protéine kinase Mζ (PKMζ) est une kinase active de manière persistante qui contribue uniquement au maintien de la potentialisation à long terme (PLT) et au stockage de la mémoire dans l'hippocampe. Cependant, il est intéressant de noter que PKMζ est régulée à la hausse dans la CDME après une nociception persistante, et son inhibition en utilisant l'inhibiteur de peptide pseudosubstrate-myristoylée ζ (IPZ) dans le cerveau ou la moelle épinière inverse la nociception. Toutefois, le rôle du IPZ et l'action de PKMζ pendant la douleur persistante, et sa régulation exacte dans la moelle épinière sont inconnus. Nous déterminons ici le rôle de PKMζ dans des modèles de douleur persistante de longue durée.

Résultats

L'inhibition pharmacologique de PKMζ chez le rat n'a eu aucun effet sur les seuils de retrait de la patte ipsilatérale (SRP) réduits observés chez les rats épargnés de lésion nerveuse (ELN), mais a transitoirement élevé les SRPs contralatérales, qui ont été réduits plusieurs semaines après la blessure. Aucun changement dans les SRPs réduits après l'inflammation de la patte arrière induite par l'adjuvant complet de Freund (ACF), n'a été observé après le traitement par IPZ ou scrIPZ. IPZ a réduit la phase tardive de la nociception induite par le formol 2% mais a affecté seulement partiellement les réponses nociceptives à 3,5 % de formol, et n'a pas affecté les réponses nociceptives à 5 % de formol. IPZ, mais pas scrIPZ, ni NPC15437, a inversé l'allodynie mécanique induite par l'injection de solution saline acide après 24 h ou 1 semaine post-induction de douleur. Le pré-traitement et post-traitement par IPZ ont évité et renversé l'allodynie mécanique induite par la capsaïcine intracolonique (CAPi.c.), alors qu'aucun effet soulageant la douleur n'a été observé

après l'injection i.t. de scrIPZ, suggérant que PKMζ peut être responsable de l'entretien de l'allodynie à distance. En outre, IPZ a empêché, et inversé l'allodynie mécanique persistante de longue durée dans un modèle modifié d'amorçage hyperalgique en utilisant la capsaïcine intracolonique et intraplantaire. Surtout, nous démontrons pour la première fois que IPZ a inversé l'allodynie durable établie tant après l'amorçage que les injections CAPi.c. La délétion génétique de *prkcz* a soutenu les conclusions pharmacologiques ci-dessus. Ainsi, les souris *prkcz*$^{-/-}$, mais pas *prkcz*$^{+/-}$ ou souris de type sauvage, ont affiché des réponses atténuées dans les modèles d'allodynie distante, mais pas dans les douleurs neuropathiques induites par ELN. Fait important, ces effets d'atténuation de la douleur de *prkcz* KO étaient significativement plus marqués chez les souris males que les souris femelles. Nous avons démontré que l'injection i.t. de 10 nmol de NMDA a produit une diminution significative de SRPs pendant 24 - 48 h qui a été renversée par l'injection i.t. de IPZ. En conséquence, une augmentation significative des taux de la protéine PKMζ a été observée 60 min après l'injection i.t. de NMDA.

En outre, nous avons aussi évalué le rôle de plusieurs éléments régulateurs de PKMζ, identifiés dans des études de l'hippocampe, de l'hyperalgésie induite par le NMDA dans la moelle via l'inhibition pharmacologique. Nous démontrons ici que l'inhibition de PIN1 dans la moelle utilisant juglone (5-hydroxynaphtho-quinone) a exacerbé l'allodynie induite par l'injection i.t. de NMDA. L'amélioration de cette allodynie a été associée à une diminution des niveaux de PIN1, et d'une augmentation particulière de PKMζ, mais pas de la forme longue de la protéine PKCζ. En plus, l'inhibition de PDK1 utilisant GSK-2334470 a réduit significativement la phosphorylation de PKMζ, mais pas les niveaux de PKMζ totale. Le prétraitement par GSK-2334470 a empêché le développement de l'allodynie induite par le NMDA. De même, le prétraitement par pep2m, un inhibiteur de l'interaction entre la protéine de fusion sensible à *N*-éthylmaléimide (FSN) et le sous-type 2 de récepteur de glutamate (GluR2), a empêché l'allodynie induite par le NMDA. Ces données suggèrent que PIN1, PDK1 et l'interaction FSN-GluR2 sont nécessaires pour la mise en

place de l'hyperalgésie induite par le NMDA dépendante de PKMζ.

Signification

Avec sa structure et sa fonction uniques, PKMζ promet d'être une cible clé pour le traitement de la douleur chronique qui dépend de la neuroplasticité. Ces études ont fourni une analyse cellulaire et de système détaillée du rôle de PKMζ dans le maintien de la neuroplasticité inadaptée contribuant à la douleur persistante. Nos résultats illustrent un rôle pour PKMζ dans le maintien de la douleur persistante de longue durée, en particulier dans les modèles d'allodynie à distance, où la contribution de la sensibilisation centrale peut être séparée du rôle des entrées périphériques. Nos résultats utilisant des concentrations variables de formol mettent en évidence la notion que les effets analgésiques de l'inhibition de PKMζ peuvent être masqués à des concentrations plus élevées qui produisent une inflammation accrue périphérique. En outre, nos études ont contribué non seulement à une meilleure compréhension de la régulation de PKMζ dans la moelle épinière, mais de la neuroplasticité vertébrale en général. Comprendre la neuroplasticité vertébrale est essentiel pour le développement de médicaments, pour le bénéfice des patients qui actuellement ne peuvent pas être traités de façon optimale avec des analgésiques de pointe.

Figures Index

Study 1

Study 2

Study 3

List of Abbreviations

ABP (AMPA receptor binding protein)

ACC (anterior cingulate cortex)

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)

ASIC (acid sensing ion channel)

BDNF (brain-derived neurotrophic factor)

CamKII (calcium calmodulin kinase II)

CCI (chronic constriction injury)

CFA (complete Freund's adjuvant)

CGRP (calcitonin gene-related protein)

CNS (central nervous system)

CSF (cerebrospinal fluid)

CPIP (chronic post-ischemia pain)

CPN (common peroneal nerve)

CREB (cAMP response element-binding protein)

DAG (diacylglycerol)

DRG (dorsal root ganglion)

EPSP (excitatory postsynaptic potential)

ERK (extracellular signal-regulated kinase)

GRIP (glutamate receptor interacting protein)

HET (heterozygous)

IASP (International Association for the Study of Pain)

IP3 (inositol trisphosphate)

i.c. (intracolonic)

i.m. (intramuscular)

i.p. (intraperitoneal)

i.pl. (intraplantar)

i.t. (intrathecal)

KO (knock-out)

LTD (long-term depression)

LTP (long-term potentiation)

MAPK (mitogen-activated protein kinase)

mGluR (metabotropic glutamate receptor)

mTOR (mammalian target of rapamycin)

NMDA (N-methyl-D-aspartate)

NK1 (neurokinin1)

PDK1 (phosphoinositide-dependent kinase 1)

PLC (phospholipase C)

PIN1 (protein interacting with NIMA1)

PI3K (phosphoinositide 3 kinase)

PKA (protein kinase A)

PKCζ (protein kinase Cζ)

PKMζ (protein kinase Mζ)

PWL (paw withdrawal latency)

PWT (paw withdrawal threshold)

SCDH (spinal cord dorsal horn)

SNI (spared nerve injury)

SNL (spinal nerve ligation)

SP (substance P)

TNF (tumor necrosis factor)

WDR (wide dynamic range)

WT (wild-type)

ZIP (ζ-pseudosubstrate inhibitory peptide)

5HT (5-hydroxytryptamin)

Acknowledgements

I would like to thank first and foremost my supervisor, Dr. Terence Coderre, for giving me the opportunity to work in his laboratory on this fascinating project. I am most thankful for his guidance, and detailed thesis edits! Your kindness towards and availability for your students is exceptional – I am truly grateful to have had the chance to work with you.

I would like to thank the people without whose support I would not have been able to start this project in the first place – Dr. Hugh Bennett and the department of Experimental Medicine, my former academic advisor Dr. J.J. Lebrun, Dr. Coimbatore Srikant for both his moral and financial support at the time, and Dr. Babykumari Chitramuthu for her continuous faith in me. Thank you to my MSc supervisor Dr. Corinne Houart, not only for your support over the past seven years, but also for being a role model of confidence, and female leadership to me.

I need to thank my fellow lab members for their help and support over the past years: Dr. Virginia Cornea, André Laferrière, Dr. Ragavendran Jagadeesan and Kathleen Vincent, who each brought to the lab a unique sense of humour, the delicious combination of which I was fortunate to witness. Although their stay in the lab was short, their help for this project was tremendous: Aiste Mickeviciute helped complete important parts of the mouse experiments, and Stefanie Ding scored rats' behavior even after she'd left – thank you for all your help!

Thank you to my thesis committee for your valuable insights and suggestions for this project: my academic advisor Dr. Xiang-Jiao Yang, members of the pain centre Dr. Philippe Seguela, who kindly agreed to function as examiner of this work, Dr. Jeffrey Mogil, and especially Dr. Laura Stone. She generously opened her lab for me to learn all things "mouse" for this project. A special thank you to Dr. Magali Millecamps, who shared some of her extensive expertise on mouse behavior with me, and Dr. Stephanie Gregoire some of hers on spared-nerve-injury surgery.

For completion of this project, we received the unique *prkcz* knock-out mice from Dr. Messing's laboratory, and Dr. Sandeep Gyawali kindly helped genotype all mice used for this project. Hicham Mahboubi, supervised by Dr. Ursula Stochaj, completed western blots, and translated this thesis abstract into French. Thank you!

I am sincerely grateful to the people who brought a lot of joy into the execution phase of this project: Flora Lopes, who is one of the most positive and effective people I have met so far, everyone else at the McGill Animal Center, and the researchers on the 7th floor of the McIntyre Medical building at the time: thank you for creating an oasis of smiles, kindness and generosity for me!

I could not have completed this work without the love and support from my friends and family. I am especially grateful to my parents Zahida & Mubashar Nasir, Natasha Pracejus for helpful comments on parts of this thesis, and Dr. Cybele Wong for all the little and big things.

Last, but not least, I would like to thank the Alan Edwards foundation for their financial support through a PhD studentship that aided completion of this project.

Contribution of Authors

The body of this thesis is presented and discussed in the introduction (chapter 1), studies 1 – 4 (chapter 2-5) and general discussion (chapter 6). Manuscripts for data in chapters 2, 3, 4, and 5 are in preparation. All work presented here was supervised and conceptualized by Dr. Coderre, who also edited this thesis.

Study 1: Effects of PKMζ inhibition in animal models of persistent pain: influence of peripheral nociceptive input

Design of experiments:	H. Nasir, T.J. Coderre
Execution of experiments:	H. Nasir, S. Ding – completed ~ 50% formalin scoring, A. Laferrière - taught catheter placement, completed ~ 50% of SNI and CFA von Frey testing, V. Cornea - taught SNI surgery
Analysis of data:	H. Nasir
Writing of text:	H. Nasir, T.J. Coderre (edits)

Study 2: Role of PKMζ inhibition in models of remote allodynia

Design of experiments:	H. Nasir, T.J. Coderre
Execution of experiments:	H. Nasir, S.Ding – completed acidic saline von Frey testing for 24 h experiment, A. Laferrière - performed all intramuscular and intrathecal injections for "24 h"experiment, as well as some injections + von Frey tests on 4 time points for "1 week" experiment, H. Mahboubi – completed acidic saline western blots
Analysis of data:	H. Nasir
Writing of text:	H. Nasir, T.J. Coderre (edits)

Study 3: Effects of *prkcz* knock-out in animal models of persistent pain: influence of sex differences

Design of experiments:	H. Nasir, T.J. Coderre
Execution of experiments:	H. Nasir, A. Mickeviciute - performed 50% of all SNI surgeries, and 2% formalin tests, S. Gyawali - genotyped all mice
Analysis of data:	H. Nasir
Writing of text:	H. Nasir, T.J. Coderre (edits)

Study 4: Regulation of PKMζ in the SCDH

Design of experiments:	H. Nasir, T.J. Coderre
Execution of experiments:	H. Nasir, H. Mahboubi - completed all presented western blots
Analysis of data:	H. Nasir
Writing of text:	H. Nasir, T.J. Coderre (edits)

Table of Contents

1 Introduction

Chronic pain is one of the leading health issues of the 21st century. Estimates suggest about 20% of the world adult population suffers from chronic pain (Goldberg and McGee, 2011). The International Association for the Study of Pain (IASP) *task force for classification of chronic pain* has recently defined chronic pain as "persistent or recurrent pain lasting longer than 3 months" (Treede et al., 2015). With a sum of $261 to $300 billion, the cost of chronic pain in the US matches costs for heart disease ($309 billion), and exceeds it for cancer ($243 billion), and diabetes ($188 billion) (Gaskin and Richard, 2012), evidencing that chronic pain is not only a debilitating disease for the affected individual, but is coupled to tremendous economic cost. Understanding thus how pain transitions into a chronic state and is maintained over time will greatly aid the development of targeted treatment options that may both decrease personal suffering, as well as the associated economic burden. The past 50 decades of pain research on animals have allowed for a deep understanding and appreciation of changes occuring within the central nervous system – both during an acute and during chronic pain states. In this thesis, by use of rat and mouse animal models, we focus on the role of central sensitization in chronic pain and provide novel evidence for an involvement of the protein kinase M zeta (PKMζ) as an important factor responsible for the maintenance of persistent pain.

1.1 The nociceptive system: an overview

The IASP broadly defines pain as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage'. This broad definition comprises acute, nociceptive pain, as well as persistent pain states. Here, we will first highlight properties of the nociceptive system that allow for this "unpleasant sensory and emotional experience".

Nociceptive pain is transmitted through nociceptors in the periphery to the central nervous system (CNS) via small-diameter, unmyelinated C-fibres and small-diameter myelinated Aδ fibres.

The cell-bodies of these primary afferent fibres are located in the dorsal root ganglia (DRG), and the central axonal end terminates in the spinal cord dorsal horn (SCDH) – synaptically connecting to SCDH neurons. From the spinal cord, stimuli are further transmitted via the spinothalamic and spinoreticulothalamic tracts to the hypothalamus and thalamus (as well as additional smaller tracts to other brain regions), and from there to the cortex, thus informing the organism of the pain stimulus in the periphery, and allowing for emotional and cognitive evaluation of the pain event (Julius and Basbaum, 2001). Importantly, descending feedback systems activated in the brainstem rostral ventral medulla, and midbrain periaqueductal gray modulate the signal propagated from the spinal cord.

The two major classes for nociceptors, C and Aδ fibres are largely responsible for transmission of different types of pain input (this nomenclature refers to nociceptors in skin, which in muscle and deep tissue are referred to as group III and IV fibres, respectively). The myelinated Aδ (group III) fibres transmit acute, localized "fast" pain, while C (group IV) fibres that consist of unmyelinated axons, conduct poorly localized, diffuse, "slow" pain (Dubin and Patapoutian, 2010). While Aδ fibres are found in both hairy and glabrous skin, C polymodal nociceptors are mostly in hairy skin (Bessou and Perl, 1969). Cutaneous nerves form units with nociceptive Aδ and C fibres that are thus easily activated during cutaneous injuries. Touch-activated Aβ-fibres also play a role in pain modulation, prominently described in the "gate control theory" of pain (Melzack and Wall, 1965), and may also contribute to allodynia (Cervero and Laird, 1996).

1.1.1 C fibres

C fibres constitute about 70% of all nociceptors and have been classified into subtypes that respond specifically to mechanical (Bessou and Perl, 1969, Kumazawa and Perl, 1977), heat (Georgopoulos, 1976), cold (LaMotte and Thalhammer, 1982) or chemical stimuli (Meyer and Campbell, 1988). The majority of C fibres act as polymodal nociceptors, i.e. possess the ability to detect all – mechanical, heat, and chemical – stimuli. Until recently, C-fibres were subdivided into

peptidergic (expressing calcitonin gene-related peptide (CGRP) and substance P (SP) (Ribeiro-da-Silva and Cuello, 1995)) and non-peptidergic fibres (binds isolectin B4 (IB4) that strongly colocalizes with P2X3 receptors in these fibres (Chen et al., 1995)). A new transcriptome analysis of 622 single mouse neurons suggests 11 categories for sensory neuron types, implying a further division of the C-fibre broad categories into nonpetidergic 1, 2 and 3 (with the common marker PLXNC1), peptidergic 1 (marker: TAC1) and 2 (marker: CNTNAP2), as well a neuron group with a tyrosin hydroxylase cluster (marked by PIEZO2) (Usoskin et al., 2015).

1.1.2 Silent nociceptors

Importantly, in addition to polymodal nociceptors there are also a group of silent or "initially mechano-insensitive" unmyelinated afferents. These are activated after injury to normal tissue by high-threshold (noxious) mechanical or heat stimuli (Schaible and Schmidt, 1988), are more sensitive to chemical irritants (e.g. capsaicin) than polymodal nociceptors, are sensitized by endogenous inflammatory substances (Klede et al., 2003), and play a major role in the initiation of central sensitization (Schmidt et al., 1995).

1.1.3 Aδ fibres

Aδ fibres are further subdivided into high-threshold mechanoreceptors (HTM, type I) and heat-sensitive nociceptors (MH, type II). The majority of type I Aδ fibres act as high-threshold mechanoreceptors, i.e. respond to highly noxious mechanical insult and only to heat stimuli if an injury has sensitized the receptors' receptive fields (Fitzgerald and Lynn, 1977). Type II Aδ fibres respond to heat stimuli with a delay of several seconds then slowly convey the message to the SCDH (Meyer and Campbell, 1981).

1.1.4 Aβ fibres

Relaying sensory information on touch and muscle spindle secondary endings, Aβ fibres play a role in dynamic tactile allodynia. For example, light stimulation (touch) of the area of

secondary hyperalgesia following intradermal capsaicin in humans caused vasodilatation – likely resulting from the interaction of C-fibre and Aβ fibre interaction through interneurons in the dorsal horn (Cervero and Laird, 1996). Thus, normally touch-activated Aβ fibres have been shown to mediate pain following injury (Campbell et al., 1988, Treede and Cole, 1993).

1.1.5 How are nociceptors activated? – normal nociception, sensitization, desensitization

Nociceptors express a wide range of receptor channels that transduce the noxious stimuli. Several different receptor channel types and subtypes exist that convey signals of different modalities: heat, mechanical, chemical, as well as cold, and acidic stimulation.

1.1.6 Voltage-gated-channels

Voltage-gated sodium, potassium and calcium channels are required for a rapid depolarization and transmission of the pain signal during an acute pain event. Accordingly, tetrodotoxin (TTX)-resistant voltage-gated sodium channels (NaV)1.8, and TTX-sensitive NaV1.7, have been found on almost all nociceptors (Agarwal et al., 2004). Further, deletion of NaV1.8 is associated with a reduction of heat sensitivity, while lack of channel NaV1.7 results in a complete loss of pain perception in humans (Cox et al., 2006) and mice with these mutations (Nassar et al., 2004).

1.1.7 Transient receptor potential (TRP) channels

The ionotropic transient receptor potential vanilloid (TRPV) 1 channel activated by capsaicin, the active ingredient in hot peppers, normally transduces noxious heat into electrical signals. Its expression was found in most heat-sensitive nociceptive afferents (Caterina et al., 1997). While TRPV1 are activated at about 43°C (Patapoutian et al., 2003), depolarization via the related channels TRPV2 require higher (>50°C), and via TRPV4 and TRPV3 lower (innocuous cool) temperatures ranges (Huang et al., 2011). In contrast TRPA1 may be activated by noxious cold

stimuli (Peier et al., 2002). Interestingly, TRPV2 is also activated by osmotic stretch and may additionally act as a mechanotransducer (Muraki et al., 2003), in addition to other putative channels.

1.1.8 Mechano– and acid-sensing channels

With the large diversity of mechanical stimuli - from light brush to severe noxious stimulation - comes a large diversity of mechanoreceptors that dissociate between stimulus intensities and characteristics. The free nerve endings of C fibres and Aδ fibres in the skin can function as high-threshold mechanoreceptors, while Aβ fibres (encapsulated by various specialized structures, such as Pacinian corpuscles and Ruffini endings), convey light touch and vibration. Acid Sensing Ion Channels (ASICs) are activated in response to an acidic (low) pH environment such as occurs after inflammation (Sutherland et al., 2000).

1.2 Central sensitization

When does acute pain become chronic? The transition from acute to chronic pain is associated with the sensitization of neurons located in the central nervous system: central sensitization. The IASP defines central sensitization as the "increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input". Indeed, central sensitization has been implicated in clinical chronic pain conditions including fibromyalgia and whiplash injury (Banic et al., 2004), irritable bowel syndrome (Verne and Price, 2002), chronic low back pain (Phillips and Clauw, 2011), osteoarthritis (Bajaj et al., 2001), endometriosis (Bajaj et al., 2003), postoperative pain (Katz and Seltzer, 2009), and neuropathic pain (Campbell and Meyer, 2006). Data obtained from animal models indicate central sensitization is critically important for spontaneous pain, as well as allodynia – painful responses to normally innocuous stimuli (Woolf, 2011). With heterogeneous etiology underlying these diverse pain conditions, we aim to understand what specific mechanisms account for sensitization of CNS neurons, and to what extend they contribute to the expression of persistent pain. While the sensitization of nociceptors or peripheral

sensitization importantly also contributes to long-lasting expression of pain, its discussion here would go beyond the scope of this thesis. The following sections will highlight the properties and molecular players of central sensitization.

1.2.1 What is central sensitization?

Central sensitization denotes the persistently enhanced sensitivity and efficacy of neurons within the central nervous system that is characterized by enlarged receptive fields, increased spontaneous activity, reduced activation threshold and increased responses to noxious and/or non-noxious stimulation (Latremoliere and Woolf, 2009). In the absence of sustained ongoing peripheral input, sensitized central neurons may or may not return to their normal state. Central sensitization was discovered mainly following either high intensity, high-frequency stimulation (e.g. highly noxious stimulation, or experimental C-fibre stimulation) (Latremoliere and Woolf, 2009) or tissue injuries (Coderre and Katz, 1997). With its long-lasting character and unique, molecularly still unclear, maintenance mechanisms, central sensitization can account for pain felt either after the healing of injured tissue or sometimes in areas remote to injury (remote pain) (Woolf, 2011).

1.2.2 History

Although the concept of sensitization or facilitation of central neurons had been described as early as 1859 (see review by Coderre and Katz (1997), the term central sensitization itself was first coined by Clifford Woolf after a set of pioneering experiments in 1983. Woolf demonstrated lowering of firing thresholds, enhanced efficacy and increased spontaneous activity in α-motoneurons of the flexor efferents (activating the flexion withdrawal reflex) of rats ipsilateral – and importantly contralateral – to a heat-injury at the hind paw. These changes persisted for several hours, during which not only nociceptive stimuli, but light touch activated this nociceptive reflex. These changes of the flexion reflex largely depended on CNS neuron alterations. This was seen first in the activation of motoneurons through stimulation of Aβ sensory fibres only following, and not before, an injury or high intensity conditioning stimulation. Second, the increased efficacy,

including enlarged receptive fields of neurons ipsilateral and contralateral to the heat injury, was not reduced following complete sensory block of the periphery including the site of injury. Although the exact neural mechanism required to initiate and maintain these neuronal changes remained elusive, Woolf first described central neuron-dependent changes in response to peripheral heat injury or C fibre stimulation (Woolf, 1983).

1.2.3 Modes of central sensitization: homosynaptic vs. heterosynaptic facilitation

Central sensitization can occur at one specific activated synapse (homosynaptic), or encompass several neighbouring synapses (heterosynaptic). Homosynaptic "use-dependent" facilitation – the classic form of long-term potentiation (LTP) in hippocampal CA1 – denotes the repeated activation of the same SCDH neuron/afferent synapse. In the spinal cord, this synaptic potentiation occurs not only within one synapse, but can spread to previously unstimulated synapses. This latter phenomenon is believed to underlie all types of pain that involve sensitization of non-injured tissues, such as in clinical conditions of referred pain/hyperalgesia, secondary hyperalgesia and allodynia and is termed heterosynaptic facilitation or potentiation (Latremoliere and Woolf, 2009).

1.2.4 Temporal summation and wind-up

Activity-dependent plasticity: The summation of depolarizing postsynaptic potentials that repeatedly arrive at the same synapse after primary afferent stimulation may be "summated" to produce a cumulative response (temporal summation). Patch-clamp recordings evidenced that both Aδ and C fibres that were repeatedly stimulated at low frequencies produced temporal summation causing a long-lasting decrease of postsynaptic membrane potential (Sivilotti et al., 1993), and with it a sensitization of CNS neurons.

The phenomenon of wind-up arises with temporal summation of depolarizations of slow synaptic potentials. For example, repeated C-fibre stimulation of the same strength produced action potential discharge that increased with each successive stimulation. The neural firing of the SCDH

outlasted the afferent input by several seconds, suggesting a "winding up" of initial input potential (Mendell and Wall, 1965). It has been suggested that wind-up may be seen as a protective mechanism to cause intense pain rapidly, by "winding up" neuronal activity during noxious stimulation (Wishik, 2005), e.g. during immersion of the hand in very hot water. Prolonged immersion would thus yield rapid high intensity activity, causing withdrawal of the hand, and continued pain for several seconds after withdrawal. Wind-up is not synonymous with central sensitization (Woolf, 1996), but the conditions that lead to wind-up (if they persist) produce central sensitization.

1.2.5 LTP in pain pathways

In the spinal cord, long-lasting potentiation of synaptic transmission between primary afferent and spinal cord neurons has been observed *in vitro* (Randic et al., 1993, Lozier and Kendig, 1995) and *in vivo* (Sandkuhler and Liu, 1998, Svendsen et al., 1999). This form of LTP between C-fibre afferent and dorsal horn neurons was elicited by direct electrical stimulation of the sural nerve, as well as noxious skin heating, noxious pinching of the skin or hind paw formalin injection. Importantly, the maintenance of LTP was sustained even after afferent nerve transection following noxious skin stimulation – strongly suggesting that a central, peripherally-independent mechanism maintains LTP (Sandkuhler and Liu, 1998). LTP has not only been observed in the spinal cord, but also in various brain regions following high-intensity noxious stimulation. Hind paw digit amputation in the rat caused LTP in neurons located in the anterior cingulate cortex (ACC) – a potentiation that could not be altered by local anesthesia at the hind paw (Wei and Zhuo, 2001). Importantly, LTP has also been attributed to the maintenance of secondary hyperalgesia in humans (Klein et al., 2004).

1.3 Mechanisms underlying central sensitization

Reminiscent of LTP in memory pathways (see below), central sensitization constitutes a "fast" (early-onset) induction phase, and a "slow" (late-onset) maintenance phase. While the former

relies on fast synaptic glutamatergic transmission, as well as phosphorylation of receptors and ion channels, the latter entails protein transcription and translation (Woolf and Salter, 2000). Activity-dependent (neuronal) central sensitization requires N-methyl-D-aspartate receptor (NMDA-R) activation for both phases (Woolf and Thompson, 1991). Non-activity-dependent central sensitization relying on immune and glial cell activation will not be discussed here.

1.3.1 Excitatory neurotransmitters

Activation of primary afferents in the periphery through noxious stimulation of peripheral nociceptors causes a release of glutamate (Kangrga and Randic, 1991, Sluka and Willis, 1998), SP (Sorkin et al., 1992, Afrah et al., 2002), CGRP (Santicioli et al., 1992) and brain-derived neurotrophic factor (BDNF) (Thompson et al., 1999, Lever et al., 2001) in the SCDH synapse. These neurotransmitters and neuromodulators are required for the development of central sensitization (Latremoliere and Woolf, 2009). Indeed, intrathecal (i.t.) SP and NMDA-R antagonists reversed contralateral hyperalgesia after heat injury (Coderre and Melzack, 1991), and neurokinin 1 and 2 antagonists prevented nociception-induced sensitization of spinothalamic tract neurons (Dougherty et al., 1994). Conversely, i.t. injection of SP, neurokinin A and NMDA into the SCDH decreased paw withdrawal latencies (PWLs) from 48°C water – thereby mimicking the contralateral hyperalgesia that developed after a heat injury (Coderre and Melzack, 1991).

1.3.1.1 Glutamate and the central sensitization - initiation cascade

The most abundant excitatory neurotransmitter of the peripheral and central nervous system, glutamate, is released from primary afferents and was shown to convey the pain message via ionotropic ligand-gated NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, as well as through metabotropic glutamate receptors (mGluRs). Through its activation of AMPA and kainate receptors, glutamate causes a depolarization in the post-synaptic neuron, gradually causing wind-up-like properties after repeated stimulation. This depolarization opens the pore within NMDA-Rs that under basal conditions is blocked by Mg^{2+} ions. This release

allows Ca^{2+} and Na^{+} influx, causing enhanced depolarization, as well as an activation of calcium calmodulin kinase II (CamKII) and other intracellular cascades linked to various phosphorylation events (Fang et al., 2002). The important role of NMDA-R activity was shown through use of competitive and non-competitive NMDA-R antagonists that prevented the development of windup, temporal summation, and depolarization of the neuron after high-frequency stimulation (Tao et al., 2003, Davies, 1987 #3091).

Evidence further suggests that binding of glutamate to its mGluRs located primarily in the superficial dorsal horn, SP to its neurokinin-1 (NK1) receptor, and CGRP to CGRP receptors are required for the initiation of central sensitization. For example, ablation of SP containing neurons in the superficial dorsal horn of the lumbar spinal cord diminished mechanical and thermal hyperalgesia in response to capsaicin (Khasabov et al., 2002). Further, inhibition of CGRP receptors abolished the arthritis-induced enhanced synaptic transmission in substantia gelatinosa neurons of rats (Bird et al., 2006), and its application enhanced synaptic transmission in amygdala neurons in an NMDA-dependent manner (Han et al., 2010). Finally, inhibition of mGluRs reduced responses of primate spinothalamic tract neurons *in vivo* (Neugebauer et al., 1999), and decreased enhanced formalin nociception in rats (Fisher and Coderre, 1996), while agonists elicited potentiated effects to noxious and innocuous stimuli. Additionally, i.t. administration of the mGluR1/5 agonist DHPG elicited spontaneous pain, mechanical allodynia and thermal hyperalgesia in rats and enhanced second phase formalin nociception (Fisher and Coderre, 1998, Fisher, 1996 #3524`).

1.3.2 Protein kinases & intracellular signaling

During the initiation phase, second-messenger-dependent kinases – most notably cAMP-dependent protein kinase (PKA), protein kinase C (PKC), CamKII, and phosphoinositide 3 kinase (PI3K) - are required for phosphorylation of receptors, contributing to a state of enhanced and persistent synaptic efficacy (Willis, 2001). Reported activators of these kinases are calcium influx, as well as glutamatergic transmission via mGluRs and SP activity at NK-receptors (Latremoliere

and Woolf, 2009). Importantly, PKA, PKC and CamKII were shown to be required for the initiation, but not for the maintenance of LTP in SCDH in response to nociceptive stimulation. Inhibitors of these kinases (KN-93 and AIP for CamKII; Rp-CPT-cAMPS for PKA; chelerythrine and Gö 6983 for PKC) blocked LTP in C-fibre evoked field potentials in SCDH when injected 30 min prior to stimulation. However, no reversal of LTP was observed if CamKII inhibitors were injected at 60 min, and PKA & PKC inhibitors at 30 min post stimulation, suggesting LTP was maintained by differential mechanisms (Yang et al., 2004a).

PKC was shown to be an important effector of the phospholipase C (PLC)/PKC pathway activated by nociceptive input that contributes to the increase in intracellular Ca^{2+} through release by intracellular stores of the endoplasmatic reticulum (Ribeiro and Putney, 1996). The Gq subunit, among others associated with mGluR Group I, and 5-hydroxytryptamin (5HT) receptors was shown to activate PLC (Tappe-Theodor et al., 2012), which is required for hyperalgesia after noxious stimulation. Indeed, pretreatment with a PLC inhibitor reduced the second phase of the formalin test (Coderre, 1992), and intraplantar (i.pl.) endothelin-1 induced hyperalgesia was prevented through PLC inhibiton (Motta et al., 2006). Following its activation, PLC cleaves phosphatidylinositol 4,5 bisphosphate into diacylglycerol (DAG) and inositoltriphosphate (IP3). DAG activates PKC, which goes on to posttranslationally modify AMPA- and NMDA-Rs to enhance their conductance, channel opening and calcium permeability (Chen and Huang, 1992, Jenkins and Traynelis, 2012). IP3 binds to IP3 receptors on the endoplasmatic reticulum causing a release of calcium from intracellular stores (Berridge, 1998).

PI3K, a lipid kinase observed in various signalling pathways, was shown to contribute to central sensitization. PI3K phosphorylation of phosphoinositides produces second messengers involved in protein kinase B (or AKT) and extracellular signal-regulated kinase (ERK) signaling. Indeed, electrophysiological experiments showed that inhibition of PI3K reduced wind-up of spinal wide dynamic range (WDR) neurons, and dose-dependently decreased first phase formalin pain,

suggesting it is required for the initiation of central sensitization. Importantly, inhibition of PI3K additionally prevented ERK and CamKII phosphorylation 15 min post formalin, together with a reduction of phosphorylation of the NMDA-R's NR2A subunit (Pezet et al., 2008), players that have been shown to contribute to late-phase central sensitization. Overall, these data suggest PI3K plays a pivotal role in the initiation and maintenance of central sensitization by affecting several necessary signalling events.

ERKs, part of the mitogen-activated kinases (MAP) kinase family, have been shown to influence pain processing. Converging evidence suggests ERK may be activated through different pathways connected to various mediators of central sensitization: NMDA-R, AMPA-R, NK-1, mGluR signaling (Krapivinsky et al., 2003, Latremoliere, 2009 #1618, Karim, 2001 #3205). Following their activation, ERKs translocate to the nucleus where they have been shown to phosphorylate transcription factors, such as cAMP response-element binding protein (CREB) (Impey et al., 1998) – thereby playing an important role in the transcriptional late phase of central sensitization. Indeed, ERKs were activated in the SCDH following intradermal formalin or capsaicin, and pharmacological inhibition of ERKs decreased both late phase formalin pain (Ji et al., 1999), as well as persistent inflammatory hyperalgesia (Ji et al., 2002). Their activation during nociceptive processing in the ACC has been described to be coupled to NMDA-R activity; thus, inhibition of NMDA-R reduced pERK and pCREB expression following 5% formalin or NMDA bath application of rat brain slices (Cao et al., 2012). In addition, ERK may also influence membrance conductance through phosphorylation of the K^+ channel Kv4.2 (Hu et al., 2007).

1.3.3 Phosphorylation of receptors

Phosphorylation of various receptors and genes has been shown to cause enhanced efficacy during synaptic transmission, as well as to aid enhanced transmission by increasing excitatory receptor expression near the membrane. Experiments conducted in hippocampal cell cultures have determined that ionotropic AMPA receptor phosphorylation through PKA or CamKII increase

calcium permeability (Keller et al., 1992), potentiate currents (Greengard et al., 1991), and increase AMPA-R expression near the plasma membrane through interaction with anchoring proteins. AMPA receptors display two main phosphorylation sites: Ser-845 on Glu1 domain by PKA, which has been shown to cause a 40% increase of current in HEK cells, and Ser-831 on GluR1 by PKC (Roche et al., 1996). The latter is also phosphorylated by CamKII *in vitro* (Mammen et al., 1997). In spinal nociception, CamKII was upregulated following intradermal injection of capsaicin, and its inhibition reduced AMPA-R phosphorylation (Fang et al., 2002).

The phosphorylation of NMDA-R subunits plays an important role in the initiation and maintenance of central sensitzation. For example, Complete Freund's Adjuvant (CFA)- induced inflammation caused an upregulation of NMDA subunit NR2B phosphorylation (Guo et al., 2002, Bu et al., 2015). Local anesthesia of the inflammed hind paw decreased NR2B phosphorylation indicating a relation between its effect and afferent glutamatergic signalling. NMDA-R phosphorylation is elicited by various kinases: PKA and PKC both phosphorylate NMDA-R subunits causing enhanced synaptic transmission. The PKC-induced phosphorylation of NMDA-R has been associated with an increase in channel opening – again yielding increased NMDA-R-mediated currents (Chen and Huang, 1992), ultimately resulting in enhanced nociceptive sensitivity. Interestingly, CFA-induced NR2B phosphorylation – and with it the corresponding rat paw hyperalgesia - was decreased by i.t. pretreatment with the full-length PKC inhibitor chelerythrine, as well as mGluR and NK1 antagonists and Src inhibitors (Guo et al., 2002). Src family members were shown to modulate NMDA-R currents during the induction of central sensitization (Salter, 2004). Further, the activity at the NR1 subunit of NMDA-R increased phosphorylation at PKC-specific site Ser-896 in the superficial dorsal horn between 2 min and 1 h following noxious heat stimulation of the hind paw. Phosphorylation of NR1 colocalized strongly with PKCδ, but rarely with PKCγ, activity. Inhibition of NMDA-R using MK-801 decreased both NR1 phosphorylation and hyperalgesia (Brenner et al., 2004).

1.3.4 Gene transcription

The late or maintenance phase of central sensitization is characterized by "slow" gene transcription and translation. Kinases associated with the MAPK-ERK pathway highlighted above activate transcription factors such as CREB, and drive the expression of genes encoding e.g. c-fos, c-jun, Nk1, TrkB, Cox2. Phosphorylated CREB in the SCDH is increased following noxious stimulation (Ma and Quirion, 2001, Mitsikostas et al., 2011), and the transcriptional changes it induces are required for the maintenance of central sensitization (Ji and Rupp, 1997, Turgeon et al., 1997, Duric and McCarson, 2007, Manna and Stocco, 2007, Hou et al., 2016). A special role has been ascribed to the transcriptional modulator CREB in hippocampal LTP. Thus, knock-out (KO) of CREB in transgenic mice was linked to decrease of LTP and AMPA-Rs' excitatory postsynaptic potentials (EPSPs) (Middei et al., 2013).

1.3.5 Trafficking of AMPA receptors

Although trafficking of NMDA and mGluR have been shown to play an important role in the context of the maintenance of central sensitization (Shanthanelson et al., 2009, Lau, 2007 #3390), considerable emphasis has been put on AMPA receptor trafficking from extrasynaptic sites to synapses as a means to enhance and sustain synaptic efficiency. AMPA-R trafficking is deemed one of the most important factors sustaining the maintenance of LTP and long-term depression (LTD) (Malinow and Malenka, 2002). Following depolarization of the postsynapse and activation of protein kinases, additional AMPA receptors are transported to and retained in the postsynaptic membrane thereby strengthening synaptic transmission.

Similarly, in spinal nociception, AMPA-R trafficking has been proposed as an important mechanism of enhanced synaptic transmission. The earliest study elucidating trafficking of AMPA-R subunits *in vivo* utilized the intracolonic (i.c.) capsaicin-induced remote pain model. The AMPA GluR1 subunit was increased in the synaptosomal membrane, and decreased in the cytosolic fraction in the lumbar spinal cord of rats subjected to i.c. capsaicin instillation, and pretreatment

with a CamKII inhibitor prevented this GluR1 trafficking (Galan et al., 2004). While a role of AMPA-R trafficking in central sensitization has by now been well established (Beattie et al., 2010, Larsson, 2008 #3534), its regulation in this context is incompletely understood. A regulatory role has been ascribed to an interaction between the N-thylmaleimide-sensitive fusion protein (NSF) and GluR2. Katano and colleagues reported a decrease of NSF, as well as GluR2/3, and a concomitant increase of GluR1 subunit following intradermal CFA – suggesting the NSF-GluR2 interaction, which is required for AMPA retention within synapses in the hippocampus, may also be important for spinal AMPA-R regulation in the context of central nociceptive sensitization (Katano et al., 2008).

During hippocampal LTP, which shares similarities with central sensitization (see below), AMPA receptor trafficking involves the activation of several regulatory proteins such as glutamate receptor-interacting protein (GRIP), AMPA-R binding protein (ABP) and protein interacting with protein kinase C α 1 (PICK1) (Dong et al., 1999, Dev, 2004 #3539). PICK1 was shown to interact with AMPA-R's GluR2 subunit, thereby targeting the subunit for recycling. During enhanced synaptic transmission, AMPA-R GluRs were more readily recycled in two processes: the first regulated by PICK1 and its interaction with anchor proteins ABP and GRIP, the second by an interaction between NSF and GluR2 (Lu and Ziff, 2005, Braithwaite, 2002 #3542). PICK1 was shown to target ABP/GRIP and GluR2 complexes, such that following GluR2 phosphorylation, the receptor is trafficked from ABP/GRIP to PICK1 (Lu and Ziff, 2005). Impairment of the PICK1 interaction with ABP/GRIP has also been shown to lead to a decrease of GluR2 phosphorylation by PKC, GluR2 surface expression and NMDA-induced GluR2 endocytosis. Similarly, impairment of the NSF-GluR2 interaction decreased AMPA-R-dependent potentiation (Zou et al., 2005). It still remains to be discovered whether these regulatory processes contribute to nociceptive central sensitization, and will be discussed further in study 4 of this thesis.

1.4 Evidence for central sensitization

The following sections will discuss the role of central sensitization in pain of different etiologies: cutaneous injury, neuropathic pain, inflammatory pain, formalin-induced chemical pain, and will highlight the differential features of remote hypersensitivity after muscle or visceral injury.

1.4.1 Cutaneous injury

Noxious stimulation of the skin (with hot, cold or mechanical stimuli) causes both hyperalgesia and allodynia (enhanced pain sensation and lowered pain threshold of nociceptors to subsequent noxious and innocuous stimulation). Hypersensitivity (hyperalgesia and allodynia) resulting from an injury to the skin is characterized by two distinct hyperalgesic zones. Primary hyperalgesia denotes hypersensitivity at the site of injury itself, while secondary hyperalgesia denotes the hypersensitivity in the uninjured skin surrounding the injury. Secondary hyperalgesia develops with a short time delay and extends in duration compared to primary hyperalgesia (Hardy et al., 1950). Experimental heat injury lowered the mechanical threshold in the human hand, both at the site of injury, between two injuries and interestingly outside the area of flare produced by the injuries: the area of mechanical secondary hyperalgesia (Raja et al., 1984). While primary hyperalgesia is thought to be due to nociceptor (peripheral) sensitization, secondary hyperalgesia has been explained by the widely accepted theory proposed by Hardy and colleages in the 1950s postulating central sensitization in the SCDH as its mechanistic underpinning.

1.4.1.1 Central sensitization

Hardy and colleagues (1950) illustrated that hyperalgesia produced by a burn injury continued to develop after a subsequent peripheral nerve block at the site of the injury – although much slower than without the local anesthetic block. They concluded that a sensitization of SCDH neurons developed from peripheral inputs associated with primary hyperalgesia, and a spreading sensitization within the SCDH resulted in hyperalgesia at sites remote from the injury (i.e., secondary hyperalgesia).

The existence of a central mechanism underlying secondary hyperalgesia is now well accepted. Early studies observed increased firing in SCDH neurons to mechanical stimulation (Kenshalo et al., 1982, Simone et al., 1991, Pertovaara, 1998) or electrical stimulation of C-fibres (Cook et al., 1987) following a cutaneous heat injury. Importantly, this increased firing was only observed following mechanical, not heat stimulation of the zone of secondary hyperalgesia. Secondary allodynia (enhanced sensitivity to normally innocuous stimuli such as light touch) has been shown in humans (LaMotte et al., 1991, Cervero et al., 1993, Klein et al., 2004) and rodents (Martinez-Rojas et al., 2014).

Apart from Woolf's historic flexor-efferent experiments, evidence for central sensitization as a key component of secondary hyperalgesia was provided by a study describing the development of contralateral hyperalgesia following a heat injury of the rat hind paw (Coderre and Melzack, 1991), since peripheral mechanisms would be unable in this instance to explain sensitization within an area so remote to the place of injury.

In an attempt to dissociate peripheral activity and the resulting possible peripheral ongoing input, Coderre and colleagues offered evidence for central processes causing secondary contralateral hyperalgesia. Prior to, or following a heat injury the injured paw was denervated in rats – thus preventing communication of the peripheral region with the spinal cord. Contralateral PWLs in rats with the ipsilateral hind paw denervated after injury, exhibited hyperalgesia, whereas those in which the ipsilateral hind paw was denervated before the injury were unaffected. These data suggest the heat injury induced changes in sensitivity of spinal cord neurons. However, if the peripheral nerves were transected before the injury occurred, central neurons were not sensitized and contralateral PWLs remained in normal range (Coderre and Melzack, 1985).

1.4.1.2 Capsaicin-induced central sensitization

Early studies showed the development of central sensitization following intradermal capsaicin. Similar to a heat injury, capsaicin produced a flare in the human forearm within seconds, and within minutes a surrounding area of mechanical and heat hyperalgesia (Simone et al., 1989). Generally, heat hyperalgesia is localized and lasts for about 2 h, while mechanical hyperalgesia spreads for several cm and lasts at least 24 h (LaMotte et al., 1991).

The development of this secondary hyperalgesia following intradermal capsaicin in the human depends on initial peripheral signalling and is maintained by central sensitization. Thus, application of an anesthetic strip 1 cm away from the capsaicin injection site prevented the spread of secondary hyperalgesia to the opposite side of the strip and interestingly, the injection of capsaicin into anesthetized skin prevented the development of hyperalgesia, suggesting the initial spread depends on peripheral signalling (LaMotte et al., 1991). However, the area of mechanical hyperalgesia following topical or intradermal injection in or outside the receptive field of neurons did not correlate with reduced nociceptor threshold, suggesting central changes were responsible for the capsaicin-induced pain (LaMotte et al., 1992).

Supporting this finding, recordings of monkey cutaneous primary afferents before and after intradermal capsaicin showed no increased firing in response to heat or mechanical stimulation, independent of whether the injection was made in- or outside of the nociceptive receptive field (Baumann et al., 1991). Additionally, it was shown that capsaicin activated and sensitized spinothalamic tract high-threshold and WDR-neurons, such that the increased neuron discharge correlated with increased pain ratings in humans (Simone et al., 1991).

Interestingly, the larger area of secondary hyperalgesia does not contain an area of exaggerated heat sensitivity. Heat stimuli applied within the zone of mechanical secondary hyperalgesia resulted in similar thresholds in and outside this zone. More importantly, the magnitude of initial capsaicin pain reported by the participant did not correlate with the area of

mechanical hyperalgesia, suggesting the central sensitization was not directly correlated with the amount of pain felt (Ali et al., 1996).

Similar effects of intradermal capsaicin on thermal and mechanical thresholds have been demonstrated in rats. Thus, following exhibiting nocifensive behaviors (lifting, guarding) for about 3 min, PWTs and PWLs were lowered ipsilateral, but not contralateral to the capsaicin injection for most of the plantar surface of the injected hind paw (Gilchrist et al., 1996) – thereby making the rat an excellent model to study the effects of capsaicin on central sensitization.

1.4.1.3 Conclusion cutaneous injury

In conclusion, thermal or chemical (e.g. capsaicin) injury of the skin induces peripheral and central sensitization and resulting hyperalgesia. Primary hyperalgesia develops in the area of injury, is restricted to the visible flare that develops in close proximity to the injury, and depends on peripheral sensitization of mainly C and A-δ fibre nociceptors. With a short time delay, secondary hyperalgesia develops in an area spanning about 10-20 cm around the site of injury. Although induced by different types of cutaneous injuries (heat, capsaicin), secondary hyperalgesia is observed mainly in response to mechanical punctate or stroking stimulation and relies on central mechanisms. Thus, neurons in the SCDH exhibiting the classic properties of central sensitization discussed above are responsible for secondary hyperalgesia.

1.4.2 Neuropathic pain & central sensitization

Neuropathic pain is a debilitating pain condition that arises from "a primary lesion or dysfunction in the peripheral or central nervous system" and may last for years (Merskey, 1994). It has been shown to develop after traumatic injury, metabolic diseases (diabetic neuropathy), neurotoxicity, infection or tumor invasion in the periphery. All of these conditions may cause a plethora of neurological and immunological changes that ultimately result in sensitization of afferent nerves and nerve terminals (peripheral sensitization), and/or result in sensitization of neurons within the central nervous system (Woolf, 2004). Neuropathic pain can also occur after

spinal cord injury, stroke or neurodegenerative diseases (e.g., multiple sclerosis) in the CNS, sometimes also called central pain.

Various animal models have been developed aiming to study the etiology of neuropathic pain (e.g. streptozotocin-induced diabetic neuropathy, injury-induced neuropathy) and the multitude of mechanistic changes they induce. Associated with neuropathic pain are changes in central neuronal tone, activity of inflammatory mediators and neurotrophins such as BDNF, which is deemed one of the most important neuromodulating factors inducing and maintaining neuropathic pain in animal models (Nijs et al., 2014). In addition, structural changes such as sprouting of A fibres from SCDH lamina III to lamina II (Woolf et al., 1992, Nakamura, 1999 #3125), contributes to the central neural hypersensitivity associated with neuropathic pain in rodents. A wealth of studies has contributed to our understanding of nociceptive signaling in models of neuropathic pain, discussing these in details would go beyond the scope of this thesis. Here, we will focus on the role of central sensitization, and in particular the involvement of AMPA-R and NMDA-R activity, in the manifestation of neuropathic pain chronicity.

1.4.2.1 Central neuroplasticity: role of glutamatergic transmission in the development and maintenance of neuropathic pain

Evidence suggests that nerve injury is associated with increased glutamatergic transmission in the SCDH. Detailed analysis using patch clamp recordings, microdialysis and immunohistochemistry in SNI rats indicated an increase in the frequency of miniature excitatory postsynaptic potentials (mEPSCs). Moreover, glutamate levels were increased in the cerebrospinal fluid (CSF) (Inquimbert et al., 2012) and SCDH (Coderre et al., 2005) after peripheral nerve injury. Importantly, the heightened glutamatergic excitatory potentials returned to baseline after mGluR5s were pharmacologically blocked (Inquimbert et al., 2012). Early studies indicated an increase of both AMPA receptor's GluR1 and GluR2/3 immunoreactive staining in ipsilateral SCDH after chronic constriction injury (CCI), but not sham-injury. Time-course analysis revealed a peak of

AMPA-R increase at 14 d that plummeted at 35 d post surgery, matching the time course of thermal hyperalgesia in CCI rats (Harris et al., 1996). Ipsilateral to CCI injury AMPA-R subunit GluR2 protein levels, as well as GluR2 mRNA, were significantly increased in laminae I and II of the SCDH, compared to naive and sham-operated rats (Garry et al., 2003).

