An investigation into the epigenetic regulation of nociceptive processing

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I, Keri Kiyomi Tochiki, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

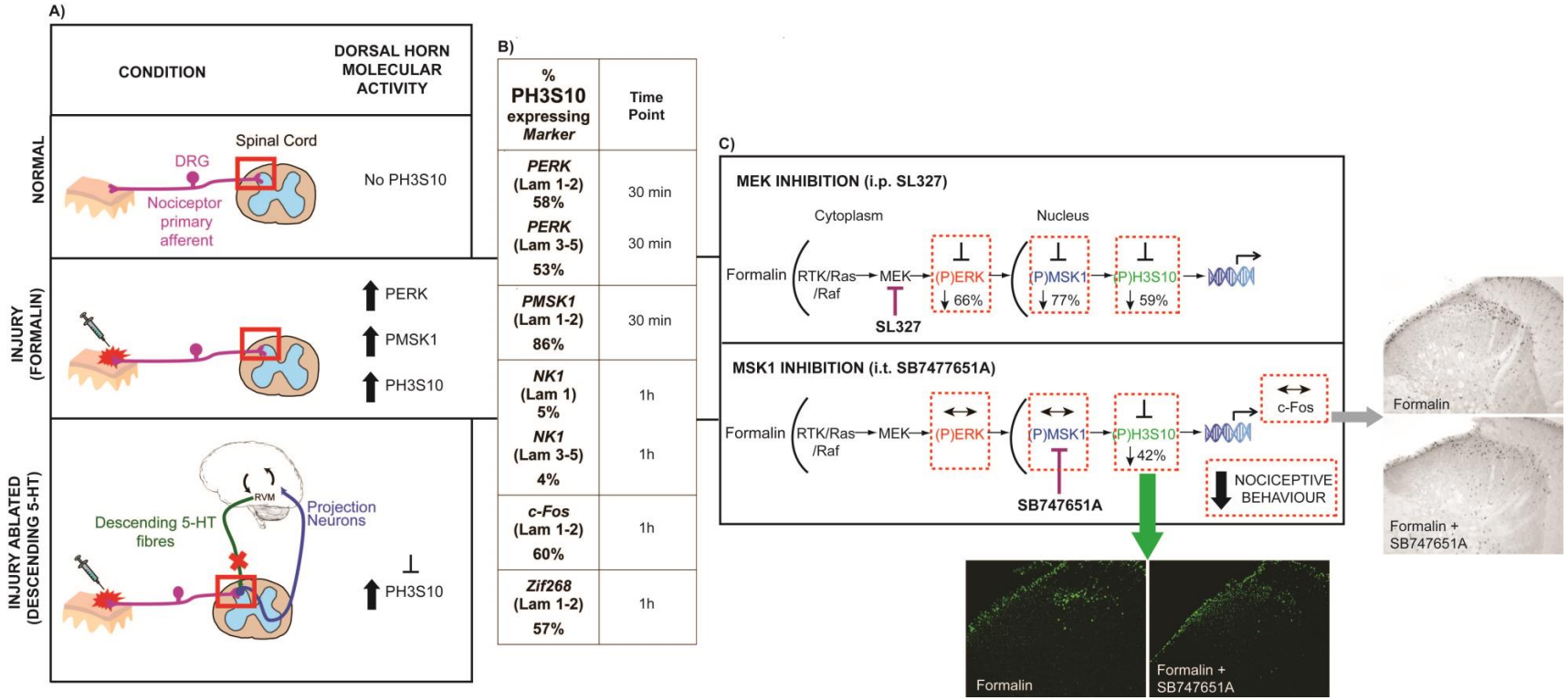
The induction of pain states is known to involve changes in gene expression in DRGs and spinal dorsal horn. These changes lead to plasticity in both peripheral and central pathways in the form of peripheral and central sensitisation, which contribute to the full manifestation of pain states.

Strong evidence exists to implicate the regulation of pain states by epigenetic mechanisms. Histone modification contributes to long-term plasticity and modulation of gene expression and histone modifying enzymes have been shown to regulate memory formation, a process thought to require neural activity similar to central sensitisation. Specifically, both neural processes engage the same molecular events such as extracellular signal-regulated kinase (ERK) activity, which induces the histone modification phosphorylation of histone H3 at serine 10 (PH3S10) *via* the nuclear mitogen and stress-activated protein kinase (MSK1).