Large scale microarray analysis 14 d following peripheral nerve axotomy demonstrated an upregulation of AMPA-R GluR3 in SCDH laminae II-III. Additionally, a concordant upregulation of important players of excitatory transmission: ERK, p38 MAPK, PKCα, βI and δ, as well as Ca^{2+}, and Na^{+} channel subtypes, was observed in SCDH (Yang et al., 2004b).

1.4.2.2 Effects of pre-emptive glutamatergic synaptic inhibition

Blocking NMDA-R activity using MK-801 prior to and up to 5 d following CCI surgery prevented the normal reduction in mechanical and cold PWTs associated with CCI injury. A similar but less profound anti-allodynic effect was observed using the mGluR antagonist (S)-4CPG. Importantly, i.t. injections of these inhibitors of glutmatergic transmission were associated with a reduction of PKC at the postsynaptic membrane as assessed by autoradiography using phorbol esthers (Yashpal et al., 2001). Anti-mGluR1 (30μg) and mGluR5 (10μg & 30μg) antibodies i.t. injected 1 h pre- and 24 h post- CCI surgery reduced cold hypersensitivity in rats (Fundytus et al., 1998). Additionally, cold, mechanical and heat hypersensitivity associated with CCI injury were reduced by 7 days of i.t. treatment with mGluR1 antisense oligonucleotides (knockdown) starting either 3 days before or 5 days after injury (Fundytus et al., 2001). These data suggest mGluRs play a role in neuropathic chronic pain.

Similarly, preemptive ketamine, an NMDA-R antagonist, prevented for at least 2 weeks the development of spinal nerve ligation (SNL)-induced cold and mechanical allodynia, as well as spontaneous pain when injected intrathecally, and to a lesser extend when injected systemically. These data suggest an NMDA-R-dependent sensitization of spinal neurons may contribute to the persistent and long-lasting character of neuropathic pain (Burton et al., 1999). In addition, the

AMPA/kainate receptor antagonist NBQX injected immediately before CCI surgery, or up to 24 h post-surgery also increased thermal response latencies (Mao et al., 1992).

1.4.2.3 Effects of glutmatergic inhibition following nerve injury

Inhibition of glutamatergic activity after nerve injury does not effectively reverse neuropathic pain-induced mechanical allodynia or thermal hyperalgesia. I.t. injections of the AMPA-kainate receptor antagonist NBQX 14 d post CCI surgery only transiently increased withdrawal latencies (up to 1 h), while NMDA-R antagonist MK-801, as well as the AMPA-R antagonist AP-5 had no effect on thermal withdrawal latencies (Yoshimura and Yonehara, 2006). Similarly, NBQX administered via i.t. catheter 28 d post spinal cord injury, only transiently (up to 180 min) attenuated mechanical thresholds and reduced WDR neuron activity (Gwak et al., 2007). Reduced thermal PWLs and mechanical PWTs in CCI rats were transiently attenuated by i.t. AMPA-R antagonists SYM2206, or myrGluR2(846-856) at the peak of allodynic symptoms (Garry et al., 2003). NMDA and AMPA-R inhibition several days post SNI surgery using MK801 and NS1209, respectively, did not alter the surgery-induced reduction in mechanical and cold PWTs (Erichsen and Blackburn-Munro, 2002).

1.4.2.4 Conclusion neuropathic pain

Together, these results suggest spinal glutamatergic transmission via NMDA, AMPA and mGlu receptors is neccessary at least for the development of nerve-injury induced thermal hyperalgesia and mechanical allodynia. It seems that while the inhibition of glutamate transmission can prevent or delay the onset of neuropathic pain expression in varying models, it is not as effective at reversing established neuropathic pain.

1.4.3 Inflammatory pain

Inflammatory pain reflects pain associated with tissue injury and infiltration of immune cells. Clinically, these processes are associated with many chronic pain conditions, such as arthritis,

cystitis, colitis, as well as autoimmune diseases (Hendrickson et al., 2002). In animal models, inflammatory pain conditions are typically modelled by injection of irritating substances, most commonly CFA or carrageenan that cause the attraction of immune cells and the release of inflammatory substances, and produce hyperalgesia and allodynia at the affected and surrounding tissue. Besides characteristic peripheral changes associated with the inflammation (most commonly observed through tissue swelling, reddening near inflamed area), substantial changes within the spinal cord contribute to the chronicity of inflammatory pain. Sustained afferent barrage following peripheral inflammatory stimulation is associated with sensitization of centrally located neurons (Herrero et al., 2000). Contributing to this sensitization are activity-dependent neuronal changes, immune cell infiltration, and altered signaling within the spinal cord.

1.4.3.1 Activity-dependent changes in the spinal cord

Following peripheral CFA injection, neuronal excitability within the SCDH changes significantly. Intradermal CFA has been shown to induce increases in receptive fields of spinal WDR neurons, which also exhibit bursts of spontaneous firing, discontinuous receptive fields, and lower thresholds that are correlated over time with thermal hyperalgesia at the hind paw. Importantly, local anesthesia post-CFA did not alter the enlarged receptive field of lamina I neurons, suggesting the excitability of centrally located neurons was not due to continuous input from sensitized peripheral afferents innervating the inflamed tissue, but to central alterations (Hylden et al., 1989).

NMDA and AMPA receptor activity plays an important role in the induction and maintenance of CFA-induced hypersensitivity. NMDA-R NR1 subunit immunoreactivity increased in the synaptosomal fraction of L4/5 SCDH segments following CFA. This increased immunoreactivity correlated with the time course of mechanical allodynia. Topical application of the NMDA-R- antagonist D-APV one day after CFA injection reversed the increase in NMDA-R immunoreactivity in the SCDH. Similarly inhibition of c-AMP-dependent PKA diminished NR1

immunoreactivity at the SCDH synapse, suggesting PKA downstream of NMDA-R may be required for increased NR1 synaptic expression (Yang et al., 2009). Thermal hyperalgesia following intradermal injection of CFA in the hind paw was attenuated by i.t. NMDA-R antagonist MK-801, which also reduced the increase of receptive field sizes of WDR neurons in the superficial and deep dorsal horn, suggesting NMDA-Rs play an important role in the spinal neuroplasticity induced by hind paw CFA injection (Ren et al., 1992). Additionally, the AMPA-R subunit GluR2 was significantly increased in SCDH crude cytosolic fraction 24 h post CFA, suggesting AMPA-Rs significantly contribute to CFA-induced long-lasting hypersensitivity (Park et al., 2008). CFA also induced an increase in GluR2, and a decrease in GluR1 AMPA-R subunits in SCDH cytosolic fractions, while it yielded the opposite changes in crude membrane fractions at 24 h post-CFA. Importantly, these changes were not present at 2 h, concordant with the conclusion of the time-course described above: central sensitization in the SCDH following inflammation is slowly induced after high-intensity peripheral activity associated with inflammation. Overall, these studies suggest AMPA-R may play a role in the induction of central sensitzation (Park et al., 2008). Additionally, p38 MAPK, which has been implicated in hippocampal LTP, was activated in SCDH following CFA, as assessed by increased spinal immunoreactivity (Liang et al., 2012). Similarly, i.t. pretreatment with the CamKII inhibitor KN93 prevented the development of thermal hyperalgesia and mechanical allodynia following CFA (Luo et al., 2008).

Time course analysis in isolated spinal preparations revealed that central sensitization depended on peripheral inputs for the first 3 h following carrageenan. However, hyperreflexia in spinal preparations fully developed at 6 h post-carrageenan, suggesting inflammation-induced peripheral inputs may have induced central sensitzation after 6 h, lasting for at least 20 h post-carrageenan (Hedo et al., 1999). Carrageenan induced a brief neuronal discharge at time of injection and an increase in the magnitude of flexor reflex, standing in contrast to formalin that did not

produce significant hyperexcitability. Thirty min post injection, carrageenan decreased the response threshold of flexor efferents to mechanical stimulation (Xu et al., 1995).

1.4.3.2 Activity-dependent changes in the brain

Besides changes in spinal neurons, inflammation-associated alterations in transmission have been shown to occur in the brain as well. Glutamate-induced spike firing in the ACC (Li et al., 2014), and phosphorylation of the NMDA-R subunit NR1 in the arcuate nucleus have both been found to increase after hind paw CFA injection (Peng et al., 2011b).

1.4.3.3 Activation of microglia in the spinal cord

In addition to paw edema and thermal hyperalgesia, hind paw CFA has been shown to induce spinal microglia activation (Raghavendra et al., 2004). Activated microglia displayed increased expression of toll-like receptor 4 and OX-42 (marker of activated glial cells). Importantly, suppression of microglia by chronic i.t. minocyclide treatment alleviated pain hypersenstivity, as well as the CFA-induced upregulation of these markers, while no changes in peripheral edema were observed (Zhao et al., 2015). Following CFA-induced peripheral inflammation, neutrophils and macrophages expressing lipocalin-2 (LCN), and its receptor 24p3R, infiltrated the spinal cord. In addition, LCN2-KO mice exhibited decreased mechanical allodynia and thermal hyperalgesia following hind paw CFA. Along these lines, additional inflammatory events such as neutrophil infiltration, myeloperoxidase activity, and expression of pro-inflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β and macrophage inflammatory protein (MIP)-2) were reduced in LCN-KO mice, suggesting LCN plays an important role in the persistent expression of inflammatory pain (Jha et al., 2014). Overall, microglial markers macrophage-1 antigen (Mac-1), toll-like receptor (TLR) 4 and glycoprotein CD14, as well as proinflammatory cytokines (IL-1β, IL-6, TNFα), were increased in the lumbar spinal cord, brainstem and forebrain in both early inflammatory and later phases following CFA injection. Additionally, following microglial

activation, there is also an activation of astrocytes in spinal cord associated with inflammatory injury (Raghavendra et al., 2004).

1.4.3.4 Conclusion inflammatory pain

In conclusion, hind paw injection of inflammatory substances such as CFA or carrageenan causes mechanical hypersensitivity. Besides the apparent peripheral inflammation, this persistent hypersensitivity depends on central sensitization that develops with a time lag of at least three hours and often outlasts peripheral edema formation. Central sensitization induced by inflammatory stimuli is sustained by activation of immune responses in the spinal cord. These include the activation of microglia and astrocytes, as well as recruitment of macrophages and neutrophils that release various cytokines and other mediators causing a sensitization of spinal neurons.

1.4.4 Formalin test

Subcutaneous injection of dilute formaldehyde to an animal's fore paw or hind paw yields long-lasting, reliable pain that can be measured robustly. The corresponding test termed the formalin test has been widely employed to assess tonic pain in animals and expands upon the hotplate test, pinch test, flinch – jump test, which all measure transient pain. The formalin test is thus an excellent test for the observation of a freely moving animal, exhibiting pain behavior in response to formalin for a prolonged period of time. It provides an advantage over inflammatory models of CFA or carrageenan, since it produces pain behavior that does not require additional stimulation.

A typical formalin test is characterized by a bi-phasic response, a first phase that lasts up to 5 minutes, and a second phase that starts around 15-20 minutes post-injection and lasts about 60 minutes. The two phases are divided by an interphase that is characterized by active inhibition – thus another injection of formalin during an existing interphase does not yield a second first phase, but rather enhanced inhibition (Henry et al., 1999).

Since the earliest experiments in the 1970s, much research has gone into the elucidation of the molecular underpinnings of these two distinct phases. Important to note, before examining results, is the wide range in formalin concentration used in these studies – from 0.02% - 5.0%. Only concentrations of 1% or more induce two phases and significantly alter the paw histologically, with the degree of inflammatory or histological changes depending on formalin concentration (Rosland et al., 1990).

Hunskaar and Hole (1987) were the first to propose distinct molecular mechanisms underlying these discrete phases by investigation of inflammatory and anti-inflammatory drugs. Morphine, codeine, nefopam and orphenadrine yielded relief of both first and second phase pain after 1% formalin, while nonsteroidal and steroid anti-inflammatory drugs only alleviated second phase nociceptive scores. However, the ability of nonsterioidal antiinflammatory drugs to reverse second phase pain is correlated with higher formalin concentration: indomethacin significantly reduced second phase pain at 5% formalin concentration, while it was ineffective at 1% formalin concentration (Yashpal and Coderre, 1998).

1.4.4.1 Central mechanisms underlying second phase pain

Coderre et al. (1990) demonstrated in a set of experiments using 2.5% formalin that second phase formalin pain strongly depends on central changes induced by first phase activity. Spinal anesthesia *prior to* formalin injection nearly abolished second phase pain, while spinal anesthesia initiated 5 min *after* formalin induction did not affect second phase pain expression. These data suggest that inflammation alone cannot be held responsible for pain perception of the second phase. Rather, changes within the CNS (i.e., central sensitization) – likely caused by an initial first phase barrage, may contribute heavily to second phase formalin pain. Yashpal et al. (1996) gave support and conceptual expansion of central sensitization in second phase formalin pain. Thus, spinal anesthesia pre- or post- first phase was induced in catheterized rats that had received varying concentrations of formalin: 2%, 3.75% and 5%. Importantly, the ability to demonstrate a role for

central sensitization in second phase formalin pain was masked as the formalin concentration increased, when significant peripheral inflammation was observed.

Clinical, as well as experimental studies have shown that analgesic agents administered *prior* to peripheral injury significantly decrease the pain following the injury, or reduce the need for further analgesic administration – a phenomenon known as pre-emptive analgesia. Pre-emptive analgesia prevents or diminishes sensitization in the CNS (Coderre and Katz, 1997). A spinal readout for neuronal activity is the expression of c-*fos*, a transcription factor responsible for many cellular functions that is induced rapidly (within 15 min) and transiently expressed in spinal dorsal horn after noxious stimulation (Bullitt, 1990). Importantly, c-*fos* expression in the spinal dorsal horn was significantly reduced if spinal lidocaine anesthesia was initiated prior to 2.5% formalin injection, but not if spinal anesthesia began 5 min after the formalin injection. The expression of c-*fos* correlated with nociception, such that spinal lidocaine-pretreated rats displayed reduced second phase pain, while post-treated rats were unaffected (Yashpal et al., 1998), reflecting the phenomenon of pre-emptive analgesia.

A further study showed that formalin-induced spinal c-*fos* was only reduced by 27% when the injected paw was locally anesthetized using the hydrophilic lidocaine derivative, QX-314, 5 min after 5% formalin. A greater reduction in c-*fos* was achieved using remifentanil anesthesia during the first and QX-314 during the second phase, thereby abolishing pain behavior during both phases (Abbadie et al., 1997). These results strongly suggest that second phase nociception depends on central hyperexcitability generated during the first phase and is reduced by pre-emptive analgesia.

Some progress has been made in an attempt to characterize the molecular underpinnings of second phase pain. In harmony with results discussed above, i.t. injection of NK1-R antagonists 10 min pre- 2% formalin attenuated second phase nociceptive scores, while simultaneously reducing c-*fos* expression in the superficial dorsal horn (King et al., 2000). NMDA-R inhibition pre-formalin using MK-801 (i.t) or memantine (i.p) reduced second phase pain strongly, with minimal effect on

first phase nocieption (Yamamoto and Yaksh, 1992, Eisenberg et al., 1993). Additionally, PKC was increased in response to 2.5% formalin – which depends on NMDA-R activity. I.t. injection of an NMDA-R antagonist 10 minutes prior to formalin reduced both first and second phase nociception (Yashpal et al., 2001). Consistent with PKC increase following formalin (Yashpal et al., 1995, Yashpal et al., 2001), and formalin yielding an increase in c-*fos* expression, PKC$\gamma^{-/-}$ mice exhibit lower c-*fos* levels 90 min post formalin (Zeitz et al., 2001). This c-*fos* reduction is in accordance with behavioral observations of reduced licking behavior in the second phase. These data provide compelling evidence for a spinal mechanism contributing to second phase formalin pain that largely depends on NMDA-R activation, as well as PKC and SP activity.

1.4.4.1 Summary formalin test

The dose used in the formalin test, determines to what degree peripheral and central components contribute to both phases. Much work has gone into deciphering the molecular components of second phase formalin pain. Players of central sensitization – NMDA, mGluRs, as well as protein kinases such as PKC – are required for second phase formalin pain expression. Altogether these studies support the view that a high-intensity input to the spinal cord causes long-lasting changes within the CNS that in turn manifests pain behavior in the experimental subject, and may benefit from pre-emptive analgesia. What the exact maintenance mechanism for second phase formalin pain is remains unclear.

1.4.5 Referred pain and remote hypersensitivity

Referred pain has been defined as "pain felt at a site remote from the site of origin/stimulation". It has been suggested input from different body parts (e.g. heart and arm during angina) converges on dorsal horn and brain stem neurons, such that higher brain centres may not correctly identify the site of stimulus origin (Arendt-Nielsen and Svensson, 2001).

Hyperalgesia and allodynia are both associated with central sensitization, and pain felt in an area remote from original tissue stimulation may result from heterosynaptic facilitation:

sensitization of unaffected neurons close to a sensitized neuron (Latremoliere and Woolf, 2009). This type of pain may not solely rely on convergence of different inputs, but also on a sensitization of neurons, and thus is better termed as *remote hypersensitivity* or *remote allodynia*.

Clinical examples of remote allodynia include irritable bowel syndrome and fibromyalgia characterized by a widespread hypersensitivity without discernible tissue injury (Verne et al., 2001, Moshiree, 2007 #3555). Experimental models of remote allodynia include i.m. acidic saline-induced persistent pain and i.c. capsaicin-induced persistent pain that will be discussed in detail in study 2. By default, remote pain models allow for a dissociation of the site of tissue injury (possible peripheral sensitization) and the site of pain expression, and as such add immense value to the study of central sensitization.

1.5 Issues related to central sensitization

Central sensitization shares unique features with long-term potentiation (LTP) in the hippocampus, which reflects a long-term strengthening of the connections within synapses required for the formation of memories and central sensitization.

1.5.1 LTP in memory and pain processing

LTP was first discovered in the perforant pathway of the hippocampal formation (Bliss and Lomo, 1973) and is deemed one of the most important models of long-term memory (Kandel et al., 2000). LTP denotes the long-lasting heightened transmission of signals between two neurons following high-frequency stimulation. LTP may in theory occur at any excitatory synapse of the central nervous system (Kandel et al., 2000). Most work advancing our understanding of LTP has been conducted in the hippocampus and has taught us that – similar to central sensitization – LTP consists of an early (initiation) and a later transcription- and translation-dependent phase (maintenance). The emerging candidate for memory maintenance is the novel protein kinase Mζ

(PKMζ), exhibiting unique properties and clearly maintaining memory and LTP *in vivo* (see below). Its role in pain pathways is incompletely understood and the primary focus of this thesis.

1.5.1.1 What are the similarities between hippocampal and spinal LTP?

Although a lot less is known about spinal LTP and central sensitization, as compared to hippocampal LTP, some important characteristics are similar for the two phenomena. The first is the action of glutamate – the most abundant excitatory neurotransmitter in the CNS, which is the key transmitter in both cases. Experiments conducted on *in vitro* horizontal splice preparation of the hamster SCDH suggest that the direct excitation of spinal cord neurons by afferent fibres depended on the activity of L-glutamate (Schneider and Perl, 1988).

Second, it has been established that the initiation of spinal LTP, hippocampal LTP and central sensitization all depend on NMDA-R activation. High-frequency sciatic nerve stimulation elicited LTP in the spinal cord – that was attenuated by spinal application of an NR2B-subunit-specific NMDA-R antagonist, suggesting that it is NMDA-Rs containing the NR2B subunit that are responsible for initiating spinal LTP (Pedersen and Gjerstad, 2008).

Lastly, calcium influx is vital for LTP induction in both the hippocampus and the spinal cord. Superficial dorsal horn lamina I neurons exhibited an LTP-like sensitized state following conditioning stimulation of C-fibres, only if intracellular calcium levels rise (Drdla and Sandkuhler, 2008), and post-synaptic increase of Ca^{2+} in the absence of presynaptic stimulus has been shown to elicit LTP in spinal cord slices (Naka et al., 2013). Although glutamate and NMDA-Rs are key, LTP in the SCDH has also been shown to require the co-activation of NK1-, NMDA-Rs and T-type calcium channels (Ikeda et al., 2003). The second phase of the formalin test has been shown to depend on calcium influx through NMDA-R (Coderre and Melzack, 1992).

1.5.2 Initiation versus maintenance

As discussed above, there are important mechanistic differences in the initiation and the maintenance of nociceptive central sensitization underlying persistent pain that share similarities

with LTP and long-term memory. The initiation of central sensitization has been shown to be linked to an activation of NMDA-R, induction of CamKII activity and subsequent c-AMP-dependent recruitment of PKA, as well as a diacylglycerol-dependent activation of PKC. Substrates of both PKA and PKC proteins are responsible for house-keeping and activation of neurons affecting such processes as neurotransmitter release and receptor channel opening (Latremoliere and Woolf, 2009). Importantly, these protein kinases are only activated transiently, and return to their inactive resting state within tens of minutes or a few hours (Sacktor, 2011). Levels of full-length PKCs (ζ, γ, β) were upregulated in pain models characterized by peripheral inflammation, i.e. CCI, mononeuropathy, CFA & carrageenan-induced inflammation, formalin, capsaicin (Yajima et al., 2003, Miletic, 2000 #67, Mao, 1995 #63, Zhou, 2003 #73, Marchand, 2011 #14, Laferriere, 2011 #2310). Importantly, their inhibition yielded analgesic effects only if administered prior to injury preventing the induction phase, while post-injury administration only resulted in transient analgesic effects (Hua et al., 1999). Moreover, inhibition of PKA at 24 h following i.m. acidic saline injection has been shown to relieve mechanical hypersensitivity (Hoeger-Bement and Sluka, 2003), but inhibition of either PKA or PKC one week following tissue injury did not reverse mechanical hypersensitivity (Hoeger-Bement and Sluka, 2003, Sluka, 2006 #2176). Thus, a role for PKA and PKC involvement in the induction phase has been well established. However, it is unclear what mechanisms maintain heightenened synaptic transmission during persistent pain.

Due to the large heterogeneity of persistent pain syndromes and models, delineating a common maintenance mechanism requires some important considerations. In many clinical syndromes, as well as in corresponding animal models, both peripheral and central components are intertwined to manifest these long-lasting, difficult to treat chronic conditions. In the quest of understanding the underpinnings of central components, isolating the central from peripheral mechanisms is both beneficial and challenging. In neuropathic pain, described above, central changes play an important role in the maintenance of the pain. However, these are by far not the

only causality for this pain perception, as evidenced by the lack of analgesia when blocking crucial components of central sensitization after, but not before, nerve injury.

1.6 PKMζ in LTP and memory maintenance

1.6.1 PKC family

The PKC family consists of three subtypes classified by their protein structure and second messenger requirements: the conventional or classical PKCs (α, βI, βII, γ), novel PKCs (δ,ε,η,θ) and atypical PKCs (aPKC) comprising PKCλ/ι (mouse λ is an ortholog to the human or rat ι), PKCζ and PKMζ (Hirai and Chida, 2003). Except PKMζ, which only consists of a catalytic domain, all PKCs consist of a C-terminal catalytic and an N-terminal regulatory domain that features a pseudosubstrate inhibitory sequence. Only in the presence of second messengers do these full-length PKCs temporarily lose their autoinhibition to further phosphorylate their substrates. For conventional and novel PKCs diacylglycerol is required for its activation. While activation of classical PKCs is also regulated by intracellular calcium and respective calcium-calmodulin kinase, novel PKC activation is independent of intracellular calcium levels. Activation of atypical PKCλ and PKCζ, however, is independent of DAG and requires phophorylation downstream of PI3K (Hirai and Chida, 2003, Suzuki et al., 2003). Because of its truncated structure, PKMζ activity is independent of second messengers.

1.6.1.1 Atypical PKC gene structure

While PKCλ is transcribed from the *prkcl* gene, PKCζ and PKMζ are both transcribed from the *prkcz* gene. Originally thought of only as a proteolytic fragment of PKCζ (Inoue et al., 1977), it is now known that a separate promoter can also transcribe PKMζ mRNA from the *prkcz* gene (Hernandez et al., 2003), and that PKMζ is more often formed by transcription than by cleavage of PKCζ, particularly in areas such as the hippocampus, which has little or no PKCζ. PKMζ and PKCζ exhibit differential mRNA structures that feature differential translational starting sites. PKMζ mRNA closely resembles the PKCζ coding sequence for the catalytic domain. Although the 3'

untranslated regions are identical, as well as the final 1864bp that are part of the coding sequence, the 5' region is unique in both isozymes (Hernandez et al., 2003). aPKCs, similar to all AGC kinases (named after the grouping of PKA, PKG and PKC) exhibit an ATP-binding region, an activation loop, a turn motif and a hydrophobic motif in their catalytic domain. PKCζ is activated through "activation loop" phosphorylation (threonine residue 410), and authophosphorylation at the turn motif (T560). In PKCζ, the catalytic domain is connected to the regulatory domain, which features a PB1 domain (Phox and Bem1), which is involved in protein-protein interaction, a pseudosubstrate domain and atypical C1 domain that together are responsible for autoinhibition in the resting state.

Following its synthesis, PKMζ, as well as most other AGCs, requires constitutive phosphorylation of its "activation loop" through phosphoinositide-dependent kinase 1 (PDK1) – unlike full-length PKCs it does not require further turn motif phosphorylation. Lacking the regulatory domain, the constitutive phosphorylation by PDK1 leaves PKMζ persistently active – a unique and highly suitable structure to exhibit a "maintenance" function within persistently active biological processes (for schematic gene structures of PKMζ and PKCζ, see Appendix Fig. 8.3).

1.6.2 PKMζ and the maintenance of memories

The earliest studies correlated PKMζ activity with long-lasting EPSPs during transcription-based hippocampal late-LTP (Osten et al., 1996), and showed its cytosolic protein levels increased in the maintenance phase of LTP (Sacktor et al., 1993). The break-through in memory and LTP research was Sacktor & colleagues' discovery that the persistently active PKMζ was necessary and sufficient for LTP maintenance. Thus, whole cell patch clamp recordings of hippocampal cells displayed EPSPs with PKMζ application, and specific inhibition of PKMζ abolished previously potentiated responses (Ling et al., 2002). PKMζ received further attention through pioneering *in vivo* experiments establishing that inhibition of PKMζ eradicated previous learned spatial memory –

and with it provided further support for LTP as the neural correlate of memory maintenance (Pastalkova et al., 2006). Other studies highlighted PKMζ's requirement for several types of memory, such as allocentric spatial memory (Serrano et al., 2008), object location (hippocampus) (Hardt et al., 2010), conditioned taste aversion (insular cortex) (Shema et al., 2007), and auditory fear conditioning (amygdala) (Serrano et al., 2008).

1.6.3 How does PKMζ maintain memories?

In memory pathways, PKMζ activity is tightly regulated. In the resting state, protein interaction with NIMA 1 (PIN1) translationally blocks PKMζ mRNA (Westmark et al., 2010). Kinases activated during LTP induction, most prominently MAPK, ERK, CamKII, PKC, PKA, mTOR act as second messengers to release the translational block on PKMζ, allowing it to be transcribed and phosphorylated and ultimately enhance the expression of AMPA-R at the postsynapse. The resulting increase in synaptic AMPA-R is thought to cause enhanced persistent synaptic transmission which is needed to encode long-term memories.

How does PKMζ increase the number of AMPA-Rs on the postsynapse? A recent study suggested that PKMζ does so by influencing the NSF-GluR2-dependent AMPA receptor trafficking, thereby increasing the available amount of postsynaptic AMPA receptors, by preventing AMPA-R endocytosis (Yao et al., 2008). NSF is a multihomomeric ATPase that is involved in vesicle fusion, membrane fusion events and neurotransmitter release (Hanson et al., 1997, Rothman, 1994). It interacts with the C-terminal domain of GluR2 to stabilize AMPA receptors in the postsynaptic membrane, preventing their endocytosis (Nishimune et al., 1998). Blocking this interaction using anti-NSF monoclonal antibody diminished synaptic transmission (Nishimune et al., 1998). Further, a small peptide pep2m that targets the binding domain of GluR2 in the post-synaptic membrane, reduced the frequency, but not the amplitude of AMPA receptor-dependent synaptic transmission (Noel et al., 1999). NSF has been shown to retain AMPA-R at the postsynapse by uncoupling of PICK1-GluA2 complexes storing a pool of these subunits near the

membrane (Hanley et al., 2002). These uncoupling events occur in the presence of rising intracellular calcium levels (Hanley, 2007). Whole-cell recordings of hippocampal CA1 slices showed enhanced EPSPs following postsynaptic PKMζ perfusion, that was blocked by simultaneous perfusion of either pep2m or pep-NSF3 (a blocker of NSF ATPase activity) (Yao et al., 2008).

Further support for PKMζ's maintenance mechanism through enhancing AMPA-R postsynaptic activity was given in several experiments. Inactivation of PKMζ in the amygdala prevented fear memory development, which correlated with reduced postsynaptic GluR2 levels. Simultaneous infusion of GluR2 endocytosis inhibitors and the PKMζ inhibitor ZIP, abolished ZIP's memory-deleting phenotype (Migues et al., 2010), suggesting PKMζ was involved in retaining GluR at the postsynapse. Indeed, *in vivo* pre- or post-treatment with ZIP, but not scrZIP, caused a reduction of high frequency stimulation-induced late-LTP, which could be prevented by co-application of GluA2. Virus-based PKMζ knockdown also caused a decay of late-LTP, a process that again was prevented by application of GluA2. Importantly, a strong stimulation, such as a foot shock during training enhanced memory, together with inducing increased PKMζ and GluA2 in the hippocampus. In a different set of memory tasks, chronic application of GluA2 prevented the decay of memories produced by ZIP treatment, such that mice spent less time finding the hidden platform in the water maze task (Dong et al., 2015). These results confirm that PKMζ maintains late-LTP EPSPs that underlie long-term memory by preventing GluA2-dependent AMPA-R endocytosis (Dong et al., 2015). Understanding how PKMζ may be regulated during maintenance of central sensitization- dependent spinal nociception is the aim of study 4 of this thesis.

1.6.4 Specificity of ZIP

Most studies that have explored the role of PKMζ in long-term memory and pain maintenance utilized pharmacological inhibition using ZIP. Questioning these results, a study overexpressing PKMζ fusion protein in cultured cells and hippocampal slices showed that ZIP did

not effectively inhibit the protein (Wu-Zhang et al., 2012). However, the amount of overexpressed PKMζ in this artificial system largely exceeded the amount normally present during a potentiation event, and the efficacy of ZIP in inhibiting PKMζ phosphorylation decreased with increasing PKMζ concentrations to a level resulting in excess "spare" kinase activity (Yao et al., 2013)

In 2013, two independent studies using constitutive and conditional knockout of the *prkcz* gene contested the specificity of ZIP *in vivo*. Mice with a genetic deletion of exon 11 of the *prkcz* gene, rendering PKM/Cζ inactive, displayed normal late-LTP in response to theta-burst and high-frequency stimulation. This long-term maintenance was reversed by application of ZIP (Volk et al., 2013). Similarly, (Lee et al., 2013) using deletion of exon 9 of the *prkcz* gene, found normal LTP maintenance in knockout mice, as well as normal spatial, fear and recognition memory that were all reversed by application of ZIP. Further, ZIP blocked not only PKMζ, but also PKClambda in this study. Together these results allow for the conclusion that it is not PKMζ, but another protein kinase that maintains long-term memories. In line with this evidence, a spinal pain study showed that not only PKMζ, but also PKCλ expression in the spinal cultured synaptoneurosomes increased 15 and 30 min after stimulation with BDNF, and ZIP inhibited PKCλ (Melemedjian et al., 2013)

A recent study allows for a different interpretation of these results: PKMζ is essential for the maintenance of long-term memories, and its loss in knockout mice is heavily compensated by the atypical aPKC isozyme PKCι/λ. Tsokas et al. (2016) convincingly showed that PKCι/λ was upregulated in prkcz$^{-/-}$ mice, and its inhibition using a PKCι/λ-antagonist reversed late-LTP, as well as acquired spatial long-lterm memories. Emphasising a compensatory mechanisms specifically after knockout, the authors showed that PKMζ antisense blocked late-LTP in wildtype, but not in knockout mice, suggesting a mechanism other than PKMζ had taken over the maintenance role (Tsokas et al., 2016). Additionally, backing the case for PKMζ, and not other isozymes, in the maintenance of long-term memories is seen in the examination of the direct effects of PKMζ

application. Injection of PKMζ-expressing virus into the neocortex enhanced taste aversion memories, and dominant-negative PKMζ reversed them (Shema et al., 2011). Direct application of PKMζ enhances AMPA-dependent synaptic transmission, and blocking AMPA-R synaptic transmission reverses late-LTP and as well as memory performance. Further it was shown that blocking GluR2-removal from the postsynapse prevents the memory- and LTP-abolishing phenotype elicited by ZIP, suggesting ZIP's memory deleting phenoype stems of disruption of PKMζ's effects on AMPA-R maintenance (Migues et al., 2010). If ZIP were acting via a non-specific mechanism, the PKMζ-dependent AMPA-transmission would not be abolished, however. Altogether these studies strongly indicate that it is PKMζ and not other PKC isozymes that maintains long-term memories and is a prime candidate for maintenance of long-lasting pain.

1.6.5 The role of full-length PKCs in persistent pain

Multiple PKC isozymes have been implicated to play a role in the induction, but not maintenance, of persistent pain states. This is in accordance with the role of PKCs in the phosphorylation and trafficking of AMPA and NMDA-Rs (see above). Indeed, several PKC isoforms are upregulated in response to neuropathic injury, inflammatory, chemical and referred pain induction (Sluka et al., 1997, Cesare et al., 1999, Zhou et al., 2003, Narita et al., 2004). For example, following adjuvant-induced inflammation, increases in thermal hyperalgesia are correlated with increased expression of PKCβII in the membrane fraction of lumbar spinal cord (Igwe and Chronwall, 2001). PKCγ KO mice showed normal acute pain responses, but reduced persistent pain following nerve injury (Malmberg et al., 1997). However, PKCγ KO does not fully abolish persistent pain perception: PKCγ KO mice displayed reduced neuronal hyperexcitability in response to mustard oil application, but intact mechanical allodynia (Martin et al., 2001).

Importantly, several studies have shown that PKC inhibition following pain induction in the maintenance phase did not reduce or reverse exisiting hypersensitivity: mechanical allodynia after

i.m. acidic saline (Sluka and Audette, 2006), second phase formalin pain (Laferriere et al., 2011), or persistent pain following hyperalgesic priming (An et al., 2015), suggesting full-length PKCs may not be responsible for the maintenance of persistent pain.

1.7 Aims

The primary goal of this thesis is to elucidate the role of PKMζ in the maintenance of persistent pain. While the induction mechanisms are relatively clear, the maintenance mechanism underlying persistent pain is unknown. Evidence suggests that PKMζ may play a role in the maintenance of short-lasting persistent pain following i.pl. capsaicin or formalin (Laferriere et al., 2011, Marchand et al., 2011, Melemedjian et al., 2013), yet its role in different types of long-lasting persistent pain models, and its regulation within the SCDH remains elusive.

This thesis targets four specific aims. The first aim is to elucidate the role of PKMζ in models of longer lasting neuropathic and inflammatory pain after pharmacological inhibition using ZIP. This aim also asssessed the influence of increased peripheral inputs on the effect of ZIP in a model of chemical pain, as well as contrasting ZIP's effects on ipsilateral and contralateral allodynia in neuropathic rats (study 1). The second aim was to elucidate the role of PKMζ (again using ZIP) in referred pain models of i.m. acidic saline and i.c. capsaicin, where there is a separation between the injury and testing sites (study 2). The third aim was to directly test the role of PKMζ in these pain models through use of constitutive PKM/Cζ KO mice, as well as studying potential sex differences in these mice (study 3). The fourth aim was to examine how PKMζ is regulated in the SCDH during NMDA-induced nociception (study 4).

2 Study 1.

Effects of PKMζ inhibition in animal models of persistent pain: influence of peripheral nociceptive input

2.1 Rationale

Chronic pain is associated with the maintenance of synaptic changes in the CNS. PKMζ has been shown to maintain synaptic changes in memory and pain pathways (Asiedu et al., 2011, Laferriere et al., 2011, Sacktor, 2011), and its inhibition following cutaneous injury or spinal glutamatergic agonist dihydroxyphenylglycine (DHPG)-induced nociception reversed lowered mechanical thresholds up to 2 h and 48 h respectively (Laferriere et al., 2011). However, PKMζ's role in long-lasting persistent pain, such as neuropathic and inflammatory pain, is less clear. On the one hand, studies suggest a role for PKMζ in the maintenance of these pain models. Thus, nerve injury increased phosphorylated PKMζ levels in the ACC at 3 days (Li et al., 2010) and 10 days (King et al., 2012), and bilateral injection of the PKMζ inhibitor ZIP into the ACC alleviated mechanical neuropathic allodynia in mice transiently (Li et al., 2010), and relieved spontaneous, but not evoked tactile and thermal hypersensitivity in nerve-injured rats (King et al., 2010). Additionally, electrophysiological experiments evidenced a role for PKMζ both in neuropathic and inflammatory pain. Thus, ZIP alleviated the nerve-injury induced increase in EPSC amplitude and number of active AMPA channels in ACC neurons (Li et al., 2010), reduced the capsaicin-induced increase in firing rates of WDR neurons in the dorsal horn (Laferriere et al., 2011), and the spinal WDR firing rate following i.pl. formalin (Marchand et al., 2011). Together, these findings suggest that PKMζ plays a role in the heightened transmission following neuropathic, inflammatory and chemical pain.

On the other hand, spinal blockade of PKMζ was ineffective in relieving long-lasting neuropathic or inflammatory pain. ZIP injected into the SCDH did not relieve allodynia in common peroneal nerve (CPN)-ligated mice (Li et al., 2010) or CCI-rats (Laferriere et al., 2011, Marchand et al., 2011, King et al., 2012), and produced only transient anti-allodynia in SNL-rats (King et al., 2012). Additionally, spinal neuron EPSCs in neuropathic and sham-operated mice displayed similar amplitudes following ZIP injection (Li et al., 2010). Moreover, PKCζ/PKMζ phosphorylation was

increased in SCDH neurons following CFA, but this increase was not reduced following i.t. ZIP. ZIP was also unable to elevate mechanical paw withdrawal thresholds (PWTs), and only transiently elevated thermal PWLs that are both reduced in CFA-treated rats (Marchand et al., 2011). Both neuropathic and inflammatory pain models exhibit significant peripheral injury and ongoing peripheral inputs to the spinal cord (Coderre et al., 1993b). In an attempt to dissociate the role of PKMζ in peripherally-dependent and centrally-dependent long-lasting pain, i.t. ZIP was injected to rats with chronic post-ischemia pain (CPIP) during the early phase characterized by strong peripheral inflammation, and in the late phase when peripheral changes have resolved and continued allodynia depends on central sensitization (Laferriere et al., 2008). Indeed, no ZIP-induced changes in PWTs were observed in early-CPIP rats, while importantly the allodynia present in late-CPIP rats was reversed by PKMζ inhibition (Laferriere et al., 2011).

Recent experiments showed an increase in total PKMζ/PKCζ/λ levels following 2% formalin injection to the rat hind paw (Laferriere et al., 2011). Marchand et al. (2011) also reported increases in phosphorylated PKCζ/PKMζ levels following 5% formalin, with ZIP treatment reducing pPKM, but not pPKC levels. Nociception in the formalin test has been shown to depend on a peripheral signaling-dependent first phase, and a central sensitization-dependent second phase. Importantly, ZIP dose-dependently reduced pain scores in the centrally-dependent late phase, but importantly, not the first phase at 2% formalin (Laferriere et al., 2011). Neither the control peptide scrZIP, nor the full-length PKC inhibitor NPC15437 yielded an analgesic effect (Laferriere et al., 2011).

It is unclear, what role PKMζ plays in persistent nociception that heavily depends on ongoing peripheral input. We expect that spinal PKMζ contribution to pain maintenance is obscured in pain models in which there is significant ongoing peripheral input. Here, we aim to further examine the role of PKMζ in the maintenance of neuropathic, inflammatory, and chemical nociception. Through pharmacological inhibition using ZIP, we propose to test the effects of PKMζ inhibition on

mechanical and thermal thresholds in rats with SNI-induced neuropathic pain and CFA-induced hind paw inflammation, and on nociceptive scores in rats receiving varying concentrations of hind paw formalin injections that yield low, medium and high levels of peripheral inflammation. We hypothesized that PKMζ inhibition will alleviate centrally-dependent (second phase formalin nociception accompanied by low peripheral input), but not largely peripherally-dependent long-lasting pain (neuropathic and inflammatory).

2.2 Materials and Methods

2.2.1 Animals

All procedures on rats and mice were approved by the McGill Animal Care Committee and all animal care guidelines were observed.

2.2.2 Subjects

Long Evans hooded rats were obtained from Charles River laboratories (Quebec, Canada) and kept in-house with a 12 h dark-light cycle, and with food and water available *ad libitum*. Following arrival, a miminum acclimatization period of 2-5 days was allowed before the start of experiments.

2.2.3 Procedures

2.2.3.1 Inflammatory pain

Rats were given a 50 µl i.pl. injection of 1 mg/ml CFA in the left hind paw. We examined the effects of i.t. ZIP on CFA-induced mechanical allodynia and thermal hyperalgesia using von Frey and Hargreaves tests (see below) 24 h after pain induction.

2.2.3.2 Neuropathic pain

Neuropathic pain was induced using the spared nerve injury (SNI) model of Decostered and Woolf (2010), established by transecting the common peroneal and tibial nerve branches, sparing

the adjacent sural and saphenous nerves. Rats received i.t. ZIP ten days or 6 weeks post SNI surgery. PWTs were measured using von Frey filaments (described below) on both contralateral and ipsilateral sides.

2.2.3.3 Chemical pain

A volume of 50 µl of 2%, 3.5% or 5% formalin (Sigma, in 0.9% saline) was injected i.pl. in to the left hind paw of i.t. catheterized rats (see below). At the peak of the second phase of the formalin test (26 min), ZIP or scrZIP was injected via i.t. catheter. Nociception was assessed for 44 min as described below in the section labelled Formalin test.

2.2.4 Drug administration

All drugs were administered in a volume of 20 µl into the i.t. space by either lumbar puncture or an indwelling i.t. catheter. ZIP, and its inactive control peptide scrZIP (Tocris), were dissolved in sterile water and administered at a dose of 10 nmol.

2.2.4.1 Lumbar puncture

A 26 gauge ½ inch needle was inserted between the L5 and L6 vertebrae into the *cauda equina* while the animal was anesthetized with isoflurane (4% induction, 2% maintenance). Correct placement of the needle was verified by flick of the tail at initial needle placement and on drug injection.

2.2.4.2 Catheter placement

A 5 cm polyethylene catheter (PE-32) was inserted through a 23 gauge needle between the L5 and L6 vertebra and pushed 4 cm to reach the the lumbar enlargement in rats under pentobarbital anesthesia. After removal of the needle, the outer end of the PE-32 catheter was glued to a 14 cm piece of PE-10 catheter. The tight connection between PE-10 and PE-32 catheters was verified by flushing saline through the connected catheters. Several stitches underneath and over the skin anchored the catheter until the time of testing. Rats were given a 24 h recovery period between catheter placement

and formalin testing. Drugs were administered i.t. in a volume of 20 µl and flushed with the quantity of saline measured to fill the catheter. Subsequently, temporary hind paw paralysis after lidocaine injection verified correct i.t. placement of the catheter.

2.2.5 Behavioral assays

2.2.5.1 von Frey test

Mechanical allodynia was determined by measuring the 50% mechanical PWT using stimulation with von Frey filaments (nylon filaments, Stoelting, Woodale, IL, USA) according to the modified "up-down" method described by Chaplan et al. (1994). Rats were placed in clear plastic cubicles on an elevated mesh-wire and allowed to acclimate between 20 and 40 minutes before applying filaments for 10 s to the plantar surface of the rat hind paw. Filament strength (0.25 g – 15 g) was increased with every negative response, and decreased with a positive response (lifting of paw), until 5 responses were recorded following the first positive response. The 50% threshold was calculated according to the response pattern following the formula: 50% g threshold = $(10^{[xf + k\delta]})/10{,}000$ (xf = strength (in log units) of von Frey hair applied last; k denotes the tabular value described in Chaplan, 1994; δ = mean difference (in log units) between stimuli).

2.2.5.2 Hargreaves test

For acclimatization, rats were placed in clear plastic cubicles placed on a glass plate. Radiant heat from a light source heated the glass under the plantar surface of the rat hind paw until the rat withdrew its hind paw. The radiant heat was set at an intensity that normally produced latencies of about 10 seconds. Both left and right PWLs were measured interspaced by 10 minute intervals. To prevent tissue damage, a cut-off of 20 seconds was set. The reported thermal latency was determined by averaging three consecutive measures of PWLs spaced 10 minutes apart.

2.2.5.3 Formalin test

Rats were acclimatized to the testing chamber for 15 minutes prior to testing. The testing chamber consisted of a Plexiglas® box allowing the rat to move freely. A mirror installed at a 45

degree angle beneath the box aided viewing of the hind paws. The formalin test employed rating of nociceptive behavior on a 4 point scale: 0 – no pain behavior, 1 – favoring of the paw, 2 – lifting of the paw, 3 – licking/shaking the paw (Dubuisson and Dennis, 1977). Total nociceptive score was determined using a weighted means method of behavioral scoring (Coderre et al., 1993a).

2.2.6 Data analysis

All data are presented as mean ± SEM. Graphs were plotted using GraphPad Prism (version 6.0). All statistical analyses were carried out using SPSS. All time course data were analysed using repeated measures ANOVA followed by multiple comparisons with Bonferroni post-hoc test.

2.3 Results

2.3.1 Effects of PKMζ inhibition on mechanical thresholds in neuropathic rats

To understand whether PKMζ plays a role in the maintenance of neuropathic pain, rats were subjected to SNI and treated with either ZIP or scrZIP 10 days post-surgery. SNI induced a decrease in PWTs at 10 d post-surgery (mean baseline/post-test difference 11.092 ± .627, $p < .001$). The graph shows that neither ZIP, nor scrZIP, elevated thresholds in the ipsilateral paw between 10 and 12 days post-surgery. This was confirmed by the nonsignificant main effect of treatment in our repeated measures ANOVA ($F(1,10) = 2.928$, $p = .118$, Figure 2.1A).

Additionally, we tested ipsilateral and contralateral mechanical PWTs for 6 weeks. PWTs on the contralateral paw remained at baseline levels 10 day post-surgery, but gradually decreased so that rats exhibited significantly lower mean PWTs in both the contralateral (4.72 ± .62) and the ipsilateral (3.94 ± 0.59) hind paw at 6 weeks post-surgery (Figure 2.1B). When ZIP was injected at 6 weeks, contralateral PWTs were transiently, but significantly increased at 30 min ($F(1,10) = 10.627$, $p = .009$) and 60 min ($F(1,10) = 12.438$, $p = .005$) post-drug application (Figure 2.1C). Importantly, PWTs in the ipsilateral paw did not increase following PKMζ inhibition using ZIP.

Only the allodynia in the contralateral paw was alleviated for at least 1 hr and returned to allodynic levels by 24 h.

2.3.2 Effects of PKMζ inhibition on mechanical and thermal thresholds in CFA-injected rats

2.3.2.1 Mechanical allodynia

Hind paw injection of CFA yielded a sustained decrease in mechanical thresholds between 24 h and 72 h. I.t. ZIP or scrZIP injection at 24 h post-CFA did not increase PWTs as compared to pre-drug value (Figure 2.2A).

2.3.2.2 Thermal hyperalgesia

PWLs were decreased significantly between 24 h and 72 h post-CFA. The reduced PWLs after CFA treatments were unaffected in either scrZIP or ZIP-treated rats. Accordingly, ANOVA revealed a non-significant main effect of treatment (Figure 2.2B, $F(1,10) = 0.005$, $p = .943$). Thus, hind paw injection of CFA results in prolonged mechanical and thermal hypersensitivity that is not reversed by PKMζ inhibition.

2.3.3 Effects of PKMζ inhibition on late-phase formalin nociception depends on formalin concentration

I.pl. injections of varying concentrations of formalin (2%, 3.5% and 5%) all produced biphasic nociceptive behaviors in scrZIP treated rats. While nociceptive responses to formalin were unaffected by scrZIP (as scores were similar to untreated rats in earlier studies), they were significantly reduced in ZIP-treated rats, in a manner that depended on the concentration of formalin used. With 2% formalin, ZIP-treated rats displayed a significant reduction of nociceptive scores in the second phase 32-44 minutes post formalin, compared to scrZIP-treated rats. Accordingly, a significant main effect of treatment was revealed by ANOVA ($F(1,10) = 17.312$, $p = .002$) (Figure 2.3A). For 3.5% formalin, rats administered ZIP 26 min after formalin displayed

reduced nociception at 38, 42 and 44 minutes post formalin, compared to scrZIP-treated rats (Figure 2.3B). Conversely, with 5% formalin, ZIP-treated rats did not display a reduction in nociceptive scores compared to scrZIP-treated rats (Figure 2.3C). ZIP thus relieved second phase nociception at low and medium, but not high concentration of peripherally-injected formalin.

2.4 Discussion

This study provides novel insight into the role of PKMζ in models of persistent pain, and the contribution of ongoing peripheral input to PKMζ-dependent nociception. In this study, we have shown that pharmacological inhibition of PKMζ using ZIP did not reverse SNI-induced ipsilateral mechanical allodynia. We have further shown the development of contralateral allodynia in SNI-subjected rats at 6 weeks after surgery that was transiently reversed by i.t. PKMζ inhibition. In line with previous findings (Marchand et al., 2011, King et al., 2012), PKMζ inhibition 24 h after CFA did not relieve mechanical allodynia and thermal hyperalgesia, suggesting PKMζ inhibition alone was not responsible for sustaining the underlying nociceptive hypersensitivity. In order to discern the role of central neuroplasticity from ongoing peripheral pathology in persistent pain, we conducted formalin tests with increasing concentrations of formalin: 2%, 3.5% and 5%. We reproduced previous findings (Laferriere et al., 2011), showing PKMζ inhibition reduced second phase nociception after 2% formalin, and importantly show that second phase pain induced by 3.5% formalin was partially reduced, while ZIP did not relieve nociception induced by 5% formalin.