MSK1 has been implicated in various animal models of neural plasticity and PH3S10 is a known marker of transcriptional activation, but neither has been investigated following noxious stimulation. The aim of this thesis was to use the rat formalin model, which induces a biphasic nocifensive behavioural response reflective of peripheral nociceptor activation followed by central sensitisation maintained by ongoing peripheral input, to investigate the role of histone modifications, in particular PH3S10, in pain processing.

This thesis demonstrated using immunohistochemistry that hindpaw formalin stimulation caused induction of PMSK1 and PH3S10 in the ipsilateral dorsal horn. PH3S10 peaked at 30 minutes and colocalised with markers of the pain pathways PERK, NK1, c-Fos, and Zif268. Formalin-induced PMSK1 and PH3S10 were prevented by inhibition of upstream ERK activity with the MEK inhibitor SL327. Moreover, intrathecal delivery of the MSK1 inhibitor SB747651A prevented full expression of PH3S10, and attenuated both phases of the formalin response. These findings are the first to elucidate key players involved in ERK/MAPK regulation of pain processing, and suggest that histone phosphorylation plays a crucial role in the response to injury.

Graphical Abstract



Main findings in this thesis. Box A) Expression of molecular targets investigated in the dorsal horn under naïve, injured, and injured-5-HT-ablated conditions. Red box indicates area of investigation. Box B) Percentage colocalisation of formalin-induced PH3S10 population with markers of the pain pathways. Box C) Pharmacological inhibition of ERK/MAPK signalling by SL327 led to a reduction in formalin-induced PERK, PMSK1, and PH3S10. In a separate experiment, inhibition of MSK1 by SB747651A reduced PH3S10 and prevented the full expression of pain states. Horizontal arrows indicate no change in injury-induced expression of molecular targets following drug treatment. Black inhibitory symbols indicate reduction in injury- induced expression of target following 5,7-DHT ablation or drug treatment.

Table of Contents

Table of Figures

Table of Tables

List of Abbreviations

5, 7-DHT 5, 7 dihydroxytryptamine creatinine sulphate
5-HT 5-hydroxytryptamine, serotonin
ABC avidin-biotin complex
ACC anterior cingulate cortex
AMPAR α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA Analysis of Variance
Arc/Arg3.1 activity-regulated cytoskeleton-associated protein
AUC area under curve
BCA bicinchoninic acid
CaMKII calcium calmodulin dependent kinase II
CHAPS 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
ChIP chromatin immunoprecipitation
ChIP-seq chromatin immunoprecipitation sequencing
CFA complete Freund's adjuvant
CNS central nervous system
CO_2 carbon dioxide
CREB cAMP response element-binding protein
CVLM caudal ventrolateral medulla
DAB 3,3' diaminobenzidine
DAMGO [D-Ala2, N-MePhe4, Gly-ol]-enkephalin
DMSO dimethyl sulfoxide
DNMT DNA methyltransferase
DRG dorsal root ganglion
EE environmental enrichment
ERK p44/42 MAPK (Thr202/Tyr204) extracellular signal-regulated kinase
EtOH ethanol
FITC fluorescein isothiocyanate avidin D
GAPDH glyceraldehyde-3-Phosphate Dehydrogenase
GDNF glial cell line-derived neurotrophic factor
GFAP glial fibrillary acidic protein
GPCR G-protein coupled receptor
H_2O_2 hydrogen peroxide
HAT histone acetyltransferase
HCl hydrochloric acid
HDAC histone deacetylase
HDACi histone deacetylase inhibitor
HRP horseradish peroxidase
IB4 isolectin B4
IEG immediate early gene
IHC immunohistochemistry
i.p. intraperitoneal
i.pl. intraplantar
i.t. intrathecal
JMJD2A Jumonji domain 2 Lysine-specific histone demethylase
LPb lateral parabrachial area
LTP long-term potentiation
MAPK mitogen-activated protein kinase
MeCP2 DNA methyl-CpG binding protein 2
mGluR metabotropic glutamate receptor
MSK mitogen and stress-activated protein kinase (T581)
MEK mitogen-activated protein kinase- also MAPKK