We have shown that inhibition of spinal PKMζ is insufficient for alleviating ipsilateral SNI-induced neuropathic pain. Our findings are in line with previous studies conducted in rats with CCI, SNL and CPN-ligation evidencing PKMζ inhibition using i.t. ZIP did not alter the hypersensitive neuropathic phenotype (Li et al., 2010, Marchand et al., 2011;Laferriere, 2011 #2384, King et al., 2012). The SNI model of neuropathic pain has been well established to produce robust and reproducible mechanical and heat hypersensitivity (Decosterd and Woolf, 2000, Mogil et al., 2010). Recent studies show that sensory afferent sensitization plays an important role in the establishment

and maintenance of SNI-induced mechanical allodynia. *Ex vivo* recordings of fibres from day 16-42 SNI rats show an enhanced firing of action potentials in Aδ and C fibres in response to mechanical stimulation (Smith et al., 2013). Similarly, partial spinal nerve ligation (pSNL) was associated with the cutaneous sensitization of fast conducting myelinated mechanoreceptors contributing to the persistent decrease in PWTs (Boada et al., 2015). Moreover, skin innervated by undamaged nerve fibres neighbouring damaged fibres exhibited hypersensitivity. Electrophysiological recordings of L4 dorsal root ganglion neurons one week following L5 dorsal root pSNL displayed sensitization properties (lowered thresholds, increased receptive field sizes), suggesting an enhanced peripheral input contributes to the hypersensitivity phenotype (Boada et al., 2015). Together, these findings indicate that not a change in spinal cord neurons *per se*, but a change within the peripheral nociceptive input is the main contributor to the maintenance of hypersensitivity after nerve injury. Thus, targeting synaptic changes within the spinal cord, i.e. reversal of central sensitization, is unlikely to cause a change in behavioral phenotype. Our results displaying a lack of analgesia following i.t. inhibition of PKMζ in the SCDH 10 days post SNI surgery is thus in line with these findings, suggesting ongoing peripheral pathology may continuously be contributing to altered mechanical thresholds. To fully understand the contribution of PKMζ to persistent pain, a differentiation of peripheral ongoing pathology from central synaptic plasticity is necessary.

Our tests for mechanical hypersensitivity from 10 d to six weeks post SNI surgery displayed a persistent ipsilateral mechanical allodynia, which is in line with previous findings (Pertin et al., 2005, Mogil et al., 2010). Contralateral thresholds have not been shown to be significantly decreased by SNI in mice and rats (Pertin et al., 2005, Polgar and Todd, 2008)) or CCI mice (Mogil et al., 2010) up to 28 d after surgery. Similarly, in our experiment, we observed non-allodynic PWTs at 10 d and 18 d following surgery. However, when testing continued to 6 weeks, we observed a gradual development of allodynia. Although there are other studies that have not reported contralateral hypersensitivity after nerve injury (Pertin et al., 2005, Polgar and Todd, 2008,

Mogil et al., 2010), this controversy could be explained by strain differences (Mogil et al., 1999, Mogil et al., 2005, Cahill et al., 2014) or by our determination that contralateral alterations may not occur until many weeks after injury, at time points most studies do not assess hypersensitivity.

Inhibition of PKMζ at six weeks transiently relieved the contralateral mechanical allodynia for 60 minutes. These results suggest ongoing peripheral inputs from the injured hind limb for several weeks cause a PKMζ-dependent central sensitization which underlies contralateral allodynia. To further delineate the role of PKMζ in the development of central sensitization, future studies may measure separate ipsi- and contralateral SCDH levels of PKMζ following SNI surgery.

We added further support to earlier studies showing that PKMζ inhibition using ZIP does not alter CFA-induced inflammatory pain during ongoing acute inflammation. We showed that ZIP relieved neither mechanical allodynia, nor thermal hyperalgesia when injected 24 h after CFA injection. In accordance with Sun et al. (2014), who showed ZIP injection into the periaqueductal gray (PAG) did not alter CFA-induced mechanical and thermal hypersensitivity, and Marchand et al. (2011), where ZIP did not reverse CFA-induced mechanical allodynia, and only had a minor, brief effect on thermal hyperalgesia. These data suggest PKMζ inhibition during an inflammatory state does not yield significant phenotypic changes. We expect that ongoing peripheral inputs from the inflamed hind paw overwhelm any reduction in nociception associated with spinal PKMζ inhibition. This conclusion is supported by experiments which showed that injecting i.t. ZIP to CPIP rats early after injury, when there is evidence of peripheral tissue damage, could not alleviate allodynia; whereas allodynia was alleviated when injecting i.t. ZIP three weeks after injury, when the peripheral tissue damage had resolved. To further delinate the contribution of peripheral inflammation to PKMζ mediated long-lasting pain, we injected ZIP via an i.t. catheter at the peak of the second phase of the formalin test following 2%, 3.5% and 5% formalin. As demonstrated previously (Laferriere et al., 2011), we again showed that nocicieption induced by 2% formalin could be significantly reversed by PKMζ inhibition. However, the antinociceptive effects of ZIP in

the formalin test were reduced as the formalin concentration increased from 3.5 to 5.0% formalin, with no antinociceptive effects at 5% formalin. These data strongly suggest, PKMζ is responsible for the maintenance of formalin-induced central sensitization – yet its pain relieving effect may be masked at higher formalin concentrations. Indeed 5% formalin concentration has been shown to result in prolonged edema associated with increased vascular permeability and cellular inflammatory responses, while lower concentrations of formalin (< 2%) produce a short-lived neurogenic inflammation (Damas and Liegeois, 1999). Since all three concentrations normally produce biphasic nociceptive responses, the behavioral readout is not sufficient to delineate the underlying mechanistic processes. These findings further add to the notion that central sensitization may underlie a variety of pain conditions. However, the sole inhibition of its maintenance may not *per se* be sufficiently analgesic, as the overall pain expression or even reactivation of maintenance mechanisms may depend more on ongoing peripheral input and signaling.

This experiment further adds support to the notion that second phase formalin pain is associated with central sensitization. Since we conducted the formalin experiments in animals with an indwelling i.t. catheter, we were able to measure the effects of differential formalin concentrations in freely moving and awake animals. This is an important advantage in the investigation of central sensitization, since we were able to direclty examine the effects of PKMζ inhibition without confounding anesthesia, and were thus able to show an important role for PKMζ in the maintenance of second phase formalin pain. Further experiments examining the regulation of PKMζ and its effects on enhanced glutamatergic transmission (the postulated mechanism of PKMζ in LTP) are required to fully understand central sensitization and its maintenance in formalin-induced pain.

In conclusion, this study provides important and novel information regarding the role of PKMζ in long-lasting persistent pain characterized by peripheral ongoing inputs. We have shown that the amount of peripheral inflammation critically determines the degree of analgesia achieved

by PKMζ inhibition. Higher peripheral inflammation coupled to increased peripheral signalling and ongoing inputs to the spinal cord thus may continuously modify pain processing and central sensitization such that PKMζ inhibition does not produce antinociceptive effects (ipsilateral mechanical allodynia following SNI, CFA-induced mechanical and thermal hypersensitivity, and high-concentration formalin nociception). Both the antinociceptive effects of PKMζ inhibition on second phase formalin pain following 2% and 3.5%, as well as its transient anti-allodynic effects on contralateral allodynia following SNI, suggest that PKMζ activity is required for sustaining central sentization-dependent pain, making PKMζ an interesting target in the treatment of pain conditions characterized by low peripheral input.

2.5 Study 1 Figures

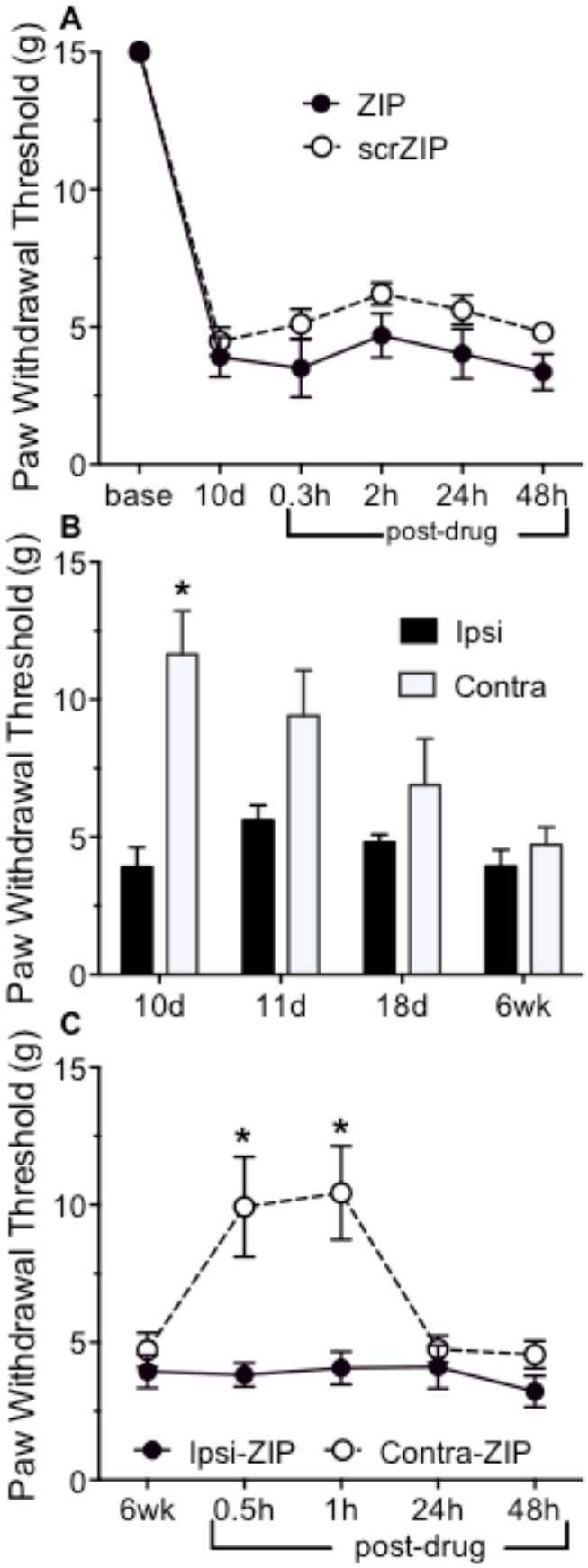

Figure 2.1. PKMζ effects on SNI-induced ipsilateral and contralateral mechanical allodynia.

A) Ipsilateral PWTs are significantly reduced 10 d after SNI-surgery (p < .001 for all timepoints compared to base). Neither i.t. ZIP, nor scrZIP, alleviate SNI-induced mechanical allodynia. **B)** Contralateral (Contra) PWTs decrease gradually from 10 d post-surgery (when contralateral PWTs are significantly higher than ipsilateral (Ipsi) PWTs, p = .001) and reach maximal allodynic values at 6 weeks. **C)** I.t. ZIP transiently relieves contra-lateral, but not ipsilateral, allodynia when injected at 6 weeks after surgery (p < .05). Repeated measures ANOVA, n = 6/group.

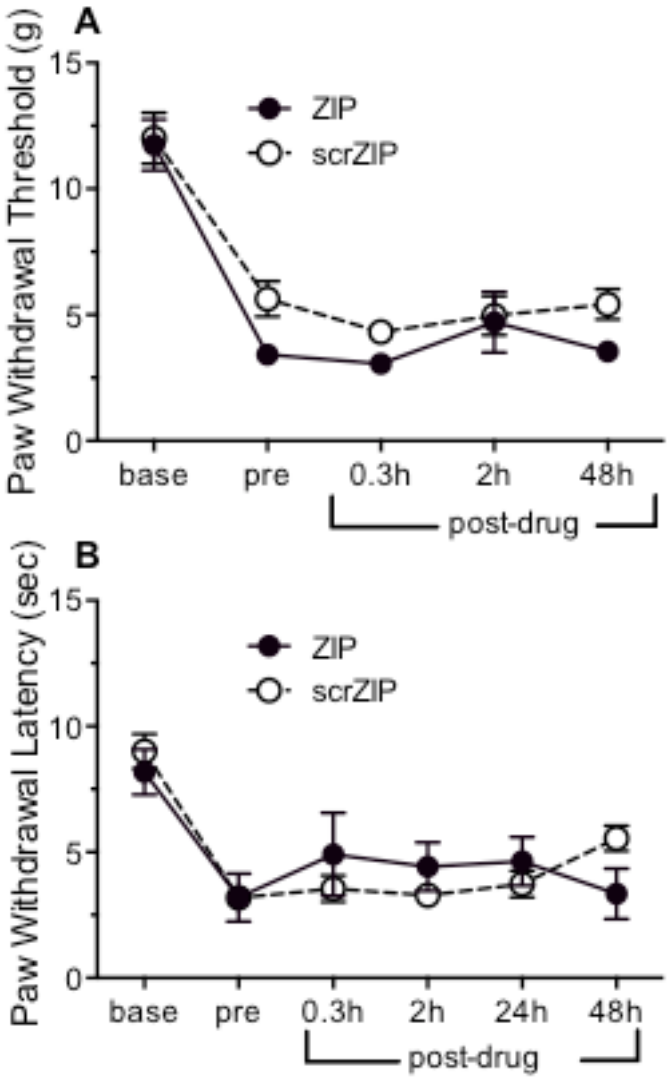

Figure 2.2. Effects of PKMζ inhibition on CFA-induced inflammatory mechanical allodynia and thermal hyperalgesia.

A) Mechanical PWTs are significantly reduced 24 h post-CFA (pre), and remain low from 20 min after i.t. ZIP or scrZIP for another 48 h, $p < .015$ for all timepoints post-base. **B)** Thermal PWLs (sec) decrease significantly 24 h after CFA (pre), and no change is observed after ZIP or scrZIP for another 48 h. Repeated measures ANOVA, n = 6/group.

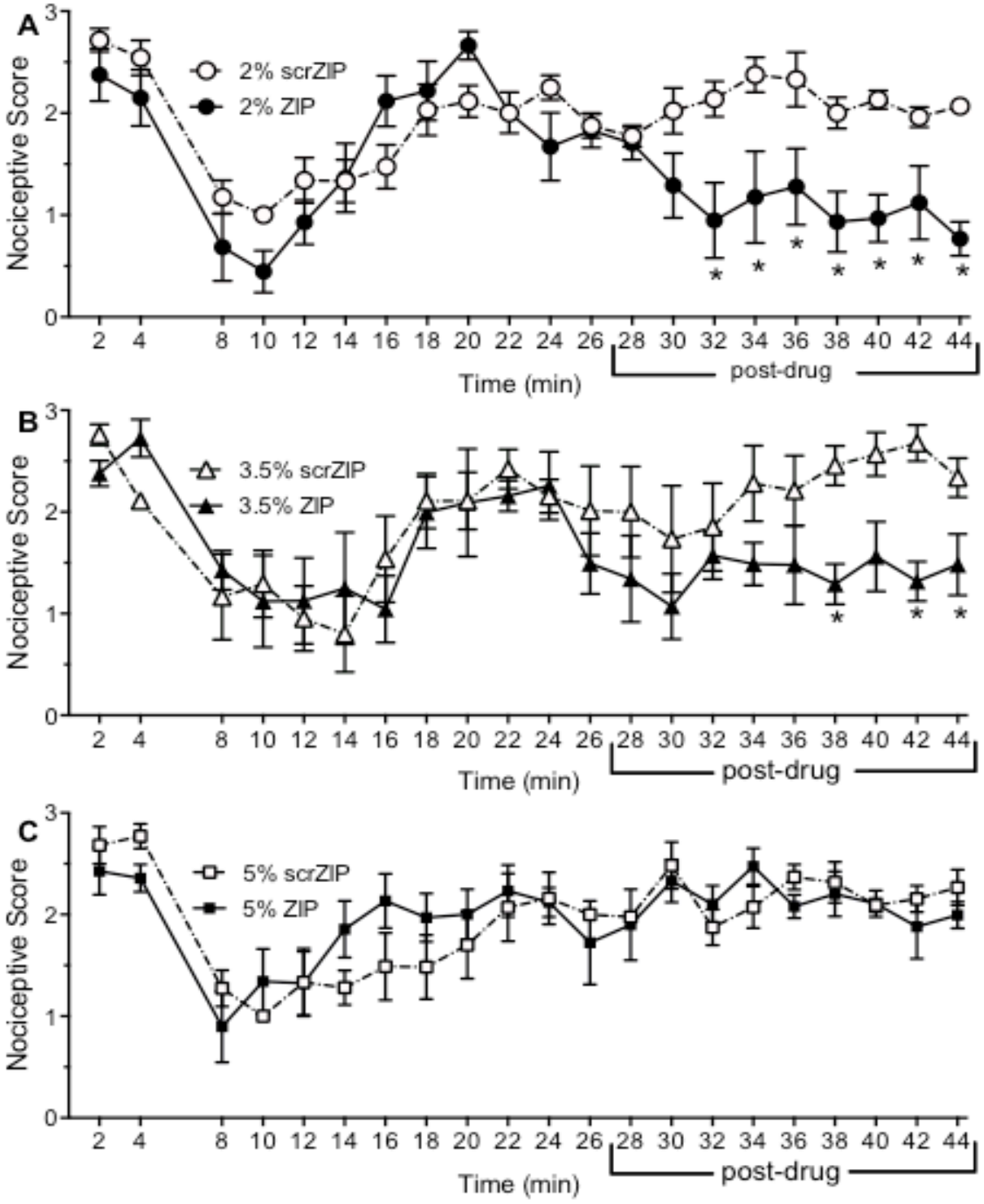

Figure 2.3. Effects of ZIP on nociception induced by 2%, 3.5 and 5% formalin.

A) Compared to scrZIP, i.t. ZIP significantly attenuates late-phase nociceptive scores between 32 and 44 minutes post-2% formalin. **B)** Compared to scrZIP, i.t. ZIP attenuates nociceptive scores associated with 3.5% formalin at 38 min, 42 min and 44 min post-3.5% formalin. **C)** Compared to scrZIP, i.t. ZIP has no effect on nociceptive scores induced by 5.0% formalin. Repeated measures ANOVA, Bonferroni adjused, $*p < 0.05$ - significantly different from scrZIP-treated groups after 2%, and 3.5% formalin.

3 Study 2.

Effects of PKMζ inhibition in animal models of remote allodynia

3.1 Rationale

Irritable bowel syndrome (Verne and Price, 2002, Verne et al., 2003) and fibromyalgia (Graven-Nielsen et al., 2000) exhibit symptoms of referred or remote pains that rely on sensitization of CNS neurons (Verne and Price, 2002, Verne et al., 2003). Understanding the mechanistic underpinning of sensitization in referred pain by use animal models of remote pain such as i.c. capsaicin or intramuscular (i.m.) acidic saline is critical for the development of adequate treatment options for such syndromes.

I.c. capsacin. Injection of 0.1% capsaicin into the rodent colon (i.c. capsaicin) has been shown to produce spontaneous pain-related behaviors such as licking, stretching and contracting the abdomen for about 20 minutes following instillation, as well as colon plasma extravasation and importantly, remote allodynia at the hind paw that lasts for at least 24 h (Laird et al., 2001, Galan et al., 2003). Mediators of central sensitization have been identified to contribute to the initiation of i.c. capsaicin-induced remote pain, with extracellular signaling-regulated kinase-1 and -2 (ERK1/2) and CamKII elevated 45-90 min following i.c. capsaicin instillation. *Pretreatment* with a pharmacological inhibitor (U0126) of ERK1/2 alleviated hind paw allodynia 3-6 h later (Galan et al., 2003), and an inihibitor of CamKII prevented the recruitment of the AMPA-R subunit GluR1 to the plasma membrane (Galan et al., 2004). Interestingly, a selective increase and recruitment of GluR1 to the plasma membrane was observed at 30 min and 2 h post i.c. capsaicin that was prevented by i.c. lidocaine or i.t. pretreatment with a PKA inhibitor (H89). The latter also prevented associated neural sensitization of colonic reflex activity captured by a urethral sphincter electromyogram (Peng et al., 2011a). I.t. pretreatment with a toxin blocking N-type calcium channels also reversibly reduced remote i.c. capsaicin hyperalgesia (Diniz et al., 2014). I.t. pretreatment with NMDA-inhibitor APV (Peng et al., 2011a), or microinjection of APV into the rostral ventromedial medulla (RVM) (Sanoja et al., 2010) prevented acute i.c. capsaicin-related

remote hypersensitivity. Together, these results highlight the importance of NMDA-R-mediated, PKA-dependent AMPA-R trafficking in initiation of central sensitization following i.c. capsaicin. However, the means by which i.c. capsaicin-remote hypersensitivity is maintained over time is unclear.

Mechanistic insights may be drawn from evidence on the central sensitization-dependent secondary hyperalgesia that develops around the site of i.pl. capsaicin-induced primary hyperalgesia. This secondary hyperalgesia was reversed 24 h post-capsaicin by inhibition of spinal PKMζ, but not by inhibiting full-length PKCs. Importantly, local anesthesia at the paw reduced primary, but not secondary hyperalgesia, suggesting secondary hyperalgesia was maintained through neuronal, PKMζ-dependent changes in the SCDH (Laferriere et al., 2011).

I.m. acidic saline. Two injections of acidic saline into the gastrocnemious muscle (i.m. acidic saline) in rats or mice produce minimal tissue injury at the site (Sluka et al., 2001), but significant bilateral *remote* mechanical allodynia, but not heat hyperalgesia, in the rodent hind paw. Remote mechanical allodynia was also observed in humans lasting for at least 30 d after muscle injection of an acidic buffer (Frey Law et al., 2008). Stimulation of the site of injection (thigh muscle), and site of testing (hind paw) elicits differential Fos staining, indicating the development of primary muscle hyperalgesia and remote hind paw hyperalgesia following i.m. acidic saline (Sharma et al., 2009).

Evidence suggests the cAMP-PKA pathway is required for the induction, and NMDA & AMPA receptor activity for the maintenance of i.m. acidic saline-induced remote allodynia – thereby indicating various mediators involved in the induction and maintenance of long-lasting remote pain. Thus, inhibition of PKA at 24 h post i.m. acidic saline, but not at one week, reversed the i.m. acidic saline-induced decrease in PWTs, and an associated cAMP-PKA-stimulated increase in pCREB and CREB immunoreactivity (Hoeger-Bement and Sluka, 2003). Spinal inhibition of

NMDA-R or AMPA-R at one week after the second i.m. acidic saline, but not prior to the first injection, decreased the remote hind paw allodynia (Skyba et al., 2002). A similar decrease of the remote allodynia was observed following microinjection of an NMDA-inhibitor to the rostroventral medulla (RVM) 24 h after the second i.m. acidic saline (Da Silva et al., 2010). Spinal inhibition of NMDA, but not non-NMDA glutamate receptors, 5 d after the first acidic saline injection, but before the second injection delayed the onset of mechanical allodynia associated with i.m. acidic saline. At the same time, microdialysis revealed a calcium-dependent increase of extracellular glutamate and aspartate levels in the SCDH and rostroventral medulla following the second, but not first, i.m. acidic saline injection (Skyba et al., 2005, Radhakrishnan and Sluka, 2009). Glutamate concentration in the SCDH has been shown to remain elevated for at least one week after i.m. acidic saline (Skyba et al., 2005). Together, these results indicate the importance of NMDA-R activity in both the induction and early maintenance phases. AMPA-R activity contributes predominantly to the maintenance of i.m. acidic saline-induced persistent remote allodynia (Skyba et al., 2005).

Other than glutamatergic receptor activation, it is unclear what downstream mediators contribute to the maintenance of i.m. acidic saline-induced remote allodynia. Inhibition of PKC at 24 h and 1 week post-i.m. acidic saline did not reverse the remote allodynia, suggesting PKC was not vital for the maintenance of this long-lasting pain (Sluka and Audette, 2006). Further, evidence shows that the involvement of spinal glial activation coupled to IL-1 release is not required for the maintenance of i.m. acidic saline-induced remote allodynia, as suppressing glial activity 11 d post i.m. acidic saline did not elevate PWTs (Ledeboer et al., 2006). Furthermore, pretreatment with the non-selective acid sensing ion channel (ASIC) blocker amiloride prevented the development of i.m. acidic saline-induced remote allodynia. In addition, ASIC3-null mice displayed very high thresholds to mechanical stimulation following i.m. acidic saline, and their dorsal horn WDR neurons displayed a lack of sensitization properties (enlargement of receptive field, enhanced firing to noxious stimulation), suggesting muscle ASIC3 activation is required for the induction of i.m.

acidic saline-induced remote allodynia and neuronal sensitization (Sluka et al., 2003). However, ASICs inhibition did not reverse persistently decreased PWTs following i.m. acidic saline, indicating the maintenance of this remote allodynia depends on other factors (Gautam et al., 2012). Additionally, the development of long-lasting acid-induced remote allodynia was prevented in mice overexpressing neurotrophin-3 (NT-3), or following i.m. injection of NT-3 concurrent with acidic saline, but not if NT-3 was administered 4 d after the first acidic saline injection (Gandhi et al., 2004).

Thus, although the initiation of i.m. acidic saline-induced remote allodynia is relatively well understood, it is unclear what mechanisms drive the maintenance of this persistent remote pain.

Here, we propose to test the role of spinal PKMζ in the maintenance of i.m. acidic saline- and i.c. capsaicin-induced remote pain by pharmacological inhibition with i.t. ZIP. Importantly, we will investigate the timing of PKMζ inhibition – before and after pain induction to delineate its involvement in both induction and maintenance phases. We hypothesize a role for PKMζ in both the initiation and maintenance of centrally-dependent i.m. acidic saline- and i.c. capsaicin-induced remote allodynia.

Hyperalgesic priming. The observation that two injections of i.m. acidic saline produce a very prolonged hind paw allodynia not observed after a single i.m. acidic saline, suggested that the first injury produces priming that affects the duration of allodynia after the second injury, a phenomenom know as hyperalgesic priming. Various models of hyperalgesic priming have been developed to study long-lasting persistent pain induced by repeated noxious stimuli, with the first "priming" stimulus typically a low-dose inflammatory agent (carrageenan, IL-6, BDNF), and the second "challenging" stimulus often PGE2, although sometimes a tissue injury (i.e., skin incision). Coupled to a priming stimulus, the challenging stimulus normally induces longer-lasting persistent hypersensitivity than if given alone – in the case of PGE2 allodynia lasting for 24 h, instead of 4 h.

Some progress has been made in understanding the central mechanisms associated with hyperalgesic priming. Price and colleagues utilized a hyperalgesic priming model constituting IL-6, followed by PGE2 four days later. By spinally inhibiting CamKII or MAPK shortly before IL-6 administration, they illustrated a requirement of these kinases for the initiation of priming-induced persistent hyperalgesia. Importantly, inhibiting CamKII or MAPK at day 4 post-IL-6, did not reverse PGE2 -induced pain hypersensitivity, suggesting these kinases were not responsible for the maintenance of this persistent pain state.

Role of PKMζ in hyperalgesic priming. Hyperalgesic priming induced by BDNF as the priming stimulus, caused prolonged PGE2-induced allodynia that was prevented by ZIP injection between priming and challenging stimuli(Melemedjian et al., 2013). Further support for a PKMζ-dependent maintenance of hyperalgesic priming was given by An et al. (2015) who induced hyperalgesia using carrageenan as the priming and i.pl. incision injury as the challenging stimulus. Inhibition of PKMζ using ZIP, but not of full-length PKC using NPC15437, following decay of carrageenan-related allodynia, prevented the incision-related prolonged decrease in mechanical PWTs. Importantly, inhibition of full-length PKCs, but not inhibition of PKMζ, prior to carrageenan, produced transient analgesia to carrageenan-induced, but not later, prolonged incision-induced allodynia (An et al., 2015). These studies demonstrate that inhibition of PKMζ in a maintenance phase when hyperalgesia induced by the priming stimulus has abated, prevents prolonged hypersensitivity following subsequent stimulation. It is not clear, however, whether inhibition of PKMζ would be sufficient to reduce the maintenance of hyperalgesia following both the priming and challenging stimuli.

Thus, in this study we aimed to understand whether repeated stimuli that induce remote allodynia (i.e., i.c. or i.pl. capsaicin) produce enhanced and prolonged allodynia (similar to hyperalgesic priming), and whether PKMζ plays a role in the establishment and maintenance of this

priming-induced long-lasting hyperalgesia. We pharmacologically inhibited PKMζ at three time points, before, in-between, and after repeated stimuli (i.c. capsaicin) spaced two weeks apart. We further tested for mechanical allodynia using the von Frey test following i.m. acidic saline, i.c. capsaicin, and compared the latter to that induced by i.pl. capsaicin. We hypothesized that long-lasting allodynia following priming was maintained by PKMζ activity, and expect that ZIP will alleviate allodynia whether it is administered in between or following the challenge injection of either i.pl. or i.c. capsaicin.

3.2 Methods

3.2.1 Animals

All experiments were conducted using adult male Long Evans rats (200-300g, Charles River Laboratories), housed in conditions following McGill Animal Care guidelines (3/cage, 12 h light/dark cycle). Food and water were available *ad libitum*.

3.2.2 Procedures

3.2.2.1 I.c. capsaicin-induced remote allodynia

In rats, we examined the effects of ZIP pre-and post-treatment to a single i.c. capsaicin instillation. A volume of 200 µl of 0.1% capsaicin was injected into the colon via the anus using a fine plastic canula (PE-20 polyethylene tubing) that had been inserted 7 cm (van den Wijngaard et al., 2009).

In the pretreatment experiment, i.t. ZIP, or its control peptide scrZIP, were injected 20 min prior to i.c. capsaicin instillation and rats were tested for mechanical allodynia using the von Frey test. In the posttreatment condition, these drugs were injected i.t. 60 min after the i.c. capsaicin instillation.

3.2.2.2 I.m. acidic saline-induced remote allodynia.

Persistent hind paw mechanical allodynia was induced following two i.m. acidic saline injections (100 μl; 32 gauge needle) to the lateral gastrocnemius muscle (Sluka et al., 2001). The conscious rats were wrapped in a towel, and the needle was inserted carefully to minimize tissue injury. We examined the effect of the cell membrane-permeable PKMζ inhibitor myristoylated – ζ - pseudosubstrate inhibitory peptide (ZIP), its scrambled inactive version (scrZIP), NPC15437 or its vehicle, on i.m. acidic saline-induced remote allodynia at different time points post-pain induction. NPC15437 is an inhibitor of all PKC isoforms containing the PKC regulatory domain (Sullivan et al., 1991). Following confirmation of i.m. acidic saline-induced allodynia, we administered these drugs 24 h or one week after the second i.m. acidic saline injection. These studies assessed the role of PKMζ/PKC in the maintenance phase of remote allodynia. We further quantified PKMζ levels using Western blot at 1 week post drug injection, which occurred 1 week post-pain induction.

3.2.2.3 Priming-induced hyperalgesia

We examined the ability of two i.c. capsaicin injections spaced two weeks apart to produce hyperalgesic priming-induced prolonged remote allodynia. We further compared i.c. capsaicin-induced priming to hyperalgesic priming induced by two injections of i.pl.capsaicin (50 μl). Next we examined the effects of ZIP and its inactive control peptide scrZIP on hyperalgesic priming when administered before the first pain induction (pretreatment), prior to the second pain induction (intermediate treatment) and following the second pain injection (post-treatment). These studies assessed both the nature of visceral capsaicin-induced and cutaneous capsaicin-induced priming, as well as the role of PKMζ in the maintenance of these different pain models.

3.2.2.4 Drug preparation

ZIP (Myr-SIYRRGARRWRKL-OH, Tocris), scrZIP (Myr-SIYRRGARRWRKL-OH, Tocris), were dissolved in sterile water at a dose of 10 nmol. NPC15437 (Sigma) was dissolved in

100 mM Tris-saline (pH 7.2), the vehicle consisted of the same solution lacking NPC15437. Acidic saline was produced by 0.9% of NaOH dissolved in sterile water with the pH adjusted to 4.0. Capsaicin (Sigma, 0.1%) was dissolved in 10% ethanol, 10% Tween 80 and 80% saline.

3.2.3 Behavioral testing

Mechanical allodynia was assessed using the von Frey test as described in section 2.2.5.1 of study 1.

3.2.4 I.t. injection

I.t. injection procedures were as described in methods of study 1 in the section Drug administration: Lumbar puncture.

3.2.5 Western blot

SCDHs were quick frozen, and homogenized twice for 5 sec each in the presence of final sample buffer (FSB) containing protease inhibitor phenylmethylsulfonyl fluoride (PMSF) crystals. Samples were incubated for 10 min in 95°C. After centrifugation (5 min at 13,000 rpm, room temperature), Trichloroacetic acid (TCA) precipitation was performed on the resulting supernatant. Samples were incubated on ice for 20 min, centrifuged and the resulting protein pellet was eluted directly in 2xFSB (100-200 µl depending on size of the pellet). Lastly, samples were incubated for 10 min at 95°C and separated on a 10% polyacrylamide gel. Proteins were blotted to nitrocellulose membrane. Membranes were incubated with primary antibody recognizing total PKCζ, PKCλ and PKMζ (1:500, Santa Cruz) or a primary actin antibody (1:400, Santa Cruz). Incubation with horseradish peroxidase-coupled secondary antibody (1:20,000) was followed by incubation with chemiluminescent reagent (Abcam). Films were developed between 5 and 60 seconds, and scanned with a Canon N650U scanner. Films were analyzed using Image J (NIH).

3.2.6 Data analysis

All data are represented as mean ± SEM, and were analyzed using Statistical Package for the Social Sciences (SPSS). Time course analyses were analyzed using repeated measures ANOVA, followed by Bonferroni post-hoc comparisons. Statistical differences between treatment groups in western blots were obtained by comparisons of numbers obtained from ImageJ analysis using Student's t-test. Area under the curve was calculated in Microsoft Excel and Graphpad Prism and the resulting values analyzed using Student's t-tests. Statistical outliers were defined as those values outside the1/3 interquartile range (SPSS) and were omitted from the analysis. Animals were also rejected from analysis if there was any question that the i.t. injection was not successful.

3.3 Results

3.3.1 Effects of PKMζ inhibition on i.c. capsaicin-induced remote allodynia

A single instillation of 0.1% capsaicin into the colon produced a significant and long-lasting reduction in PWTs at the hind paw (remote allodynia), as evidenced by a significant main effect of treatment revealed by ANOVA ($F(1,10) = 21.08$, $p = .001$). Post hoc analysis demonstrated significantly reduced PWTs in capsaicin-treated rats, as compared to vehicle-treated, at 30 min, 60 min and 2 h post-instillation (Figure 3.1).

We next compared the effects of a pre- and post-treatment of the PKMζ inhibitor ZIP, or its control peptide scrZIP on PWTs after i.c. capsaicin. I.t. injection of ZIP 20 min prior to i.c. capsaicin completely prevented the capsaicin-induced reductions in PWTs, as indicated by a significant main effect of treatment in our ANOVA ($F(1,10) = 148.01$, $p < .0001$, Figure 3.2A). Capsaicin-induced reductions in PWTs were also rapidly (within 30 min) and persistently restored to baseline values following post-treatment with ZIP ($F(1,10) =102.303$, $p < .0001$, Figure 3.2B).

These data strongly suggest that PKMζ inhibition is sufficient to both prevent and reverse persistent remote mechanical allodynia induced by i.c. capsaicin.

3.3.2 Effects of PKMζ inhibition at 24 h and one week post repeated acidic saline injections on PWTs and PKMζ expression

3.3.2.1 Drug injection 24 h post i.m. acidic saline

Two injections of acidic saline into the thigh muscle significantly lowered hind paw mechanical PWTs from 24 h to at least 3 weeks after the second injection of acidic saline. Rats injected with i.t. ZIP 24 h later displayed a significant, and persistent increase in PWTs between 30 min and 3 weeks following its administration. Accordingly, there was a significant main effect of treatment revealed by repeated measures ANOVA ($F(1,3) = 12.93$, $p < .0001$). Conversely, treatment with NPC15437 at the same time point had no significant effect on acidic saline-induced reduction of PWTs, as assessed by Bonferroni post-hoc comparisons (see Figure 3.3).

3.3.2.2 Drug injection 1 week post i.m. acidic saline

In a separate experiment, we tested the effects of ZIP one week after i.m. acidic saline. Rats injected with acidic saline in the thigh muscle twice displayed decreased hind paw PWTs at 24 and 1 week following the injections. Administration of ZIP one week after the second acidic saline injection significantly and persistently elevated PWTs for at least three weeks post-drug. scrZIP treated rats, however, continued to display lowered PWTs until the end of testing at 4 weeks. Bonferroni-post-hoc comparisons revealed significant differences marked by an asterisk in Figure 3.4A. NPC15437 injected at one week post-acidic saline, similar to vehicle treatment, had no PWT-elevating effect. Accordingly, repeated measures ANOVA revealed a significant main effect of treatment ($F(3,20) = 21.2$, $p < .0001$, see significance differences yielded by Bonferroni comparisons in Figure 3.4B). These results demonstrate the unique ability of ZIP to reverse already

established referred allodynia; thus, only ZIP-targeted PKMζ, and not full-length PKC, plays a role in the maintenance of remote allodynia.

3.3.2.3 Effects of i.m. acidic saline on phosphorylated and total aPKC protein levels

Spinal cords of rats were taken out two weeks following the second acidic saline injection, and compared to untreated control samples. Two antibodies, one recognizing activation-loop phosphorylation (T410) at all aPKCs, the other recognizing total aPKC levels were used to assess the effects of acidic saline. Phosphorylated PKMζ ($t(6.19) = 3.35$, $p = .015$), as well as of phosphorylated full-length aPKCs ($t(10) = 11.23$, $p < .0001$) were significantly higher following acidic saline compared to control. However, both total PKMζ ($t(10) = -.940$, $p = .37$) and total full-length PKCζ/λ ($t(10) = .49$, $p = .63$) remained unchanged following acidic compared to their respective levels in control samples. Interestingly, within the untreated control group total protein levels were significantly higher than phosphorylated PKMζ ($t(6.312) = 4.117$, $p = .006$) or PKCζ/λ ($t(10) = 3.269$; $p = .008$). However, following acidic saline treatment, phosphorylated exceeded total PKCζ/λ levels ($t(10) = 5.330$, $p < .0001$). Also following acidic saline, pPKMζ was upregulated to the point that it was equal to total PKMζ ($t(10) = .392$, $p = .703$), Figure 3.4C.

3.3.2.4 Effects of PKMζ inhibition at 1 week on phosphorylated and total aPKC protein levels

Next, we tested the effects of ZIP on phosphorylation of PKMζ, and full-length aPKCs, as well as on total PKMζ and PKCζ/λ levels. Rats were injected with ZIP or scrZIP 1 week after acidic saline and their spinal cords removed 1 week after the i.t. injection. We western blotted for T410-pPKM/Cζ/λ and total aPKC. Homogenates from ZIP-treated rats contained significantly lower levels of pPKMζ than those obtained from scrZIP-treated rats ($t(6) = 2.521$, $p = .045$). Importantly, no significant difference for these groups was discernible for pPKCζ/λ ($t(6) = 1.853$, $p = .113$). Total PKMζ levels, however, were not significantly decreased following ZIP ($t(6) = -2.131$, $p =$

.077). Likewise, no difference between ZIP and scrZIP-treated samples in total PKCζ/λ levels was observed (t(6) = -.255, p = .808, Figure 3.4D). Thus, the persistent increase in PWTs of i.m. acidic saline-treated rats following ZIP at 1 week is associated with a decrease in PKMζ phosphorylation.

3.3.3 Effects of PKMζ inhibition on repeated i.c. capsaicin injections

Two injections of i.c. capsaicin spaced two weeks apart produced a prolonged, persistent reduction in PWTs. PWTs following 1Xi.c. capsaicin or 2Xi.c. capsaicin were similarly low up to 24 h. While PWTs of rats injected with 1Xi.c. capsaicin returned to baseline at 48 h, PWTs in the 2Xi.c. capsaicin group remained decreased for at least 6 days, and recovered at 8d. These differences were confirmed by significant main effects of injury condition ($F(1,9) = 14{,}933$, $p = .004$), and time ($F(2.215, 19.932) = 67.097$, $p < .0001$), as well as a significant time*injury condition interaction ($F(2.215, 19.932) = 12{,}907$, $p < .0001$) in our repeated measures ANOVA. Bonferroni post-hoc comparisons revealed significant differences marked by asterisks in Figure 3.5.

3.3.3.1 Pretreatment 2Xi.c. capsaicin

We next tested the effects of the PKMζ inhibitor ZIP, or its inactive control peptide scrZIP, on persistently decreased PWTs after 2Xi.c. capsaicin. I.t. injection of ZIP 20 min prior to the first i.c. capsaicin injection prevented the development of the prolonged allodynia, exhibiting a transient decrease of PWTs between 30 min and 2 h post 2Xi.c. capsaicin (Figure 3.6A). ScrZIP-treated rats continued to display lowered PWTs until 6 d post 2Xi.c. capsaicin. These differences were verified by repeated measures ANOVA yielding significant main effects of treatment ($F(1,9) = 16.242$, $p = .003$) and time ($F(4.128, 37.149) = 10.106$, $p < .0001$), but not treatment*time interaction ($F(4.128, 37.149) = 1.171$, $p = .34$). Bonferroni post-hoc comparisons revealed significant differences at 48 h ($p = .009$), and 6 d ($p = .015$) and a trend towards significance at 2 h ($p = .057$). Thus pre-emptive PKMζ inhibition protects from development of long-lasting pain following repeated stimulations with i.c. capsaicin.

3.3.3.2 Intermediate treatment 2Xi.c. capsaicin

Injecting ZIP 2 weeks following the first i.c. capsaicin, when its associated allodynia had resolved and 20 min prior to 2Xi.c. capsaicin, rats exhibited a decrease in PWTs only between 30 min and 2 h, while the associated PWT reduction in scrZIP treated rats remained until at least 4 d. Confirming these differences, repeated measures ANOVA revealed significant main effects of treatment ($F(1,10) = 95{,}817$, $p < .0001$) and time ($F(3.869, 38.692) = 14{,}327$, $p < .0001$), as well as a significant treatment*time interaction ($F(3.869, 38.692) = 9{,}401$, $p < .0001$). Bonferroni post-hoc comparisons detected significantly higher PWTs in the ZIP-treated group at 2 h ($p < .0001$) and 4 d ($7.705 \pm .866$, $p < .0001$). Thus, we show that inhibiting PKMζ following the first (priming) stimulus prevented the subsequent development of a prolonged allodynic response to the challenging stimulus (Figure 3.6B).

3.3.3.3 Post-treatment 2Xi.c. capsaicin

Last, we tested the effects of pharmacological PKMζ inhibition on the maintenance of allodynia after priming and challenge injections that normally cause allodynia for up to 4-6 d. Injecting ZIP 24 h post 2Xi.c. capsaicin abolished the persistent decrease of PWTs between 30 min and 4 d post-ZIP, while scrZIP had no effect on the lowered PWTs after 2Xi.c. capsaicin (Figure 3.6C). Correspondingly, our repeated measures ANOVA revealed significant main effects of treatment ($F(1,10) = 24.409$, $p = .001$) and time ($F(3.673, 36.727) = 22.861$, $p < .0001$), as well as a significant treatment*time interaction ($F(3.673, 36.727) = 4.994$, $p = .003$). Compared to scrZIP treatment, ZIP-treated rats displayed significantly higher PWTs at 30 min ($p = .010$), 120 min ($p = .003$), and 4 d ($p = .003$) according to Bonferroni post-hoc comparisons. These results suggest PKMζ is required for the maintenance of priming-induced sensitization.

3.3.4 Effects of PKMζ inhibition on allodynia after repeated i.pl. capsaicin

3.3.4.1 1Xi.pl.capsaicin vs 2Xi.pl.capsaicin

We compared the effects of a single i.pl. (1Xi.pl. capsaicin) to two injections of i.pl. capsaicin (2Xi.pl. capsaicin). Rats injected with 1Xi.pl. capsaicin displayed PWTs that were lowered for at least 2 h, but were returned to baseline by 8 days. In contrast, rats injected with 2Xi.pl. capsaicin showed PWTs that were significantly greater at 30 min and 2 h and were still lowered at 8 days (Figure 3.7A). Repeated measures ANOVA revealed significant main effects of group ($F(1,10) = 84.161$, $p < .0001$) and time ($F(1,10) = 1458.6$, $p < .0001$), as well as a significant group*time interaction ($F(1,10) = 6.246$, $p = .031$). Significant differences in PWTs between groups 1Xi.pl. capsaicin and 2Xi.pl. capsaicin were observed at 30 min ($p = .016$) , 2 h ($p = .002$), and 8 d ($p < .0001$).

3.3.4.2 Pretreatment 1Xi.pl. capsaicin

Pretreatment with the PKMζ inhibitor ZIP 20 min prior to 1Xi.pl. capsaicin significantly elevated PWTs at 1 h and 2 h post i.pl.capsaicin, compared to PWTs of scrZIP-treated rats that increased only at 24 h (Figure 3.7B). The corresponding repeated measures ANOVA detected significant main effects of treatment ($F(1,10) = 25.563$, $p < .0001$) and time ($F(4,40) = 61.224$, $p < .0001$), as well as a significant time*treatment interaction ($F(4,40) = 8.943$, $p < .0001$). PWTs in ZIP-treated rats were significantly higher at 1 h ($p = .044$) and 2 h ($p < .0001$).

3.3.4.3 Pretreatment 2Xi.pl.capsaicin

PWTs decreased significantly following 2 injections of i.pl. capsaicin spaced 2 weeks apart. Rats pretreatment with ZIP prior to the first i.pl. capsaicin injection displayed a transient reduction in PWTs between 30 min and 2 h after the second pain induction. However, ZIP prevented the persistent lowering of PWTs that lasted up to 8 d in the scrZIP-treated group (Figure 3.7C), as

reflected in significant main effects of treatment ($F(1,10) = 100.272$, $p < .0001$) and time ($F(2,212) = 99.606$, $p < .0001$), as well as a significant time*treatment interaction ($F(2.212, 22.123) = 25.079$, $p < .0001$) in our repeated measures ANOVA. Bonferroni-post-hoc comparisons highlight significant differences between ZIP and scrZIP groups at 60 min ($p = .001$), 120 min ($p < .0001$) and 8 d ($p < .0001$).

3.4 Discussion

We have investigated whether PKMζ activity plays a role in the maintenance of long-lasting persistent, CNS-dependent pain. Here we show for the first time that spinal PKMζ maintains i.c. capsaicin-induced & i.m. acidic saline-induced remote allodynia, as well as i.c. capsaicin- and i.pl. capsaicin-induced hyperalgesic priming. These data suggest spinal PKMζ plays a vital role in the maintenance of central sensitization-dependent long-lasting persistent pain.

I.c. capsaicin not only causes secondary allodynia on the abdomen of the animal, but also remote allodynia at the hind paw (Laird et al., 2001). Reproducing this data we showed, in comparison to vehicle instillations, profound mechanical allodynia lasting for at least 24 h following i.c. capsaicin. Previously, a persistent PKA-dependent enhancement of AMPA-R GluR1 was observed following i.c. capsaicin, as well as an increase in urethral reflex spike frequency (Peng et al., 2011a), clearly indicating the sensitization of CNS-neurons. Additionally, PKMζ critically maintains LTP through a persistent enhancement of AMPA-R maintenance at, and trafficking to the postsynapse (Ling et al., 2002, Li et al., 2010). In line with these data, we show that a pretreatment with the PKMζ inhibitor ZIP prevents the development of i.c. capsaicin-induced remote allodynia at the hind paw. A similar pretreatment effect was observed using a PKA inhibitor that prevented i.c. capsaicin-induced sensitization (Peng et al., 2011a). In hippocampal observations PKA phosphorylates AMPA-R's GluR1 and GluR4 and promotes AMPA trafficking into synapses (Esteban et al., 2003). Additionally, a hippocampal study showed PKMζ interaction with PICK1

and NSF to retain AMPA subunits at the postsynapse (Yao et al., 2008). Our findings add to the picture of differential and equally important roles for PKA and PKMζ in the initiation of sensitization and persistent pain.

Our data show that posttreatment with the PKMζ inhibitor ZIP 24 h after i.c. capsaicin instillation reversed previously established sensitization – thereby highlighting a unique role of PKMζ in the maintenance of allodynia unseen for previously explored protein kinases. These data are consistent with a former study on central sensitization-dependent secondary hyperalgesia after hind paw i.pl. capsaicin, showing PKMζ inhibtion 24 h post-capsaicin injection reversed secondary hyperalgesia, while full-length PKC inhibition was ineffective (Laferriere et al., 2011).

The present study does not elaborate on the precise mechanism by which PKMζ maintains central sensitization-dependent long-lasting pain. Drawing from our understanding of PKMζ regulation and activity in hippocampal long-term-memory, we may speculate it causes a persistent increase of synaptic AMPA receptors coupled to a persistent enhancement of synaptic transmission. Several players are involved in AMPA receptor trafficking, some of which will be discussed in study 4. Future experiments quantifying AMPA's GluR 1 and 2 in postsynaptic and cytosolic fractions from e.g. i.c. capsaicin-treated rats will further clarify the picture of PKMζ-dependent maintenance. However, our data establish a role for PKMζ in the maintenance of long-lasting persistent, central sensitization-dependent remote pain.

Repeated application of pain-inducing stimuli cause a state of hyperalgesic priming. Previous reports of PKMζ in hyperalgesic priming elicited through IL-6/BDNF and challenged by PGE2 determined it responsible for prolonging the allodynia associated with the second (challenging) stimulus (Asiedu et al., 2011, Melemedjian et al., 2013). In these studies PKMζ was inhibited stimultaneously with the priming stimulus IL-6, or 2 days prior to the challenging stimulus PGE2. Expanding on these two earlier studies, we present novel evidence that ZIP reverses established

long-lasting hyperalgesia following priming and challenging stimuli using repeated injections of i.m. acidic saline into the thigh muscle, and following repeated i.c. capsaicin applications.

While the initiation of i.m acidic saline-induced persistent pain is relatively well understood (enhanced glutamatergic transmission, NMDA-R, PKA and PKC activity), we had an incomplete understanding of how this pain is maintained for several weeks. Both NMDA-R and AMPA-R activity was required for the maintenance at 1 week post-second i.m. acidic saline injection (Skyba et al., 2005), yet one study showed that PKA, which was pivotal for allodynia expression at 24 h, was not the protein kinase underlying this maintenance mechanism at 1 week post-drug (Hoeger-Bement and Sluka, 2003). This crucial timing led to the assumption that at 24 h post i.m. acidic saline an induction phase may be in place, while one week post i.m. acidic saline a pure maintenance mechanism was dependent on unknown factors. Our experiments thus add important information to this picture: our first experiment - when inhibitors for PKMζ or full-length PKC were administered at 24 h post second i.m. acidic saline-injection – shows that PKMζ, but not full-length PKC, is required for the maintenance of allodynia. With PKCs playing an important role for the induction of central sensitization (Latremoliere and Woolf, 2009) and i.m. acidic saline-remote pain (Sluka and Audette, 2006), we thus conclude the time-point 24 h post i.m. acidic saline constitutes a PKMζ-dependent maintenance phase. When injected at one week post i.m. acidic saline, the PKMζ inhibitor ZIP, but not full-length PKC inhibitor NPC15437, reversed the established mechanical allodynia. Thus, PKMζ is required for the maintenance of remote pain also at 1 week after the second injection. This role of PKMζ in long-term maintenance resembles its role in the AMPA/NMDA-R-dependent mechanism underlying late-LTP maintenance in the hippocampus (Lu et al., 2001, Watt et al., 2004).

PKMζ, but not other PKC isoforms, is upregulated during LTP (Osten et al., 1996), and PKMζ administration boosts the late-LTP phase (Ling et al., 2002), while dominant-negative PKMζ

disrupts previously acquired memories and late-LTP (Shema et al., 2011). Importantly, only spinal PKMζ, not PKCζ or λ, was upregulated 2 - 24 h post i.pl.capsaicin (Laferriere et al., 2011), and viral PKCζ/PKMζ administration has been shown to induce long-lasting persistent mechanical allodynia (Asiedu et al., 2011). Our behavioral data show PKMζ-dependent remote allodynia lasts for at least 3 - 4 weeks following the second i.m. acidic saline injection. Our western blot data examining effects of i.m. acidic saline indicate a persistent increase in PKMζ, as well as PKCζ/λ phosphorylation, while total PKMζ and PKCζ/λ levels remained close to control values. As expected, in untreated animals total expression of PKCζ/λ and PKMζ were significantly higher than the proportion of phosphorylated protein. Following activation with i.m. acidic saline, this picture shifted towards higher phosphorylation than total levels for PKCζ/λ. Also, pPKMζ, which was significantly lower than total PKMζ in untreated rats, was significantly elevated in i.m. acid saline-treated rats to the point that it now equaled that of total PKMζ. Our western blot data further highlights a decrease of pPKMζ, but not pPKCζ/λ, nor total PKM/Cζ/λ levels following ZIP, compared to that produced by scrZIP – thereby matching ZIP's behavioral anti-allodynic properties. These data further indicate that a single injection of ZIP is sufficient for the disruption of PKMζ's persistent activity (Kelly et al., 2007).

Timing is crucial for the establishment for i.m. acidic saline-induced priming, with 5 days between injections producing weeks-long allodynia, with 10 days between injections reducing the duration of remote allodynia (Sluka et al., 2001). Here, we spaced two injections of i.c. capsaicin two weeks apart. When preceded by a previous stimulation, the i.c. capsaicin instillation induced remote allodynia lasting for at least 6 days instead of 48 h after a single instillation. This prolonged pain response was PKMζ dependent. Thus, pretreatment with ZIP prior to the first i.c. capsaicin instillation only allowed for transient, but not long-lasting allodynia, suggesting PKMζ activity during the priming phase is required for the expression of long-lasting pain weeks later. Our

intermediate treatment, when ZIP was injected following recovery of the first i.c. capsaicin instillation and prior to the second one, prevented the development of the prolonged allodynia and is in line with previous reports on intermediate treatment in hyperalgesic priming using IL-6/BDNF + PGE2 (Asiedu et al., 2011, Melemedjian et al., 2013) or IL-6 + plantar incision (An et al., 2015). Lastly, our post-treatment, with ZIP injected *after* the second i.c. capsaicin instillation, reversed this established long-lasting remote allodynia. To our knowledge the role of PKMζ after establishment of long-lasting priming-induced pain has not been explored previously. We show that pain manifested after priming with weeks-long intervals can be reversed through a single i.t. injection of ZIP following priming and challenge.

Two conclusions can be drawn from these experiments: first, unlike i.m. acidic saline, that requires a crucial timing of less than 10 d between repeated injections into the thigh muscle, a single injection of capsaicin into the colon sensitizes spinal cord neurons in a manner that allows for altered neuronal responses at least two weeks later. Second, PKMζ is required, for the mantenance of long-lasting persistent pain, but not for the transient expression of allodynia immediately following pain induction. Thus, in our pretreatment experiment transient allodynia developed at 30 min after the second i.c. capsaicin instillation that gradually disappeared by latest 48 h, similar to the transient allodynia in Asiedu et al. (2011). These conclusions point to a potential mechanistic model reminiscent of "synaptic tagging" explored in hippocampal neurons. Synaptic tagging denotes the process of "marking" a synapse through specific "plasticity related proteins (PRP)" for enhanced potentiation following high or low intensity stimulation. It was shown that PKMζ may act as such a PRP, such that PKMζ boosts the enhancement of synaptic transmission at synapses that had previously received even low tetanization (Sajikumar et al., 2005). Similarly, if PKMζ is blocked following stimulation, the synaptic tag was disrupted and late-LTP in these previously stimulated synapses could not be elicited. Thus, it was shown, PKMζ is required for the transition

from early to late LTP. Applying these findings to our data, it may be suggested that disruption of PKMζ using ZIP before the initiation of priming, may prevent the proper "synaptic tagging" which subsequently fails to elicit long-lasting persistent pain. Further, we reproduced data demonstrating spinal PKMζ-dependent sensitization following i.pl. capsaicin (Laferriere et al., 2011). Additionally, we established hyperalgesic priming using two injections of i.pl. capsaicin spaced two weeks apart resulting in at least 8 d long allodynia that would otherwise have lasted 24 h.

Overall these studies have revealed important mechanisms to help answer the question how a single or repeated pain event may be maintained over time. Central sensitization has been explored since the 1980s, strongly improving our understanding of pain maintenance and introducing the notion of "neuronal memory" that alters sensitivity based on previous insults – here, PKMζ has been determined a key component of central sensitization-dependent long-lasting persistent pain models. Critically, PKMζ, unlike other protein kinases, was shown to reverse already established remote allodynia – thereby making it an excellent target for clinical treatment of long-lasting pain. In this study, we have closely modeled clinically relevent long-lasting pain phenomena, i.e. muskoloskeletal pain through i.m. acidic saline, and through i.c. capsaicin the irritable bowel syndrome - a common visceral disorder with increasing prevalence, yet with inadequate treatment options (Verne and Price, 2002). This study thus opens an avenue for the exploration of central sensitization and its reversal in clinically prevalent – central sensitization-dependent – conditions.

3.5 Study 2 Figures

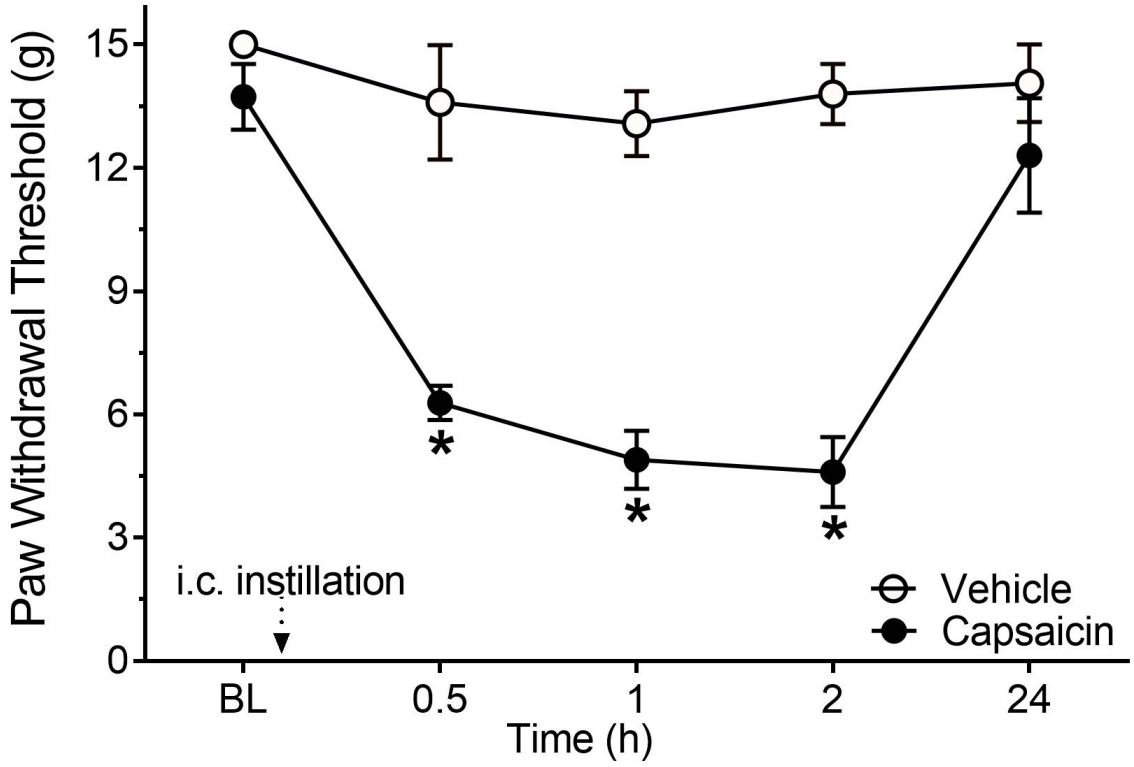

Figure 3.1. Effects of i.c. capsaicin on hind paw PWTs.

PWTs are significantly reduced from 30 min to 2 h after 0.1% i.c. capsaicin and return to BL values at 24 h. Two-way ANOVA, Bonferroni adjusted, * $p < .001$, as compared to vehicle-treated group, n = 6/group.

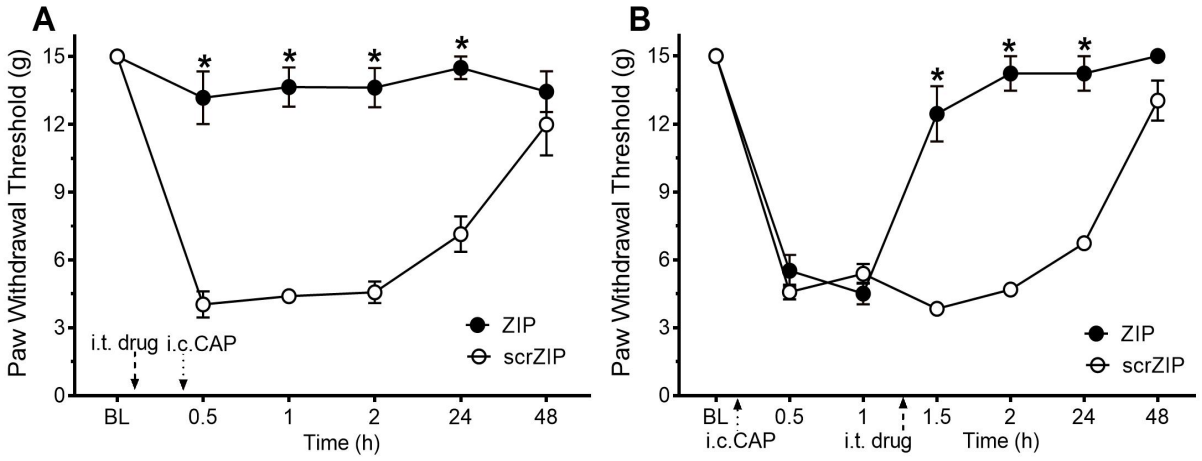

Figure 3.2. Effects of inhibition of PKMζ on intracolonic capsaicin (i.c.CAP)-induced remote allodynia.

A) PWTs are reduced between 0.5 and 24 h after i.c.CAP and scrZIP, but remain at baseline (BL) levels in rats pre-treated with i.t. ZIP 20 min prior to 0.1% i.c.capsaicin. **B)** Again PWTs are reduced between 0.5 and 24 h after i.c.CAP and scrZIP. However, i.t ZIP given after the 1 h test reversed i.c.CAP-induced reduction in PWTs between 30 min and 24 h post-drug. * $p < .01$, repeated measures ANOVA, Bonferroni.

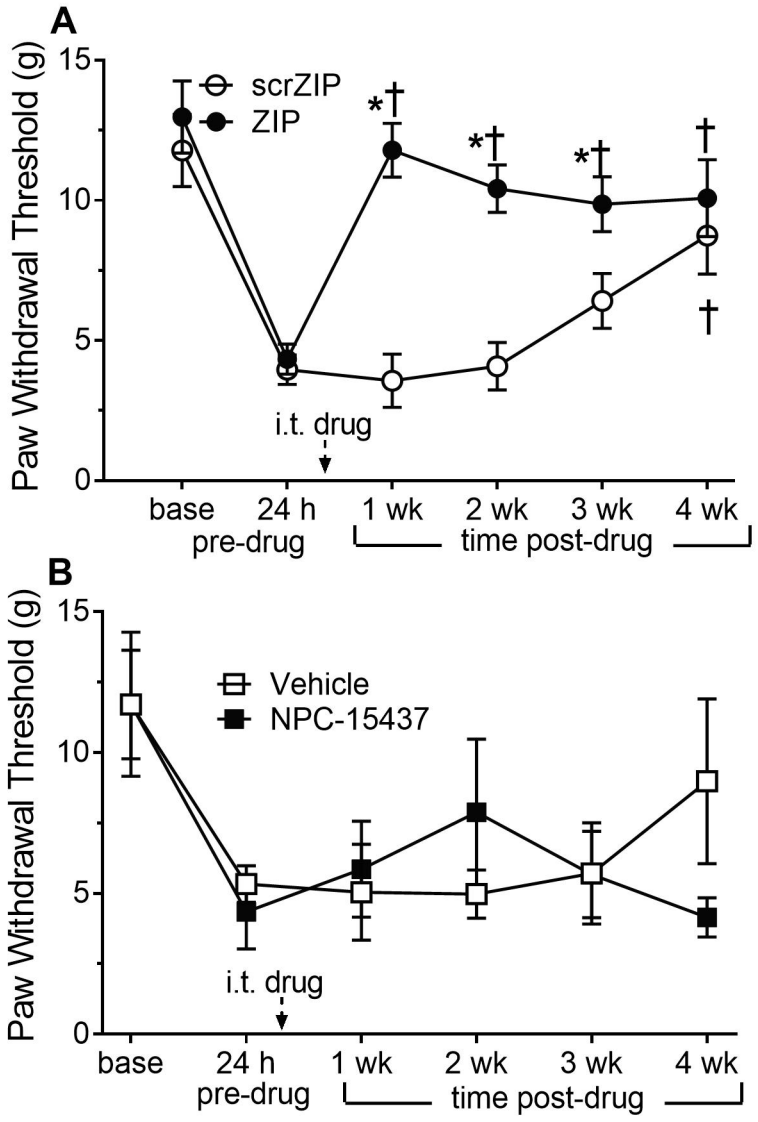

Figure 3.3. Effects of inhibition of PKMζ and full-length PKCs on i.m. acidic saline-induced remote mechanical allodynia.

A) PWTs are significantly reduced following 2 injections of i.m. acidic saline in both groups. Compared to scrZIP, i.t. ZIP injected at 24 h post i.m. acidic saline restores PWTs to baseline levels. **B)** PWTs are again significantly reduced following 2 injections of i.m. acidic saline in both groups. Compared to Vehicle, NPC15437 yields no change in PWTs when injected at 24 h post-i.m. acidic saline. Repeated measures ANOVA, $p < .05$, Bonferroni adjusted. † significantly different from pre-drug, * significantly different from scrZIP.

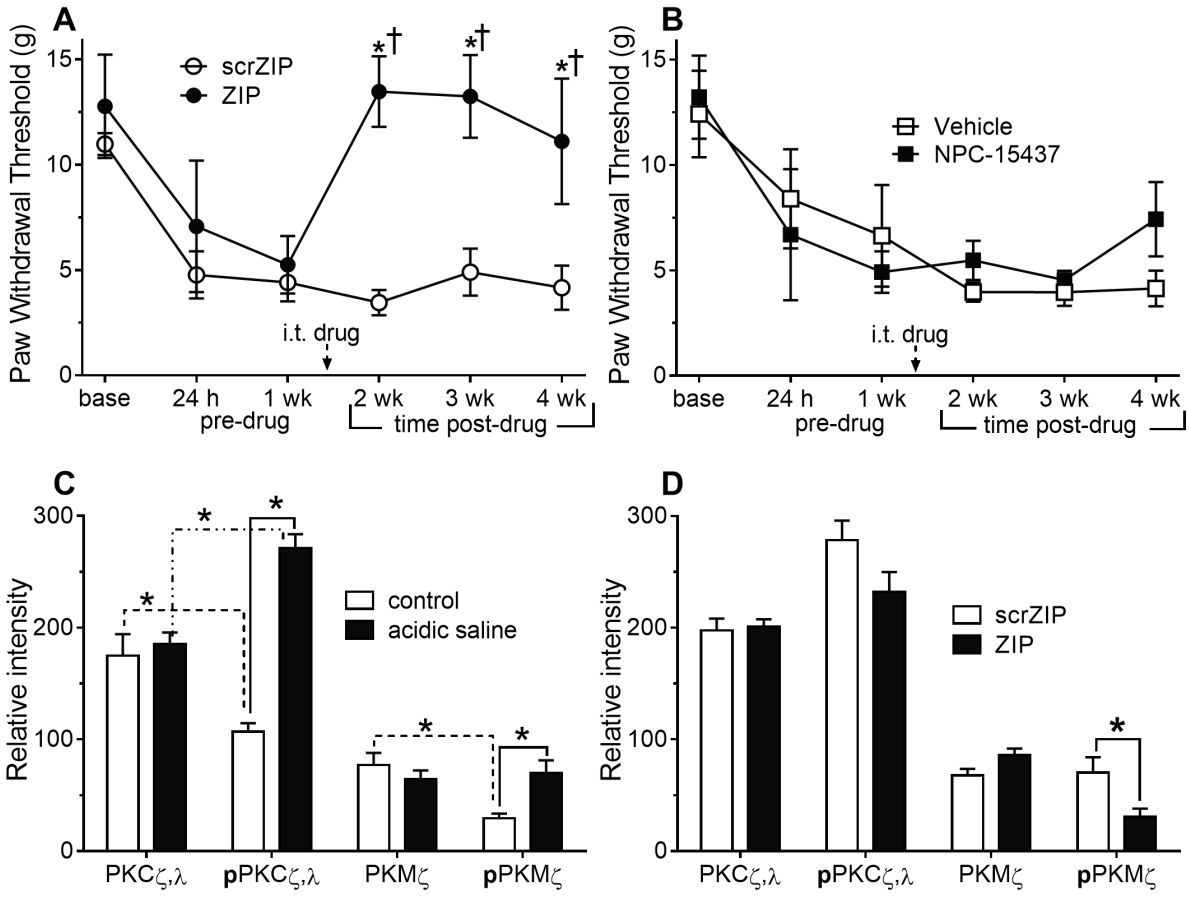
A
Paw Withdrawal Threshold (g)
scrZIP
ZIP
*†
i.t. drug
base
24 h
1 wk
2 wk
3 wk
4 wk
pre-drug
time post-drug
B
Vehicle
NPC-15437
C
Relative intensity
control
acidic saline
PKCζ,λ
pPKCζ,λ
PKMζ
pPKMζ
D
scrZIP
ZIP

Figure 3.4. Effects of inhibition of PKMζ and full-length PKCs on i.m. acidic saline-induced remote mechanical allodynia.

A) PWTs are significantly reduced between 24 h and 4 weeks following 2 injections of i.m. acidic saline in scrZIP-treated rats. I.t. ZIP injected at 1 week restores PWTs to baseline levels between 2 and 4 weeks. **B)** PWTs are again significantly reduced between 24 h and 4 weeks following 2 injections of i.m. acidic saline in both groups. Compared to Vehicle, NPC15437 yields no change in PWTs when injected at 1 week post i.m. acidic saline. Repeated measures ANOVA, $p < .05$, Bonferroni adjusted. † significantly different from pre-drug, * significantly different from scrZIP. **C)** Two weeks following i.m. acid saline injection, the relative intensity of pPKM and pPKC was significantly higher in treated rats compared to untreated controls (solid line). Total PKM and PKC levels were higher than their respective phosphorylated protein levels in untreated control SCDH (dashed line), and pPKC levels were significantly higher than total PKC following i.m. acidc saline (dot-interspersed line). However, in the i.m. acidic saline-treated group, there was no difference between total and phosphorylated PKMζ levels. **D)** ZIP-treated rats display significantly lower levels of pPKMζ, but not pPKC, total PKM or total PKC. T-test, $p < .05$.

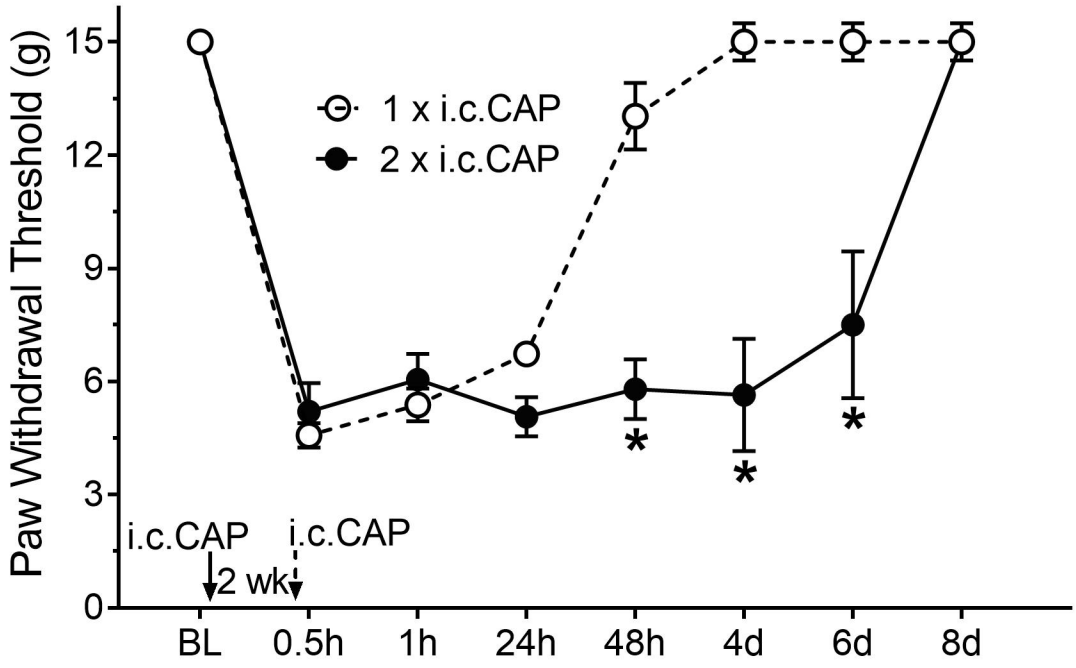

Figure 3.5. Effects of single or repeated i.c. capsaicin on hind paw PWTs.

One injection of i.c. capsaicin results in mechanical allodynia at the hind paw that resolves by 48 h, while two injections of i.c. capsaicin spaced 2 weeks apart result in prolonged allodynia lasting up to 6 d. * $p < .005$, Bonferroni-adjusted, repeated measures ANOVA. Note 1 x i.c.CAP data repeated from Figure 3.2, 2 x i.c.CAP data from Figure 3.6A.

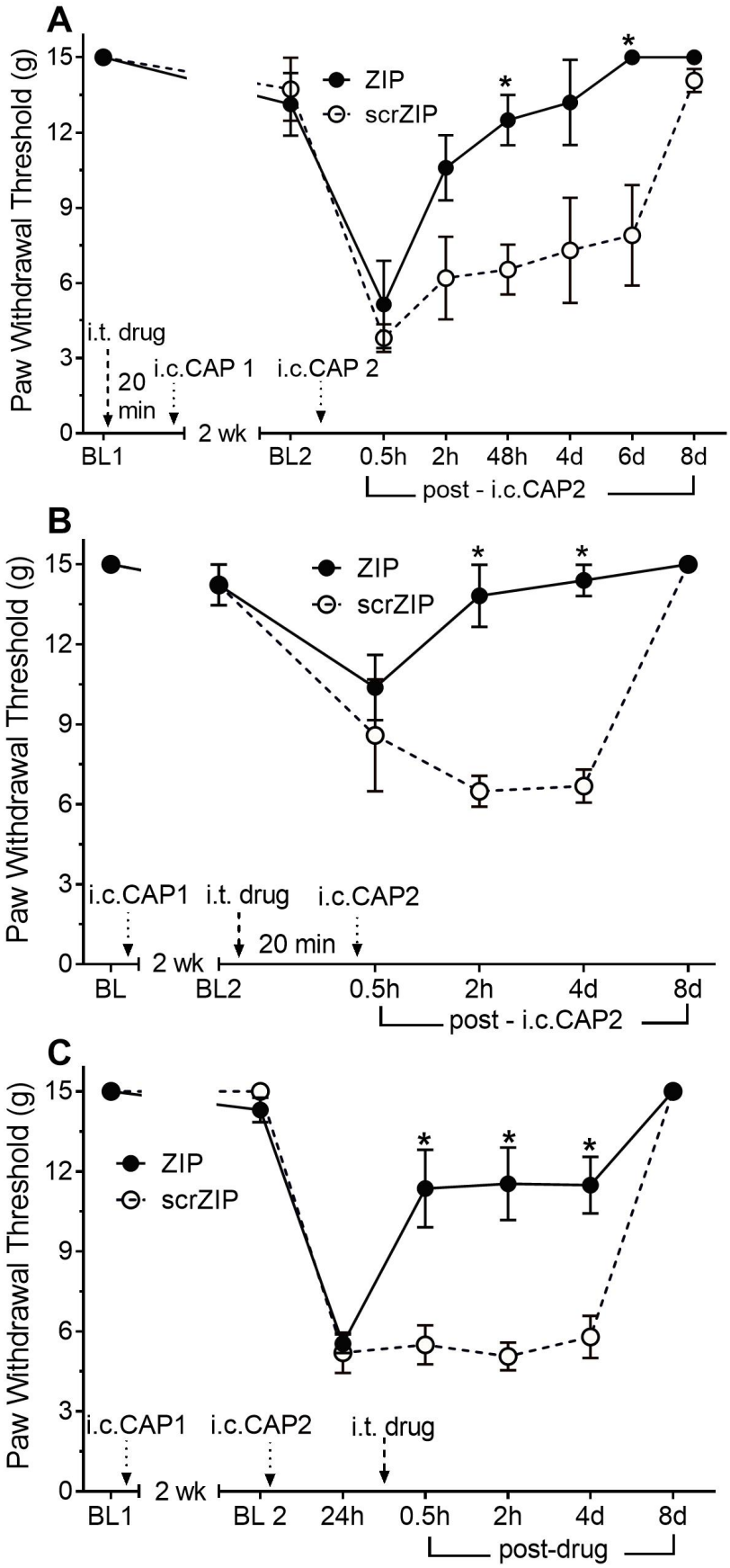
A
Paw Withdrawal Threshold (g)
ZIP
scrZIP
i.t. drug
20 min
i.c.CAP 1
i.c.CAP 2
2 wk
BL1
BL2
0.5h
2h
48h
4d
6d
8d
post - i.c.CAP2
B
Paw Withdrawal Threshold (g)
ZIP
scrZIP
i.c.CAP1
i.t. drug
i.c.CAP2
20 min
2 wk
BL
BL2
0.5h
2h
4d
8d
post - i.c.CAP2
C
Paw Withdrawal Threshold (g)
ZIP
scrZIP
i.c.CAP1
i.c.CAP2
i.t. drug
2 wk
BL1
BL 2
24h
0.5h
2h
4d
8d
post-drug

Figure 3.6. Effects of inhibition of PKMζ before, between, or after two instillations of i.c. capsaicin on priming-induced hyperalgesia.

A) scrZIP administered 20 min prior to the first of two instillations of i.c.CAP did not affect the reduction of PWTs lasting between 0.5 and 6 days after the second instillation i.c.CAP2. Rats pretreated with i.t. ZIP, 20 min prior to the first i.c. capsaicin injection exhibited a transient, but not persistent, reduction of PWTs when challenged with a second i.c. capsaicin injection 2 weeks later.

B) scrZIP administered 20 min prior to the second of two instillations of i.c.CAP did not affect the reduction of PWTs lasting between 0.5 and at least 4 days after the second instillation i.c.CAP2. In contrast, i.t. ZIP at this time point prevented the development of long-lasting mechanical allodynia (i.e., between 2 h and 4 days).

C) scrZIP administered 24 h after the second of two instillations of i.c.CAP did not affect the reduction of PWTs lasting between 24 h and at least 4 days after the second instillation i.c.CAP2. In contrast, i.t ZIP reversed the i.c. capsaicin-induced mechanical allodynia and prevented further development of priming-induced long-lasting hyperalgesia (between 30 min and 4 days).* $p < .05$, Bonferroni, repeated measures ANOVA, n = 6/group.

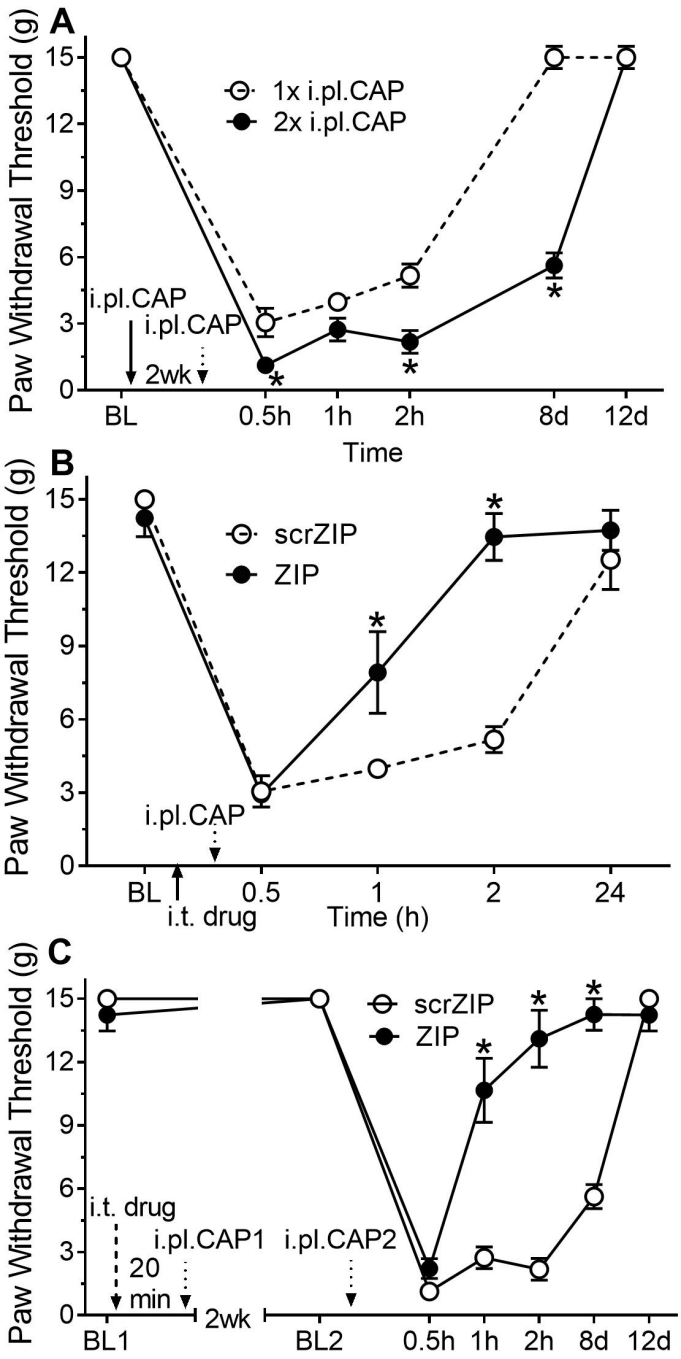
A
Paw Withdrawal Threshold (g)
15
12
9
6
3
0
1x i.pl.CAP
2x i.pl.CAP
i.pl.CAP
i.pl.CAP
2wk
*
*
*
BL
0.5h
1h
2h
8d
12d
Time
B
Paw Withdrawal Threshold (g)
scrZIP
ZIP
*
*
*
i.pl.CAP
BL
i.t. drug
0.5
1
2
24
Time (h)
C
Paw Withdrawal Threshold (g)
scrZIP
ZIP
*
*
*
i.t. drug
20
min
i.pl.CAP1
i.pl.CAP2
2wk
BL1
BL2
0.5h
1h
2h
8d
12d

Figure 3.7. Effects of ZIP on hyperalgesic priming induced by repeated i.pl. capsaicin.

A) Two injections of i.pl. capsaicin (i.pl.CAP) produce significant PWT-reduction for at least 8 d that returns to BL by 12 d, while the PWT reduction associated with a single injection has resolved by 8 d. Note repeated data from sections B and C. **B)** PWTs are significantly reduced from 30 min to at least 2 h following 1Xi.pl. capsaicin in scrZIP-pretreated rats, and this reduction is significantly reduced at 1 and 2 hrs in ZIP-treated rats. **C)** PWTs are significantly reduced between 30 min and 8 days following 2Xi.pl. capsaicin in scrZIP-pretreated rats. Pretreatment with ZIP prevents the development of long-lasting persistent (1h to 8days), but not transient (30 min), allodynia. *$p < .05$, Bonferroni, repeated measures ANOVA.

4 Study 3.

Effects of *prkcz* knock-out in animal models of persistent pain: influence of sex difference

4.1 Rationale

PKMζ, a unique autonomously active PKC isozyme, is either cleaved from PKCζ or transcribed from the *prkcz* gene from its own internal promoter. Using pharmacological inhibition with ZIP, as well as viral administration of PKMζ in hippocampal slices, PKMζ was found necessary and sufficient for the maintenance of late LTP, and also the maintenance of long-term memories (Serrano et al., 2005, Pastalkova et al., 2006, Serrano et al., 2008, Yao et al., 2008). Pharmacological inhibition using ZIP has further shown PKMζ to be essential in the maintenance of cental sensitization-dependent nociception (Asiedu et al., 2011, Laferriere et al., 2011, Marchand et al., 2011, Melemedjian et al., 2013, An et al., 2015).

Genetic deletion of prkcz. Using genetic deletion of exon 9 or 11 of the *prkcz* gene, recent studies demonstrated that hippocampal memory maintenance was preserved in conventional PKMζ KO mice, and it was still reversed by ZIP. Despite their lack of PKMζ, these mice developed normal LTP (Volk et al., 2013), and successfully completed spatial, fear and other hippocampal-dependent memory tasks (Lee et al., 2013; Volk et al., 2013) – both LTP and reward memories were successfully reversed by ZIP injection (Volk et al., 2013; Lee et al., 2013). Volk et al. conditionally ablated PKMζ from forebrain neurons in the adult, yielding the same LTP-phenotype on acute hippocampal slices as in the conventional KO. However, pain researchers (Laferriere et al., 2011; Marchand et al.2011) demonstrated a specific prolonged increase of PKMζ, and not PKCλ/ι, in response to cutaneous injuries causing persistent pain behaviors that are prevented by ZIP administration *in vivo*. In contrast, however, PKCλ/ι was upregulated at 15 min and 30 min in response to BDNF in cultured synaptoneurosomes (Melemedjian et al., 2013). In previous studies 1 and 2, we have shown that pharmacological inhibition of PKMζ using ZIP was ineffective in alleviating pain in models characterized by continued ongoing peripheral inputs (neuropathic, inflammatory, and first phase formalin pain), while pre- and post-treatment of ZIP in models with low-ongoing inputs and strong involvement of spinal neuroplasticity (i.m. acidic saline and i.c.

capsaicin remote allodynia models, second phase formalin pain) respectively, prevented and reversed the hypersensitivity in rats.

In this study, we aim to directly test the role of PKMζ in neuropathic and formalin pain, as well as in models of remote allodynia by using mice with constitutive KO of the *prkcz* gene. We hypothesized that *prkcz* KO mice may show an attenuated response compared to wild-type (WT) and heterozygous (HET) mice in remote allodynia, and second phase formalin pain, but not neuropathic pain. We tested for mechanical allodynia using the von Frey test, and performed formalin testing with increasing concentrations of formalin.

Sex differences in pain perception. Sex differences regarding both pain perception and PKMζ-dependent learning and memory tasks have been well established. In humans, women are reported to exhibit higher sensitivity to nociceptive thermal and mechanical stimuli, and more often develop chronic pain conditions such as fibromyalgia or irritable bowel syndrome (Chang and Heitkemper, 2002, Yunus, 2002). These differences in human pain perception between sexes can be traced back to psychosocial, sociocultural, as well as partly biological, e.g. hormonal cyclical, factors (Wiesenfeld-Hallin, 2005). Animal studies report confounding evidence that is in some cases due to experimenter bias (Mogil, 2012, Sorge et al., 2014). They also hint towards an important role of genotype in the perception of pain. For instance, significant differences in thermal hyperalgesia were observed between genetically different mouse strains and in some cases these differences were sex-specific (Mogil et al., 2000).

Sex difference, memory & PKMζ. In humans, men out-perform females in spatial memory tasks (Astur et al., 1998), while women perform better in memory tasks related to emotions or verbal skills (Helmstaedter et al., 1999). Male rodents exhibit reliable advantages in spatial working and reference memory, independent of strain genotype (Jonasson, 2005). Similar to sex differences in pain perception, the underlying mechanism for these differences in memory are not well understood. Recent evidence suggests PKMζ may play an important role in the differential retention

of spatial memories between sexes. Male rats out-performed females in radial maze test when tested 60 days after initial training, but not when tested 1 d after training. This greater performance correlated with elevated synaptic PKMζ, as well as synaptic GluR2 levels. PKMζ thus plays a greater role in the maintenance of spatial memories in male than in female rats (Sebastian et al., 2013).

It is unknown whether PKMζ-dependent long-lasting pain is differentially manifested in male and female mice. Thus, we further aim to investigate whether sex differences in centrally-dependent remote allodynia exist, and whether these differences are PKMζ-dependent. Concordant with previous studies described above, we hypothesized that female mice may display enhanced pain behavior, and that PKMζ may play a larger role in nociceptive maintenance in male than in female mice. To this end we utilized WT, *prkcz* HET and KO mice, as well as performing pharmacological inhibition using ZIP in Long-Evans rats of both sexes. For these sex difference trials, we utilized the i.m. acidic saline- and i.c. capsaicin-induced remote allodynia models and tested for differences in mechanical allodynia using the von Frey test.

4.2 Methods

4.2.1 Subjects

4.2.1.1 Wild-type (WT) mice

WT mice on a C57BL6 background were obtained by breeding of *prkcz*$^{+/-}$ mice.

4.2.1.2 Prkcz$^{-/-}$ *and Prkcz*$^{+/-}$ *mice*

Prkcz$^{-/-}$ (KO) mice lack exon 9 of the *prkcz* gene, rendering the transcription of PKMζ and PKCζ mRNA incomplete. *Prkcz*$^{+/-}$ (HET) exhibit one WT and one modified *prkcz* allele. A vector targeting exon 9 of the mouse *prkcz* gene was generated using a 129S6/SvEvTac mouse BAC clone from the RPC1-22 library and the plasmid pK-11 Frt-PGKNeo-Frt-LoxP-pBSSK (Meyers et al.,

1998). Following chimera generation, exon 9 was deleted with help of the cre-lox p-system and backcrossed for 11 generations to C57BL6 background. These breeder mice were obtained from the laboratory of Professor Messing, University of Texas, USA (Lee et al., 2013) for development of our experimental mouse colony.

4.2.1.3 Colony maintenance

Prkcz$^{-/-}$, as well as *prkcz*$^{+/-}$ male and female mice were bred in-house to produce KO, HET and WT offspring. Offspring used for experiments were genotyped both at the start and end of experiments. To maintain genetic quality, breeding pairs were control genotyped at the beginning and end of experiments.

4.2.1.4 Genotyping

Using a commercially available ear punch that was cleaned using 70% ethanol, a 2 mm sample from the ear of awake restrained mice was obtained. Ear punches were incubated overnight at 55°C in 50 µl of lysis buffer in presence of protein kinase K (20mg/ml). After vortexing, the samples were placed for 10 minutes at 100°C in a PCR machine and subsequently centrifuged at maximal compatible speed to pellet the undissolved tissue. The resulting gDNA was used directly for PCR amplification (0.5 – 2µl/10 – 20 µl reaction).

4.2.2 Pain models

4.2.2.1 Remote allodynia

<u>*I.m. acidic saline*</u>: An injection of 20 µl of acidic saline (pH 4.0) was administered to the gastrocnemious muscle of isoflurane anesthetized mice, with a modification of the procedure of Sharma et al. (2009).

<u>*I.c. capsaicin*</u>: 50 µl of capsaicin (Sigma, 0.1%, 10% ethanol, 10% tween 80, 80% saline) was instilled into the colon of awake restrained mice using a thin plastic cannula (PE-10 catheter). The perianal area was covered with petroleum jelly prior to the 4 cm deep insertion of the cannula

to avoid pre-instillation irritation. Mice were placed in a Plexiglas® box on elevated mesh wire immediately following capsaicin injection, as described previously (Laird et al., 2001).

4.2.2.2 Neuropathic pain

Mice were subjected to SNI according to the methods of Decosterd and Woolf (2000). Briefly, under isoflurane anesthesia (2% induction, 0.8% maintenance), the common peroneal and tibial branches of the sciatic nerve were transected, leaving the sural and saphenous nerves intact.

4.2.3 Behavioral testing

4.2.3.1 Time of testing

Male and female mice were tested on the same testing days for i.m. acidic saline experiments, such that male mice were habituated and tested first, followed by cleaning of the testing equipment, and subsequent habituation and testing of female mice. For all other experiments, male and female mice were time matched such that the testing was performed at the same time of day on separate days.

4.2.3.2 Formalin test

Following acclimatization within their home cages in the testing room for 1 h, mice were acclimatized to the testing chamber for 15 minutes, and subsequently received an i.pl. injection of 25 μl 1% or 2% formalin. The testing chamber consisted of a clear plastic cubicle with a mirror placed at a 45% angle below the cubicle for easy observation of paws. The formalin test employed timed rating of nociceptive behavior on a 4 point scale: 0 – no pain behavior, 1 – favoring of the paw, 2 – lifting of the paw, 3 – licking/shaking (Dubuisson and Dennis, 1977). Total nociceptive score was determined using a weighted means method of behavioral scoring (Coderre et al., 1993a).

4.2.3.3 von Frey test

Mice were acclimatized within their home cages in the testing room for 1 h before acclimatization in the test cubicles placed on an elevated mesh wire for 45 minutes. Calibrated von

Frey filaments (Stoelting, Woodale, IL) were applied for 4 sec, or until paw withdrawal. Stimulus intensities ranged between 0.1 and 4g, corresponding to filaments 2.36 – 4.56. For each mouse the appropriate range of stimulus intensities to be used was determined by applying five consecutive up/down stimulations (Dixon, 1991) after determining the lowest filament to evoke a positive response. The 50% threshold (grams) was calculated as described by Chaplan et al. (1994). All testing was performed blind to the genotype group.

4.2.4 Data analysis

All data are presented as mean ± SEM. Graphs were plotted using GraphPad Prism (version 6.0). Statistical outliers were defined as those values outside the 1/3 interquartile range (SPSS) and were removed from the data set. All time course analyses were carried out using Statistical Package for the Social Sciences (SPSS). All time course data were analysed using repeated measures ANOVA followed by multiple comparisons with Bonferroni post-hoc test. Percent change from baseline was calculated using the formula (post-drug PWT – BL PWT)/(BL PWT)*100 for each animal. The resulting percent changes were analyzed for statistical differences using two-way ANOVA.

4.3 Results

4.3.1 Prkcz KO and the influence of peripheral activity on persistent pain

4.3.1.1 Effects of PKM/Cζ KO on ipsilateral and contralateral SNI-induced allodynia

SNI induced a significant and persistent reduction in PWTs at 10 d post-surgery on the ipsilateral side for male mice of all genotypes. Although there was a significant main effect of time ($F(5,65) = 259.85$, $p < .0001$), no difference between genotypes was observed for the testing period of 6 weeks, as confirmed by non-significant effect of genotype ($F(2,13) = 0.67$, $p = .525$), as well as

a non-significant geneotype*time interaction ($F(10,65) = 1.59$, $p = 0.13$), in our repeated measures ANOVA (Figure 4.1A).

On the contralateral side, PWTs in all genotypes were not significantly different from baseline at 10 d post-surgery. However, at all following time points PWTs were significantly reduced across all genotypes compared to the respective baseline values (marked † in Figure 4.1B). KO mice (n = 5) showed significantly higher PWTs than HET (n= 6) and WT (n=5) at 6 weeks (** in Figure 4.1B), and significantly higher PWTs than HET at 5 weeks post-surgery (* in Figure 4.1B). These differences were confirmed by significant main effects of genotype ($F(2,13) = 5.75$, $p = .016$) and time ($F(5,65) = 13.41$, $p < .0001$), and a significant genotype * time interaction ($F(10,65) = 2.809$, $p = .006$).

4.3.1.2 Effects of PKM/Cζ KO on late phase formalin pain

I.pl. injections of 1% or 2% formalin produced similar biphasic nociceptive behaviors in WT and HET male mice. Interestingly, KO mice displayed significantly reduced nociceptive scores at 1%, but these effects were less robust at the higher formalin concentration.

1% formalin: Repeated measures ANOVA with Greenhouse-Geisser correction determined significant main effects of genotype ($F(2,28) = 94.617$, $p < .001$) and time ($F(25.25,353.5) = 3.23$, $p < .001$). Bonferroni post-hoc tests revealed significant differences between KO and WT groups (Figure 4.2A).

2% formalin: Following the inhibitory interphase, KO mice, similar to HET and WT mice, developed second phase pain starting 16 min post-formalin, reaching its peak between 20 - 28 min. Toward the end of the test the nociceptive scores for KO mice decreased significantly compared to WT and HET mice, corresponding to significant main effects of genotype ($F(2,26) = 17.37$, $p < .001$) and time ($F(44, 1144) = 8.566$, $p < .0001$), and a significant genotype * time interaction ($F(88,1144) = 1.894$, $p < .0001$). Significant differences following Bonferroni post-hoc comparisons are indicated in Figure 4.2B).

4.3.1.3 Effects of PKM/Cζ KO on PWTs following i.c. capsaicin

I.c. capsaicin induced a significant decrease in PWTs between 30 min and 2 h after capsaicin instillation, with the reduction depending on mouse genotype. WT and HET mice displayed a long-lasting decrease in mechanical thresholds that were only elevated by 24 h ($p < .006$ for all time points). KO mice, however, displayed a transient decrease at 0.5 h ($p < .0001$) and 1 h ($p = .02$) after i.c. capsaicin that was alleviated by 2 h after i.c. capsaicin. However, this decrease was significantly less than that seen in WT and HET mice between 0.5 - 2 h. These differences were evidenced by significant main effects of genotype ($F(2,25) = 68.740$, $p < .0001$) and time ($F(4,100) = 120.721$, $p < .0001$), as well as a significant time * genotype interaction ($F(8,100) = 12.853$, $p < .0001$) following repeated measures ANOVA. Bonferroni post-hoc comparisons detected significances marked in the graph from 0.5 h to 2 h after capsaicin (Figure 4.3). Thus, PKM/Cζ KO prevents the development of otherwise greater and longer-lasting mechanical allodynia in male mice.

Next, we assessed whether pharmacological inhibition using ZIP could reverse i.c. capsaicin-induced mechanical allodynia in HET and WT mice, and importantly, whether ZIP treatment had further analgesic effects in KO mice. ZIP was injected following the reduction in PWTs 30 min post-i.c. capsaicin in WT, HET and KO mice. The drug significantly and persistently elevated PWTs in both WT and HET mice (Figure 4.4A,B)

However, the effect of ZIP was less impressive in KO mice. Although PWTs are elevated at 1 h post-i.c. capsaicin in ZIP-treated mice, the increase reflects largely the significantly lower predrug PWTs in untreated KO mice (at 0.5 h; Figure 4.4C).

Thus, the percentage change in PWTs from pre-drug (0.5 h after i.c. capsaicin) to post-drug (60 min and 120 min after i.c. capsaicin) did not significantly increase in KO mice injected with i.t. ZIP. Untreated KO mice increased PWTs by 259% at 60 min and by 405% at 120 min compared to

PWTs at 0.5 h after i.c. capsaicin. Comparatively, KO mice that had received an injection of ZIP showed smaller increase of PWTs at 60 min (156%) and 120 min (175.5%) compared to their untreated counterparts, indicating ZIP had unimpressive PWT alleviating effects in KO mice (Figure 4.4D).

4.3.1.4 Prkcz KO reduces i.m. acidic-saline induced remote allodynia

Both WT and HET male mice displayed significantly lowered PWTs compared to baseline persisting from one week post-i.m. acidic saline (see Figure 4.5 for significances). PWTs of WT mice dropped the lowest of the three groups between week 1 and week 4 after i.m. acidic saline ($p \leq .001$ for weeks 1 - 4).

At week 1, a transient allodynia developed in the KO group (mean difference to baseline = 0.54 ± 0.155, $p = .017$), that was no longer present at weeks 2, 3, and 4. Despite this transient allodynia at week 1 after i.m. acidic saline, KO animals displayed significantly higher PWTs than WT mice at all weeks ($p < .05$, Bonferroni) and PWTs that were significantly higher than those of HET mice at week 4 (Figure 4.5). In accordance with the results depicted in the graph, repeated measures ANOVA revealed significant main effects of genotype ($F(2,25) = 25.688$, $p < .001$) and time ($F(4, 100) = 19.481$, $p < .0001$), as well as a significant time * genotype interaction ($F(8,100) = 2.37$, $p = .022$).