MOPS 3-(N-Morpholino) propanesulfonic acid, 4-Morpholinepropanesulfonic acid
NaB sodium butyrate
NaF sodium fluoride
NGF nerve growth factor
NK1R neurokinin 1 receptor
NMDAR N-methyl-D-aspartate receptor
NRM nucleus raphe magnus
NTS nucleus of the solitary tract
O/N overnight
p38 p38 MAPK (mitogen-activated protein kinase)
PAG periaqueductal grey matter
PB phosphate buffer
PBS phosphate buffered saline
PCREB phospho- cAMP response element-binding protein
PERK phospho-p44/42 MAPK (Thr202/Tyr204) extracellular signal-regulated kinase
PFA paraformaldehyde
PGE_2 prostaglandin E2
PKA protein kinase A
PKC protein kinase C
PMeCP2 phospho methyl-CpG binding protein 2
PMSK1 phospho mitogen and stress-activated protein kinase (Thr581)
PP1 protein Serine/Threonine phosphatase 1
pp38 phospho p38 MAPK (mitogen-activated protein kinase)
PVDF polyvinylidine difluoride
RNAi RNA interference
RSK2 ribosomal s6 kinase/ p70S6K
RVM rostral ventromedial medulla
RT room temperature
SAHA suberoyl anilide hydroxamic acid
SDS-PAGE sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SGK1 serum- and glucocorticoid-inducible kinase 1
SP substance P
SP-SAP substance P- saporin
SST somatostatin
TPH tryptophan hydroxylase
TRK tyrosine kinase receptor
TRP transient receptor potential
TSA trichostatin A
TSA tyramide signal amplification
TTBS triton- tris buffered saline
TTX tetrodotoxin
VPL ventral posterolateral nucleus (of the thalamus)
VPM ventral posteromedial nucleus (of the thalamus)
WT wild type

N.B. There is a lack of consistency regarding the nomenclature used for the abbreviation of histone modifications. In this thesis the nature of the modification is listed first, followed by the specified histone tail, followed by the specific residue and position. For example, Phosphorylation of histone H3 at serine 10 would be PH3S10. Likewise, Acetylation of histone H4 at lysine 8 would be referred to as AcH4K8.

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Finally, I want to dedicate this Thesis to my Mom and Dad, for their love and support, and for believing in me always.

Chapter 1 General Introduction

Pain is a complex and subjective experience encompassing both sensory and affective-motivational components that are contextually dependent. The International Association for the Study of Pain (IASP) describes pain as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.' Somewhat counter-intuitively, pain is ultimately protective, and the physiological processes underlying the pain response have in fact been proven to have an evolutionarily advantageous outcome in organism survival (Crook et al., 2014). The generation of unpleasant sensations associated with pain manifest in a complex behavioural response that serves as a warning to promote the avoidance of further potentially dangerous encounters, in addition to preventing further damage to the original site of injury to promote tissue healing. While the inability to feel pain might seem enticing, the importance of having an intact pain response is easily highlighted through cases of individuals bearing genetic mutations that prevent them from appropriately sensing and responding to harmful stimuli, which can lead to a lifetime of severe injuries (Cox et al., 2006; Goldberg et al., 2007).

Negative societal associations/perceptions of pain are not unwarranted as pain can adopt a pathological form when it becomes no longer protective and can be extremely debilitating. Behaviourally, pain can manifest as touch-evoked pain (allodynia) and/or as an exaggerated response to noxious stimulation (hyperalgesia), which can spread beyond the original site of injury. Pain can persist beyond the point at which the injury has healed, illustrating one example in which pain is not purely sensory-driven as it can be experienced even in the absence of obvious tissue damage (Latremoliere and Woolf, 2009; Scholz and Woolf, 2002). In maladaptive/pathological pain states, pain can arise spontaneously and is often chronic in nature. Chronic pain- lasting 3 months or longer- is a major problem, with 1 in 5 individuals in Europe having reported moderate to severe pain at some point in their lives (Breivik et al., 2006). Furthermore, many of these individuals (40%) also report an inadequate management of their pain. Severe consequences arise due to the lack of suitable treatments, including compromised quality of living and well-being, in addition to economic burden on society (Breivik et al., 2006; Reid et al., 2011). Thus, a clear and progressive understanding of the neurobiological processes underlying normal and aberrant nociceptive processing remains ever crucial. In particular, epigenetic mechanisms have been identified as potential targets for the regulation of pain processing, because they are required for long-term synaptic plasticity and the regulation of gene expression; key characteristics necessary for the full expression of persistent pain states.

The contents of this General Introduction will firstly illustrate the classification of pain types and underlying neurobiology and neurophysiology of pain processing.

Subsequently, the field of neuroepigenetics will be introduced and a case for neuroepigenetic regulation of pain states will be made.

1.1 Classification of pain types

It is useful to understand the different types of pain that exist. Pain can be separated into three categories (Scholz and Woolf, 2002; Woolf, 2010), described below.

Nociceptive pain

Nociception is the ability of the nervous system to encode noxious stimuli (International Association for the Study of Pain). Nociceptive pain describes the normal, protective response to intense, high-threshold noxious stimuli that elicits a physiological response (i.e. withdrawal reflex, unpleasant sensory and emotional sensation) which serves as a warning system to limit further damage to the body.