4.3.2 Influences of sex on remote allodynia in WT, HET and KO mice

4.3.2.1 Influences of sex on i.c. capsaicin-induced decrease of PWTs

Female mice. WT, HET and KO mice subjected to a single instillation of i.c. capsaicin displayed a long-lasting decrease in PWTs starting at 30 min post-instillation such that PWTs differed significantly over time ($F(4, 92) = 53.22$, $p < .0001$). A trend towards a significant main effect of genotype was detected in our repeated measures ANOVA ($F(2,23) = 3.221$, $p = .058$). No significant genotype * time interaction was observed ($F(8, 92) = .849$, $p = .562$). Together these

results suggest KO, HET and WT all displayed allodynic PWTs that did not differ between genotypes (Figure 4.6).

Comparison of time course effects between female and male mice. WT male ($p < .0001$) and female ($p = .01$) mice both exhibited a significant persistent decrease of PWTs starting 30 min after- i.c. capsaicin (Figure 4.7A). Thus, no significant difference of PWT values between the sexes was observed at 1 h ($p = 1.00$) or 2 h ($p = 1.00$). Male mice showed significantly increased PWTs at 24 h compared to female PWTs ($p = .022$).

Similarly, HET male ($p < .0001$) and female mice ($p = .001$) displayed significantly reduced PWTs at 30 min after i.c. capsaicin compared to baseline values that persisted until the end of testing. Thus, no significant difference in PWTs between the sexes was observed (Figure 4.7B). At 30 min after i.c. capsaicin the mean PWT values of KO male and female mice were significantly lower than their respective baseline values (Figure 4.7C). Interestingly, at 1 h after i.c. capsaicin this PWT decrease was attenuated in male, but not in female mice, so that female mice displayed significantly lower PWT values than their male counterparts. At 2 h male mice had fully recovered, leading to a significant difference between male and female PWTs. Female mice continued to display allodynic PWTs at 24 h. These differences were confirmed by significant main effects of genotype ($F(5,45) = 10.08$, $p < .0001$) and time ($F(4,180) = 76.413$, $p < .0001$), and genotype*time interaction ($F(20,180) = 2.282$, $p = .002$). These data strongly suggest a PKMζ-dependent sex difference that may be accountable for differential pain perception in male and female mice.

Comparison of overall percent change from baseline PWTs within female and male mice. Next, we calculated the overall percent change from baseline in PWTs across all time points for KO, HET and WT male and female mice. We compared how much the percent change from baseline PWTs in HET and WT male and female mice differed from that of their KO counterparts. Male KO mice displayed significantly lower PWT percent changes from baseline than male HET,

and WT mice, and than their female counterparts (see * in Figure 4.7D). In female mice, no significant difference between the overall percent change from baseline PWT was observed. Thisdata highlights that PKM/Cζ KO prevents the reduction in PWTs or heightened sensitivity in male, and not in female mice (Figure 4.7D).

4.3.2.2 Influences of sex on prkcz KO in i.m. acidic saline-induced remote pain

Female mice. Female mice in each genotype group displayed a significant and persistent reduction in mechanical thresholds starting 1 week after i.m. acidic saline ($p < .0001$, Bonferroni). The extent of mechanical allodynia was slightly, but significantly less pronounced in KO than in HET female mice at 2 week after i.m. acidic saline ($p = .008$), and less than both HET and WT at week 3 - 4 of testing ($^*p < 0.05$, Figure 4.8). In accordance with these observations, there were significant main effects of genotype ($F(2,24) = 5.791$, $p = .009$) and time ($F(4,96) = 328.2$, $p < .0001$), as well as a significant geneotype*time interaction ($F(8,96) = 2.887$, $p = .006$). These results indicate KO female mice develop allodynia that is slightly, but significantly, less than in WT and HET mice.

Comparison of time course effects between female and male mice. Next, we examined the differences between male and female PWTs within each of the three distinct genotypes (WT, HET, KO) for each of the four testing time points. Starting 1 week post-pain induction, WT male and female PWTs were persistently lowered and did not differ significantly (Figure 4.9A). Interestingly, HET female mice developed significantly lower PWTs than HET male (Figure 4.9B), and importantly, female KO mice displayed significantly lower PWTs than their male counterparts at all time points after pain-induction ($p < .007$, Bonferroni, Figure 4.9C). These sex differences across genotypes were verified by significant main effects of group ($F(5,49) = 29.748$, $p < .0001$), and time ($F(4,196) = 147.903$, $p < .0001$), as well as a significant sex accross genotype* time interaction ($F(20,196) = 6.284$, $p < .0001$). These results indicate PKMζ KO prevents the development of remote allodynia in male, but not female mice, and partial KO of PKMζ elicits an

intermediary pain response in male, but not female mice, suggesting PKMζ may be differentially expressed or significant for nociception across sexes.

Comparison of overall percent change from baseline PWTs beween male and female mice. We first examined the overall percent change in PWTs from baseline for each genotype within each sex. Bonferroni post-hoc test indicates no difference in % change from baseline PWTs between female KO and WT ($p = 1.00$), or female HET mice ($p = 1.00$). However, KO male percent change from baseline differs significantly from WT ($p < .0001$), but not from HET ($p = 1.00$) male mice. Additionally, HET and KO male percent changes were significantly lower than those of their respective female counterparts (Figure 4.9D). These data suggest PKM/Cζ KO provides greater analgesia in male than in female mice.

4.3.2.3 Comparison of the effects of pharmacological inhibition of PKMζ on i.c. capsaicin-induced referred allodynia in male and female rats

Next, we tested whether sex differences in the contribution of PKMζ to i.c. capsaicin-induced remote allodynia could be confirmed in another species. The following experiments were conducted in rats with pharmacological inhibiton of PKMζ using ZIP.

Female rat: Female rats displayed a decrease of PWTs starting at 30 min after i.c. capsaicin and i.t. scrZIP ($p < .0001$) that resolved by 72 h. Treatment with the PKMζ inhibitor ZIP reduced the full development of i.c. capsaicin-induced remote allodynia, leading to significant main effects of treatment ($F(1,10) = 99.03$, $p < .0001$) and time ($F(3.096,30.961) = 32.71$, $p < .0001$), as well as a significant time*treatment interaction ($F(3.096, 30.961) = 6.82$, $p = .001$) in our repeated measures ANOVA. Bonferroni post-hoc comparisons revealed a significant increase in PWTs in the ZIP-treated group from from 30 min to 48 h ($p \leq .007$, see asterisks in Figure 4.10).

Comparison of i.c. capsaicin-induced remote allodynia in female and male rats. Although both male and female rats exhibited i.c. capsaicin-induced reductions in PWTs, the remote

allodynia was more pronounced in females than males. As shown in Figure 4.11, male, but not female, rats started recovery at 24 h ($p < .0001$), and were fully recovered by 48 h while female rats required 72 h for full recovery, leading to a significant difference between the sexes ($p = .001$). Accordingly, we discovered significant main effects of sex ($F(1,10) = 10.708$, $p = .008$) and time ($F(1.91, 19.19) = 137.016$, $p < .0001$) as well as a significant time*sex interaction ($F(1.91, 19.19) = 4.917$, $p = .02$) in our repeated measures ANOVA.

Comparison effect of ZIP on i.c. capsaicin-induced remote allodynia in female and male rats: Although ZIP reduced i.c. capsaicin-induced remote allodynia in both male and female rats (see Figure 4.12 compared to allodynia exhibited in rats in Figure 4.11), the effects of ZIP were much greater in males than females - especially at 30 min ($p = .045$) and 1 h ($p = .001$) post-ZIP. Accordingly, our repeated measures ANOVA revealed significant main effects of sex ($F(1,10) = 29.447$, $p < .0001$) and time ($F(2.69, 26.90) = 7.765$, $p = .001$), as well as a significant sex*time interaction ($F(2.69, 26.90) = 2.102$, $p = .129$).

4.4 Discussion

Using genetic deletion of the *prkcz* gene, this study confirms previous results highlighting an important role for PKMζ in the maintenance of remote pain obtained after pharmacological inhibition using ZIP. We further elucidated sex differences in the maintenance of remote pain, and show for the first time that PKMζ is more strongly utilized in male than in female mice for the maintenance of spinally dependent long-lasting pain.

4.4.1 Neuropathic pain

Weeks-long persistent ipsilateral allodynia at the rodent hind paw following SNI has been well established (Decosterd and Woolf, 2000). Although conducted using small sample sizes, our data showing significant allodynia in WT, HET and KO mice confirms the robustness of this model (Jarvis and Boyce-Rustay, 2009), as well as the notion that a role for PKM/Cζ in the expression of

ipsilateral pain may be masked by ongoing peripheral inputs, as discussed in study 2. Although contralateral allodynia following SNI is not always reported (Decosterd and Woolf, 2000, Mogil et al., 2010), our data shows the gradual development of contralateral allodynia which is significant 4 weeks following surgery. Importantly, KO mice showed significantly less reduction in contralateral PWTs than HET and WT mice, suggesting PKM/Cζ is required for the development of contralateral allodynia. These preliminary data complement our findings using ZIP on rats (study 1), where PKMζ inhibition at 6 weeks after SNI-surgery was able to transiently reverse the established contralateral allodynia. Together these results allow for the speculation that strong peripheral input causes a PKMζ-dependent sensitization of spinal neurons, which can be detected when assessing contralateral allodynia.

4.4.2 Formalin test

The formalin test provides an important tool for testing of centrally-dependent (second phase) pain (Coderre et al., 1990). A previous study has highlighted the neccessity for PKMζ activity for the maintenance of second phase pain after PKMζ inhibition using ZIP in rats (Laferriere et al., 2011). In study 1, we have shown that ZIP's pain reducing effect at 2% and 3.5% formalin is masked at a higher formalin concentration. Here, we show by use of *prkcz* KO, HET and WT mice that PKM/Cζ KO substantially reduces the development of second phase formalin pain at 1%, but the effect is less obvious at a higher formalin concentration. Conversely, WT and HET mice developed second phase pain regardless of formalin concentration. Our data showing the development of first phase pain in all three genotype groups suggests that the first phase of the formalin test likely does not depend on central sensitization and is independent of PKM/Cζ activity. Similarly, a pretreatment of ZIP was not able to reduce first phase formalin pain up to a dose of 20 nmol, with higher doses of ZIP leading to sedative effects (Laferriere et al., 2011). Our data thus show for the first time dose-independently that PKMζ is not required for first phase, but is for second phase formalin pain although it is observed at a low, and not high, formalin concentration.

It may be suggested higher formalin concentrations produce an increased peripheral drive that continues to signal pain regardless of central sensitization. It remains to be explored what concentrations of formalin produce how much peripheral input to the spinal cord to understand what level of ongoing peripheral input masks the protective effects of PKMζ inhibition. Generally, however, this study highlights the important role of the degree of peripheral injury signalling onto the spinal cord, as well as the role of spinal PKMζ in persistent pain maintenance.

4.4.3 PKMζ is required for maintenance of remote allodynia

I.c. capsaicin. KO mice that received an injection of i.c. capsaicin recovered from a transient allodynia by 1 h following the injections, while WT and HET mice remained allodynic for longer periods. These data are in line with our previous experiments on rats – pretreatment with the PKMζ inhibitor ZIP showed a transient allodynia that faded away by 1 h after injection. It may be speculated that capsaicin's early activation of nociceptors may induce changes responsible for the initial allodynia that could, however, not be sustained for longer periods without PKMζ activity. Together with results obtained on rats using ZIP our data using two remote allodynia models in KO mice suggest PKMζ is required for the maintenance of long-lasting persistent pain. Since HET mice developed both i.m. acidic saline, and i.c. capsaicin – induced pain, even partial expression of PKMζ may be sufficient for the maintenance of long-lasting pain.

Acidic saline. Two injections of i.m. acidic saline produce long-lasting remote allodynia. Previous reports show PKC and PKA are required for the induction (Hoeger-Bement and Sluka, 2003, Sluka and Audette, 2006), but unknown players for the maintenance of this persistent allodynia. We showed in study 2 that inhibition of PKMζ either at 24 h or 1 week following i.m. acidic saline reverses the allodynia – an effect previously unseen with any other protein kinase inhibitor. Here, we show that KO mice lacking PKM/Cζ did not develop long-lasting allodynia in response to i.m. acidic saline, while both HET and WT mice did. Together with our pharmacological inhibition using ZIP discussed in study 2, showing both a decrease of mechanical

allodynia, as well as a decrease of PKMζ protein levels following ZIP, these data strongly suggest PKMζ is required for the maintenance of remote pain induced by i.m. acidic saline.

4.4.4 Prkcz KO in long-term memory & pain

Studies on constitutive KO (Lee et al., 2013, Volk et al., 2013), as well as conditional forebrain ablation of PKM/Cζ (Volk et al., 2013) revealed normal LTP expression, as well as normal performance in a spatial memory task. Importantly, in these studies, ZIP was able to abolish late-LTP and reverse previously acquired memories. However, more recent data highlights a compensatory mechanism involving PKCι/λ. Thus, *prkcz*$^{-/-}$ mice showed increased expression of the full-length atypical PKCι/λ, as well as of its truncated form consisting only of its catalytic domain. Importantly, postsynaptic perfusion of truncated PKCι/λ resulted in enhanced AMPA-mediated potentiation, similar to the effects of PKMζ postsynaptic perfusion (Yao, 2013). Additionally, blocking PKMζ specifically using antisense oligonucleotides, or conditional KO prevented the development of normal LTP and formation of long-term memories (Tsokas, 2013). While our study cannot elaborate on spinal LTP in *prkcz*$^{-/-}$ mice, our data show reduced remote allodynia in PKM/Cζ mice and no ZIP-induced alterations in the %change from baseline PWT in these KO mice. Conversely, WT and HET mice exhibit greater, ZIP-reversible remote allodynia. Thus, the compensatory mechanism in place for hippocampal LTP may either not apply to PKMζ-dependent spinal nociception, or the maintenance of spinal nociception depends on an LTP-independent mechanism. Our data show that *prkcz* KO prevents from the development of central sensitization-dependent long lasting pain (second phase formalin, and remote allodynia) that is in line with previous data using ZIP.

4.4.5 PKMζ & sex differences

We show here that female mice develop remote allodynia in response to i.c. capsaicin, and i.m. acidic saline. We further show that male *prkcz* KO mice develop less profound allodynia than

their HET or WT littermates, while no differential pain expression between genotypes is observed in female mice. Importantly, our data show that female and male WT mice develop profound allodynia, suggesting that the sex difference observed here depends on PKMζ expression or utilization, suggesting female mice exhibit a differential regulation of pain behavior, which depends on other genetic components or environmental factors (Mogil, 2012).

The sex discrepancy in allodynia was most profound in *prkcz* KO mice – females developed persistent allodynia, while their male counterparts did not. This finding suggests PKM/Cζ may either contribute more strongly to the maintenance of pain and remote allodynia in male mice than in female mice; or a potential compensatory mechanism following constitute KO is more efficient in fulfilling the maintenance role in female mice. Support for the former may be drawn from work on GluR1$^{-/-}$ mice. AMPA-R's GluR1 is required for hippocampal LTP and formation of long-term memories (Kessels and Malinow, 2009). GluR1$^{-/-}$ male mice showed a deficit in contextual fear conditioning, while their female counterparts did not (Dachtler et al., 2011). Nitric oxide (NO) plays an important role in GluR1-independent LTP and memory formation (Hardingham and Fox, 2006), and NO formation is sexually dimorphic as it was shown to depend on estrogen signaling (Weiner et al., 1994). It has been suggested that female mice may preferentially utilize NO signalling for LTP, while males preferentially use GluR1 (Dachtler et al., 2011). Our results may be related to these findings. Since NO is produced more strongly in females, and NO-signalling is required for LTP during spinal nociception (Zhang et al., 2005), spinal LTP and central sensitization in female mice may rely more heavily on NO-signalling than on PKMζ activity as is used by males. Our data support this idea, since KO females did develop allodynia (unlike their male counterparts), but less profoundly than in HET or WT females.

A previous report on long-term memory tested with the radial arm maze-retention task reported male rat test scores were significantly higher than those of females when tested 60 d after training, but not 1 d after training – which correlated with increased synaptic PKMζ and synaptic

GluR2 levels. Importantly, male rats displayed higher synaptic PKMζ expression compared to females irrespective of memory retention task/timing, suggesting that PKMζ is critical for differential memory expression between sexes (Sebastian et al., 2013). In line with the notion that PKMζ may be more strongly expressed and utilized in the maintenance of spinal nociception in males, the analgesic effects of ZIP following i.c. capsaicin were significantly stronger in male than in female rats. The finding that ZIP post-treatment did significantly alleviate PWTs following capsaicin in female rats, while *prkcz* KO did not have a pain preventing effect in female mice may be traced back to constitutive KO compensation, differential post-treatment effect or species differences. The possibility that ZIP may be targeting a player other than PKM/Cζ cannot be ruled out. In accordance with the recently described compensatory mechanism by PKCι/λ in these constitutive knockout mice (Yao, 2013), and our findings that ZIP is more analgesic in male rats allows for the speculation that PKCι/λ may be more strongly utilized for compensatory mechanism in female rats – again thereby bringing up the question how central sensitization-dependent long-lasting pain is maintained in females.

4.4.6 Experimental confounding factors

Hormonal changes influence pain behavior in females (Riley et al., 1999, Fillingim and Ness, 2000). When testing for sex differences, it is recommended to test females at the same stage of their cycle to obtain a unitary result of "female" pain perception compared to males. Although here we did not control for cycle stages, we were able to obtain significant sex differences in the expression of remote allodynia after PKM/Cζ KO. It could be that cycle-matched females may have produced even greater differences in pain behavior across genotypes. Here, all female subjects were between 8 and 16 weeks old, and thus at an age in which menstrual cycling occurs. Litter numbers allowed for an age-matching of female and male mice, such that age-differences defining sex-differences can be ruled out.

4.4.7 Conclusion

By use of genetic manipulation of *prkcz* this study provides novel information on the role of PKMζ in the maintenance of neuropathic and chemical pain, as well as remote allodynia. This study confirms and complements previous findings obtained by pharmacological inhibition using ZIP, suggesting PKMζ is required for the maintenance of central sensitization-dependent pain, but that the ability to demonstrate its role may be masked in cases of strong peripheral drive. Further, this study shows for the first time that sex differences in models of remote allodynia depend on PKMζ activity. Future studies may discern the underlying mechanisms related to both PKMζ's role in persistent pain maintenance, and in differential contribution to remote pain in female mice.

4.5 Study 3 Figures

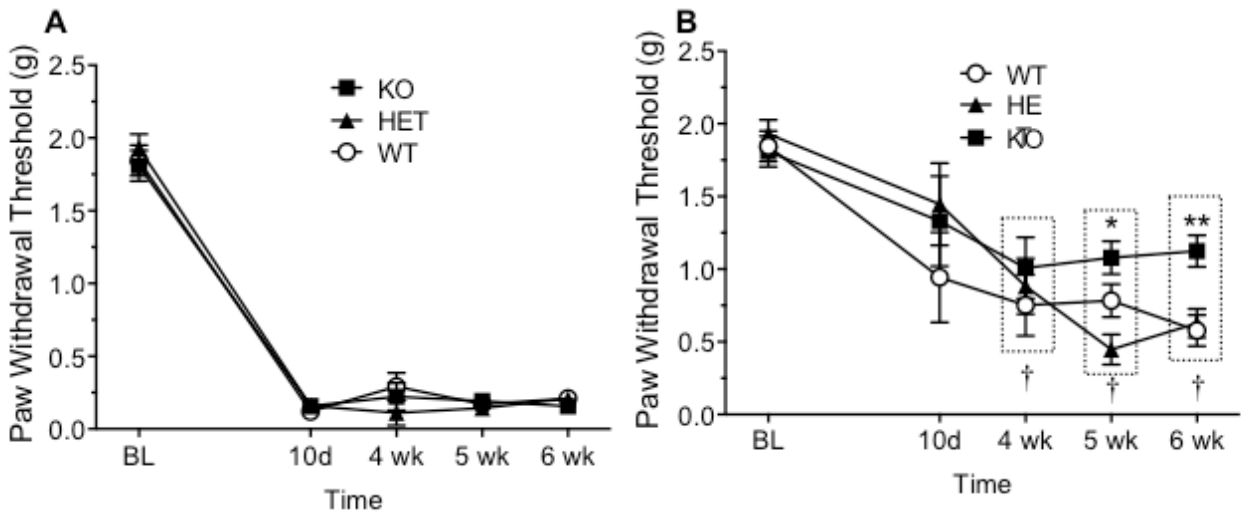

Figure 4.1. Effects of SNI on ipsilateral (A) and contralateral (B) PWTs in WT, HET and KO mice.

A) KO, HET and WT male mice showed significantly reduced ipsilateral PWTs from 10 d to 6 weeks post-surgery compared to baseline. No difference between genotypes was observed. **B)** Contralateral PWTs in WT (n=5), HET (n=6) and KO (n=5) mice were not significantly reduced at 10 d, but were from 4 weeks – 6 weeks post-surgery (†). KO mice displayed significantly higher PWTs than HET (*$p < .005$) at 5 weeks, and both WT and HET (**$p < .02$) at 6 weeks, repeated measures ANOVA.

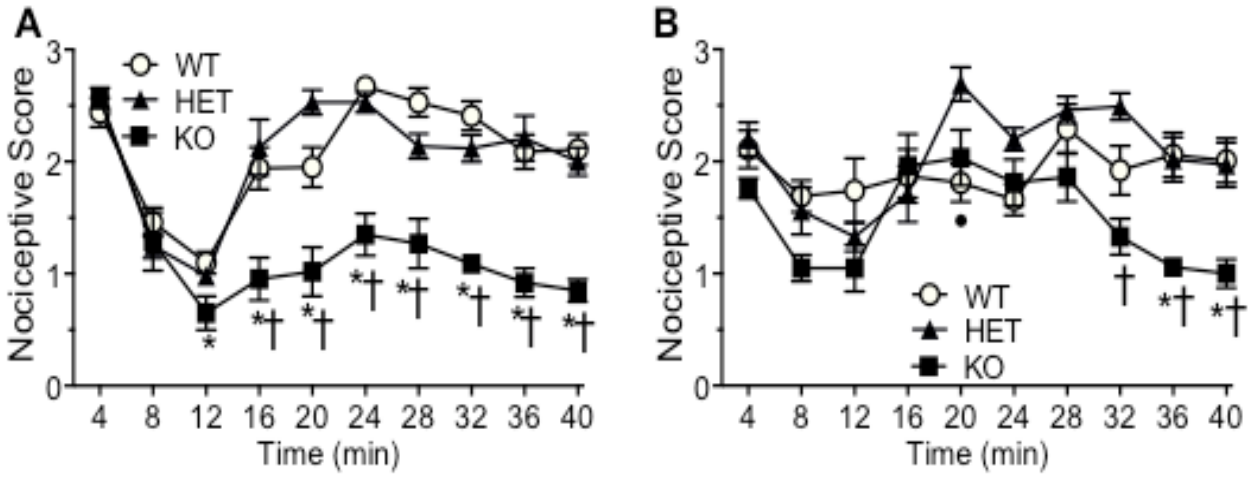

Figure 4.2. Effects of genetic manipulation of the *prkcz* gene on formalin pain.

A) *prkcz* KO male mice show reduced late-phase pain compared to WT and HET mice after 1% formalin. **B)** 2% formalin induces pain in KO, HET and WT mice, with late phase pain reduced in KO mice, particularly at late time points. Comparison KO to WT*, to HET †, between WT and HET •, $p < .05$, Bonferroni adjusted, repeated measures ANOVA.

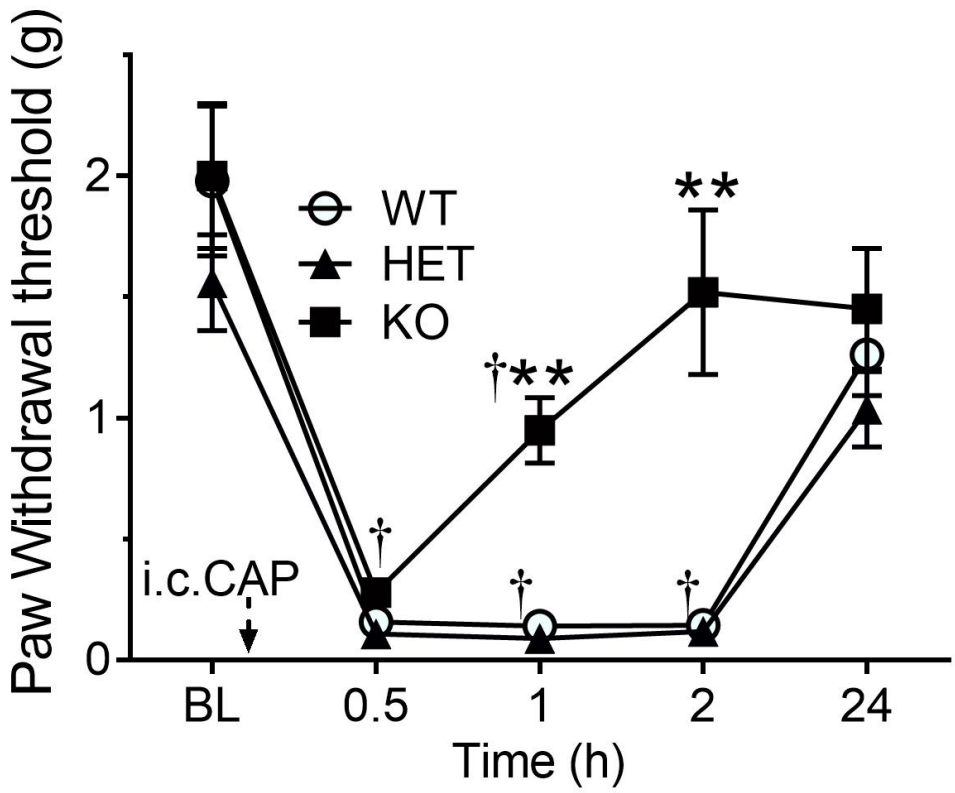

Figure 4.3. Effects of genetic manipulation of the *prkcz* gene on i.c. capsaicin-induced remote allodynia.

WT and HET male mice display a significant decrease in PWTs between 30 min and 2 h after i.c. capsaicin. KO mice show a significant decrease in PWTs from baseline (BL) at 30 min and 1 h after i.c. capsaicin, but this allodynia is transient and significantly less profound in KO mice compared to WT and HET mice at 1 h and 2 h after i.c. capsaicin. Repeated measures ANOVA, Bonferroni adjusted (**$p < .01$, compared to WT and HET, $^{\dagger}p < .05$ compared to BL).

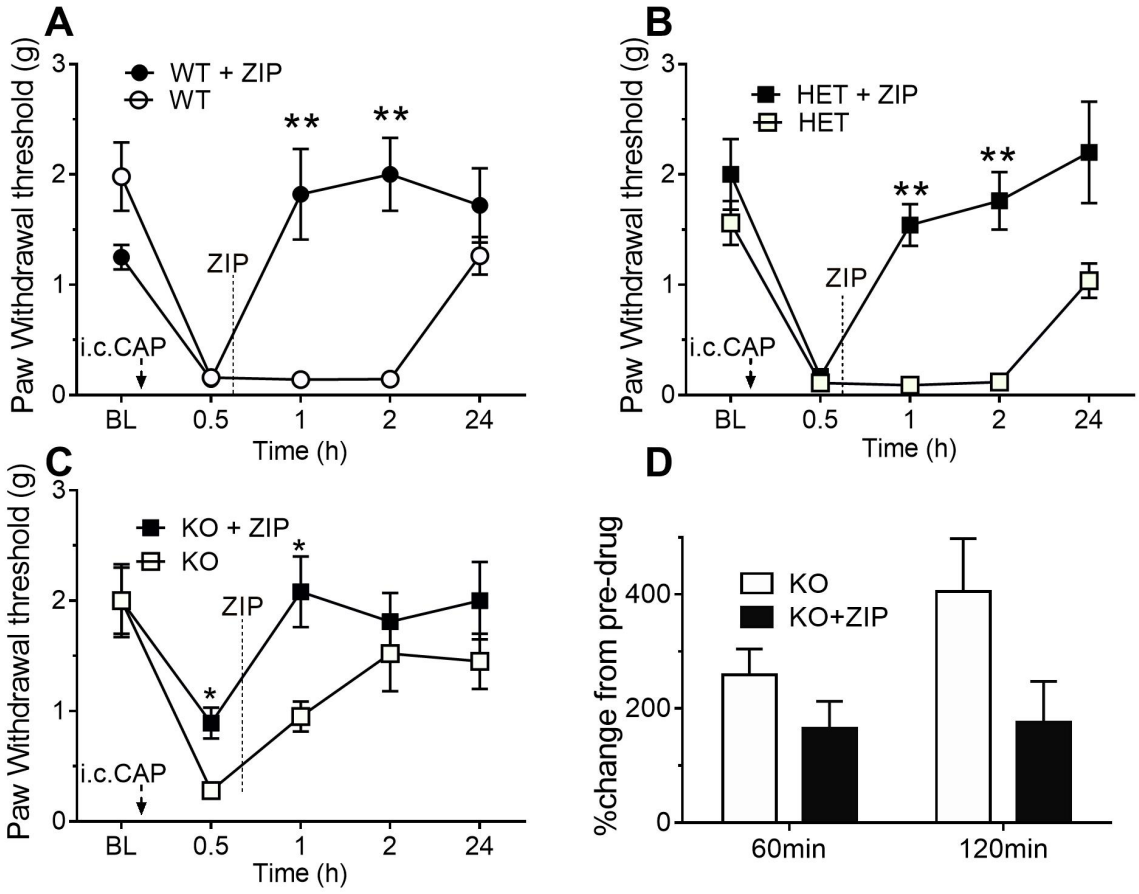
A
WT + ZIP
WT
Paw Withdrawal threshold (g)
ZIP
i.c.CAP
BL
0.5
1
2
24
Time (h)
B
HET + ZIP
HET
C
KO + ZIP
KO
D
%change from pre-drug
60min
120min

Figure 4.4. Effects of PKMζ inhibition on i.c. capsaicin-induced remote allodynia in mice with genetic alterations in the *prkcz* gene.

I.t ZIP 40 min after i.c. capsaicin increased PWTs significantly at 60 min and 120 min after i.c. capsaicin in **A)** WT mice and **B)** HET mice. **C)** Although PWTs are elevated at 1 h post-i.c. capsaicin in ZIP-treated KO mice, the increase reflects largely the significantly lower 0.5 h PWTs in untreated mice compared to the same time (i.e., pre-drug) in ZIP-treated KO mice. While protein kinases other than PKMζ may be responsible for early allodynia, the pre-drug allodynia in untreated KO mice is even lower than expected likely due to random variability. Thus, the percentage change in PWTs from pre-drug (0.5 h after i.c. capsaicin) to post-drug (60 min and 120 min after i.c. capsaicin) was not higher in KO mice i.t. injected with ZIP than the percentage changes in untreated KO mice over the same time period **(D)**. Repeated measures ANOVA, Bonferroni adjusted, *$p <$ .05, **$p <$ .005.

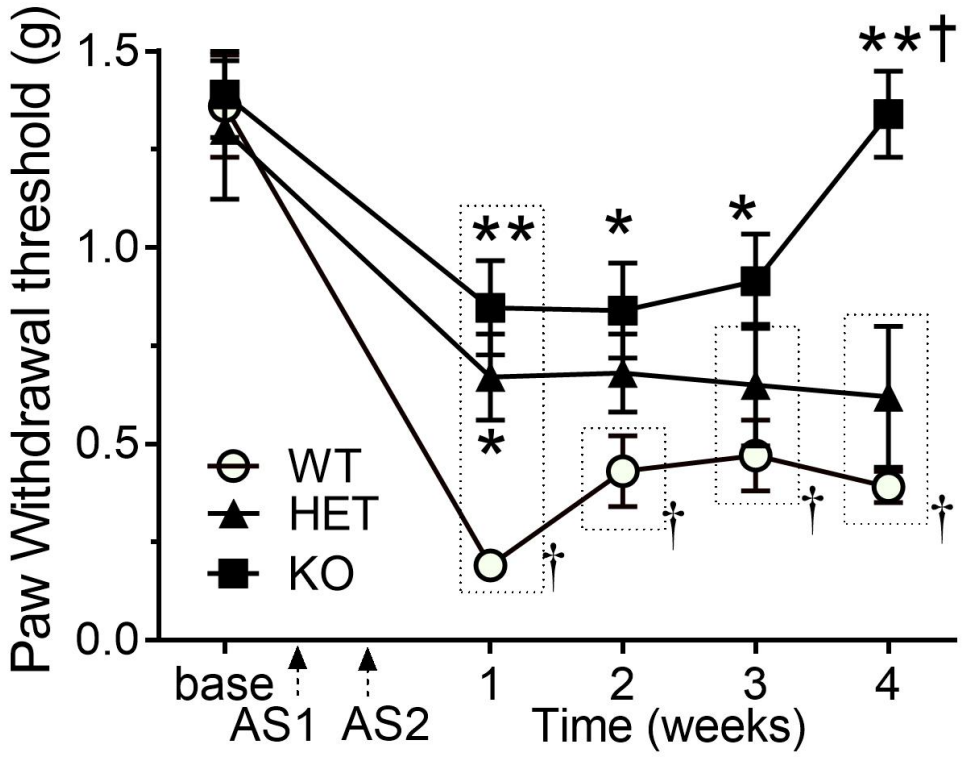

Figure 4.5. Effects of *prkcz* KO on i.m. acidic saline-induced remote mechanical and cold allodynia.

WT male mice display reduced PWTs starting 1 week after i.m. acidic saline lasting for at least 4 weeks. Compared to WT mice, allodynia is reduced in HET mice from week 1 to 4, with a significant elevation at week 1. Mechanical PWTs in KO mice were significantly higher than in WT throughout weeks 1-4, and higher than HET mice at week 4. Repeated measures ANOVA, Bonferroni-adjusted (*$p < .05$, **$p < .001$ compared to WT, †$p < .05$ compared to HET, †$p < .05$ compared to baseline).

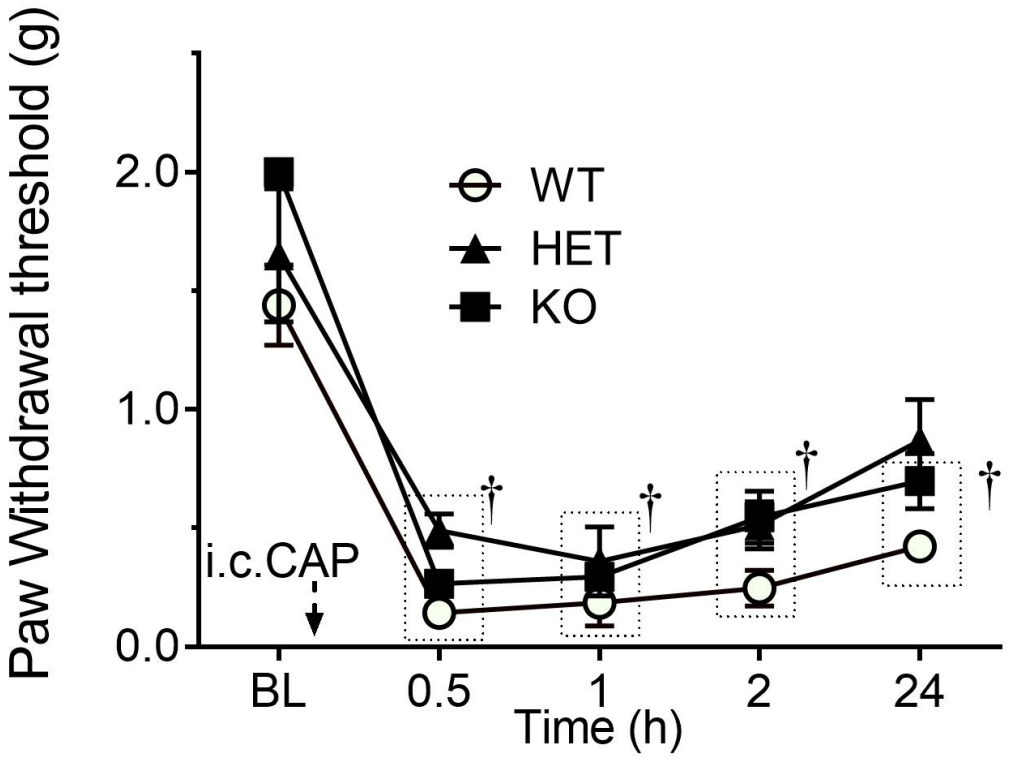

Figure 4.6. Comparison of the remote mechanical allodynia induced by i.c. capsaicin in WT, HET and KO female mice.

Female mice subjected to a single injection of i.c. capsaicin exhibited a significant decrease from BL from 30 min to 24 h after i.c. capsaicin. No difference between genotypes was observed. Repeated measures ANOVA, Bonferroni, †$p < .05$ compared to baseline.

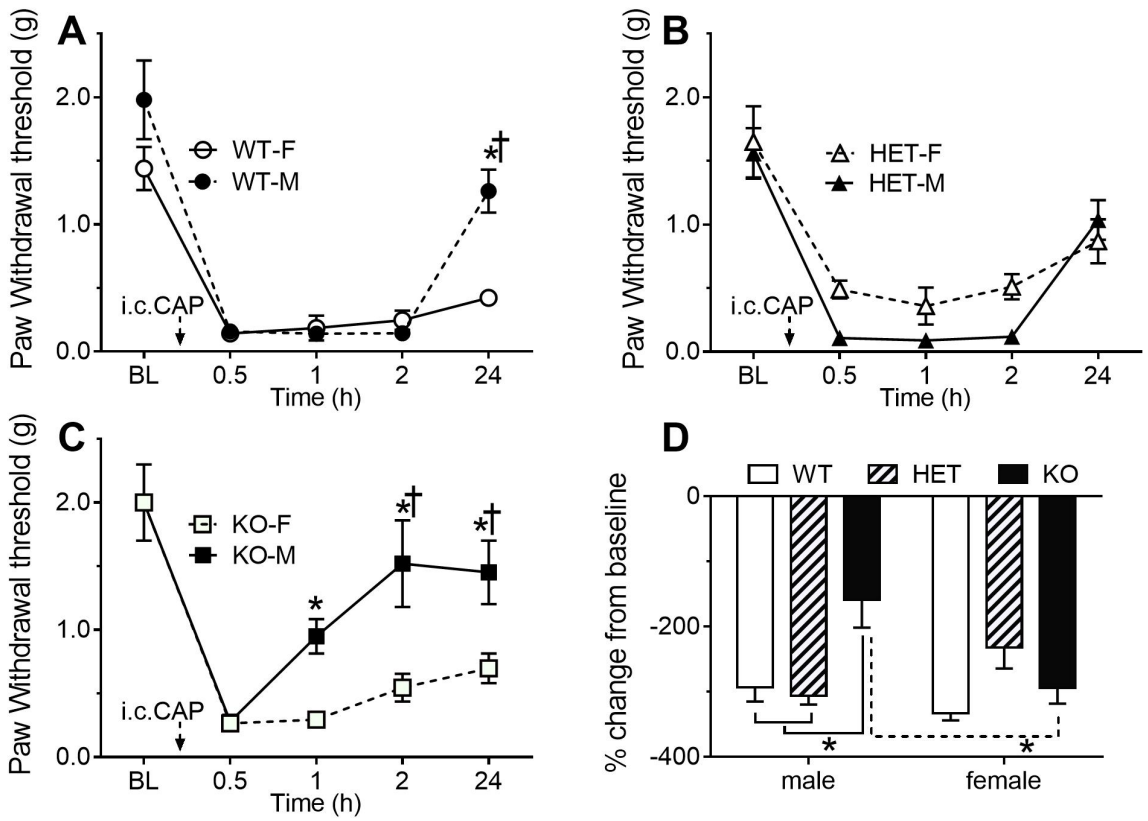

Figure 4.7. Influence of sex on the effects of *prkcz* KO on i.c. capsaicin-induced remote allodynia.

A) Both male and female WT mice display significantly and persistently reduced PWTs from 30 min to 2 h after i.c. capsaicin. However, male mice recover at 24 h, while allodynia persists to 24 h in females. **B)** Both male and female HET mice exhibit significantly and persistently reduced PWTs from 30 min to 2 h after i.c. capsaicin that are increased at 24 h. **C)** KO male and female mice exhibit significantly reduced PWTs 30 min after i.c. capsaicin. However, PWTs in male mice increase at 1 h and are restored to baseline levels by 2 h after i.c. capsaicin, while allodynia persists in female mice up to 24 h. **D)** Male KO mice show significantly smaller percent (%) change from baseline than either HET or WT male mice, or their female counterparts, while no significant differences in % change from baseline were detected in WT, HET and KO female mice. $^{*}p < .05$ from female value, $^{\dagger}p > .05$, non-significant compared to baseline, repeated measures ANOVA, Bonferroni-adjusted. Note female data from Figure 4.6, and male data from Figure 4.3 is repeated in A-C.

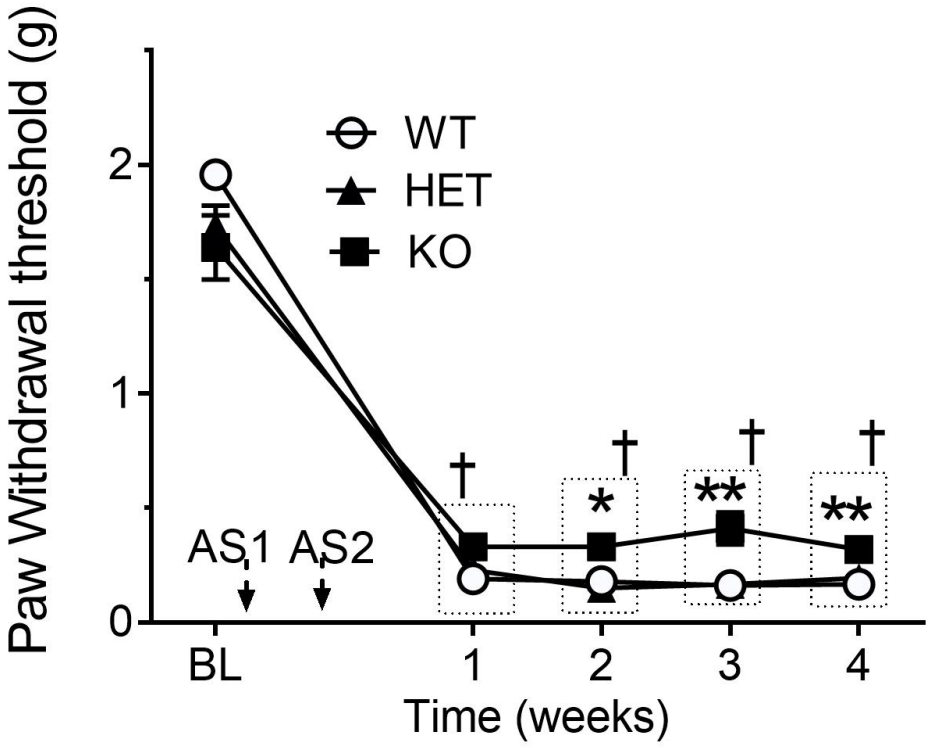

Figure 4.8. Effects of *prkcz* gene manipulation on the development of i.m. acidic saline-induced remote allodynia in female mice.

WT, HET and KO female mice exhibit allodynic PWTs starting 1 week after i.m. acidic saline. PWTs for all mice remain reduced through 4 weeks, although KO mice show a slight, but significant, increase in PWTs from HET at week 2 (*), and from both WT and HET at 3 – 4 weeks (**). Repeated measures ANOVA, Bonferroni, */**$p < .05$ from WT and HET, $^{\dagger}p < .05$ from baseline.

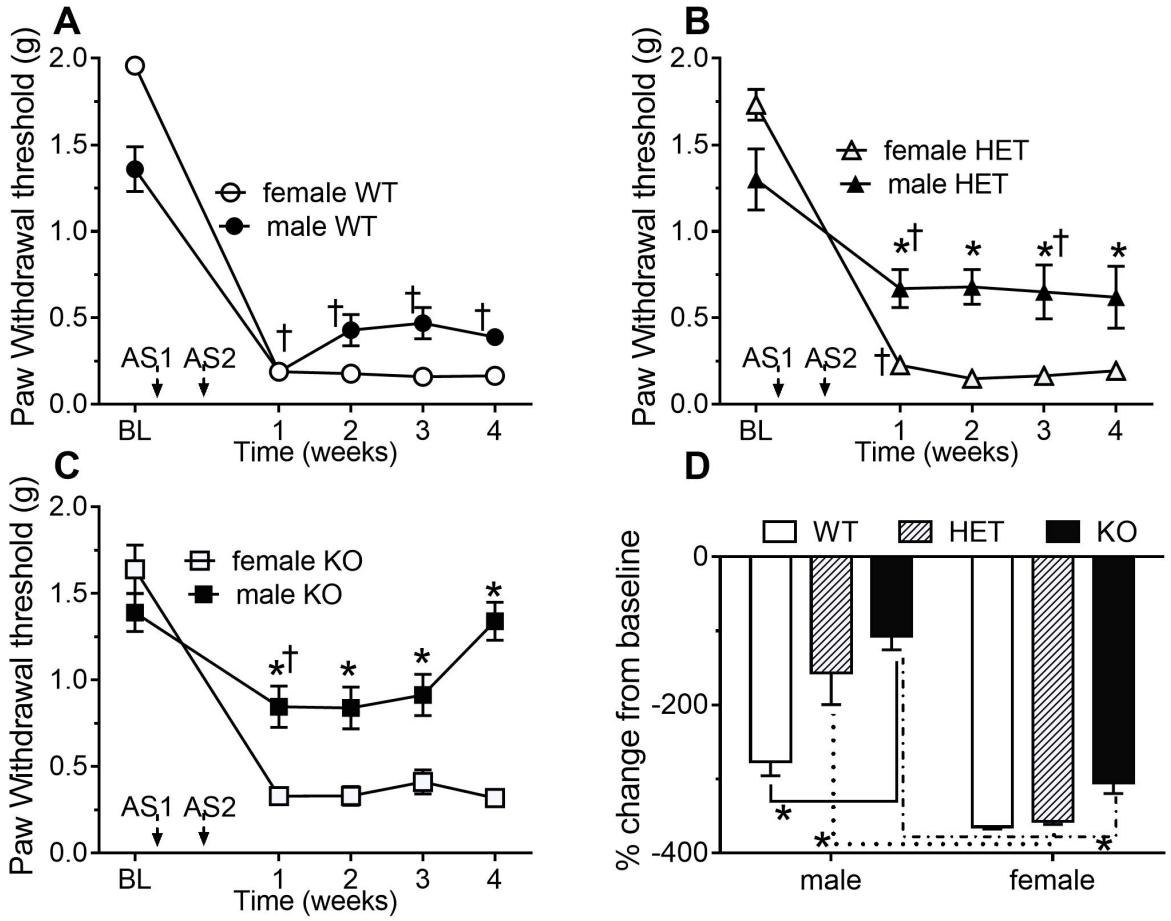

Figure 4.9. Influence of sex on the effects of *prkcz* gene manipulation on i.m. acidic saline-induced remote allodynia.

A) Both male and female WT mice display i.m. acidic saline-induced allodynia from 1 - 4 weeks. **B)** Female HET mice displayed a significant and persistent decrease in PWTs extending from 1 to 4 weeks after i.m. acidic saline. Although reduced from 1-4 weeks, the PWTs of male HET are significantly elevated compared to females throughout the 4-week testing period. **C)** Female KO mice displayed reduced PWTs from week 1 to 4, while the PWTs of male KO mice are significantly elevated compared to females from week 1-4 week, and are not different from baseline (BL) in weeks 2, 3 and 4. **D)** For female mice the percentage (%) change from baseline in PWT summed over 4 weeks does not differ between genotypes, while male KO mice display significantly smaller percent change from baseline PWTs, than WT male mice, or their female counterparts (dot-interspersed line). Similarly HET male mice exhibit smaller % change from BL PWTs than female HET (dotted line). A-C, repeated measures ANOVA; D, Two-way ANOVA; $^{*}p < .05$ from females, $^{\dagger}p < .05$ from BL value. Note female data from Figure 4.8 and male data from Figure 4.5 is repeated in A-C (AS: acidic saline).

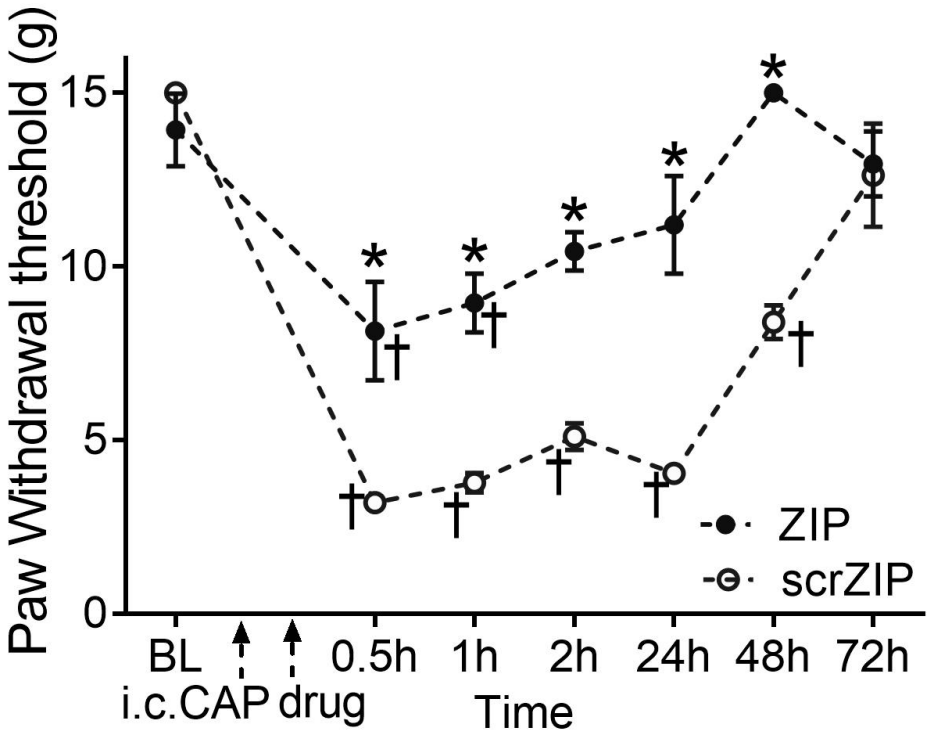

Figure 4.10. Effects of ZIP on i.c. capsaicin-induced remote allodynia in female rats.

scrZIP-treated female rats displayed a significant reduction in PWTs following i.c.CAP between 30 min and 48 h post-drug that was restored to baseline levels at 72 h. ZIP treatment 60 min after i.c. capsaicin prevented the lowering of PWTs from 30 min post-drug. *$p < .05$ from scrZIP, $^{\dagger}p < .05$ from BL value, repeated measures ANOVA, Bonferroni-adjusted

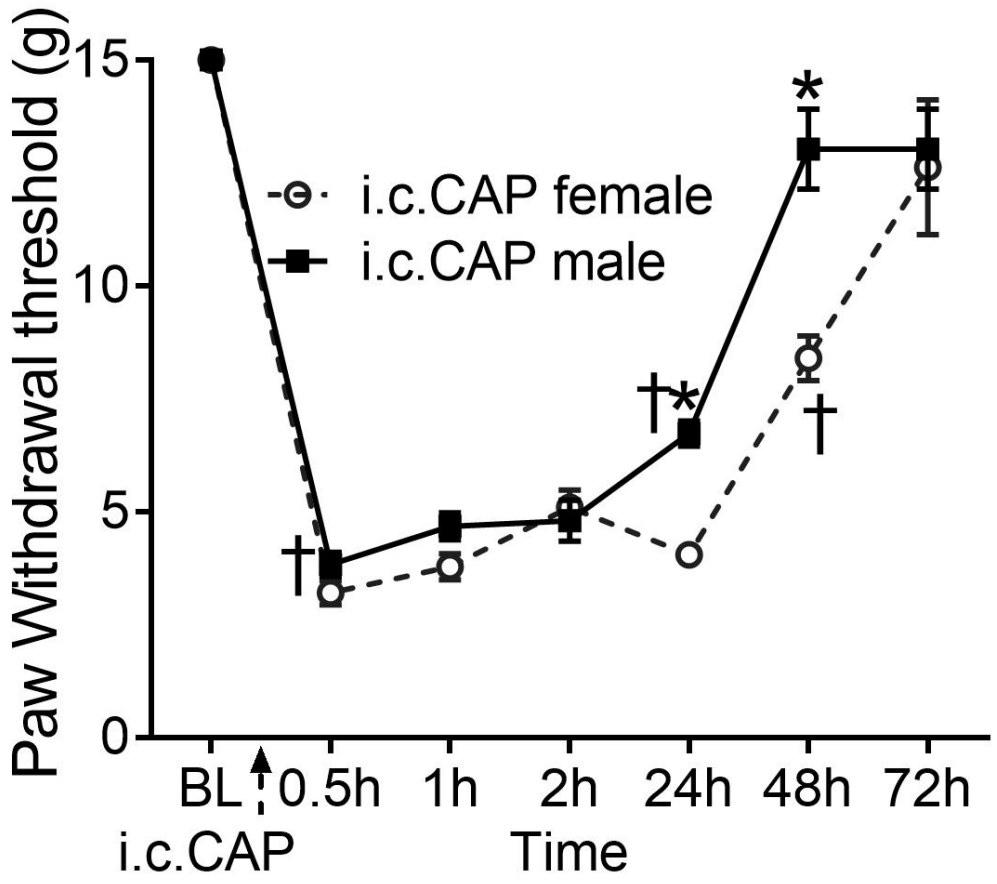

Figure 4.11. Differential effects of i.c. capsaicin in male and female rats.

Both male and female rats exhibit a significant reduction in PWTs from baseline after i.c. capsaicin. This reduction persists in female mice for at least 48 h, while male mice exhibit a gradual increase in PWTs at 24 h and a full restoration to baseline levels at 48 h, resulting in a significant increase in PWTs compared to female PWT values, $^{\dagger}p < .05$ compared to baseline, $^{*}p < .05$ compared to females; repeated measures ANOVA, Bonferroni. Note data for i.c. cap females is repeated from Figure 4.10, and for males from Figure 3.2B.

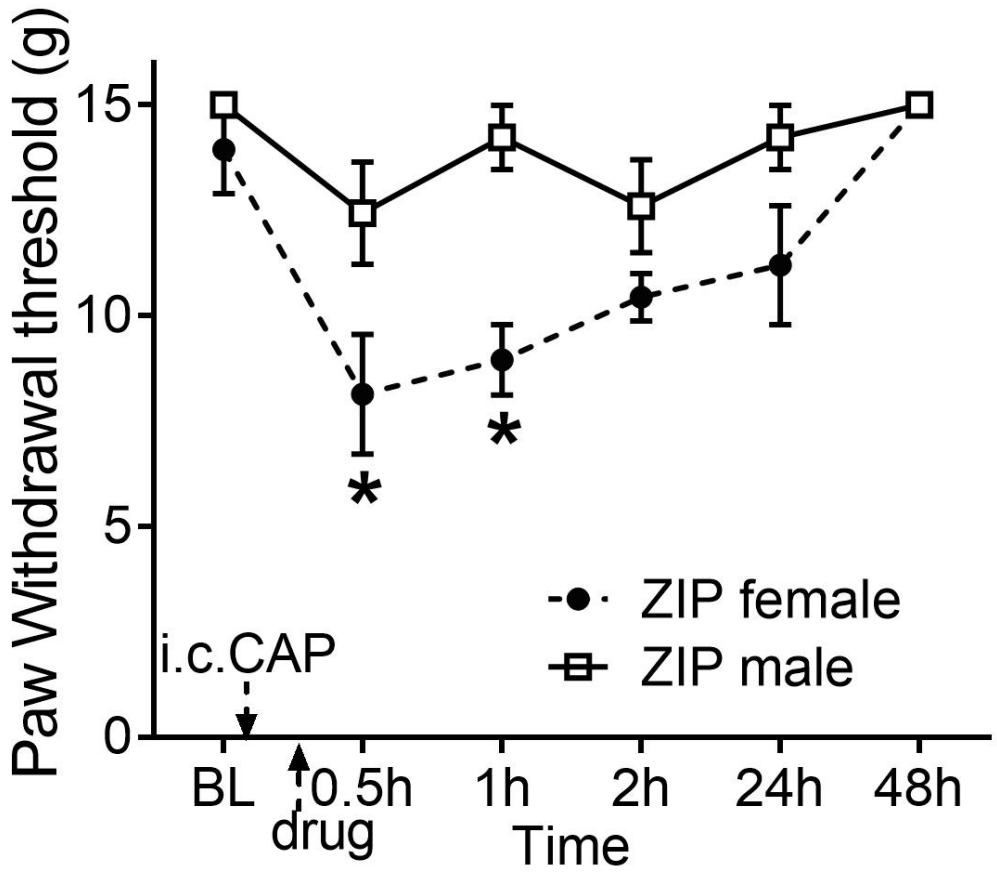

Figure 4.12. Comparison of the effects of ZIP on i.c. capsaicin-induced remote allodynia.

Although ZIP post-treatment to a single i.c. capsaicin-injection prevents the reduction in PWTs relative to baseline in male rats, its effect in female rats is less pronounced leading to significanly lower PWTs up to 1 h post-drug. *$p < .05$ compared to males, repeated measures ANOVA, Bonferroni. Note data for ZIP females is repeated from Figure 4.10, for ZIP males from Figure 3.2B.

5 Study 4

Regulation of PKMζ in the spinal cord dorsal horn

5.1 Rationale

PKMζ, a constitutively active PKC isozyme, is required for the maintenance of LTP underlying long-term memories (Ling et al., 2002, Pastalkova et al., 2006, Li et al., 2011, Sacktor, 2011) and persistent pain states (Li et al., 2010, Laferriere et al., 2011, Marchand et al., 2011, Melemedjian et al., 2013). Hippocampal studies have proposed a model of PKMζ regulation and memory maintenance (Kelly et al., 2007, Yao et al., 2008). Transcribed from the *prkcz* gene through a promoter with its own internal starting site, the PKMζ mRNA, coding only for PKCζ's catalytic domain (Hernandez et al., 2003), is translocated from the nucleus near dendrites, where it is translationally repressed in the resting state through PIN1 (Liou et al., 2011).

Pin1. PIN1 is a peptidyl-prolyl cis-trans isomeraze that catalyzes the rotation of the bond between a phosphorylated serine or threonine before proline in proteins. PIN1 was shown to suppress the translation of proteins in synaptoneurosomes, one of them being PKMζ. In the hippocampus, increases in glutamate release during LTP induction leads to activation of second messengers MAPK, ERK, CamKII, mTOR that relieve the translational block of PKMζ by PIN1, allowing its synthesis (Sacktor, 2010). Importantly, activated and phosphorylated PKMζ in turn forms a negative feedback loop further inhibiting PIN1 activity by phosphorylating its Ser^{16} site – resulting in further increase of PKMζ levels (Sacktor, 2010). 5-hydroxynaphthoquinone (juglone), a compound extracted from the dark walnut tree, irreversibly inhibits PIN1 by forming a covalent bond with a free cysteine residue on PIN1 leading to its partial unfolding (Hennig et al., 1998).

PDK1. The persistent activity of PKMζ requires activation-loop phophorylation at the T-410 site by PDK1 (Kelly et al., 2007). PDK1 is a member of the AGC protein kinase family that is responsible for activation loop phosphorylation of protein kinase C (PKC) isoforms. Only after phosphorylation by PDK1, does PKMζ attain its persistently active state. PDK1 can be inhibited specifically with the small molecule GSK-2334470 that exibits an IC50 of about 10 nM for PDK1, while leaving related AGC kinases active (Najafov et al., 2011).

NSF-GluR2 interaction. NSF is a multihomomeric ATPase that is involved in vesicle fusion, membrane fusion events and neurotransmitter release (Hanson et al., 1997, Rothman, 1994). It also interacts with GluR2 to stabilize AMPA receptors in the postsynaptic membrane, preventing their endocytosis (Nishimune et al., 1998). By regulating NSF-GluR2-dependent AMPA receptor trafficking in the hippocampus, PKMζ increases the available amount of postsynaptic AMPA receptors (Yao et al., 2008). Blocking this interaction using the small peptide pep2m, that targets the binding domain of GluR2 in the post-synaptic membrane, reduces the frequency, but not the amplitude of AMPA receptor-dependent synaptic transmission, while the corresponding control peptide pep4c has no effect (Noel et al., 1999). PKMζ activity has been proposed to maintain late-LTP by preventing the endocytosis of AMPA receptors by strengthening the receptor stabilizing NSF-GluR2 interaction (Yao et al., 2008).

All the above factors are critical to the ability of PKMζ to influence synaptic plasticity in hippocampus by influencing AMPA receptor availability. Additional studies of both memory processing and LTP reinforce the idea that PKMζ effects depend on its interaction with AMPA receptors. Indeed, a strong stimulation, such as a foot shock during memory training enhanced memory, and induced an increase of PKMζ and GluA2 in the hippocampus. In a different set of memory tasks, chronic application of GluA2 prevented the decay of memories produced by ZIP treatment, such that mice spent less time finding the hidden platform in the water maze task (Dong et al., 2015). Moreover, using the GluR2 endocytosis inhibitor GluR2/3Y to block GluR2 removal from synapses prevented memory deficits produced by PKMζ inhibiton with ZIP (Migues et al., 2010). Alternatively, inactivation of PKMζ in the amygdala prevented fear memory development, and this correlated with reduced postsynaptic GluR2 levels. In the case of LTP, *in vivo* pre- or post-treatment with ZIP, but not scrZIP, caused a reduction of high frequency stimulation-induced late-LTP, which was prevented by co-application of GluA2. Similarly, virus-based PKMζ knockdown caused decay of late-LTP, a process that again was prevented by application of GluA2 (Dong et al.,

2015). These results confirm that PKMζ maintains late-LTP EPSPs that underlie long-term memory by preventing GluA2-dependent AMPA-R endocytosis.

Overall, PKMζ regulation in hippocampal late LTP is relatively well understood – it is unclear, however, whether these regulatory players are equally important within SCDH during PKMζ-dependent long-lasting pain. In study 4, we aimed to illuminate the regulators of PKMζ activity in the SCDH. We hypothesized that similar to hippocampal regulation, PIN1 may suppress PKMζ mRNA and hypothesized that juglone will reduce PIN1 levels, while enhancing PKMζ levels and associated NMDA-induced allodynia. We also hypothesize that PDK1, as well as the NSF-GluR2 interaction are required for enhanced PKMζ activity and thus persistent sensitization, and expect that that spinal inhibition of these two mechanisms may reduce NMDA-induced allodynia.

5.2 Materials and Methods

5.2.1 Animals

All experiments were conducted using adult male Long Evans rats (200-300g, Charles River Laboratories), housed in conditions in line with the McGill Animal Care guidelines (3/cage, 12h light/dark cycle). Food and water were were available *ad libitum*.

5.2.2 Procedures

5.2.2.1 NMDA-induced allodynia

NMDA was delivered intrathecally to produce a relatively short-lasting (up to 24 h) mechanical allodynia. ZIP was injected i.t. at various time points either before or after i.t. NMDA to inhibit PKMζ, and was compared with its inactive control scrZIP, as well as with the full-length PKC inhibitor NPC15437.

5.2.2.2 PIN1 Inhibition

PIN1 was inhibited by four intraperitoneal (i.p.) injections of juglone (1 mg/kg) spaced two days apart. The last injection was given 60 min prior to spinal NMDA stimulation (Hennig et al., 1998).

5.2.2.3 PDK1 inhibition:

GSK-2334470 (1-10 µM) was i.t. injected 30 min prior to stimulation with 10 nmol i.t. NMDA to inhibit PDK1 (Najafov et al., 2011).

5.2.2.4 Inhibition of NSF-GluR2-interaction:

pep2m (10 µg) was i.t. injected 20 min prior to 10 nmol i.t. NMDA to inhibit the interaction between NSF and GluR2, and was compared with its inactive control pep4c (Noel et al., 1999).

5.2.3 Drug preparation

All drugs were injected i.t. unless otherwise indicated in a volume of 20 µl. NMDA (N-Methyl-D-aspartate, 10 nmol, Tocris) was dissolved in sterile water. ZIP (Myr-SIYRRGARRWRKL-OH, Tocris) and scrZIP (Myr-SIYRRGARRWRKL-OH, Tocris) were dissolved in sterile water in a dose of 10 nmol. NPC15437 (Sigma) was dissolved in 100 mM Tris-saline (pH 7.2), the vehicle consisted of the same solution lacking NPC15437. GSK-2334470 (1 & 10 µM) was dissolved in 5% dimethylsulfoxide (DMSO) and sterile water. Juglone (5–hydroxynaphtho-quinone) was dissolved in 5% DMSO and sterile water and injected intraperitoneally (1 mg/kg). pep2m (10 µg; KRMKVAKNAQ, Tocris) and its control pep4c (10 µg; KRMKVAKSAQ, Tocris) were dissolved in sterile water.

5.2.4 Western blot

Western blots were performed as described in study 2 with the following modifications for PIN1 detection: proteins were separated on a 15% polyacrylamide gel. Nitrocellulose membranes were incubated with primary antibody recognizing PIN1 (1:500, Abcam).

5.3 Results

5.3.1 Effects of i.t. NMDA on mechanical and thermal thresholds

5.3.1.1 Thermal hyperalgesia

As shown in Figure 5.1A, 10 nmol i.t. NMDA or i.t. vehicle did not significantly decrease PWLs from baseline over the time course spanning 30 min to 24 h post-NMDA injection. A slight decrease at 4 h in NMDA-treated rats produced a trend towards hyperalgesia when compared to vehicle-controls, as was confirmed by non-significant main effects of treatment ($F(1,10) = 4.76$, $p = .054$) and time ($F(5,50) = .968$, $p = .413$) and a non-significant treatment*time interaction ($F(5,50) = 2.074$, $p = .134$). Thus, 10 nmol i.t. NMDA did not induce significant thermal hyperalgesia in male rats.

5.3.1.2 Mechanical allodynia

Rats injected with 10 nmol i.t. NMDA exhibited a significant decrease in PWTs from 30 min to 4 h post-drug, while i.t. vehicle did not induce PWT changes (depicted in Figure 5.1B). These differences were confirmed by significant main effects of treatment ($F(1,10) = 42.407$, $p < .0001$) and time ($F(5,50) = 9.483$, $p < .0001$) and a significant treatment*time interaction ($F(5,50) = 9.98$, $p < .0001$) in our repeated measures ANOVA. Bonferroni post-hoc test revealed significantly lower PWTs for NMDA-treated rats at 30 min, 60 min, 120 min and 4 h. Thus, 10 nmol i.t. NMDA induced significant mechanical allodynia lasting for at least 4 h.

5.3.2 Effects of i.t. NMDA on PKM/Cζ protein levels and effects of ZIP on mechanical thresholds during i.t. NMDA-induced persistent pain

5.3.2.1 Effects of i.t. NMDA on spinal PKM/Cζ protein levels

We first examined whether the dose of spinal NMDA that produces persistent pain also produces changes in expression of PKMζ and PKCζ compared to vehicle treatment in rat SCDH using an antibody raised against the PKCζ C-terminal catalytic domain. Rats were injected with 10 nmol i.t. NMDA or vehicle, and spinal cords were removed 60 min post injection for western blot analysis. Compared to vehicle, PKMζ levels were highly significantly ($t(10) = 3.52$, $p = .006$), and PKCζ levels significantly ($t(10) = 2.51$, $p = .031$) increased in i.t. NMDA treated rats (Figure 5.2A/B).

5.3.2.2 Effects of ZIP on mechanical PWTs

Next, we tested whether i.t. NMDA-induced reduction of PWTs was maintained by PKMζ activity. As depicted in Figure 5.2C, 10 nmol i.t. NMDA significantly reduced PWTs in control-treated rats from 4 – 24 h post-NMDA, and compared to control, i.t. ZIP post-treatment 1 h post-NMDA significantly and persistently elevated PWTs at 4 h ($p < .0001$) and 24 h ($p = .002$), as confirmed by significant main effects of treatment ($F(1,12) = 26.685$, $p < .0001$) and time ($F(27.98, 38.58) = 27.98$, $p < .0001$), as well as a significant treatment * time interaction ($F(3.215, 38.58) = 4.385$, $p = .008$, Figure 5.2C; n = 6 in NMDA + ZIP group, n = 8 in NMDA + vehicle) in our repeated measures ANOVA.

5.3.3 Effects of PIN1 inhibition using juglone on PKM/Cζ protein levels in the SCDH

First, we assessed whether i.p. juglone effectively reduced PIN1 in the rat SCDH and whether this reduction had an effect on PKCζ and PKMζ protein levels. Rats received 4 injections of i.p. juglone spaced 2 days apart and SCDHs were collected 60 min following the last i.p.

injection. Western blots were conducted using antibodies raised against PIN1 and the C-terminal of PKCζ that recognizes both PKCζ and PKMζ. PIN1 protein levels were significantly decreased following juglone-treatment compared to control (Figure 5.3A,B) as detected by t-test ($t(7.66) = 2.768$, $p = .025$). Juglone treatment also significantly increased total PKMζ levels in western blot, causing a signficant difference in relative intensities ($t(8) = 2.7$, $p = .026$, Figure 5.3C). Importantly, no changes in full-length PKCζ levels were observed in western blots ($t(7.97) = .995$, $p = .349$, Figure 5.3D).

5.3.4 Effects of PIN1 inhibition on NMDA-induced PWT reduction

Next, we compared the effects of PIN1 inhibition on NMDA-induced mechanical allodynia. Thus, we compared four groups of rats that were pretreated i.p. with the PIN1 inhibitor juglone or a vehicle, followed by i.t. NMDA or its vehicle 60 min after the last i.p. injection. As depicted in Figure 5.3E, rats in all groups displayed a decrease in PWTs starting at 4 h post i.t. injection, with differing durations of prolonged allodynia (see below) – with the exception of the double vehicle control group for which PWTs were not reduced. Repeated measures ANOVA revealed significant main effects of treatment ($F(3,22) = 171.65$, $p < .0001$) and time ($F(5.17, 113.7) = 55.57$, $p < .0001$), and a significant treatment*time interaction ($F(15.5, 113.7) = 72.63$, $p < .0001$, Greenhouse-Geisser).

5.3.4.1 Juglone treatment

Rats treated with i.p. juglone + i.t. vehicle displayed a significant decrease in PWTs starting when first tested at 4 h after juglone/vehicle treatment ($p < .0001$), lasting for at least 4 d ($p < 0.0001$), and resolving by 8 d ($p = .540$, Figure 5.3E).

5.3.4.2 NMDA-treatment

Consistent with results shown in Figure 5.1B, rats treated with i.p vehicle and i.t. NMDA displayed a significant decrease in PWTs at 4 h ($p < .0001$), which resolved by 4 d post-NMDA ($p = .50$, Figure 5.3E).

5.3.4.3 Juglone + NMDA-treatment

Rats receiving a combination of i.p juglone and i.t. NMDA exhibited reduced PWTs by 4 h post drug, that decreased further from 24 h to 4 days, remained decreased up to 8 d, before it resolved at 12 d. Bonferroni post-hoc test revealed juglone + NMDA treatment reduced PWTs to a significantly greater extent compared to NMDA alone at 4 d ($p < .001$), and compared to juglone or NMDA alone at 8 d (both $p < .0001$, Figure 5.3E).

Thus, inhibition of PIN1 using juglone induces mechanical allodynia, and a combination of PIN1 inhibition and i.t. NMDA produces even more persistent allodynia than either NMDA or juglone alone.

5.3.5 Effects of ZIP on juglone-induced mechanical allodynia, and on enhanced mechanical allodynia in rats treated with juglone + NMDA

Next, we asked whether the decrease in PWTs following i.p juglone, and importantly, whether the enhanced and prolonged decrease in PWTs at 8 d in rats treated with juglone and NMDA was prevented by inhibiting PKMζ with ZIP. Repeated measures ANOVA performed over groups juglone-vehicle, juglone-vehicle-ZIP, juglone-NMDA, juglone-NMDA-ZIP revealed significant main effects of group ($F(5,32) = 36.42$, $p < .0001$), and time ($F(4.64, 148.4) = 89.21$, $p < .0001$), and a significant group*time interaction ($F(23.1, 148.4) = 6.845$, $p < .0001$, Greenhouse-Geisser).

5.3.5.1 Juglone-induced mechanical allodynia is ZIP-irreversible

As depicted in Figure 5.3F, PWTs following i.p. juglone + i.t. vehicle were persistently decreased from initial testing at 4 h up to 4 d after juglone/vehicle treatment (i.e. associated with juglone). ZIP was injected 60 min before the time of lowest PWTs at 4 h after i.t. vehicle. In ZIP-treated rats, PWTs remained decreased up to 4 d and returned near baseline at 8 d, similar to the group that had not received ZIP. Bonferroni post-hoc comparisons detected no significant differences between groups. Thus, juglone-induced mechanical allodynia that was not ZIP-reversible.

5.3.5.2 Enhanced and prolonged reduction in PWTs following juglone + NMDA is ZIP-reversible

Rats that had received 4 injections of i.p. juglone followed by i.t. NMDA developed the enhanced and prolonged reduction in PWTs that was significantly lower than in juglone-alone group at 8 d (discussed above). Rats injected with ZIP 60 min prior to the 8 d time point, exhibited a significant increase in PWTs compared to rats that had received juglone + NMDA only (Figure 5.3G). This was confirmed by Bonferroni post-hoc test revealing significant difference following ZIP-treatment at 8 d ($p < .0001$). Thus, the enhanced NMDA-induced allodynia in rats after inhibition of PIN1 is dependent on PKMζ.

5.3.6 Effects of PDK1 inhibtion on NMDA-induced allodynia & phosphorylation of PKMζ

5.3.6.1 GSK-2334470 dose-dependently decreases NMDA-induced allodynia.

Rats received i.t. GSK-2334470 at 1 μM or 10 μM concentration 30 min prior to i.t. NMDA. Rats treated with i.t. NMDA displayed a decrease in PWTs from 30 min to 24 h post-drug as in earlier experiments. Similarly, rats that were pretreated with 1 μM GSK displayed a significant decrease in PWTs 30 min post NMDA that persisted for at least 24 h. Importantly,

pretreatment with GSK at 10 μM concentration prevented the prolonged decrease in PWTs following NMDA (Figure 5.4A). Repeated measures ANOVA revealed significant main effects of treatment ($F(2,18) = 15.299$, $p < .0001$) and time ($F(4,72) = 18.401$, $p < .001$), but no significant treatment * time interaction ($F(8, 72) = 1.935$, $p = .008$). Bonferroni post-hoc comparisons reveal significantly higher PWTs in rats treated with 10 μM GSK+NMDA (marked by $*p < .05$ in Figure 5.4A).

5.3.6.2 Effects of PDK1 inhibition on total and pPKMζ protein levels

In hippocampal studies, PKMζ requires activation-loop phosphorylation by PDK1 for sustained activity during memory maintenance. Thus, we next asked whether the prevention of NMDA-induced allodynia after pretreatment with 10 μM GSK was associated with reduced PKMζ phosphorylation in the SCDH. Western blotting using an anti-phospho-PKCζ-T410 antibody that recognizes the activation loop phosphorylation in PKCζ and PKMζ revealed that rats treated with i.t. NMDA + 10 μM GSK exhibited significantly lower pPKMζ levels than rats pre-treated with NMDA + vehicle ($t(10) = 5.48$, $p < .0001$, Figure 5.4B,C). Total PKMζ levels were not affected by GSK pretreatment ($t(10) = 1.47$, $p = .17$; Figure 5.4B,D). Thus, the reduction of NMDA-induced allodynia by GSK is associated with a reduction of pPKMζ.

5.3.7 Effects of the disruption of the NSF-GluR2 interaction using pep2m on i.t. NMDA-induced allodynia

The interaction of NSF and GluR2 in the hippocampus is responsible for maintaining AMPA receptors at the postsynapse during LTP. This interaction is strengthened by PKMζ activity, which assists in preventing AMPA-R endocytosis. Here, we tested, whether the NSF-GluR2 interaction played a role in the maintenance of NMDA-induced allodynia. Rats were pretreated with pep2m, a peptide disrupting the NSF-GluR2 interaction, or its inactive control peptide pep4c, followed by an i.t. injection of NMDA 30 min later. As depicted in Figure 5.5, rats pretreated with

the control peptide pep4c displayed a significant decrease in PWTs from 30 min to at least 24 h post NMDA. However, rats treated with pep2m did not develop allodynia in response to i.t. NMDA, and PWTs were significantly elevated compared to rats that received NMDA + pep4c at all testing time points (Figure 5.5). Accordingly, repeated measured ANOVA revealed significant main effects of treatment ($F(1,10) = 366.01$, $p < .0001$) and time ($F(1.5,15.9) = 34.267$, $p < .0001$, Greenhouse Geisser adjusted), and a significant treatment*time interaction ($F(1.5, 15.9) = 25.16$, $p < .0001$, Greenhouse Geisser adjusted). Bonferroni post-hoc comparisons revealed significant differences ($p \leq .001$ for all time points, see asterisks in Figure 5.5). Thus, the interaction between NSF and AMPA-R's GluR2 domain is required for the expression of NMDA-induced allodynia.

5.4 Discussion

Although PKMζ regulation during hippocampal late-LTP is relatively well understood, its regulation during spinal sensitization is largely unexplored. This study illuminates important players of PKMζ regulation in the SCDH following short lasting pain induction using i.t. NMDA. Through pharmacological inhibtion, we have shown that the peptidyl-prolyl-cis-trans-isomeraze PIN1 negatively regulates PKMζ protein levels, and that phosphorylation of PKMζ by the master kinase PDK1 is required for long-lasting PKMζ-dependent pain. We also show that an interaction between NSF and GluR2 is required for the establishment of PKMζ-dependent long-lasting pain.

Central sensitization typically requires activation of NMDA-Rs (Latremoliere and Woolf, 2009). Here, we elicited allodynia through direct activation of these receptors using i.t. NMDA injection. We have shown that 10 nmol i.t. NMDA induces mechanical allodynia that is ZIP-reversible. The role of PKMζ in NMDA-induced allodynia was further confirmed by our western blot data that show a highly significant increase of total PKMζ and significant increase of PKCζ levels at 1 h following i.t.NMDA injection compared to control. Since full-length PKCs are required for the induction of central sensitization (Yashpal et al., 1995, Kawasaki et al., 2004), the

timing of cord-extraction (1 h) may have targeted the end of the induction, and the early maintenance phase, explaining why both PKCζ and PKMζ were elevated.

Evidence from hippocampal studies suggests PIN1 and PKMζ sequentially control PKMζ protein synthesis such that PIN1 represses PKMζ mRNA in the resting state, while activated PKMζ in turn further represses PIN1 activity (Westmark et al., 2010). Our data is in line with this model. We pharmacologically inhibited PIN1 using the compound juglone, and confirmed in our western blot data that PIN1 levels were significantly decreased compared to control, while total PKMζ, but not PKCζ/λ, levels were significantly increased. Rats with inhibited PIN1 that received an injection of NMDA, displayed significantly longer-lasting mechanical allodynia than rats with only PIN1 inhibition, or only i.t. NMDA injection. We further showed that this enhanced allodynia was PKMζ-dependent, as it was reversed by ZIP. Drawing from the mechanistic demonstration of PKMζ/PIN1 regulation in the production of LTP, our data suggest repressing PIN1 similarly led to enhanced PKMζ levels that in turn accounted for the enhanced allodynia. Further support for this mechanistic regulation comes from PIN1$^{-/-}$ mice that displayed normal early-, but enhanced late-LTP, as well as increased PKMζ levels compared to WT controls (Westmark et al., 2010).

While we have confirmed the inhibitory effect of juglone on PIN1, and its opposite effect on PKMζ, our behavioral data cannot fully elaborate on the mechanisms underlying the mechanical allodynia resulting from juglone treatment alone that was not ZIP-reversible. The allodynia resulting from juglone alone may be related to plasticity associated with redox cycling or reactions with glutathione, previously also attributed to juglone (Inbaraj and Chignell, 2004).

PKMζ requires activation loop phosphorylation by PDK1 to initiate its persistent activity (Kelly et al., 2007). Here, we show that pretreatment with the PDK1 inhibitor GSK-2334470 dose-dependently prevented the development of NMDA-induced allodynia. Our western blot data match the behavioral results displaying a significant reduction in phosphorylated, but not total, PKMζ

levels. Thus, a lack of PKMζ phosphorylation by PDK1 prevents the development of central-sensitization-dependent pain.

Perfusion of pep2m together with PKMζ to CA1 hippocampal slices has been shown to attenuate the enhanced AMPA-R responses obtained by administration of PKMζ, suggesting PKMζ potentiates AMPA-R responses by mediating an interaction between GluR2 and NSF (Yao et al., 2008), and i.t. pep2m between two priming stimuli prevented the elongated expression of PGE2-induced sensitization (Asiedu et al., 2011). We show here that i.t. injection of pep2m prior to i.t NMDA injection prevents the development of long-lasting allodynia. This suggests, an NSF-GluR2 interaction is required for the development of NMDA-induced allodynia, and thus matches previous reports on the importance of NSF-GluR2 interactions for long-term memories.

PKMζ maintains late-LTP by boosting AMPA-R-dependent transmission at the postsynapse (Ling et al., 2002, Ling et al., 2006). In hippocampal pyramidal cells, it does so through two known mechanisms. One mechanism, discussed above, is PKMζ ability to regulate the interaction between the fusion protein NSF with AMPA-R's GluR2 to maintain these receptors at the synapse. Two, PKMζ regulates the release of AMPA-R held at extrasynaptic stores bound to PICK1. Future experiments investigating the role of PICK1 inhibition in NMDA-induced allodynia, will help complete the picture of PKMζ-dependent maintenance. Overall, these studies have illuminated the regulation of PKMζ in the SCDH using direct activation of NMDA-Rs. We have not only confirmed the previously established roles for PIN1, PDK1 and an NSF-GluR2 interaction in the regulation of PKMζ, but have also advanced the understanding the maintenance of nociceptive central sensitization. Further studies exploring the direct effects of these players on synaptic transmission, as well as on AMPA-R trafficking and subcellular localization will help complete the first picture we have drawn.

5.5 Study 4 Figures

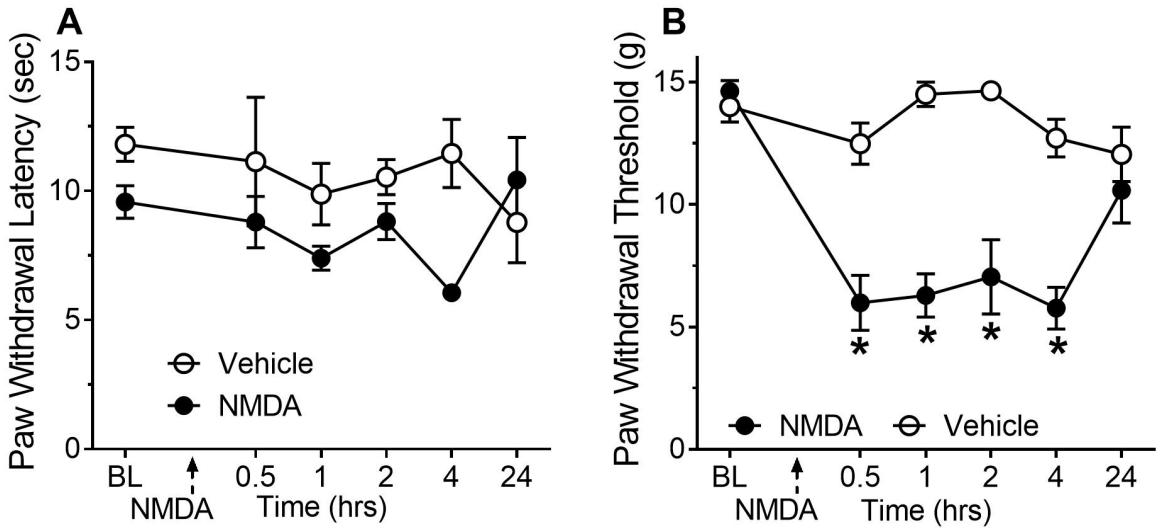

Figure 5.1. Effects of 10 nmol i.t. NMDA on thermal paw PWLs and mechanical PWTs.

A) Thermal paw-withdrawal latencies (PWLs) are not significantly affected in response to i.t. NMDA compared to vehicle. **B)** Mechanical paw-withdrawal thresholds (PWTs) are significantly decreased from 30 min to 4 h post-i.t. NMDA as compared to vehicle. * $p < .05$, Bonferroni-adjusted after repeated measures ANOVA, n = 6/group.

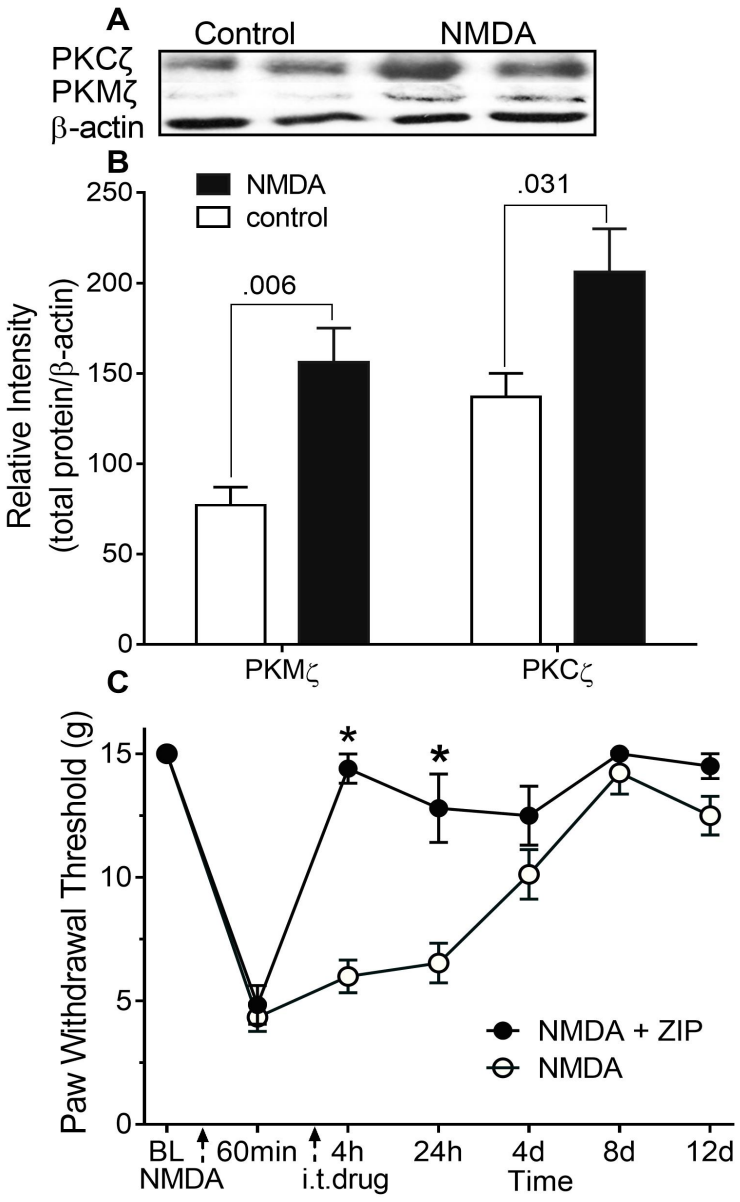

Figure 5.2. Effects of i.t. NMDA on spinal PKMζ/Cζ and effects of ZIP on NMDA-induced allodynia.

Western blots (**A**) and histograms (**B**) depicting significant increase in PKMζ and PKCζ levels following i.t. NMDA compared to control. P values following Student's t-test are shown. **C)** Rats injected with i.t. NMDA display a significant reduction in PWTs between 60 min and 24 h post-injection. Inhibition of PKMζ using ZIP significantly elevates PWTs from 4 - 24 h compared to untreated control. *$p \leq .002$, Bonferroni-adjusted, repeated measures ANOVA, n = 6/group.

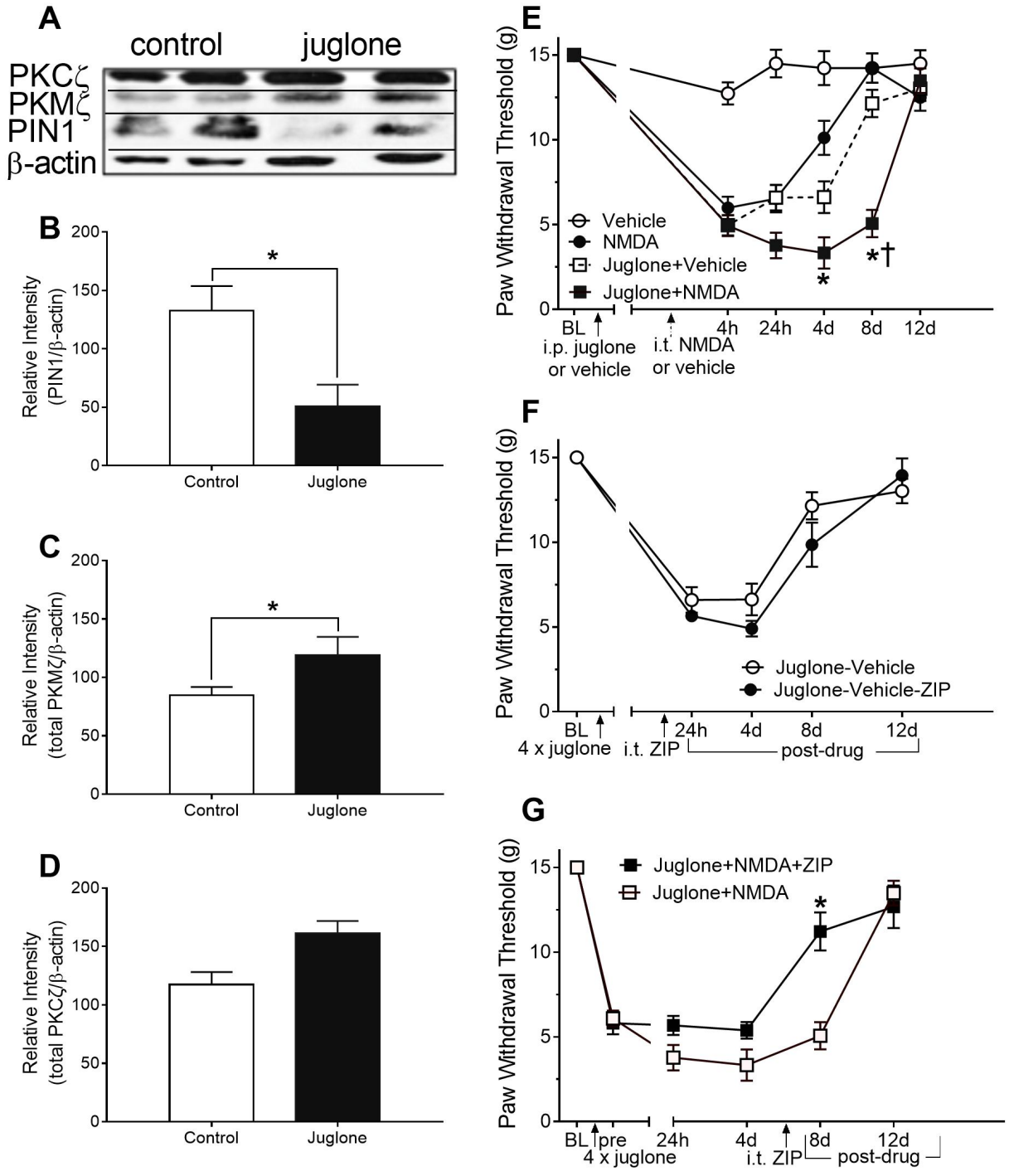

Figure 5.3. Effects of i.t. juglone on spinal PIN1, PKMζ and PKCζ protein levels and i.t. NMDA-induced allodynia.

Western blots (**A**); and histograms (**B-D**) depicting protein levels of PKCζ, PKMζ and PIN1 in juglone-treated rats compared to control. **B)** Spinal PIN1 is significantly decreased in juglone-treated rats compared to controls **C)** Total PKMζ is significantly increased in juglone-treated rats compared to controls. **D)** Total PKCζ protein levels do not differ between juglone-treated rats and controls. *$p < .05$, Student's t-test. **E)** Paw-withdrawl thresholds (PWTs) are significantly decreased for 24 h, 4 d or 8 d after treatment with NMDA, juglone or NMDA + juglone, respectively (*$p < .001$ comparison to NMDA; $^{\dagger}p < .0001$ comparison to juglone). **F)** Juglone-induced reduction in PWTs is not affected by i.t. ZIP. **G)** The prolongation of i.t. NMDA-induced allodynia by juglone is reversed by ZIP treatment, repeated measures ANOVA, Bonferroni adjusted, $p < .05$.

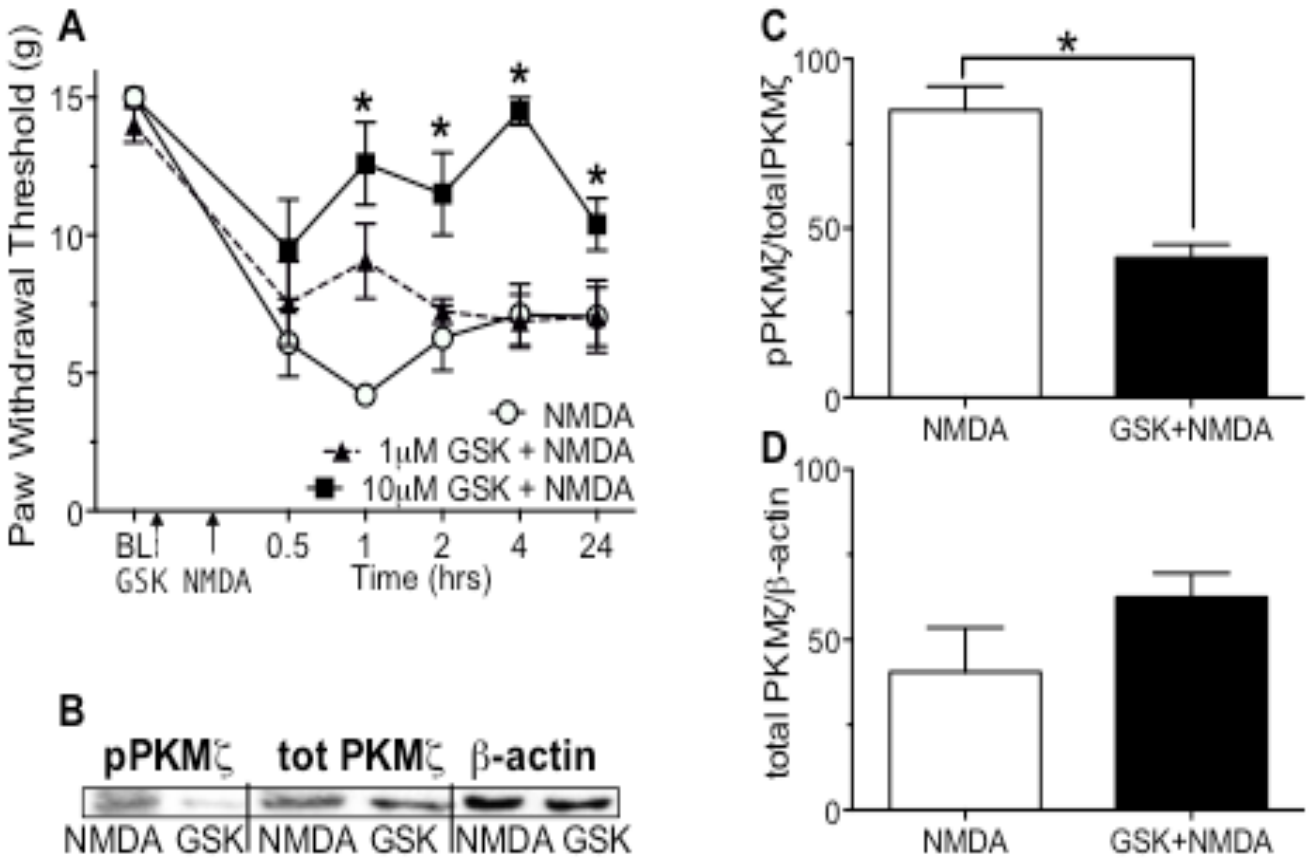

Figure 5.4. Effects of PDK1-inhibition on NMDA-induced allodynia and PKMζ phosphorylation.

A) The i.t. NMDA-induced reduction of paw-withdrawal thresholds between 0.5 and 24 h is dose-dependently prevented by GSK pretreatment, $*p \leq .018$, Bonferroni, repeated measures ANOVA. Western blot (**B**) and histograms (**C,D**) displaying a significant decrease in pPKMζ (**C**), but not total PKMζ (**D**) in rats pretreated with GSK ($*p < .0001$, Student's t-test).

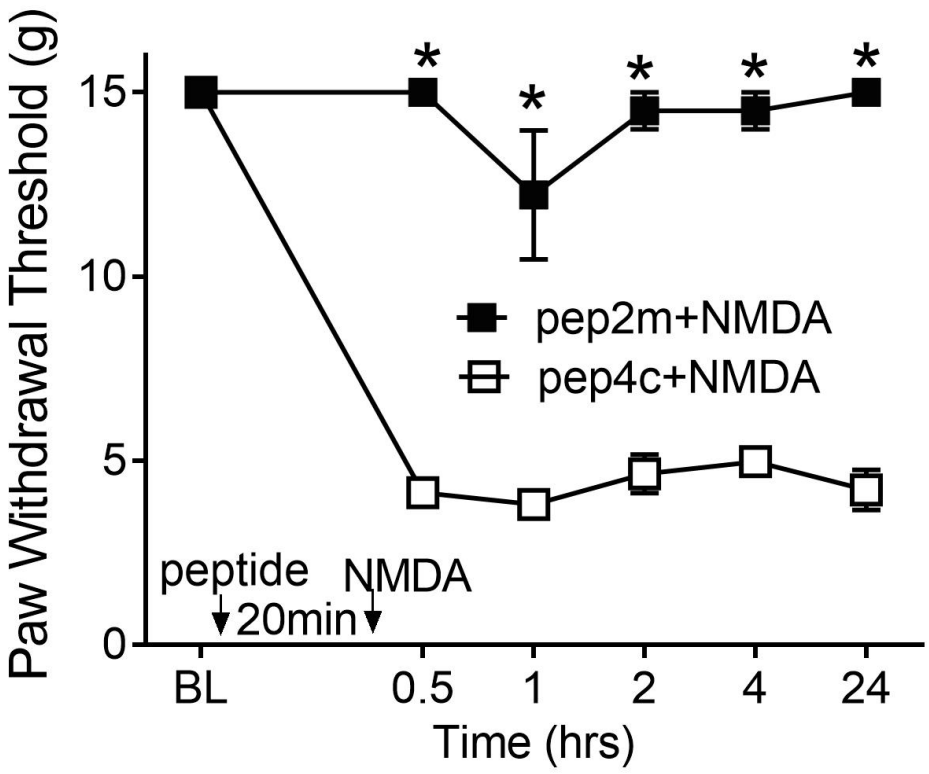

Figure 5.5. Effects of NSF-GluR2 disruption on i.t. NMDA-induced allodynia.

NMDA induces a significant reduction in paw-withdrawal thresholds (PWTs) that is unaffected by pretreatment with the control peptide pep4c. PWTs in rats pretreated with the inhibitory peptide pep2m are not significantly different from baseline, but are significantly elevated compared to those in rats treated with the control peptide (*$p < .0001$ compared to control, Bonferroni, repeated measures ANOVA).

6 General Discussion

This thesis advances our knowledge of spinal nociceptive plasticity in the maintenance of chronic pain. We provide novel evidence that PKMζ plays a pivotal role in the establishment, and importantly in the maintenance of long-lasting persistent pain. While we illuminate on the one hand highly significant analgesic effects of post-maintenance PKMζ inhibition, unseen with other protein kinase inhibitors, we on the other hand highlight an important feature -- the manner by which peripheral inputs mask the ability to demonstrate the role of spinal PKMζ in the maintenance of persistent nociception.