Inflammatory pain

Inflammatory pain concerns hypersensitivity as a result of tissue damage, which is mediated by the immune response-driven release of inflammatory mediators, also collectively called the 'inflammatory soup' (Basbaum et al., 2009; Julius and Basbaum, 2001). Inflammation is an attempt to initiate repair at the site of injury, and inflammatory pain is considered an adaptive, protective pain. If inflammatory pain is persistent following tissue injury, it can become maladaptive in nature.

Maladaptive/pathological pain

When pain no longer serves a protective function it adopts a maladaptive/pathological state, indicating aberrant nociceptive processing and a 'disease state of the nervous system' (Woolf, 2010). Pathological pain can occur following injury (i.e. neuropathic pain), but also in the absence of any obvious injury or noxious stimuli (i.e. fibromyalgia, irritable bowel syndrome).

1.2 Nociceptive pathways

The nociceptive pathways involve a network of peripheral and central nervous system relay components, which can demonstrate extensive plasticity in response to noxious input (Figure 1.1). The peripheral and central mechanisms of nociception and pain processing are described below; plasticity-related sensitisation processes are described subsequently in 1.3.

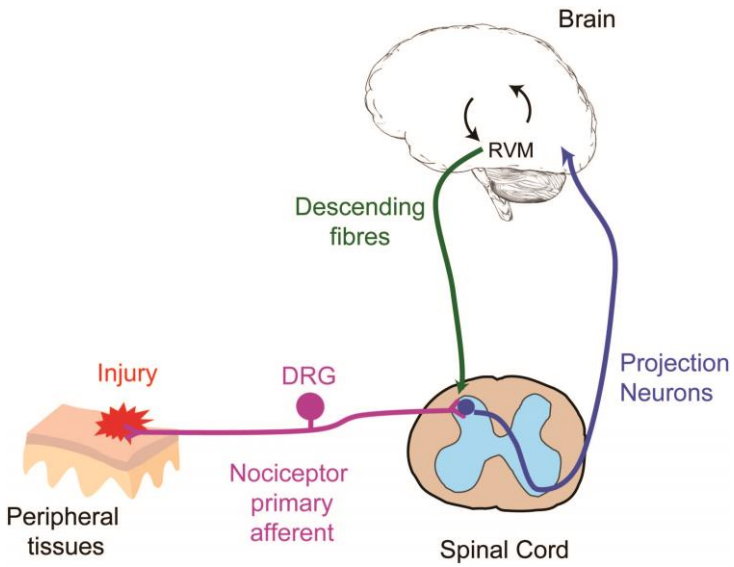


Figure 1.1 Nociceptive pathways. Information encoding noxious stimulation is relayed from the periphery (skin, viscera, and joints) to the central nervous system (spinal cord, brain).

1.2.1 Nociceptors

A subset of sensory fibres responsible for the signal transduction of noxious stimuli, called nociceptors, originate from soma housed in DRG or trigeminal ganglion and have free endings that innervate peripheral tissues including skin, joints and visceral tissue, as well as a central terminus in the dorsal horn. First identified by Sherrington (1906, 1903), the primary role of nociceptors is to transduce and relay information regarding the nature of noxious stimuli to the spinal cord. The abundance of studies that have followed in the past century has determined that primary afferent nociceptors have different properties and respond to different modalities.

The majority of nociceptors are small diameter (0.4-1.2μm) unmyelinated C-fibres, which have a conduction velocity of <2m/s (Woolf and Fitzgerald, 1983). The majority of C-fibres are polymodal (73% rat hairy skin), that is, they respond to a variety of noxious stimuli- typically mechanical, thermal and chemical stimuli (Bessou and Perl, 1969; Lynn and Carpenter, 1982).

A smaller proportion of nociceptors are A-fibres, including both larger diameter (2-5μm) myelinated Aδ-fibres (conduction velocity of 12-30m/s) (Burgess and Perl, 1967; Lawson and Waddell, 1991), as well as myelinated Aβ-fibres (5-14μm). Although often overlooked due to the fact that there are no distinctive differences in conduction velocities between Aδ and Aβ-fibres, in the rat, Aβ-fibres have been reported to comprise more than 50% of A-fibre nociceptors, and 20% of the Aβ-fibre population are nociceptors (Djouhri and Lawson, 2004). Functionally, A-fibre nociceptors can be

classified by their response to mechanical and thermal stimulation. Some A-fibre nociceptors are high threshold mechanoreceptors (20% in rat hairy skin), although the majority respond to noxious thermal stimulation as well (Djouhri and Lawson, 2004; Lynn and Carpenter, 1982). In primates, heat-responsive A-fibre nociceptors can be separated into two groups defined by action potential firing in response to noxious heat, specifically, Type I-mechano-heat Aδ and Aβ-fibres (low mechanical, higher heat thresholds), and type II-mechano-insensitive Aδ-fibres (higher mechanical, lower heat thresholds; mediate first response to heat) (Treede et al., 1998). A group of capsaicin-insensitive Type I fibres and A-fibre high threshold mechanoreceptors are primarily responsible for pinprick pain, and central sensitisation occurring from this peripheral input underlies mechanical secondary hyperalgesia (Magerl et al., 2001).

The remaining majority of non-nociceptive Aδ-fibres are low-threshold mechanoreceptors (also called D-hair), although there are a small minority that are low-threshold thermoreceptors (Djouhri and Lawson, 2004). The majority of non-nociceptive Aβ-fibres respond to tactile low-threshold mechanical stimuli such as touch (also called Aβ-LTMRs) (Menétrey et al., 1977) and also play an important role in pathological pain states (Lu et al., 2013; Torsney and MacDermott, 2006). There is also a group of C-LTMR which respond to low intensity, slow moving tactile stimulation, and play a role in mechanical hypersensitivity following injury (Bessou et al., 1971; Seal et al., 2009).

Due to myelination and conduction properties, Aδ-fibres are responsible for initial fast, sharp (pricking) pain, whereas C-fibres are responsible for the slow, dull pain that follows (Handwerker and Kobal, 1993). C-fibres respond to noxious mechanical stimulation at higher discharge frequencies compared to A-fibres; the latter is thought to encode the quality or nature of stimulation whereas the former is thought to encode stimulus intensity (Garell et al., 1996).

In the nociceptive response, the first important function of the nociceptor is the transduction of high-intensity noxious stimuli in response to noxious stimulation or tissue damage. Transduction of sensory stimuli occurs via a variety of transducer ligand-gated ion channels and G-protein coupled receptors (GPCRs) in the surface of the peripheral terminal, and lead to influx of calcium upon activation (Figure 1.2). The inward current generated by transducer activation may be sufficient to generate action potential firing, which initiates the relay of the nociceptive response to the central nervous system and encodes information about the activating stimulus. Importantly, the specific distribution of ion channels and GPCRs are responsible for the sensory

modality specificity of nociceptors, for example noxious heat, mechanical, or chemical stimuli (Ramsey et al., 2006).

There are a family of nine voltage gated sodium channels (Nav1.1-1.9) that have been shown to play a crucial role in nociceptive signalling (Raouf et al., 2010) (Figure 1.2). Sufficient activity-driven membrane depolarisation due to transducer activation causes voltage gated sodium channels to activate, which in turn conduct the flow of Na+ ions into the neuron, leading to the depolarisation upstroke of the action potential. The use of genetically modified mice in particular have contributed to the proven role of sodium channels in pain processing; Mice deficient in Nav1.7 (*SCN9A*) in peripheral nociceptors have impairments in acute mechanical and heat nociception, and inflammatory pain (Nassar et al., 2004). Behavioural responses to neuropathic pain, however, are unaffected (Minett et al., 2012). Interestingly, deletion of *SCN9A* in sympathetic as well as sensory neurons leads to a pain-free phenotype, indicating an important role for Nav1.7-expressing sympathetic neurons in neuropathic pain conditions (Minett et al., 2012). Similarly, mice genetically deficient for the TTX-resistant sodium channel Nav1.8 exhibit altered responses to noxious mechanical and thermal stimuli, and show delayed onset of inflammatory hyperalgesia (Abrahamsen et al., 2008; Akopian et al., 1999; Papadatos et al., 2002). In humans, loss-of-function mutations in *SCN9A* can lead to pain insensitivity and thus severe injuries due to the inability to detect harmful stimuli (Cox et al., 2006; Goldberg et al., 2007). Conversely, *SCN9A* gain-of-function mutations are associated with pain syndromes such as paroxysmal extreme pain disorder or erythromelalgia, the latter of which is often characterised by a burning sensation of the limbs (Bennett and Woods, 2014). Due to their importance in nociception, sodium channels in particular Nav1.7, have been of interest as pharmacological targets for the treatment of inflammatory and neuropathic pain (England and de Groot, 2009; Lee et al., 2014; McCormack et al., 2013; Nardi et al., 2012; Yang et al., 2013).