In studies 1 and 2, we showed the lack of anti-allodynic effects of PKMζ inhibition on ipsilateral neuropathic and inflammatory pain, but significant analgesic effects on contralateral neuropathic pain and remote pain after muscle or visceral injury. The differential elements seem to be the remoteness of the testing site to the original injury and the intensity of peripheral signalling to SCDH neurons. Thus, ipsilateral allodynia in both neuropathic and inflammatory pain models used are characterized by strong peripheral activity at the site of testing. In contrast, remote allodynia after i.m. acidic saline injury to the thigh muscle and i.c. capsaicin exhibit no peripheral input from the testing site and very much reduced peripheral inputs from the injury site, at the time of testing hours or days later. These findings suggest that high levels of peripheral input (particularly from the site of testing) mask the ability to demonstrate analgesic effects following PKMζ inhibition. Crucial support for the latter was given in our formalin experiments presented in studies 1 (rat using ZIP) and 3 (*prkcz* KO mouse). There, PKMζ inhibition reduced centrally-dependent second phase pain at low formalin, but not higher concentrations. With increasing formalin concentrations, the amount of peripheral inflammation increases, and with it, interestingly, the ability of ZIP or *prkcz* KO to reduce the nociceptive score in the formalin test decreases. It

seems thus that although ZIP reduces PKMζ's activity, its ability to reduce nociception after higher formalin concentrations is masked by the greater peripheral signaling.

Support for this phenomenon was seen when we assessed contralateral allodynia 6 weeks after SNI-surgery in rats. PKMζ inhibition in these rats transiently reversed contralateral, but not ipsilateral, allodynia. It seems that ZIP is able to alleviate contralateral allodynia, which we contend depends largely on a PKMζ –dependent central sensitization, but is unable to affect ipsilateral allodynia, which conversely depends to a greater extent on ongoing peripheral ipsilateral signaling. ZIP's transient effects on contralateral allodynia may be due to continuous activation from ipsilateral injury re-sensitizing the neurons involved in heterosynaptic facilitation, ultimately re-establishing the contralateral allodynia. This heterosynaptic facilitation process in SNI seems time sensitive and gradual, as no contralateral allodynia was detected at time points earlier than 6 weeks. Together these results highlighting the important interplay of peripheral and central components resulting in a complex pain phenotype open the avenue for exciting research on central sensitization, and the possibilities for adequate treatment combining both spinal and peripheral analgesia.

In study 3, we provided genetic confirmation of our pharamacological findings on the role of PKMζ in central nociceptive sensitization. We also further discovered PKMζ as a novel distinguishing factor between the sexes in the expression of remote pain, opening up interesting questions on whether PKMζ may be differentially expressed or differentially utilized by males and females.

In study 4, we highlighted for the first time some of the regulatory elements affecting persistent PKMζ activity in SCDH, opening avenues for continued exciting research on the upstream and downstream molecular effects of PKMζ in spinal nociceptive processing.

Thus, this thesis provides substantial advancement in the understanding of how acute pain transitions into a chronic state, and provides an interesting starting point for the development of therapies aimed at alleviating chronic pain by disrupting the maintenance of central sensitization in SCDH.

6.1 Central sensitization, LTP and persistent pain

Our studies have shown a role for PKMζ both in the induction, and importantly, in the maintenance of persistent pain. Pretreatment scenarios, inhibiting PKMζ prior to pain induction, prevented the development of long-lasting pain, similar to full-length PKC and PKA inhibition. Post-treatment reversed already established, otherwise long-lasting, remote pain (studies 1-4), a novel and remarkable phenomenon in protein kinase inhibition. Our conclusions for the role of PKMζ in these "induction" and "maintenance" phases are rooted in the known distinction of these phases in both the phenomona of central sensitization and LTP. As previously noted, the IASP defines central sensitization as the "increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input". While our experiments have not directly examined the responsiveness of neurons, (e.g. using electrophysiology), we have utilized behavioral nociception (formalin pain or mechanical allodynia after various injuries) as a read-out for this "increased responsiveness to normal or subthreshold afferent input". We measured paw licking/favoring after hind paw formalin and PWTs in response to mechanical stimulation of the paw after various tissue injuries, indicating an indirect measurement of nociceptive firing thresholds. The nature of our models where the injury occurred either in the thigh muscle or the colon indicates more clearly an involvement of spinal nociceptive plasticity with the expected expression of heterosynaptic facilitation, and the corresponding lowering of PWTs a lowering of neuronal firing threshold.

Can we conclude that a reversal of mechanical allodynia following spinal PKMζ inhibition thus mean a reversal of central sensitization? Central sensitization has been directly measured

following intradermal capsaicin (Biurrun Manresa et al., 2014), it requires NMDA-R activitation for its initiation (Coderre and Melzack, 1992, Brenner et al., 2004, D'Mello et al., 2011), and a great number of studies have linked central sensitization to long-lasting pain (see review by Woolf (2011). We have also previously demonstrated that PKMζ contributes to the maintenance of sensitization of WDR neurons following i.pl. capsaicin (Laferriere et al., 2011). While further central sensitization correlates have not been directly measured following PKMζ inhibition, there is considerable evidence for the role of PKMζ in synaptic plasticity. PKMζ is necessary and sufficient to maintain the transcription-dependent late phase of hippocampal long term potentiation (Ling et al., 2002, Pastalkova et al., 2006, Serrano et al., 2008). LTP was defined as "the rapidly induced long lasting increase in excitatory postsynaptic potentials lasting for at least 30 minutes", can occur at any given synapse in the CNS (Sandkuhler, 2010), and has been observed in spinal dorsal horn following i.pl. formalin (Ikeda et al., 2006), CFA (Yang et al., 2014), capsaicin (Ikeda et al., 2006), and heat injury (Sandkuhler and Liu, 1998). Are hippocampal and spinal LTP similar? Although a lot less is known about spinal LTP, they share core qualities (enhanced synaptic transmission, initiation and maintenance phase, NMDA involvement) (Latremoliere and Woolf, 2009, Sandkuhler, 2010), yet possibly some differential gene expression (Pedersen et al., 2010). Current research aims at unraveling the specific characteristics and effects of spinal LTP. A recent study investigated spinal LTP using low frequency stimulation – that was not ZIP reversible (Drdla-Schutting et al., 2012). Yet, it is known that spinal LTP at C-fibre synapses may have different forms and activate different types of signalling pathways (Ikeda et al., 2006, Drdla and Sandkuhler, 2008), such that for example high frequency stimulation may induce a different form of LTP (Ikeda et al., 2006). This study aids this research by adding an important starting point for the understanding of spinal LTP expression and maintenance.

Combining our knowledge of PKMζ required for late-LTP in the hippocampus, PKMζ required for centrally-dependent remote pain (studies 2 and 3), and remote pain being linked to

central sensitization, our studies further back the case for central sensitization underlying persistent, centrally-dependent pain. Future electrophysiological experiments examining the properties of neurons pre- and post- PKMζ inhibition in pain conditions of high and low peripheral drive would complete the picture. Our research allows for the speculation that PKMζ inhibition indeed may parallel a decrease of firing thresholds, decrease of receptive field size and other characteristics of central sensitization.

6.2 PKMζ in the literature – where do we stand?

Our findings support and advance the current literature – Pubmed lists 15 primary entries - on PKMζ in nociception. Our findings are concordant with the previously described notions that PKMζ inhibition in neuropathic and inflammatory pain is not anti-allodynic. The current literature reports no reversal of ipsilateral allodynia in neuropathic pain models following spinal ZIP injection. Marchand et al. (2011) and Laferrière et al. (2011) demonstrated this finding using CCI-rats, King et al. (2012) utilized SNL-rats, and we have shown this lack of analgesic effect in SNI-rats and *prkcz*$^{-/-}$ mice. Interestingly, inhibiting PKMζ relieved mechanical allodynia transiently after pSNL in the ACC (Li et al., 2010), and after SNI in the insular cortex (Han et al., 2015), suggesting pain processing and maintenance in the brain either rely more strongly on PKMζ, or are their effects are masked to a smaller degree than in the spinal cord. These differential PKMζ effects were further backed by western blot showing no change in total/pPKMζ levels following nerve injury in the SCDH, but significant increase in the ACC (Li et al., 2010). Interestingly, electrophysiological recordings demonstrating ZIP was only effective in reducing EPSPs in ACC in nerve-injured and not sham-operated mice, while it reduced both in the SCDH, without producing behavioral analgesic effects in the intact animal. It may not be surprising that ZIP can reduce these *in vitro* physiological responses, since these EPSP-measurements were taken from brain and spinal cord slices devoid of ongoing peripheral signalling (Li et al., 2010). Thus, the discordant behavioral and electrophysiological effects of ZIP are in line with our findings suggesting ZIP is unable to

overcome ongoing peripheral signalling and thus repeated activation of PKMζ in *in vivo* behavioral models.

We show here that ZIP was unable to relieve CFA-induced mechanical allodynia. This is in line with a previous report demonstrating a lack of persistent anti-allodynic effects of ZIP in CFA-treated rats – although a 30-min transient improvement was seen (Marchand et al., 2011). Further, we reproduced previous findings showing that second phase formalin pain was ZIP-reversible at 2% formalin, and extended the picture showing that higher formalin concentrations are not reversed by ZIP. Our findings seemingly stand in contrast to Marchand et al.(2011) who utilized 5% formalin and described a dose-dependent reversal of both first and second phase pain, achieving suppression of nociception with 10 μg of ZIP. However, in that study ZIP was administered as a pretreatment, and the subsequent induction of otherwise longer-lasting pain was prevented. In our experiments, post-treatment with ZIP allowed the induction and initial expression of pain, and specifically targeted the maintenance phase. Thus, we show for the first time a formalin-concentration-dependent mechanism masking the analgesic effects of spinal PKMζ inhibition. The importance of peripheral ongoing signaling on the analgesic effects of ZIP had been demonstrated previously using the CPIP model. ZIP injected during the early stage CPIP, characterized by strong peripheral pathology, did not reserve the decreased PWTs, while ZIP injected 12 days after CPIP, when the peripheral pathology had subsided, reversed the remaining mechanical allodynia (Laferrière et al., 2011).

Similarly, we reproduced previous findings on ZIP-reversibility of mechanical allodynia following i.pl. capsaicin (Laferriere et al., 2011), and extended it by use of repeated i.pl. capsaicin injections – showing ZIP prevented the development of long-lasting spinal sensitization.

Regarding the role of PKMζ in long lasting spinal sensitization through a “priming” model, we both support and importantly, advance current knowledge. The role of PKMζ in hyperalgesic

priming has been previously explored in pretreatment (prior to first pain induction) and intermediate (between first and second pain induction) treatment scenarios. Asiedu et al. (2011) described ZIP pretreatment to IL-6/PGE2 priming reduced the IL-6 induced mechanical allodynia, and the persistent sensitization following challenging stimulus PGE2. A similar effect was reported for intermediate treatment when ZIP was injected after IL-6-associated allodynia had subsided that resulted in the lack of otherwise longer-lasting PGE2-elongated allodynia. Similarly, intermediate treatment of ZIP in the BDNF/PGE2-2 priming model prevented long-lasting sensitization (Melmedjian, et al., 2013), and intriguingly, analgesic effects were also observed in ZIP pre- or intermediate treatment in a priming model combining IL-6 and plantar incision (An et al., 2015). Thus, our results of pre-and intermediate treatment with ZIP in priming models induced through either two i.c. capsaicin, or two i.pl. capsaicin injections spaced two weeks apart support previous findings that PKMζ is required for the maintenance of priming-induced spinal sensitization. Our post-treatment experiment advances our knowledge showing that already established long-lasting spinal sensitization can be reversed by i.t. ZIP. A role for the NSF-GluR2 interaction in the maintenance of spinal sensitization was first described in the attenuation of otherwise longer-lasting priming-induced nociception (Asiedu et al., 2011). Our results showing a prevention of NMDA-induced allodynia in rats pretreated with the NSF-GluR2 inhibitor pep2m are in harmony with these previous findings, suggesting spinal PKMζ-dependent sensitization is strongly associated with a retention of GluR2 at the postsynapse.

This thesis thus builds on previous knowledge and advances it with important considerations about the impact of peripheral signalling to the spinal cord masking analgesic effects of PKMζ inhibition, and the possible mechanistic regulatory elements of PKMζ-dependent spinal sensitization.

6.3 Specificity of ZIP for PKMζ

ZIP consists of the amino acid sequence present in the regulatory domain of full-length PKCζ and a myristoyl group at its N-terminal facilitating the entry into cells. Since PKMζ consists of only the catalytic domain, ZIP thus mimicks the inhibitory effects of the missing regulatory domain. In studies 1 and 2, we concluded that PKMζ is required for the maintenance of remote pain induced by i.m. acidic saline and i.c. capsaicin, while neuropathic and inflammatory pain may also be maintained by PKMζ, but the role of PKMζ is masked by additional contributions of mediators generated by ongoing peripheral input. These conclusions were drawn from pharmacological inhibition of PKMζ using ZIP that showed a clear pain relieving effect in models of remote allodynia, where ongoing peripheral pathology is less important. However, recent studies have also challenged the specificity of ZIP.

In vitro, 1 μM ZIP did not significantly reduce the normalized FRET ratio of PKMζ-RFP in COS7-cells overexpressing PKMζ, arguing for a lack of ZIP specificity or efficacy (Wu-Zhang et al., 2012). This *in vitro* discrepancy was convincingly refuted by Yao et al. (2013), who demonstrated that ZIP was an effective inhibitor of PKMζ with a Ki of 76nM, and that ZIP was effective in preventing PKMζ phosphorylation up to a concentration of at least 7 nM of PKMζ in hippocampal slices, but ineffective if PKMζ expression is artifically increased to a concentration of 28 nM, a concentration exceeding the endogenous amout of PKMζ by 30-40 fold. Thus, artifically created "spare" kinase, exceeding the amount endogenously produced, may be masking the effects of ZIP in Wu-Zhang's experiments (2012).

More surprisingly, *in vivo* studies on hippocampal LTP brought up the question whether ZIP effects may truly indicate a role of PKMζ. Volk et al. (2013) demonstrated the development of normal LTP in PKM/Cζ KO mice (lacking exon 11 of the *prkcz* gene) that was reversed by application of ZIP - an effect that should not occur in mice lacking PKMζ. Additionally, Volk et al.

(2013) performed conditional ablation of forebrain PKMζ that yielded similar normal LTP, again reversed by ZIP, and these mice performed normally on spatial and fear memory tasks. Lee et al. (2013), who used KO mice with deletion of exon 9 of the *prkcz* gene, reported similar results in spatial and fear memory performance, LTP development and again showed ZIP reduced LTP in these mice.

In part, these results have been put in perspective through one study indicating a compensatory mechanism of PKCλ in *prkcz*$^{-/-}$ mice (Yao, 2013). There, full-length PKCλ, as well as its truncated form PKMλ were significantly increased in *prkcz*$^{-/-}$ mice compared to WT, and postsynaptic perfusion of activated PKCλ elicited AMPA-mediated EPSPs similar to PKMζ postsynaptic perfusion. ZIP was reported to also inhibit PKCλ, in line with previous reports that PKCλ is essential for the expression of AMPA-mediated early-LTP (Ren et al., 2013), and PKCλ levels increase during LTP induction (Kelly et al., 2007). Importantly, Tsokas et al. (2013) demonstrated that conditional KO of forebrain PKMζ using locally restricted AAV-Cre injections reduced PKMζ following tetanization in stratum radiatum, as well as reducing late-LTP, and consolidation of short-term memory in active place avoidance in these mice. Specific block of PKMζ using antisense-oligonucleotides elicited similar results (Tsokas et al., 2013). Together these data back the case for PKMζ's requirement in late-LTP and long-term memory – although the compensatory mechanism cannot fully account for the effects of ZIP on LTP in the conditional forebrain KO reported by Volk et al. (2013).

To move beyond this debate about ZIP's specificity, we complemented our ZIP-studies with studies on *prkcz* KO mice generated by Lee et al. (2013). In contrast to the surprising hippocampal results, our behavioral results indicate that these male KO mice do not develop "normal" allodynia. This poses the question as to whether these mice do not develop "normal" spinal LTP (in contrast to hippocampal LTP), or whether the role of PKMζ in spinal nociception is fundamentally different from its role in the hippocampus. That it is indeed PKMζ, and not a similar isozyme, that is required

for the maintenance of spinal nociception, was indicated in previous studies showing a specific upreglation of PKMζ, not PKCζ/λ following i.pl. capsaicin (Laferriere et al., 2011), and formalin (Marchand et al., 2011). Although Melemedjian et al (2013) showed that a ZIP-sensitive persistent hyperalgesia induced by spinal BDNF was associated with increases in both PKMζ and PKCλ. However, in this thesis we have shown a specific reduction of pPKM, and not pPKC or total PKM/PKC 1 week following ZIP injection in i.m. acidic-saline treated rats. Morever, i.t. NMDA induced a specific increase in total PKM and not total PKCζ levels. These data indicate a clear role for PKMζ in these long-lasting pain conditions – it is not clear, however, whether this role is late-LTP dependent. To fully unravel spinal utilization of PKM/Cζ for nociception and spinal LTP, future experiments may explore the electrophysiologal underpinnings following pain induction in KO, HET and WT mice. Current research is being done exploring the effects of PKCλ KO on hippocampal LTP and spinal nociception (personal communication with Dr. Ghosh), as well as PKCλ/PKMζ double KOs. These mice will greatly aid our understanding of spinal nociception, as well as delineate the roles of PKMζ and PKCλ in persistent pain.

6.4 Future experiments

6.4.1 Delineation of mechanism masking PKMζ activity

In study 1 and 3, we showed that PKMζ inhibition was ineffective in relieving SNI-induced ipsilateral neuropathic, CFA-induced inflammatory and first phase formalin pain. Since ZIP was effective in relieving second phase formalin pain at lower concentrations, and transiently reversed contralateral allodynia following SNI, we concluded that strong peripheral inputs to the spinal cord may be overriding or masking the effect of PKMζ inhibition. In this scenario, continued peripheral inputs, as are characteristic to inflammatory and neuropathic pain, may cause a state of continued induction, not allowing for a pure maintenance state in which the allodynia stems exclusively from central neuronal sensitization. In order to complete this picture, future experiments may measure PKMζ levels during neuropathic and inflammatory pain. It would be interesting to see whether in

these models, PKMζ is upregulated, but its inhibition alone is insufficient in relieving the pain. Further, the formalin experiments could further be expanded measuring the level of local inflammation (e.g. Evans blue) in the paw, as well as activity in peripheral nociceptive fibres, following the three different concentrations of formalin we used (2%, and 3.5% where ZIP was effective in relieving second phase pain; and 5%, where ZIP was ineffective). Although local inflammation has been measured with increasing concentrations of formalin (Yashpal et al., 1996), it would be interesting to see to what extend local inflammation and associated peripheral nociceptor activity correlates with ZIP-analgesia.

Since we have discovered a potential masking mechanism overriden by continued peripheral inputs, the implicit assumption is that these continued inputs may cause a state of continued induction. It may thus be interesting to examine neuropathic and inflammatory pain models using a combination of both inhibitors of "induction" kinases, e.g. full-length PKCs, CamKII, mTOR, MAPK, together with PKMζ inhibitor ZIP that has been implied in both the induction and maintenance phase of spinal sensitization.

6.4.2 Further exploration of our remote pain models

Remote allodynia is characterized by low-peripheral input, and has been shown to depend on central sensitization. It would be interesting to see in the i.c. capsaicin model if inhibition of full-length PKCs, e.g. by pharmacological inhibition with NPC-15437, may relieve this remote pain. We hypothesize, it may be effective in a pretreatment, and in accordance to our results, less effective than ZIP in a post-treatment targeting specifically the maintenance phase. Similarly, we showed i.m. acidic saline induced long-lasting pain that was reversed by a single injection of ZIP. It would be interesting to examine the effects of ZIP post-treatment on this remote pain to understand if inhibition of PKMζ prior to remote pain induction may also prevent the development of long-lasting pain. Based on our previous results from i.c. capsaicin and consitutive KO, we hypothesize the lack of PKMζ during the induction phase would prevent the development of long-lasting pain.

6.4.3 Regulation of PKMζ and its effects: how does PKMζ maintain persistent pain?

PKMζ was shown to uphold its LTP maintenance mechanisms by increasing the number of AMPA receptors at the postsynaptic membrane (Yao et al., 2008). It remains to be shown if this mechanism holds true in central sensitization-dependent persistent pain. Measurement of AMPA receptors, i.e. GluR1 and 2 subunits in the SCDH synaptic and cytosolic fractions would give us a detailed understanding of, not only PKMζs mechanism, but possibly the interplay of the factors regulating AMPA-R trafficking known from hippocampal studies – such as the NSF-GluR2 interaction, PICK1-GluR2 interaction. Highly important in the understanding of PKMζ's maintenance of nociceptive sensitization in the SCDH would be electrophysiological exploration of SCDH neurons pre-and post-sensitization through e.g. NMDA, i.c. capsaicin, formalin and the subsequent application of ZIP. While we have described interesting behavioral results, it is unclear whether these results stem from a reversal of late-LTP. In light of recent report describing no effect of ZIP on LTP induced by low-frequency stimulation of the sciatic nerve *in vivo* (Drdla-Schutting et al., 2012), it is hard to speculate on the outcome of above suggested experiments – thereby making them even more interesting.

Further, we showed that the PIN1-PKMζ feedback loops discovered in the hippocampus, seems to be in place in the SCDH such that inhibition of PIN1 correlates with an increase of PKMζ protein levels. Since PIN1 is thought to translationally repress the PKMζ mRNA, it may be interesting to block PKMζ synthesis by application of a protein synthesis inhibitor such as cycloheximide together with PIN1 inhibitor juglone (or in $pin1^{-/-}$ mice). This type of experiment would allow for the definite conclusion that PIN1 represses the synthesis of PKMζ itself: thus, the here described enhanced PKMζ activity (as seen with prolonged allodynia and elevated PKMζ protein levels) following PIN1 inhibition should not occur in presence of a protein synthesis inhibitor.

Our behavioral results from pretreatment experiments using the inhibitor pep2m indicate that an NSF-GluR2 interaction is required for the successful expression of long-lasting pain. Hippocampal studies have shown that pep2m decreases AMPA-R-dependent potentiation similar to ZIP, and is the only inhibitor to date to mimick ZIPs reversal of late LTP (Yao et al., 2008). Thus, to expand our understanding of the maintenance of sensitization, a post-treatment experiment with pep2m would be able to uncover the role of NSF-GluRs interactions in the maintenance of an established pain state. If PKMζ contributes to persistent pain by maintaining AMPA-Rs at the postsynapse, a post-treatment inhibition of the interaction that links AMPA subunits to the membrane should elicit similar results as PKMζ inhibition itself.

6.4.4 Post-treatment by conditional KO

In our studies, we used constitutive *prkcz* KO mice and demonstrated a lack of allodynia following remote pain induction in male mice. Constitutive KO per definition only allows for modelling of a "pretreatment" scenario. It would be interesting to control temporal ablation of PKM/Cζ to elicit a "post-treatment" KO following pain induction, as well as understand the activity of PKM/Cζ in specific neuronal populations in the maintenance of persistent pain. For this purpose, a Cre-line coupled to the promoter of a general neuronal marker, such as ROSA-26-CRE-ERT(2) could be crossed with mice exhibiting floxed exon 9 (or 11) of the *prkcz* gene. Since this specific Cre-line is coupled to the estrogen receptor T2, the excision of this exon could be activated by injection of synthetic estrogen receptor antagonist tamoxifen allowing the Cre-ERT construct to enter the nucleus at a desired time, e.g. post-pain induction. The standard route of tamoxifen administration is by i.p. injection, or oral gavage (Whitfield et al., 2015). If 4-hydroxy-tamoxifen could specifically be injected into the SCDH, this set up could in theory also be used for spatially restricted ablation of PKM/Cζ. The classic spatial ablation using a locally expressed promoter for specific ablation of SCDH PKM/Cζ does not seem feasible with current too ubiquitously expressed Cre-lines (e.g. PKCγ-CRE, Tac1/2-Cre that are also expressed in brain) and the lack of a SCDH-

neuron-specific Cre-line. However, we could achieve spatial ablation through specific injection of AAV-Cre (Ahmed et al., 2004) in the SCDH, allowing Cre to enter only spinal cord neurons – without affecting brain PKMζ. We would thus be able to understand the role of neuronal PKMζ in the maintenance of pain in an adult mouse – free of confounding compensatory mechanisms.

6.4.5 Exploration of PKMζ expression

Current commercial and research antibodies pick up at least both PKCζ and PKMζ, and although these can be separated on western blots, no detailed histological expression analysis of PKMζ in the spinal cord has been performed to date since the antibodies do not differentiate the separate isoforms. Although attempts at understanding PKMζ localisation in the spinal cord (An et al., 2015, Marchand et al., 2011), these reports are limited by specificity of the antibody. From hippocampal studies, where PKCζ is not expressed, we have gained an understanding of PKMζ localization specifically to dendrites and spines (Hernandez et al., 2014). It would be highly interesting to understand PKMζ localization and expression pattern change in a chronic pain maintenance state. Is PKMζ preferentially localized to dendrites, spines and postsynaptic density, does it parallel observations of structural plasticity in a chronic pain state? Antisense oligonucleotides for specific PKMζ KO have been utilized (Tsokas et al., 2013) – currently nucleotide labelling techniques are commercially available. Coupled with live cell imaging (Xue et al., 2015), this technology promises advances in the understanding of PKMζ and it's role of nociceptive plasticity.

6.5 Clinical Relevance

Our studies highlight a role for PKMζ in the maintenance of long-lasting remote allodynia. So-called functional pain conditions (often involving muscle (e.g., fibromyalgia) or visceral tissues (e.g., irritable bowel syndrome)) are those that are normally not easily be treated, because no lasting peripheral tissue damage can be found. For these conditions patients are often prescribed nonspecific strong analgesics, or alternatively patients are told that their pain is not real (Cervero,

2014). Central sensitization has been recognized as one explanation for otherwise unexplainable long-lasting pain conditions (Woolf, 2011). Preemptive analgesia to prevent or minimize post-operative pain is already widely performed as a consequence of our understanding of central sensitization (Woolf, 2011). This thesis elucidates a protein kinase that if inhibited, reverses this persistent pain. Tremendous implications for the treatment of above mentioned pain conditions arise: would it be possible to eliminate chronic pain in patients that suffer without any discernible tissue injury. Further, experimental central sensitization has been described in humans (Magerl et al., 1998), this thesis gives a promising description of what could be possible in pain treatment in the future.

6.6 Contributions to original knowledge

While PKMζ has been well studied in memory pathways, the current literature on spinal PKMζ is still its infancy. This thesis expands our knowledge about the role of this kinase in spinal nociception.

Study 1. This study has extended our previous knowledge on the contribution of PKMζ in the maintenance of neuropathic, inflammatory pain, and highlighted the important concept of peripheral activity masking central sensitization and its maintenance in SCDH.

Study 2. This study provides the first description of pain reversal following the induction of remote allodynia. It further expands our knowledge on the maintenance of i.m. acidic saline and i.c. capsaicin-induced remote allodynia. This study further provides the first report of the reversal of priming-induced allodynia following two injections of the same pain stimulus spaced weeks apart. Last, it provides the first description of the establishment of long-lasting priming-induced pain using a remote pain induction protocol.

Study 3. This work presents the first report on the role of *prkcz* KO in spinal nociception, and highlights for the first time a PKMζ-dependent sex difference in the expression of remote pain.

Although explored in hippocampal study, no information about the effects of this deletion on spinal nociception was previously reported.

Study 4. This study advances the first comprehensive insights of PKMζ regulation in SCDH. We show that NMDA-induced allodynia requires PKMζ activity, and is correlated with increased PKMζ protein levels. We further showed a role for PIN1 in regulating PKMζ protein levels, and its inhibition reduces the expression of long-lasting persistent pain. Further, we demonstrate a requirement of PDK1 for the initiation of PKMζ-dependent NMDA-induced allodynia, and that PDK1 inhibition results in a reduction of PKMζ phosphorylation. Last, we demonstrated a requirement for an interaction of NSF and GluR2 in the expression of NMDA-induced allodynia, showing that a specific inhibitor of this interaction reduces the allodynia.

7 Final conclusion and summary

The studies presented in this thesis investigated the role of the structurally unique protein kinase Mζ in the maintenance of spinal nociception. Through pharmacological inhibition of PKMζ in rats and constitive PKM/Cζ knockout mice, our studies elucidated a requirement for protein kinase Mζ in the maintenance of remote pain induced through i.m. acidic saline or i.c. capsaicin. We further highlighed a role for PKMζ in models characterized by increased peripheral pathology (neuropathic, inflammatory, chemical pain) – with the analgesic effects of its inhibition likely masked by ongoing peripheral activity. Our studies with *prkcz* KO mice supported our findings observed using pharmacological inhibition of PKMζ in rats, as well as demonstrating an important role of sex differences in the contribution of PKMζ to persistent pain – with PKMζ playing a greater role in nociceptive sensitization in males than females. Finally, we provided evidence for the regulation of spinal PKMζ by PIN1, PDK1 and an interaction between NSF and GluR2, in rats exhibiting nociception induced by spinal NMDA injection. We provide a first comprehensive analysis of PKMζ in these pain models and expect future studies may elucidate the molecular

underpinnings of PKMζ-dependent maintenance of spinal nociception and establish its role in chronic pain.

8 Appendix

8.1.1 Table

Primers used to test for exon 9 KO of *prkcz*.

Allele	Oligonucleotide sequence	Base pairs
Prkcz exon 9	SG2-F: AACAGGCCATGCTCCCAAG JD2708-R: TCCTGCCTCAGCCAGAAAACAAACCACACGG	KO: 3kb WT: 2kb HET: 3&2kbs

8.1.2 Figures

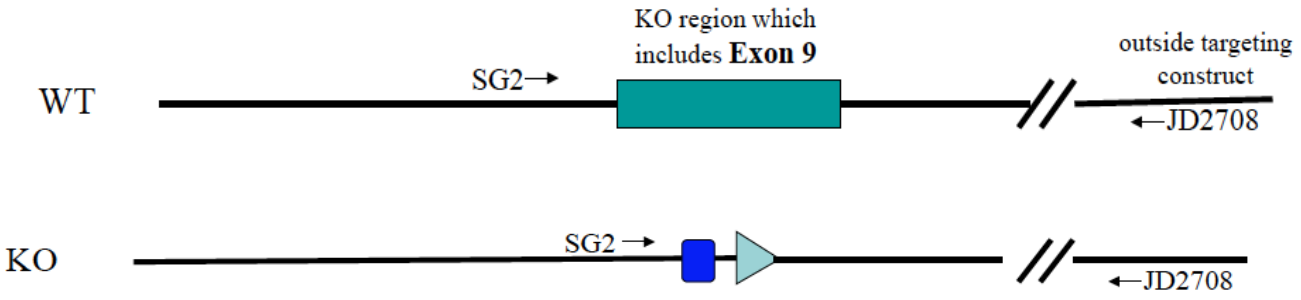

Figure 8.1. Schematic presentation of PCR strategy.

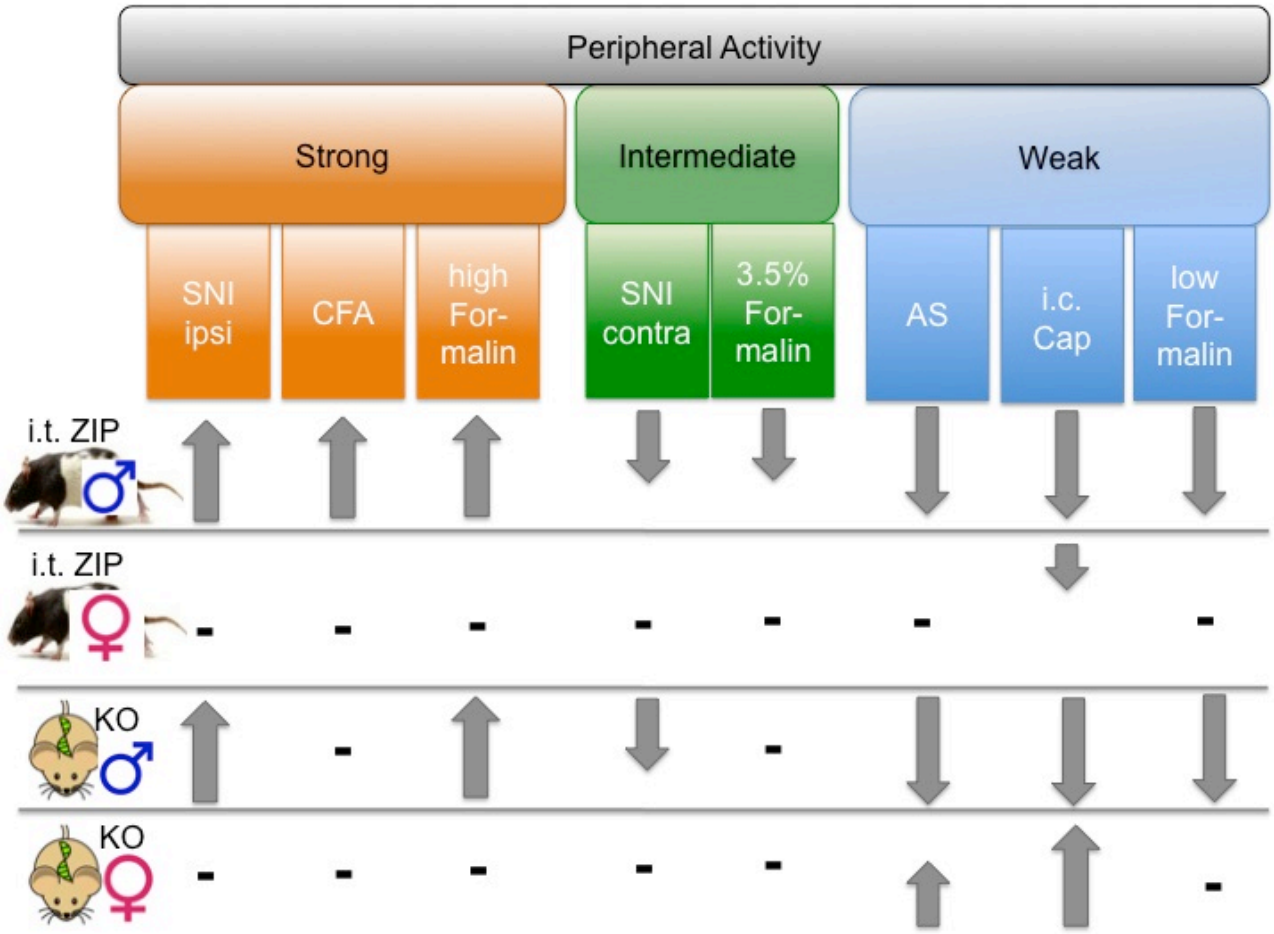

Figure 8.2. Schematic summary diagram of behavioural data.

I.t. ZIP was unable to reverse ipsilateral SNI-, CFA-induced allodynia, and high formalin concentration (5% rat, 2% mouse) pain, while weak and transient analgesic effects were observed in models of intermediate peripheral activity (contralateral SNI, 3.5% formalin), and strong analgesic effects were observed in remote pain models (AS, i.c. Cap), and low formalin concentration (2% rat, 1% mouse) in male rats. Similar analgesic effects were observed in male prkcz $^{-/-}$ mice (KO). ZIP post-treatment was analgesic in female rats, however, to less extend than in males, and female KO mice developed greater remote allodynia. Upward –facing arrows indicate display of continued pain behaviour, downward facing arrows a reduction in allodynia following ZIP, or in KO mice. SNI, spared nerve injury; CFA, Complete Freund's Adjuvant; i.m. AS, intramuscular acidic saline; i.c. Cap, intracolonic capsaicin, KO, knockout.

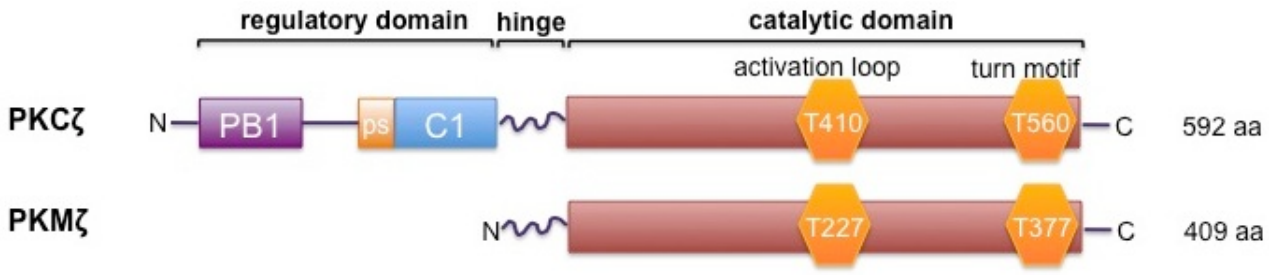

Figure 8.3. Schematic representation of PKCζ and PKMζ protein structures.

While PKMζ consists only of a catalytic domain, PKCζ features both regulatory and catalytic domains. PKMζ and PKCζ share an identical C-terminus, as well as the final 1864bp that include part of the coding sequence. While PKMζ only requires activation loop phosphorylation for persistent activity, PKCζ requires activation loop phosphorylation at threonione residue T410, as well as subsequent turn motif auto-phosphorylation. The PKCζ regulatory domain consists of a PB1 domain (Phox and Bem1) responsible for protein-protein interaction, as well as a pseudosubstrate (ps) and C1 domain that together are responsible for autoinhibition through interaction with the catalytic domain (Hartsink-Segers et al., 2015).

9 References

Abbadie C, Taylor BK, Peterson MA, Basbaum AI (1997) Differential contribution of the two phases of the formalin test to the pattern of c-fos expression in the rat spinal cord: studies with remifentanil and lidocaine. Pain 69:101-110.

Afrah AW, Fiska A, Gjerstad J, Gustafsson H, Tjolsen A, Olgart L, Stiller CO, Hole K, Brodin E (2002) Spinal substance P release in vivo during the induction of long-term potentiation in dorsal horn neurons. Pain 96:49-55.

Agarwal N, Offermanns S, Kuner R (2004) Conditional gene deletion in primary nociceptive neurons of trigeminal ganglia and dorsal root ganglia. Genesis 38:122-129.

Ahmed BY, Chakravarthy S, Eggers R, Hermens WT, Zhang JY, Niclou SP, Levelt C, Sablitzky F, Anderson PN, Lieberman AR, Verhaagen J (2004) Efficient delivery of Cre-recombinase to neurons in vivo and stable transduction of neurons using adeno-associated and lentiviral vectors. BMC neuroscience 5:4.

Ali Z, Meyer RA, Campbell JN (1996) Secondary hyperalgesia to mechanical but not heat stimuli following a capsaicin injection in hairy skin. Pain 68:401-411.

An K, Zhen C, Liu ZH, Zhao Q, Liu HP, Zhong XL, Huang WQ (2015) Spinal protein kinase Mzeta contributes to the maintenance of peripheral inflammation-primed persistent nociceptive sensitization after plantar incision. Eur J Pain 19:39-47.

Arendt-Nielsen L, Svensson P (2001) Referred muscle pain: basic and clinical findings. The Clinical journal of pain 17:11-19.

Asiedu MN, Tillu DV, Melemedjian OK, Shy A, Sanoja R, Bodell B, Ghosh S, Porreca F, Price TJ (2011) Spinal protein kinase M zeta underlies the maintenance mechanism of persistent nociceptive sensitization. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:6646-6653.

Astur RS, Ortiz ML, Sutherland RJ (1998) A characterization of performance by men and women in a virtual Morris water task: a large and reliable sex difference. Behavioural brain research 93:185-190.

Bajaj P, Bajaj P, Graven-Nielsen T, Arendt-Nielsen L (2001) Osteoarthritis and its association with muscle hyperalgesia: an experimental controlled study. Pain 93:107-114.

Bajaj P, Bajaj P, Madsen H, Arendt-Nielsen L (2003) Endometriosis is associated with central sensitization: a psychophysical controlled study. The journal of pain : official journal of the American Pain Society 4:372-380.

Banic B, Petersen-Felix S, Andersen OK, Radanov BP, Villiger PM, Arendt-Nielsen L, Curatolo M (2004) Evidence for spinal cord hypersensitivity in chronic pain after whiplash injury and in fibromyalgia. Pain 107:7-15.

Baumann TK, Simone DA, Shain CN, LaMotte RH (1991) Neurogenic hyperalgesia: the search for the primary cutaneous afferent fibers that contribute to capsaicin-induced pain and hyperalgesia. Journal of neurophysiology 66:212-227.

Beattie MS, Ferguson AR, Bresnahan JC (2010) AMPA-receptor trafficking and injury-induced cell death. The European journal of neuroscience 32:290-297.

Berridge MJ (1998) Neuronal calcium signaling. Neuron 21:13-26.

Bessou P, Perl ER (1969) Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. Journal of neurophysiology 32:1025-1043.

Bird GC, Han JS, Fu Y, Adwanikar H, Willis WD, Neugebauer V (2006) Pain-related synaptic plasticity in spinal dorsal horn neurons: role of CGRP. Molecular pain 2:31.

Biurrun Manresa JA, Finnerup NS, Johannesen IL, Biering-Sorensen F, Jensen TS, Arendt-Nielsen L, Andersen OK (2014) Central sensitization in spinal cord injured humans assessed by reflex receptive fields. Clin Neurophysiol 125:352-362.

Bliss TV, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. The Journal of physiology 232:331-356.

Boada MD, Gutierrez S, Aschenbrenner CA, Houle TT, Hayashida K, Ririe DG, Eisenach JC (2015) Nerve injury induces a new profile of tactile and mechanical nociceptor input from undamaged peripheral afferents. Journal of neurophysiology 113:100-109.
Brenner GJ, Ji RR, Shaffer S, Woolf CJ (2004) Peripheral noxious stimulation induces phosphorylation of the NMDA receptor NR1 subunit at the PKC-dependent site, serine-896, in spinal cord dorsal horn neurons. The European journal of neuroscience 20:375-384.
Bu F, Tian H, Gong S, Zhu Q, Xu GY, Tao J, Jiang X (2015) Phosphorylation of NR2B NMDA subunits by protein kinase C in arcuate nucleus contributes to inflammatory pain in rats. Sci Rep 5:15945.
Bullitt E (1990) Expression of c-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. The Journal of comparative neurology 296:517-530.
Burton AW, Lee DH, Saab C, Chung JM (1999) Preemptive intrathecal ketamine injection produces a long-lasting decrease in neuropathic pain behaviors in a rat model. Regional anesthesia and pain medicine 24:208-213.
Cahill LS, Laliberte CL, Liu XJ, Bishop J, Nieman BJ, Mogil JS, Sorge RE, Jones CD, Salter MW, Henkelman RM (2014) Quantifying blood-spinal cord barrier permeability after peripheral nerve injury in the living mouse. Molecular pain 10:60.
Campbell JN, Meyer RA (2006) Mechanisms of neuropathic pain. Neuron 52:77-92.
Campbell JN, Raja SN, Meyer RA, Mackinnon SE (1988) Myelinated afferents signal the hyperalgesia associated with nerve injury. Pain 32:89-94.
Cao H, Ren WH, Zhu MY, Zhao ZQ, Zhang YQ (2012) Activation of glycine site and GluN2B subunit of NMDA receptors is necessary for ERK/CREB signaling cascade in rostral anterior cingulate cortex in rats: implications for affective pain. Neuroscience bulletin 28:77-87.
Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816-824.
Cervero F, Gilbert R, Hammond RG, Tanner J (1993) Development of secondary hyperalgesia following non-painful thermal stimulation of the skin: a psychophysical study in man. Pain 54:181-189.
Cervero F, Laird JM (1996) Mechanisms of touch-evoked pain (allodynia): a new model. Pain 68:13-23.
Cesare P, Dekker LV, Sardini A, Parker PJ, McNaughton PA (1999) Specific involvement of PKC-epsilon in sensitization of the neuronal response to painful heat. Neuron 23:617-624.
Chang L, Heitkemper MM (2002) Gender differences in irritable bowel syndrome. Gastroenterology 123:1686-1701.
Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. Journal of neuroscience methods 53:55-63.
Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN (1995) A P2X purinoceptor expressed by a subset of sensory neurons. Nature 377:428-431.
Chen L, Huang LY (1992) Protein kinase C reduces Mg2+ block of NMDA-receptor channels as a mechanism of modulation. Nature 356:521-523.
Coderre TJ (1992) Contribution of protein kinase C to central sensitization and persistent pain following tissue injury. Neuroscience letters 140:181-184.
Coderre TJ, Fundytus ME, McKenna JE, Dalal S, Melzack R (1993a) The formalin test: a validation of the weighted-scores method of behavioural pain rating. Pain 54:43-50.
Coderre TJ, Katz J (1997) Peripheral and central hyperexcitability: differential signs and symptoms in persistent pain. The Behavioral and brain sciences 20:404-419; discussion 435-513.
Coderre TJ, Katz J, Vaccarino AL, Melzack R (1993b) Contribution of central neuroplasticity to pathological pain: review of clinical and experimental evidence. Pain 52:259-285.
Coderre TJ, Kumar N, Lefebvre CD, Yu JS (2005) Evidence that gabapentin reduces neuropathic pain by inhibiting the spinal release of glutamate. Journal of neurochemistry 94:1131-1139.
Coderre TJ, Melzack R (1985) Increased pain sensitivity following heat injury involves a central mechanism. Behavioural brain research 15:259-262.
Coderre TJ, Melzack R (1991) Central neural mediators of secondary hyperalgesia following heat injury in rats: neuropeptides and excitatory amino acids. Neuroscience letters 131:71-74.
Coderre TJ, Melzack R (1992) The role of NMDA receptor-operated calcium channels in persistent nociception after formalin-induced tissue injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 12:3671-3675.

Coderre TJ, Vaccarino AL, Melzack R (1990) Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain research 535:155-158.
Cook AJ, Woolf CJ, Wall PD, McMahon SB (1987) Dynamic receptive field plasticity in rat spinal cord dorsal horn following C-primary afferent input. Nature 325:151-153.
Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, Karbani G, Jafri H, Mannan J, Raashid Y, Al-Gazali L, Hamamy H, Valente EM, Gorman S, Williams R, McHale DP, Wood JN, Gribble FM, Woods CG (2006) An SCN9A channelopathy causes congenital inability to experience pain. Nature 444:894-898.
D'Mello R, Marchand F, Pezet S, McMahon SB, Dickenson AH (2011) Perturbing PSD-95 interactions with NR2B-subtype receptors attenuates spinal nociceptive plasticity and neuropathic pain. Molecular therapy : the journal of the American Society of Gene Therapy 19:1780-1792.
Da Silva LF, Desantana JM, Sluka KA (2010) Activation of NMDA receptors in the brainstem, rostral ventromedial medulla, and nucleus reticularis gigantocellularis mediates mechanical hyperalgesia produced by repeated intramuscular injections of acidic saline in rats. The journal of pain : official journal of the American Pain Society 11:378-387.
Dachtler J, Fox KD, Good MA (2011) Gender specific requirement of GluR1 receptors in contextual conditioning but not spatial learning. Neurobiology of learning and memory 96:461-467.
Damas J, Liegeois JF (1999) The inflammatory reaction induced by formalin in the rat paw. Naunyn-Schmiedeberg's archives of pharmacology 359:220-227.
Decosterd I, Woolf CJ (2000) Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 87:149-158.
Diniz DM, de Souza AH, Pereira EM, da Silva JF, Rigo FK, Romano-Silva MA, Binda N, Castro CJ, Jr., Cordeiro MN, Ferreira J, Gomez MV (2014) Effects of the calcium channel blockers Phalpha1beta and omega-conotoxin MVIIA on capsaicin and acetic acid-induced visceral nociception in mice. Pharmacology, biochemistry, and behavior 126:97-102.
Dixon WJ (1991) Staircase bioassay: the up-and-down method. Neuroscience and biobehavioral reviews 15:47-50.
Dong H, Zhang P, Song I, Petralia RS, Liao D, Huganir RL (1999) Characterization of the glutamate receptor-interacting proteins GRIP1 and GRIP2. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:6930-6941.
Dong Z, Han H, Li H, Bai Y, Wang W, Tu M, Peng Y, Zhou L, He W, Wu X, Tan T, Liu M, Wu X, Zhou W, Jin W, Zhang S, Sacktor TC, Li T, Song W, Wang YT (2015) Long-term potentiation decay and memory loss are mediated by AMPAR endocytosis. The Journal of clinical investigation 125:234-247.
Dougherty PM, Palecek J, Paleckova V, Willis WD (1994) Neurokinin 1 and 2 antagonists attenuate the responses and NK1 antagonists prevent the sensitization of primate spinothalamic tract neurons after intradermal capsaicin. Journal of neurophysiology 72:1464-1475.
Drdla R, Sandkuhler J (2008) Long-term potentiation at C-fibre synapses by low-level presynaptic activity in vivo. Molecular pain 4:18.
Drdla-Schutting R, Benrath J, Wunderbaldinger G, Sandkuhler J (2012) Erasure of a spinal memory trace of pain by a brief, high-dose opioid administration. Science 335:235-238.
Dubin AE, Patapoutian A (2010) Nociceptors: the sensors of the pain pathway. The Journal of clinical investigation 120:3760-3772.
Dubuisson D, Dennis SG (1977) The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 4:161-174.
Duric V, McCarson KE (2007) Neurokinin-1 (NK-1) receptor and brain-derived neurotrophic factor (BDNF) gene expression is differentially modulated in the rat spinal dorsal horn and hippocampus during inflammatory pain. Molecular pain 3:32.
Eisenberg E, Vos BP, Strassman AM (1993) The NMDA antagonist Memantine blocks pain behavior in a rat model of formalin-induced facial pain. Pain 54:301-307.
Erichsen HK, Blackburn-Munro G (2002) Pharmacological characterisation of the spared nerve injury model of neuropathic pain. Pain 98:151-161.
Esteban JA, Shi SH, Wilson C, Nuriya M, Huganir RL, Malinow R (2003) PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. Nature neuroscience 6:136-143.