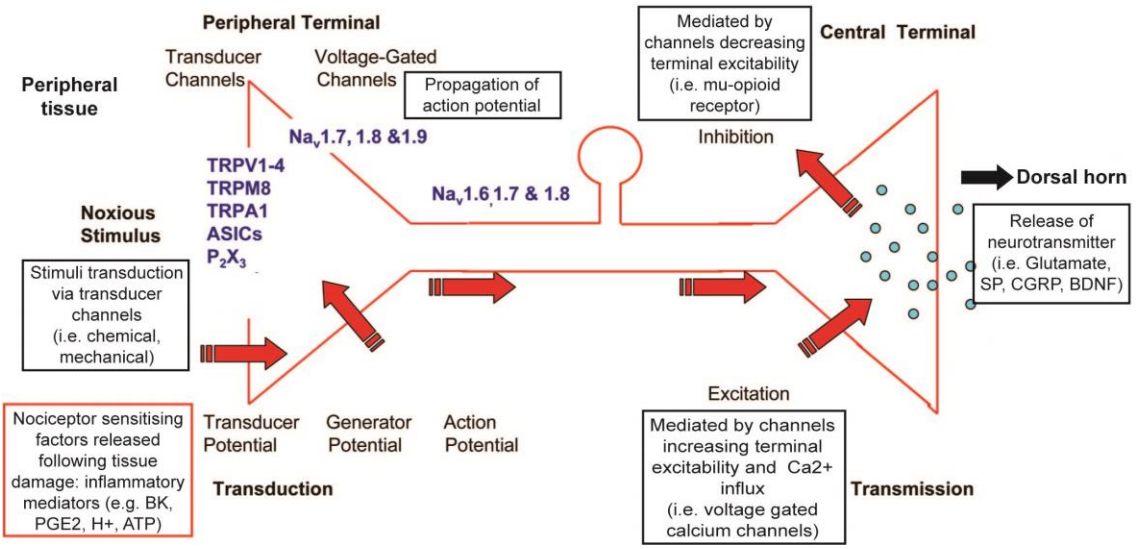


Figure 1.2 Activity of the nociceptor. Adapted from Neuron, Vol 55, Woolf C.J. and Ma Q., Nociceptors- Noxious Stimulus Detectors, pp. 353-364, Copyright (2007), with permission from Elsevier. A variety of ligand-gated ion channels in the peripheral terminal mediate the transduction of noxious stimuli. The transient-receptor-potential (TRP) channels are a group of non-selective cation channels with polymodal activation properties that mediate the response to noxious heat, pressure, or specific molecules/chemicals. TRPV1 has polymodal properties, it is the receptor for capsaicin and allicin among other molecules and is also activated by noxious heat (above 42˚C) and plays a role in inflammation and thermal hyperalgesia (Caterina et al., 2000, 1999; Davis et al., 2000). TRPV2, TRPV3, and TRPV4 mediate the nociceptive response to heat (Dhaka et al., 2006). TRPM8 mediates innocuous cooling, or cooling by agents such as menthol (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). TRPA1, expressed in a subgroup of unmyelinated peptidergic TRPV1-expressing nociceptors, is activated by noxious cold (<17°C) although this is controversial (Bandell et al., 2004; Bautista et al., 2006; Fajardo et al., 2008; Karashima et al., 2009; Kwan et al., 2009, 2006; Story et al., 2003). TRPA1 is also polymodal in activation, and responds to allyl isothiocyanate and allicin, among other agents, and mechanotransduction. Additional ligand gated ion channels mediating the transduction of noxious stimuli include acid-sensing ion channels (ASICs), activated by protons released in tissue acidosis following injury (Deval et al., 2010), and P2X receptors that activate in response to binding of ATP which is abundant in tissues post-injury (Chizh and Illes, 2001; Ding et al., 2000). The more recently identified mechanically activated PIEZO ion channels also play a key role in mechanosensory nociception (Coste et al., 2013, 2010; Kim et al., 2012), as well as nerve injury-induced mechanical allodynia (Eijkelkamp et al., 2013). PIEZO is also found in merkel cells and plays a key role in the slowly adapting merkel cell mediated response to non-noxious touch mechanosensation (Woo et al., 2014).

Classification by signalling molecules used in central neurotransmission

Propagation of an action potential along the axon of the nociceptor and its entry into the central terminal will lead to calcium influx into the terminal and subsequent neurotransmitter release into the dorsal horn (Neher and Sakaba, 2008). Glutamate is the main fast excitatory neurotransmitter used by all primary afferents, although other signaling molecules are released in addition, depending on fibre type.

Quite usefully, nociceptors can be neurochemically grouped by the type of peptide neurotransmitters that they release at their central terminals. In particular, nociceptors

can be separated into two groups, peptidergic and non-peptidergic, which indicate that one group of nociceptors synthesize peptide molecules such as substance P (SP) and calcitonin-gene related peptide (CGRP) as signaling molecules (Hunt and Rossi, 1985; Nagy and Hunt, 1982). Importantly, the peptidergic and non-peptidergic categories of nociceptors differ in their expression patterns of ion channels and receptors (Chen et al., 2006; Woolf and Ma, 2007), and have different patterns of peripheral and central terminal target innervation (Braz et al., 2005; Zylka et al., 2005). Specifically, non-peptidergic C-fibres are localised superficially in peripheral target innervations, terminating in the epidermis of the skin, while peptidergic C-fibres, including some peptidergic Aδ fibres (Lawson et al., 1997), terminate in deeper tissues (Todd, 2010). The identity of these fibres is determined in embryonic development [E12.5-P14]. In particular, TrkA-positive peptidergic neurons go on to express neuropeptides SP and CGRP (Chen et al., 2006), undergo downregulation of *Runx1* and require NGF for survival. The receptor tyrosine kinase RET appears to downregulate TrkA in the non-peptidergic neurons, and survival of the non-peptidergic population depends on glial cell line-derived neurotrophic factor (GDNF) signaling (Molliver et al., 1997). Importantly, the non-peptidergic nociceptor population can be identified immunohistochemically by the ability to bind isolectin B4 (IB4) (Plenderleith and Snow, 1993), and when characterised by their electrophysiological properties are functionally distinct from non-IB4 positive nociceptors (Stucky and Lewin, 1999). It is important to note that roughly 75% of all IB4 positive neurons also express the mas-related G-protein coupled receptor member D (Mrgprd) (Dong et al., 2001), which is particularly useful for genetic targeting of the non-peptidergic, unmyelinated C-fibre population (Cavanaugh et al., 2009; Shields et al., 2010; Zhang et al., 2013). The selective ablation of Mrgprd results in the loss of noxious mechanical but not thermal sensitivity (Cavanaugh et al., 2009; Zhang et al., 2013).

1.2.2 Spinal cord: first central hub of nociceptive activity modulation

Primary afferent nociceptors project to the dorsal horn of the spinal cord, the first key synapse of the pain pathway. The dorsal horn is a crucial site of the pain pathway, and a key structure where incoming nociceptive activity is modulated by a complex network of synaptic circuits. It is from the dorsal horn that transmission of nociceptive activity to the brain occurs, where pain is ultimately experienced. Dorsal horn activity is also subject to modulation by descending activity from the brain, which will be described later (see 1.2.3).

1.2.2.1 Central termination of primary afferents and dorsal horn circuitry

The grey matter of the dorsal horn is organised in a laminar fashion with a total of ten layers, originally described by Rexed in cat spinal cord (1954, 1952) (Figure 1.3). This

organisation is conserved in the rat (Molander et al., 1989, 1984), and includes a somatotopic distribution which relates to peripheral innervations of primary afferent fibres (Molander and Grant, 1986; Swett and Woolf, 1985). The majority of primary afferents terminate in the dorsal horn ipsilaterally, although some may project contralaterally (Culberson et al., 1979).

Central terminals of Aδ and C-nociceptors terminate in the most superficial layers of the dorsal horn, laminae I-II. Peptidergic C and Aδ-fibres terminate in laminae I-II outer (IIo), while non-peptidergic IB4 positive C-fibres, the majority of which can also be identified as Mrgprd positive, terminate primarily in inner lamina II (Iii) (Lorenzo et al., 2008; Snider and McMahon, 1998; Todd, 2010). SP-expressing nociceptors terminate within lamina I and IIo. Additionally, a small subset of unmyelinated C-fibres conveying tactile cutaneous sensory input, called C low-threshold mechanoreceptors (LTMR) have been identified, and terminate in lamina II (Liu et al., 2007; Seal et al., 2009). Deeper laminae IIi-V are primarily occupied by LTMR arborisations; specifically, Aβ tactile and hair follicle afferents primarily terminate within laminae III-V, and Aδ hair follicle afferents terminate within laminae Iii-III (Brown et al., 1981; Light and Perl, 1979; Shortland et al., 1989; Todd, 2010) (Figure 1.3). The majority of cells in lamina V are wide-dynamic range neurons that receive polymodal input and respond to all somatosensory modalities including mechanical, thermal and chemical stimuli (Mendell, 1966).