Fang L, Wu J, Lin Q, Willis WD (2002) Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:4196-4204.
Fillingim RB, Ness TJ (2000) Sex-related hormonal influences on pain and analgesic responses. Neuroscience and biobehavioral reviews 24:485-501.
Fisher K, Coderre TJ (1996) The contribution of metabotropic glutamate receptors (mGluRs) to formalin-induced nociception. Pain 68:255-263.
Fisher K, Coderre TJ (1998) Hyperalgesia and allodynia induced by intrathecal (RS)-dihydroxyphenylglycine in rats. Neuroreport 9:1169-1172.
Fitzgerald M, Lynn B (1977) The sensitization of high threshold mechanoreceptors with myelinated axons by repeated heating. The Journal of physiology 265:549-563.
Frey Law LA, Sluka KA, McMullen T, Lee J, Arendt-Nielsen L, Graven-Nielsen T (2008) Acidic buffer induced muscle pain evokes referred pain and mechanical hyperalgesia in humans. Pain 140:254-264.
Fundytus ME, Fisher K, Dray A, Henry JL, Coderre TJ (1998) In vivo antinociceptive activity of anti-rat mGluR1 and mGluR5 antibodies in rats. Neuroreport 9:731-735.
Fundytus ME, Yashpal K, Chabot JG, Osborne MG, Lefebvre CD, Dray A, Henry JL, Coderre TJ (2001) Knockdown of spinal metabotropic glutamate receptor 1 (mGluR(1)) alleviates pain and restores opioid efficacy after nerve injury in rats. British journal of pharmacology 132:354-367.
Galan A, Cervero F, Laird JM (2003) Extracellular signaling-regulated kinase-1 and -2 (ERK 1/2) mediate referred hyperalgesia in a murine model of visceral pain. Brain research Molecular brain research 116:126-134.
Galan A, Laird JM, Cervero F (2004) In vivo recruitment by painful stimuli of AMPA receptor subunits to the plasma membrane of spinal cord neurons. Pain 112:315-323.
Gandhi R, Ryals JM, Wright DE (2004) Neurotrophin-3 reverses chronic mechanical hyperalgesia induced by intramuscular acid injection. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:9405-9413.
Garry EM, Moss A, Rosie R, Delaney A, Mitchell R, Fleetwood-Walker SM (2003) Specific involvement in neuropathic pain of AMPA receptors and adapter proteins for the GluR2 subunit. Molecular and cellular neurosciences 24:10-22.
Gaskin DJ, Richard P (2012) The economic costs of pain in the United States. The journal of pain : official journal of the American Pain Society 13:715-724.
Gautam M, Benson CJ, Ranier JD, Light AR, Sluka KA (2012) ASICs Do Not Play a Role in Maintaining Hyperalgesia Induced by Repeated Intramuscular Acid Injections. Pain research and treatment 2012:817347.
Georgopoulos AP (1976) Functional properties of primary afferent units probably related to pain mechanisms in primate glabrous skin. Journal of neurophysiology 39:71-83.
Gilchrist HD, Allard BL, Simone DA (1996) Enhanced withdrawal responses to heat and mechanical stimuli following intraplantar injection of capsaicin in rats. Pain 67:179-188.
Goldberg DS, McGee SJ (2011) Pain as a global public health priority. BMC Public Health 11:770.
Graven-Nielsen T, Aspegren Kendall S, Henriksson KG, Bengtsson M, Sorensen J, Johnson A, Gerdle B, Arendt-Nielsen L (2000) Ketamine reduces muscle pain, temporal summation, and referred pain in fibromyalgia patients. Pain 85:483-491.
Greengard P, Jen J, Nairn AC, Stevens CF (1991) Enhancement of the glutamate response by cAMP-dependent protein kinase in hippocampal neurons. Science 253:1135-1138.
Guo W, Zou S, Guan Y, Ikeda T, Tal M, Dubner R, Ren K (2002) Tyrosine phosphorylation of the NR2B subunit of the NMDA receptor in the spinal cord during the development and maintenance of inflammatory hyperalgesia. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:6208-6217.
Gwak YS, Kang J, Leem JW, Hulsebosch CE (2007) Spinal AMPA receptor inhibition attenuates mechanical allodynia and neuronal hyperexcitability following spinal cord injury in rats. Journal of neuroscience research 85:2352-2359.

Han J, Kwon M, Cha M, Tanioka M, Hong SK, Bai SJ, Lee BH (2015) Plasticity-Related PKMzeta Signaling in the Insular Cortex Is Involved in the Modulation of Neuropathic Pain after Nerve Injury. Neural plasticity 2015:601767.
Han JS, Adwanikar H, Li Z, Ji G, Neugebauer V (2010) Facilitation of synaptic transmission and pain responses by CGRP in the amygdala of normal rats. Molecular pain 6:10.
Hanley JG (2007) NSF binds calcium to regulate its interaction with AMPA receptor subunit GluR2. Journal of neurochemistry 101:1644-1650.
Hanley JG, Khatri L, Hanson PI, Ziff EB (2002) NSF ATPase and alpha-/beta-SNAPs disassemble the AMPA receptor-PICK1 complex. Neuron 34:53-67.
Hardingham N, Fox K (2006) The role of nitric oxide and GluR1 in presynaptic and postsynaptic components of neocortical potentiation. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:7395-7404.
Hardt O, Migues PV, Hastings M, Wong J, Nader K (2010) PKMzeta maintains 1-day- and 6-day-old long-term object location but not object identity memory in dorsal hippocampus. Hippocampus 20:691-695.
Hardy JD, Wolff HG, Goodell H (1950) Experimental evidence on the nature of cutaneous hyperalgesia. The Journal of clinical investigation 29:115-140.
Harris JA, Corsi M, Quartaroli M, Arban R, Bentivoglio M (1996) Upregulation of spinal glutamate receptors in chronic pain. Neuroscience 74:7-12.
Hartsink-Segers SA, Beaudoin JJ, Luijendijk MW, Exalto C, Pieters R, Den Boer ML (2015) PKCzeta and PKMzeta are overexpressed in TCF3-rearranged paediatric acute lymphoblastic leukaemia and are associated with increased thiopurine sensitivity. Leukemia 29:304-311.
Hedo G, Laird JM, Lopez-Garcia JA (1999) Time-course of spinal sensitization following carrageenan-induced inflammation in the young rat: a comparative electrophysiological and behavioural study in vitro and in vivo. Neuroscience 92:309-318.
Helmstaedter C, Kurthen M, Elger CE (1999) Sex differences in material-specific cognitive functions related to language dominance: an intracarotid amobarbital study in left temporal lobe epilepsy. Laterality 4:51-63.
Hendrickson BA, Gokhale R, Cho JH (2002) Clinical aspects and pathophysiology of inflammatory bowel disease. Clin Microbiol Rev 15:79-94.
Hennig L, Christner C, Kipping M, Schelbert B, Rucknagel KP, Grabley S, Kullertz G, Fischer G (1998) Selective inactivation of parvulin-like peptidyl-prolyl cis/trans isomerases by juglone. Biochemistry 37:5953-5960.
Henry JL, Yashpal K, Pitcher GM, Coderre TJ (1999) Physiological evidence that the 'interphase' in the formalin test is due to active inhibition. Pain 82:57-63.
Hernandez AI, Blace N, Crary JF, Serrano PA, Leitges M, Libien JM, Weinstein G, Tcherapanov A, Sacktor TC (2003) Protein kinase M zeta synthesis from a brain mRNA encoding an independent protein kinase C zeta catalytic domain. Implications for the molecular mechanism of memory. The Journal of biological chemistry 278:40305-40316.
Herrero JF, Laird JM, Lopez-Garcia JA (2000) Wind-up of spinal cord neurones and pain sensation: much ado about something? Progress in neurobiology 61:169-203.
Hirai T, Chida K (2003) Protein kinase Czeta (PKCzeta): activation mechanisms and cellular functions. Journal of biochemistry 133:1-7.
Hoeger-Bement MK, Sluka KA (2003) Phosphorylation of CREB and mechanical hyperalgesia is reversed by blockade of the cAMP pathway in a time-dependent manner after repeated intramuscular acid injections. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:5437-5445.
Hou B, Cui X, Liu Y, Zhang W, Liu M, Sun YE, Ma Z, Gu X (2016) Positive feedback regulation between microRNA-132 and CREB in spinal cord contributes to bone cancer pain in mice. Eur J Pain.
Hu HJ, Alter BJ, Carrasquillo Y, Qiu CS, Gereau RWt (2007) Metabotropic glutamate receptor 5 modulates nociceptive plasticity via extracellular signal-regulated kinase-Kv4.2 signaling in spinal cord dorsal horn neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:13181-13191.

Hua XY, Chen P, Yaksh TL (1999) Inhibition of spinal protein kinase C reduces nerve injury-induced tactile allodynia in neuropathic rats. Neuroscience letters 276:99-102.
Huang SM, Li X, Yu Y, Wang J, Caterina MJ (2011) TRPV3 and TRPV4 ion channels are not major contributors to mouse heat sensation. Molecular pain 7:37.
Hunskaar S, Hole K (1987) The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30:103-114.
Hylden JL, Nahin RL, Traub RJ, Dubner R (1989) Expansion of receptive fields of spinal lamina I projection neurons in rats with unilateral adjuvant-induced inflammation: the contribution of dorsal horn mechanisms. Pain 37:229-243.
Igwe OJ, Chronwall BM (2001) Hyperalgesia induced by peripheral inflammation is mediated by protein kinase C betaII isozyme in the rat spinal cord. Neuroscience 104:875-890.
Ikeda H, Heinke B, Ruscheweyh R, Sandkuhler J (2003) Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. Science 299:1237-1240.
Ikeda H, Stark J, Fischer H, Wagner M, Drdla R, Jager T, Sandkuhler J (2006) Synaptic amplifier of inflammatory pain in the spinal dorsal horn. Science 312:1659-1662.
Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, Storm DR (1998) Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB-dependent transcription and ERK nuclear translocation. Neuron 21:869-883.
Inbaraj JJ, Chignell CF (2004) Cytotoxic action of juglone and plumbagin: a mechanistic study using HaCaT keratinocytes. Chem Res Toxicol 17:55-62.
Inoue M, Kishimoto A, Takai Y, Nishizuka Y (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. The Journal of biological chemistry 252:7610-7616.
Inquimbert P, Bartels K, Babaniyi OB, Barrett LB, Tegeder I, Scholz J (2012) Peripheral nerve injury produces a sustained shift in the balance between glutamate release and uptake in the dorsal horn of the spinal cord. Pain 153:2422-2431.
Jarvis MF, Boyce-Rustay JM (2009) Neuropathic pain: models and mechanisms. Current pharmaceutical design 15:1711-1716.
Jenkins MA, Traynelis SF (2012) PKC phosphorylates GluA1-Ser831 to enhance AMPA receptor conductance. Channels (Austin) 6:60-64.
Jha MK, Jeon S, Jin M, Ock J, Kim JH, Lee WH, Suk K (2014) The pivotal role played by lipocalin-2 in chronic inflammatory pain. Experimental neurology 254:41-53.
Ji RR, Baba H, Brenner GJ, Woolf CJ (1999) Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nature neuroscience 2:1114-1119.
Ji RR, Befort K, Brenner GJ, Woolf CJ (2002) ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:478-485.
Ji RR, Rupp F (1997) Phosphorylation of transcription factor CREB in rat spinal cord after formalin-induced hyperalgesia: relationship to c-fos induction. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:1776-1785.
Jonasson Z (2005) Meta-analysis of sex differences in rodent models of learning and memory: a review of behavioral and biological data. Neuroscience and biobehavioral reviews 28:811-825.
Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. Nature 413:203-210.
Kandel ER, Schwartz JH, Jessell TM (2000) Principles of neural science. New York: McGraw-Hill, Health Professions Division.
Kangrga I, Randic M (1991) Outflow of endogenous aspartate and glutamate from the rat spinal dorsal horn in vitro by activation of low- and high-threshold primary afferent fibers. Modulation by mu-opioids. Brain research 553:347-352.
Katano T, Furue H, Okuda-Ashitaka E, Tagaya M, Watanabe M, Yoshimura M, Ito S (2008) N-ethylmaleimide-sensitive fusion protein (NSF) is involved in central sensitization in the spinal cord through GluR2 subunit composition switch after inflammation. The European journal of neuroscience 27:3161-3170.

Katz J, Seltzer Z (2009) Transition from acute to chronic postsurgical pain: risk factors and protective factors. Expert review of neurotherapeutics 9:723-744.
Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der Meer C, Befort K, Woolf CJ, Ji RR (2004) Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:8310-8321.
Keller BU, Hollmann M, Heinemann S, Konnerth A (1992) Calcium influx through subunits GluR1/GluR3 of kainate/AMPA receptor channels is regulated by cAMP dependent protein kinase. The EMBO journal 11:891-896.
Kelly MT, Crary JF, Sacktor TC (2007) Regulation of protein kinase Mzeta synthesis by multiple kinases in long-term potentiation. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:3439-3444.
Kenshalo DR, Jr., Leonard RB, Chung JM, Willis WD (1982) Facilitation of the response of primate spinothalamic cells to cold and to tactile stimuli by noxious heating of the skin. Pain 12:141-152.
Kessels HW, Malinow R (2009) Synaptic AMPA receptor plasticity and behavior. Neuron 61:340-350.
Khasabov SG, Rogers SD, Ghilardi JR, Peters CM, Mantyh PW, Simone DA (2002) Spinal neurons that possess the substance P receptor are required for the development of central sensitization. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:9086-9098.
King T, Qu C, Okun A, Melemedjian OK, Mandell EK, Maskaykina IY, Navratilova E, Dussor GO, Ghosh S, Price TJ, Porreca F (2012) Contribution of PKMzeta-dependent and independent amplification to components of experimental neuropathic pain. Pain 153:1263-1273.
King TE, Cheng J, Wang S, Barr GA (2000) Maturation of NK1 receptor involvement in the nociceptive response to formalin. Synapse 36:254-266.
Klede M, Handwerker HO, Schmelz M (2003) Central origin of secondary mechanical hyperalgesia. Journal of neurophysiology 90:353-359.
Klein T, Magerl W, Hopf HC, Sandkuhler J, Treede RD (2004) Perceptual correlates of nociceptive long-term potentiation and long-term depression in humans. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:964-971.
Krapivinsky G, Krapivinsky L, Manasian Y, Ivanov A, Tyzio R, Pellegrino C, Ben-Ari Y, Clapham DE, Medina I (2003) The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. Neuron 40:775-784.
Kumazawa T, Perl ER (1977) Primate cutaneous receptors with unmyelinated (C) fibres and their projection to the substantia gelatinosa. Journal de physiologie 73:287-304.
Laferriere A, Pitcher MH, Haldane A, Huang Y, Cornea V, Kumar N, Sacktor TC, Cervero F, Coderre TJ (2011) PKMzeta is essential for spinal plasticity underlying the maintenance of persistent pain. Molecular pain 7:99.
Laird JM, Martinez-Caro L, Garcia-Nicas E, Cervero F (2001) A new model of visceral pain and referred hyperalgesia in the mouse. Pain 92:335-342.
LaMotte RH, Lundberg LE, Torebjork HE (1992) Pain, hyperalgesia and activity in nociceptive C units in humans after intradermal injection of capsaicin. The Journal of physiology 448:749-764.
LaMotte RH, Shain CN, Simone DA, Tsai EF (1991) Neurogenic hyperalgesia: psychophysical studies of underlying mechanisms. Journal of neurophysiology 66:190-211.
LaMotte RH, Thalhammer JG (1982) Response properties of high-threshold cutaneous cold receptors in the primate. Brain research 244:279-287.
Latremoliere A, Woolf CJ (2009) Central sensitization: a generator of pain hypersensitivity by central neural plasticity. The journal of pain : official journal of the American Pain Society 10:895-926.
Ledeboer A, Mahoney JH, Milligan ED, Martin D, Maier SF, Watkins LR (2006) Spinal cord glia and interleukin-1 do not appear to mediate persistent allodynia induced by intramuscular acidic saline in rats. The journal of pain : official journal of the American Pain Society 7:757-767.
Lee AM, Kanter BR, Wang D, Lim JP, Zou ME, Qiu C, McMahon T, Dadgar J, Fischbach-Weiss SC, Messing RO (2013) Prkcz null mice show normal learning and memory. Nature 493:416-419.
Lever IJ, Bradbury EJ, Cunningham JR, Adelson DW, Jones MG, McMahon SB, Marvizon JC, Malcangio M (2001) Brain-derived neurotrophic factor is released in the dorsal horn by distinctive patterns of

afferent fiber stimulation. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:4469-4477.
Li XY, Ko HG, Chen T, Descalzi G, Koga K, Wang H, Kim SS, Shang Y, Kwak C, Park SW, Shim J, Lee K, Collingridge GL, Kaang BK, Zhuo M (2010) Alleviating neuropathic pain hypersensitivity by inhibiting PKMzeta in the anterior cingulate cortex. Science 330:1400-1404.
Li XY, Wang N, Wang YJ, Zuo ZX, Koga K, Luo F, Zhuo M (2014) Long-term temporal imprecision of information coding in the anterior cingulate cortex of mice with peripheral inflammation or nerve injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 34:10675-10687.
Li YQ, Xue YX, He YY, Li FQ, Xue LF, Xu CM, Sacktor TC, Shaham Y, Lu L (2011) Inhibition of PKMzeta in nucleus accumbens core abolishes long-term drug reward memory. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:5436-5446.
Liang Y, Fang JQ, Du JY, Fang JF (2012) Effect of Electroacupuncture on Activation of p38MAPK in Spinal Dorsal Horn in Rats with Complete Freund's Adjuvant-Induced Inflammatory Pain. Evidence-based complementary and alternative medicine : eCAM 2012:568273.
Ling DS, Benardo LS, Sacktor TC (2006) Protein kinase Mzeta enhances excitatory synaptic transmission by increasing the number of active postsynaptic AMPA receptors. Hippocampus 16:443-452.
Ling DS, Benardo LS, Serrano PA, Blace N, Kelly MT, Crary JF, Sacktor TC (2002) Protein kinase Mzeta is necessary and sufficient for LTP maintenance. Nature neuroscience 5:295-296.
Lozier AP, Kendig JJ (1995) Long-term potentiation in an isolated peripheral nerve-spinal cord preparation. Journal of neurophysiology 74:1001-1009.
Lu W, Man H, Ju W, Trimble WS, MacDonald JF, Wang YT (2001) Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. Neuron 29:243-254.
Lu W, Ziff EB (2005) PICK1 interacts with ABP/GRIP to regulate AMPA receptor trafficking. Neuron 47:407-421.
Luo F, Yang C, Chen Y, Shukla P, Tang L, Wang LX, Wang ZJ (2008) Reversal of chronic inflammatory pain by acute inhibition of Ca2+/calmodulin-dependent protein kinase II. The Journal of pharmacology and experimental therapeutics 325:267-275.
Ma W, Quirion R (2001) Increased phosphorylation of cyclic AMP response element-binding protein (CREB) in the superficial dorsal horn neurons following partial sciatic nerve ligation. Pain 93:295-301.
Magerl W, Wilk SH, Treede RD (1998) Secondary hyperalgesia and perceptual wind-up following intradermal injection of capsaicin in humans. Pain 74:257-268.
Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. Annu Rev Neurosci 25:103-126.
Malmberg AB, Chen C, Tonegawa S, Basbaum AI (1997) Preserved acute pain and reduced neuropathic pain in mice lacking PKCgamma. Science 278:279-283.
Mammen AL, Kameyama K, Roche KW, Huganir RL (1997) Phosphorylation of the alpha-amino-3-hydroxy-5-methylisoxazole4-propionic acid receptor GluR1 subunit by calcium/calmodulin-dependent kinase II. The Journal of biological chemistry 272:32528-32533.
Manna PR, Stocco DM (2007) Crosstalk of CREB and Fos/Jun on a single cis-element: transcriptional repression of the steroidogenic acute regulatory protein gene. J Mol Endocrinol 39:261-277.
Mao J, Price DD, Hayes RL, Lu J, Mayer DJ (1992) Differential roles of NMDA and non-NMDA receptor activation in induction and maintenance of thermal hyperalgesia in rats with painful peripheral mononeuropathy. Brain research 598:271-278.
Marchand F, D'Mello R, Yip PK, Calvo M, Muller E, Pezet S, Dickenson AH, McMahon SB (2011) Specific involvement of atypical PKCzeta/PKMzeta in spinal persistent nociceptive processing following peripheral inflammation in rat. Molecular pain 7:86.
Martin WJ, Malmberg AB, Basbaum AI (2001) PKCgamma contributes to a subset of the NMDA-dependent spinal circuits that underlie injury-induced persistent pain. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:5321-5327.

Martinez-Rojas VA, Barragan-Iglesias P, Rocha-Gonzalez HI, Murbartian J, Granados-Soto V (2014) Role of TRPV1 and ASIC3 in formalin-induced secondary allodynia and hyperalgesia. Pharmacological reports : PR 66:964-971.
Melemedjian OK, Tillu DV, Asiedu MN, Mandell EK, Moy JK, Blute VM, Taylor CJ, Ghosh S, Price TJ (2013) BDNF regulates atypical PKC at spinal synapses to initiate and maintain a centralized chronic pain state. Molecular pain 9:12.
Melzack R, Wall PD (1965) Pain mechanisms: a new theory. Science 150:971-979.
Mendell LM, Wall PD (1965) Responses of Single Dorsal Cord Cells to Peripheral Cutaneous Unmyelinated Fibres. Nature 206:97-99.
Merskey HB, N. (1994) Classification of Chronic Pain. Seattle: IASP Press.
Meyer RA, Campbell JN (1981) Evidence for two distinct classes of unmyelinated nociceptive afferents in monkey. Brain research 224:149-152.
Meyer RA, Campbell JN (1988) A novel electrophysiological technique for locating cutaneous nociceptive and chemospecific receptors. Brain research 441:81-86.
Middei S, Houeland G, Cavallucci V, Ammassari-Teule M, D'Amelio M, Marie H (2013) CREB is necessary for synaptic maintenance and learning-induced changes of the AMPA receptor GluA1 subunit. Hippocampus 23:488-499.
Migues PV, Hardt O, Wu DC, Gamache K, Sacktor TC, Wang YT, Nader K (2010) PKMzeta maintains memories by regulating GluR2-dependent AMPA receptor trafficking. Nature neuroscience 13:630-634.
Mitsikostas DD, Knight YE, Lasalandra M, Kavantzas N, Goadsby PJ (2011) Triptans attenuate capsaicin-induced CREB phosphorylation within the trigeminal nucleus caudalis: a mechanism to prevent central sensitization? The journal of headache and pain 12:411-417.
Mogil JS (2012) Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. Nature reviews Neuroscience 13:859-866.
Mogil JS, Chesler EJ, Wilson SG, Juraska JM, Sternberg WF (2000) Sex differences in thermal nociception and morphine antinociception in rodents depend on genotype. Neuroscience and biobehavioral reviews 24:375-389.
Mogil JS, Graham AC, Ritchie J, Hughes SF, Austin JS, Schorscher-Petcu A, Langford DJ, Bennett GJ (2010) Hypolocomotion, asymmetrically directed behaviors (licking, lifting, flinching, and shaking) and dynamic weight bearing (gait) changes are not measures of neuropathic pain in mice. Molecular pain 6:34.
Mogil JS, Miermeister F, Seifert F, Strasburg K, Zimmermann K, Reinold H, Austin JS, Bernardini N, Chesler EJ, Hofmann HA, Hordo C, Messlinger K, Nemmani KV, Rankin AL, Ritchie J, Siegling A, Smith SB, Sotocinal S, Vater A, Lehto SG, Klussmann S, Quirion R, Michaelis M, Devor M, Reeh PW (2005) Variable sensitivity to noxious heat is mediated by differential expression of the CGRP gene. Proceedings of the National Academy of Sciences of the United States of America 102:12938-12943.
Mogil JS, Wilson SG, Bon K, Lee SE, Chung K, Raber P, Pieper JO, Hain HS, Belknap JK, Hubert L, Elmer GI, Chung JM, Devor M (1999) Heritability of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception. Pain 80:67-82.
Motta EM, Calixto JB, Rae GA (2006) Mechanical hyperalgesia induced by endothelin-1 in rats is mediated via phospholipase C, protein kinase C, and MAP kinases. Exp Biol Med (Maywood) 231:1141-1145.
Muraki K, Iwata Y, Katanosaka Y, Ito T, Ohya S, Shigekawa M, Imaizumi Y (2003) TRPV2 is a component of osmotically sensitive cation channels in murine aortic myocytes. Circulation research 93:829-838.
Najafov A, Sommer EM, Axten JM, Deyoung MP, Alessi DR (2011) Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1. The Biochemical journal 433:357-369.
Naka A, Gruber-Schoffnegger D, Sandkuhler J (2013) Non-Hebbian plasticity at C-fiber synapses in rat spinal cord lamina I neurons. Pain 154:1333-1342.
Narita M, Oe K, Kato H, Shibasaki M, Narita M, Yajima Y, Yamazaki M, Suzuki T (2004) Implication of spinal protein kinase C in the suppression of morphine-induced rewarding effect under a neuropathic pain-like state in mice. Neuroscience 125:545-551.
Nassar MA, Stirling LC, Forlani G, Baker MD, Matthews EA, Dickenson AH, Wood JN (2004) Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain.

Proceedings of the National Academy of Sciences of the United States of America 101:12706-12711.
Neugebauer V, Chen PS, Willis WD (1999) Role of metabotropic glutamate receptor subtype mGluR1 in brief nociception and central sensitization of primate STT cells. Journal of neurophysiology 82:272-282.
Nijs J, Meeus M, Versijpt J, Moens M, Bos I, Knaepen K, Meeusen R (2014) Brain-derived neurotrophic factor as a driving force behind neuroplasticity in neuropathic and central sensitization pain: a new therapeutic target? Expert opinion on therapeutic targets 1-12.
Nishimune A, Isaac JT, Molnar E, Noel J, Nash SR, Tagaya M, Collingridge GL, Nakanishi S, Henley JM (1998) NSF binding to GluR2 regulates synaptic transmission. Neuron 21:87-97.
Noel J, Ralph GS, Pickard L, Williams J, Molnar E, Uney JB, Collingridge GL, Henley JM (1999) Surface expression of AMPA receptors in hippocampal neurons is regulated by an NSF-dependent mechanism. Neuron 23:365-376.
Osten P, Valsamis L, Harris A, Sacktor TC (1996) Protein synthesis-dependent formation of protein kinase Mzeta in long-term potentiation. The Journal of neuroscience : the official journal of the Society for Neuroscience 16:2444-2451.
Park JS, Yaster M, Guan X, Xu JT, Shih MH, Guan Y, Raja SN, Tao YX (2008) Role of spinal cord alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in complete Freund's adjuvant-induced inflammatory pain. Molecular pain 4:67.
Pastalkova E, Serrano P, Pinkhasova D, Wallace E, Fenton AA, Sacktor TC (2006) Storage of spatial information by the maintenance mechanism of LTP. Science 313:1141-1144.
Patapoutian A, Peier AM, Story GM, Viswanath V (2003) ThermoTRP channels and beyond: mechanisms of temperature sensation. Nature reviews Neuroscience 4:529-539.
Pedersen LM, Gjerstad J (2008) Spinal cord long-term potentiation is attenuated by the NMDA-2B receptor antagonist Ro 25-6981. Acta physiologica 192:421-427.
Pedersen LM, Jacobsen LM, Mollerup S, Gjerstad J (2010) Spinal cord long-term potentiation (LTP) is associated with increased dorsal horn gene expression of IL-1beta, GDNF and iNOS. Eur J Pain 14:255-260.
Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A (2002) A TRP channel that senses cold stimuli and menthol. Cell 108:705-715.
Peng HY, Chang CH, Tsai SJ, Lai CY, Tung KC, Wu HC, Lin TB (2011a) Protein kinase A-dependent spinal alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate-receptor trafficking mediates capsaicin-induced colon-urethra cross-organ reflex sensitization. Anesthesiology 114:70-83.
Peng JM, Xu LS, Zhu Q, Gong S, Yu XM, Guo SY, Wu GC, Tao J, Jiang XH (2011b) Enhanced NMDA receptor NR1 phosphorylation and neuronal activity in the arcuate nucleus of hypothalamus following peripheral inflammation. Acta pharmacologica Sinica 32:160-166.
Pertin M, Ji RR, Berta T, Powell AJ, Karchewski L, Tate SN, Isom LL, Woolf CJ, Gilliard N, Spahn DR, Decosterd I (2005) Upregulation of the voltage-gated sodium channel beta2 subunit in neuropathic pain models: characterization of expression in injured and non-injured primary sensory neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:10970-10980.
Pertovaara A (1998) A neuronal correlate of secondary hyperalgesia in the rat spinal dorsal horn is submodality selective and facilitated by supraspinal influence. Experimental neurology 149:193-202.
Pezet S, Marchand F, D'Mello R, Grist J, Clark AK, Malcangio M, Dickenson AH, Williams RJ, McMahon SB (2008) Phosphatidylinositol 3-kinase is a key mediator of central sensitization in painful inflammatory conditions. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:4261-4270.
Phillips K, Clauw DJ (2011) Central pain mechanisms in chronic pain states--maybe it is all in their head. Best Pract Res Clin Rheumatol 25:141-154.
Polgar E, Todd AJ (2008) Tactile allodynia can occur in the spared nerve injury model in the rat without selective loss of GABA or GABA(A) receptors from synapses in laminae I-II of the ipsilateral spinal dorsal horn. Neuroscience 156:193-202.

Radhakrishnan R, Sluka KA (2009) Increased glutamate and decreased glycine release in the rostral ventromedial medulla during induction of a pre-clinical model of chronic widespread muscle pain. Neuroscience letters 457:141-145.
Raghavendra V, Tanga FY, DeLeo JA (2004) Complete Freunds adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. The European journal of neuroscience 20:467-473.
Raja SN, Campbell JN, Meyer RA (1984) Evidence for different mechanisms of primary and secondary hyperalgesia following heat injury to the glabrous skin. Brain : a journal of neurology 107 (Pt 4):1179-1188.
Randic M, Jiang MC, Cerne R (1993) Long-term potentiation and long-term depression of primary afferent neurotransmission in the rat spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience 13:5228-5241.
Ren K, Hylden JL, Williams GM, Ruda MA, Dubner R (1992) The effects of a non-competitive NMDA receptor antagonist, MK-801, on behavioral hyperalgesia and dorsal horn neuronal activity in rats with unilateral inflammation. Pain 50:331-344.
Ribeiro CM, Putney JW (1996) Differential effects of protein kinase C activation on calcium storage and capacitative calcium entry in NIH 3T3 cells. The Journal of biological chemistry 271:21522-21528.
Ribeiro-da-Silva A, Cuello AC (1995) Organization of peptidergic neurons in the dorsal horn of the spinal cord: anatomical and functional correlates. Progress in brain research 104:41-59.
Riley JL, 3rd, Robinson ME, Wise EA, Price DD (1999) A meta-analytic review of pain perception across the menstrual cycle. Pain 81:225-235.
Roche KW, O'Brien RJ, Mammen AL, Bernhardt J, Huganir RL (1996) Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. Neuron 16:1179-1188.
Rosland JH, Tjolsen A, Maehle B, Hole K (1990) The formalin test in mice: effect of formalin concentration. Pain 42:235-242.
Sacktor TC (2010) PINing for things past. Science signaling 3:pe9.
Sacktor TC (2011) How does PKMzeta maintain long-term memory? Nature reviews Neuroscience 12:9-15.
Sacktor TC, Osten P, Valsamis H, Jiang X, Naik MU, Sublette E (1993) Persistent activation of the zeta isoform of protein kinase C in the maintenance of long-term potentiation. Proceedings of the National Academy of Sciences of the United States of America 90:8342-8346.
Sajikumar S, Navakkode S, Sacktor TC, Frey JU (2005) Synaptic tagging and cross-tagging: the role of protein kinase Mzeta in maintaining long-term potentiation but not long-term depression. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:5750-5756.
Salter MW (2004) Cellular neuroplasticity mechanisms mediating pain persistence. Journal of orofacial pain 18:318-324.
Sandkuhler J (2010) Central sensitization versus synaptic long-term potentiation (LTP): a critical comment. The journal of pain : official journal of the American Pain Society 11:798-800.
Sandkuhler J, Liu X (1998) Induction of long-term potentiation at spinal synapses by noxious stimulation or nerve injury. The European journal of neuroscience 10:2476-2480.
Sanoja R, Tortorici V, Fernandez C, Price TJ, Cervero F (2010) Role of RVM neurons in capsaicin-evoked visceral nociception and referred hyperalgesia. Eur J Pain 14:120 e121-129.
Santicioli P, Del Bianco E, Tramontana M, Geppetti P, Maggi CA (1992) Release of calcitonin gene-related peptide like-immunoreactivity induced by electrical field stimulation from rat spinal afferents is mediated by conotoxin-sensitive calcium channels. Neuroscience letters 136:161-164.
Schaible HG, Schmidt RF (1988) Time course of mechanosensitivity changes in articular afferents during a developing experimental arthritis. Journal of neurophysiology 60:2180-2195.
Schmidt R, Schmelz M, Forster C, Ringkamp M, Torebjork E, Handwerker H (1995) Novel classes of responsive and unresponsive C nociceptors in human skin. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:333-341.
Schneider SP, Perl ER (1988) Comparison of primary afferent and glutamate excitation of neurons in the mammalian spinal dorsal horn. The Journal of neuroscience : the official journal of the Society for Neuroscience 8:2062-2073.
Sebastian V, Vergel T, Baig R, Schrott LM, Serrano PA (2013) PKMzeta differentially utilized between sexes for remote long-term spatial memory. PloS one 8:e81121.

Serrano P, Friedman EL, Kenney J, Taubenfeld SM, Zimmerman JM, Hanna J, Alberini C, Kelley AE, Maren S, Rudy JW, Yin JC, Sacktor TC, Fenton AA (2008) PKMzeta maintains spatial, instrumental, and classically conditioned long-term memories. PLoS biology 6:2698-2706.
Serrano P, Yao Y, Sacktor TC (2005) Persistent phosphorylation by protein kinase Mzeta maintains late-phase long-term potentiation. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:1979-1984.
Shanthanelson M, Arvanian VL, Mendell LM (2009) Input-specific plasticity of N-methyl-D-aspartate receptor-mediated synaptic responses in neonatal rat motoneurons. The European journal of neuroscience 29:2125-2136.
Sharma NK, Ryals JM, Liu H, Liu W, Wright DE (2009) Acidic saline-induced primary and secondary mechanical hyperalgesia in mice. The journal of pain : official journal of the American Pain Society 10:1231-1241.
Shema R, Haramati S, Ron S, Hazvi S, Chen A, Sacktor TC, Dudai Y (2011) Enhancement of consolidated long-term memory by overexpression of protein kinase Mzeta in the neocortex. Science 331:1207-1210.
Shema R, Sacktor TC, Dudai Y (2007) Rapid erasure of long-term memory associations in the cortex by an inhibitor of PKM zeta. Science 317:951-953.
Simone DA, Baumann TK, LaMotte RH (1989) Dose-dependent pain and mechanical hyperalgesia in humans after intradermal injection of capsaicin. Pain 38:99-107.
Simone DA, Sorkin LS, Oh U, Chung JM, Owens C, LaMotte RH, Willis WD (1991) Neurogenic hyperalgesia: central neural correlates in responses of spinothalamic tract neurons. Journal of neurophysiology 66:228-246.
Sivilotti LG, Thompson SW, Woolf CJ (1993) Rate of rise of the cumulative depolarization evoked by repetitive stimulation of small-caliber afferents is a predictor of action potential windup in rat spinal neurons in vitro. Journal of neurophysiology 69:1621-1631.
Skyba DA, King EW, Sluka KA (2002) Effects of NMDA and non-NMDA ionotropic glutamate receptor antagonists on the development and maintenance of hyperalgesia induced by repeated intramuscular injection of acidic saline. Pain 98:69-78.
Skyba DA, Lisi TL, Sluka KA (2005) Excitatory amino acid concentrations increase in the spinal cord dorsal horn after repeated intramuscular injection of acidic saline. Pain 119:142-149.
Sluka KA, Audette KM (2006) Activation of protein kinase C in the spinal cord produces mechanical hyperalgesia by activating glutamate receptors, but does not mediate chronic muscle-induced hyperalgesia. Molecular pain 2:13.
Sluka KA, Kalra A, Moore SA (2001) Unilateral intramuscular injections of acidic saline produce a bilateral, long-lasting hyperalgesia. Muscle & nerve 24:37-46.
Sluka KA, Price MP, Breese NM, Stucky CL, Wemmie JA, Welsh MJ (2003) Chronic hyperalgesia induced by repeated acid injections in muscle is abolished by the loss of ASIC3, but not ASIC1. Pain 106:229-239.
Sluka KA, Rees H, Chen PS, Tsuruoka M, Willis WD (1997) Capsaicin-induced sensitization of primate spinothalamic tract cells is prevented by a protein kinase C inhibitor. Brain research 772:82-86.
Sluka KA, Willis WD (1998) Increased spinal release of excitatory amino acids following intradermal injection of capsaicin is reduced by a protein kinase G inhibitor. Brain research 798:281-286.
Smith AK, O'Hara CL, Stucky CL (2013) Mechanical sensitization of cutaneous sensory fibers in the spared nerve injury mouse model. Molecular pain 9:61.
Sorge RE, Martin LJ, Isbester KA, Sotocinal SG, Rosen S, Tuttle AH, Wieskopf JS, Acland EL, Dokova A, Kadoura B, Leger P, Mapplebeck JC, McPhail M, Delaney A, Wigerblad G, Schumann AP, Quinn T, Frasnelli J, Svensson CI, Sternberg WF, Mogil JS (2014) Olfactory exposure to males, including men, causes stress and related analgesia in rodents. Nat Methods 11:629-632.
Sorkin LS, Westlund KN, Sluka KA, Dougherty PM, Willis WD (1992) Neural changes in acute arthritis in monkeys. IV. Time-course of amino acid release into the lumbar dorsal horn. Brain Res Brain Res Rev 17:39-50.
Sullivan JP, Connor JR, Tiffany C, Shearer BG, Burch RM (1991) NPC 15437 interacts with the C1 domain of protein kinase C. An analysis using mutant PKC constructs. FEBS letters 285:120-123.

Sun J, Lu B, Yao J, Lei W, Huang Y, Zhang H, Xiao C (2014) Intra-periaqueductal gray infusion of zeta inhibitory peptide attenuates pain-conditioned place avoidance in rats. Brain research 1582:55-63.
Sutherland SP, Cook SP, McCleskey EW (2000) Chemical mediators of pain due to tissue damage and ischemia. Progress in brain research 129:21-38.
Suzuki A, Akimoto K, Ohno S (2003) Protein kinase C lambda/iota (PKClambda/iota): a PKC isotype essential for the development of multicellular organisms. Journal of biochemistry 133:9-16.
Svendsen F, Rygh LJ, Gjerstad J, Fiska A, Hole K, Tjolsen A (1999) Recording of long-term potentiation in single dorsal horn neurons in vivo in the rat. Brain research Brain research protocols 4:165-172.
Tao F, Tao YX, Mao P, Johns RA (2003) Role of postsynaptic density protein-95 in the maintenance of peripheral nerve injury-induced neuropathic pain in rats. Neuroscience 117:731-739.
Tappe-Theodor A, Constantin CE, Tegeder I, Lechner SG, Langeslag M, Lepcynzsky P, Wirotanseng RI, Kurejova M, Agarwal N, Nagy G, Todd A, Wettschureck N, Offermanns S, Kress M, Lewin GR, Kuner R (2012) Galpha(q/11) signaling tonically modulates nociceptor function and contributes to activity-dependent sensitization. Pain 153:184-196.
Thompson SW, Bennett DL, Kerr BJ, Bradbury EJ, McMahon SB (1999) Brain-derived neurotrophic factor is an endogenous modulator of nociceptive responses in the spinal cord. Proceedings of the National Academy of Sciences of the United States of America 96:7714-7718.
Treede RD, Cole JD (1993) Dissociated secondary hyperalgesia in a subject with a large-fibre sensory neuropathy. Pain 53:169-174.
Treede RD, Rief W, Barke A, Aziz Q, Bennett MI, Benoliel R, Cohen M, Evers S, Finnerup NB, First MB, Giamberardino MA, Kaasa S, Kosek E, Lavand'homme P, Nicholas M, Perrot S, Scholz J, Schug S, Smith BH, Svensson P, Vlaeyen JW, Wang SJ (2015) A classification of chronic pain for ICD-11. Pain 156:1003-1007.
Tsokas P, Hsieh C, Yao Y, Lesburgueres E, Wallace EJ, Tcherepanov A, Jothianandan D, Hartley BR, Pan L, Rivard B, Farese RV, Sajan MP, Bergold PJ, Hernandez AI, Cottrell JE, Shouval HZ, Fenton AA, Sacktor TC (2016) Compensation for PKMzeta in long-term potentiation and spatial long-term memory in mutant mice. Elife 5.
Tsokas P, Hsieh, C, Wallace, E.J.C, Pan, L., Hartley, B.R., Tcherepanov, A., Hernandez, A.I., Cottrell, J.E., Fenton, A.A., Sacktor, T.C. (2013) Conditional knockout of the PKC/PKM ζ gene and acute translational blockade by PKM ζ antisense show new synthesis of PKM ζ protein is crucial for LTP and long-term memory. Society for Neuroscience Abstract #23321/G59.
Turgeon SM, Pollack AE, Fink JS (1997) Enhanced CREB phosphorylation and changes in c-Fos and FRA expression in striatum accompany amphetamine sensitization. Brain research 749:120-126.
Usoskin D, Furlan A, Islam S, Abdo H, Lonnerberg P, Lou D, Hjerling-Leffler J, Haeggstrom J, Kharchenko O, Kharchenko PV, Linnarsson S, Ernfors P (2015) Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. Nature neuroscience 18:145-153.
van den Wijngaard RM, Welting O, Bulmer DC, Wouters MM, Lee K, de Jonge WJ, Boeckxstaens GE (2009) Possible role for TRPV1 in neomycin-induced inhibition of visceral hypersensitivity in rat. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 21:863-e860.
Verne GN, Himes NC, Robinson ME, Gopinath KS, Briggs RW, Crosson B, Price DD (2003) Central representation of visceral and cutaneous hypersensitivity in the irritable bowel syndrome. Pain 103:99-110.
Verne GN, Price DD (2002) Irritable bowel syndrome as a common precipitant of central sensitization. Curr Rheumatol Rep 4:322-328.
Verne GN, Robinson ME, Price DD (2001) Hypersensitivity to visceral and cutaneous pain in the irritable bowel syndrome. Pain 93:7-14.
Volk LJ, Bachman JL, Johnson R, Yu Y, Huganir RL (2013) PKM-zeta is not required for hippocampal synaptic plasticity, learning and memory. Nature 493:420-423.
Watt AJ, Sjostrom PJ, Hausser M, Nelson SB, Turrigiano GG (2004) A proportional but slower NMDA potentiation follows AMPA potentiation in LTP. Nature neuroscience 7:518-524.
Wei F, Zhuo M (2001) Potentiation of sensory responses in the anterior cingulate cortex following digit amputation in the anaesthetised rat. The Journal of physiology 532:823-833.

Weiner CP, Lizasoain I, Baylis SA, Knowles RG, Charles IG, Moncada S (1994) Induction of calcium-dependent nitric oxide synthases by sex hormones. Proceedings of the National Academy of Sciences of the United States of America 91:5212-5216.
Westmark PR, Westmark CJ, Wang S, Levenson J, O'Riordan KJ, Burger C, Malter JS (2010) Pin1 and PKMzeta sequentially control dendritic protein synthesis. Science signaling 3:ra18.
Whitfield J, Littlewood T, Soucek L (2015) Tamoxifen administration to mice. Cold Spring Harb Protoc 2015:269-271.
Wiesenfeld-Hallin Z (2005) Sex differences in pain perception. Gend Med 2:137-145.
Willis WD (2001) Role of neurotransmitters in sensitization of pain responses. Annals of the New York Academy of Sciences 933:142-156.
Wishik J (2005) Medical and Legal Aspects of Neurology. Tucson, AZ: Lawyers & Judges Publishing Company, Inc.
Woolf CJ (1983) Evidence for a central component of post-injury pain hypersensitivity. Nature 306:686-688.
Woolf CJ (1996) Windup and central sensitization are not equivalent. Pain 66:105-108.
Woolf CJ (2004) Dissecting out mechanisms responsible for peripheral neuropathic pain: implications for diagnosis and therapy. Life sciences 74:2605-2610.
Woolf CJ (2011) Central sensitization: implications for the diagnosis and treatment of pain. Pain 152:S2-15.
Woolf CJ, Salter MW (2000) Neuronal plasticity: increasing the gain in pain. Science 288:1765-1769.
Woolf CJ, Shortland P, Coggeshall RE (1992) Peripheral nerve injury triggers central sprouting of myelinated afferents. Nature 355:75-78.
Woolf CJ, Thompson SW (1991) The induction and maintenance of central sensitization is dependent on N-methyl-D-aspartic acid receptor activation; implications for the treatment of post-injury pain hypersensitivity states. Pain 44:293-299.
Wu-Zhang AX, Schramm CL, Nabavi S, Malinow R, Newton AC (2012) Cellular pharmacology of protein kinase Mzeta (PKMzeta) contrasts with its in vitro profile: implications for PKMzeta as a mediator of memory. The Journal of biological chemistry 287:12879-12885.
Xu XJ, Elfvin A, Wiesenfeld-Hallin Z (1995) Subcutaneous carrageenan, but not formalin, increases the excitability of the nociceptive flexor reflex in the rat. Neuroscience letters 196:116-118.
Xue L, Karpenko IA, Hiblot J, Johnsson K (2015) Imaging and manipulating proteins in live cells through covalent labeling. Nat Chem Biol 11:917-923.
Yajima Y, Narita M, Shimamura M, Narita M, Kubota C, Suzuki T (2003) Differential involvement of spinal protein kinase C and protein kinase A in neuropathic and inflammatory pain in mice. Brain research 992:288-293.
Yamamoto T, Yaksh TL (1992) Comparison of the antinociceptive effects of pre- and posttreatment with intrathecal morphine and MK801, an NMDA antagonist, on the formalin test in the rat. Anesthesiology 77:757-763.
Yang F, Guo J, Sun WL, Liu FY, Cai J, Xing GG, Wan Y (2014) The induction of long-term potentiation in spinal dorsal horn after peripheral nociceptive stimulation and contribution of spinal TRPV1 in rats. Neuroscience 269:59-66.
Yang HW, Hu XD, Zhang HM, Xin WJ, Li MT, Zhang T, Zhou LJ, Liu XG (2004a) Roles of CaMKII, PKA, and PKC in the induction and maintenance of LTP of C-fiber-evoked field potentials in rat spinal dorsal horn. Journal of neurophysiology 91:1122-1133.
Yang L, Zhang FX, Huang F, Lu YJ, Li GD, Bao L, Xiao HS, Zhang X (2004b) Peripheral nerve injury induces trans-synaptic modification of channels, receptors and signal pathways in rat dorsal spinal cord. The European journal of neuroscience 19:871-883.
Yang X, Yang HB, Xie QJ, Liu XH, Hu XD (2009) Peripheral inflammation increased the synaptic expression of NMDA receptors in spinal dorsal horn. Pain 144:162-169.
Yao Y, Kelly MT, Sajikumar S, Serrano P, Tian D, Bergold PJ, Frey JU, Sacktor TC (2008) PKM zeta maintains late long-term potentiation by N-ethylmaleimide-sensitive factor/GluR2-dependent trafficking of postsynaptic AMPA receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:7820-7827.
Yao Y, Shao C, Jothianandan D, Tcherepanov A, Shouval H, Sacktor TC (2013) Matching biochemical and functional efficacies confirm ZIP as a potent competitive inhibitor of PKMzeta in neurons. Neuropharmacology 64:37-44.

Yao Y, Tsokas, P., Jothianandan, D., Tcherepanov, A., Van De Nes, P., Sacktor, T. (2013) Compensation by PKC ι / λ activation and PKM ι / λ formation in PKM ζ knock-out mice. Society for Neuroscience Abstract #23322/G60.

Yashpal K, Coderre TJ (1998) Influence of formalin concentration on the antinociceptive effects of anti-inflammatory drugs in the formalin test in rats: separate mechanisms underlying the nociceptive effects of low- and high-concentration formalin. Eur J Pain 2:63-68.

Yashpal K, Fisher K, Chabot JG, Coderre TJ (2001) Differential effects of NMDA and group I mGluR antagonists on both nociception and spinal cord protein kinase C translocation in the formalin test and a model of neuropathic pain in rats. Pain 94:17-29.

Yashpal K, Katz J, Coderre TJ (1996) Effects of preemptive or postinjury intrathecal local anesthesia on persistent nociceptive responses in rats. Confounding influences of peripheral inflammation and the general anesthetic regimen. Anesthesiology 84:1119-1128.

Yashpal K, Mason P, McKenna JE, Sharma SK, Henry JL, Coderre TJ (1998) Comparison of the effects of treatment with intrathecal lidocaine given before and after formalin on both nociception and Fos expression in the spinal cord dorsal horn. Anesthesiology 88:157-164.

Yashpal K, Pitcher GM, Parent A, Quirion R, Coderre TJ (1995) Noxious thermal and chemical stimulation induce increases in 3H-phorbol 12,13-dibutyrate binding in spinal cord dorsal horn as well as persistent pain and hyperalgesia, which is reduced by inhibition of protein kinase C. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:3263-3272.

Yoshimura M, Yonehara N (2006) Alteration in sensitivity of ionotropic glutamate receptors and tachykinin receptors in spinal cord contribute to development and maintenance of nerve injury-evoked neuropathic pain. Neuroscience research 56:21-28.

Yunus MB (2002) Gender differences in fibromyalgia and other related syndromes. J Gend Specif Med 5:42-47.

Zeitz KP, Malmberg AB, Gilbert H, Basbaum AI (2001) Reduced development of tolerance to the analgesic effects of morphine and clonidine in PKC gamma mutant mice. Pain 94:245-253.

Zhang XC, Zhang YQ, Zhao ZQ (2005) Involvement of nitric oxide in long-term potentiation of spinal nociceptive responses in rats. Neuroreport 16:1197-1201.

Zhao XH, Zhang T, Li YQ (2015) The up-regulation of spinal Toll-like receptor 4 in rats with inflammatory pain induced by complete Freund's adjuvant. Brain research bulletin 111:97-103.

Zhou Y, Li GD, Zhao ZQ (2003) State-dependent phosphorylation of epsilon-isozyme of protein kinase C in adult rat dorsal root ganglia after inflammation and nerve injury. Journal of neurochemistry 85:571-580.

Zou S, Li L, Pei L, Vukusic B, Van Tol HH, Lee FJ, Wan Q, Liu F (2005) Protein-protein coupling/uncoupling enables dopamine D2 receptor regulation of AMPA receptor-mediated excitotoxicity. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:4385-4395.