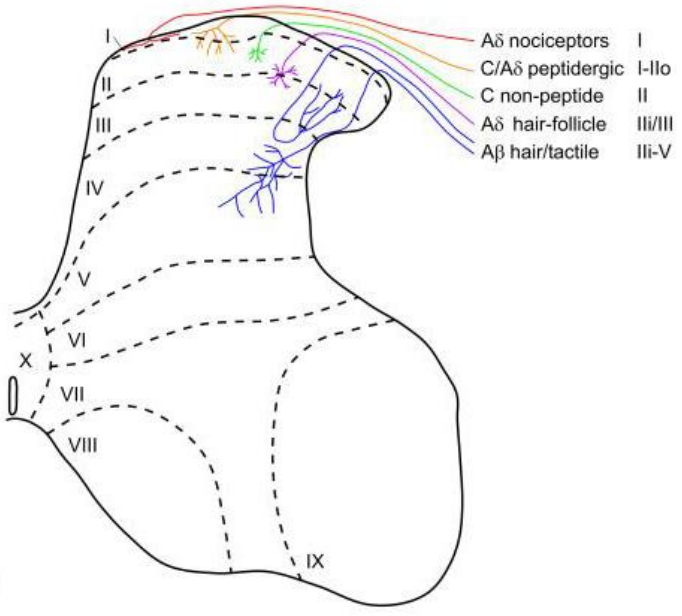


Figure 1.3 Termination of primary afferent fibres in the dorsal horn of the spinal cord. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Vol 11, Todd, A.J., Neuronal circuitry for pain processing in the dorsal horn, pp. 823–836, copyright (2010).

Interneurons

The majority of primary afferents synapse onto interneurons, which have local dendritic arborisations and axon projections within dorsal horn laminae I-III (Todd, 2010). Interneurons are either inhibitory or excitatory depending on their main neurotransmitter, which is glycine and/or GABA, or glutamate, respectively. In the rat spinal cord, GABA is present in 25 and 30% of lamina I and II neurons, respectively (Polgár et al., 2003), and glycinergic neurons in laminae I-II predominantly co-express with the GABAergic population (Polgár et al., 2003; Todd and Sullivan, 1990). Due to the complexity of spinal cord circuitry, it is likely that all spinal cord neurons receive primary afferent input, as well as input from both inhibitory and excitatory interneurons (Todd, 2010).

Interneurons constitute the majority of neurons in lamina I and III, and nearly all of the neurons in lamina II. Indeed, lamina II interneurons have been the most extensively studied. Lamina II interneurons can be categorised based on their neurochemical identity, dendritic morphology (islet, radial, central, vertical neurons), or electrophysiological properties in response to stimuli (Grudt and Perl, 2002; Todd, 2010). Islet and central cells are innervated primarily by C-fibres; radial and vertical cells are innervated by C and Aδ-fibres. While there are correlations between interneuron dendritic morphology and neurotransmitter identity (GABA/Glycinergic, Glutamatergic), this is not completely straightforward. Further, there is little correlation between dendritic morphology and firing patterns; firing pattern is more closely correlated with neurotransmitter (Todd, 2010); for example, Kv4 mediated A-type potassium currents can be found exclusively in glutamatergic interneurons (Yasaka et al., 2010).

When using immunohistochemistry to identify interneurons, it is difficult to achieve immunostaining for GABA or glutamatergic cell bodies. Therefore, recent studies have taken to identifying interneurons using a neurochemical approach. Neuropeptide Y (NPY), galanin, parvalbumin (PV), and nNOS are four neurochemical markers known to label GABAergic interneurons, with the latter two also labelling cotransmitting GABA and glycinergic interneurons (Laing et al., 1994; Polgár et al., 2011; Sardella et al., 2011; Tiong et al., 2011). The appeal of using these four markers is that they are easily identifiable using IHC, and are non-overlapping populations with some functional distinctiveness in response to stimuli (Polgár et al., 2013). Conversely, the proteins somatostatin (SST), neurotensin, calretinin, SP, NKB, and for the most part calbindin and PKCγ can be used to label glutamatergic excitatory interneurons. PKCγ in particular is expressed by a set of neurons in lamina II inner and lamina III; these cells

receive input from low threshold mechanoreceptors (Lu et al., 2013; Malmberg et al., 1997; Miraucourt et al., 2007; Neumann et al., 1996).

Lamina I interneurons are less well-studied, although they have also been identified by morphology (fusiform, pyramidal, multipolar) (Han et al., 1998; Lima and Coimbra, 1986). Notably, however, this classification does not distinguish interneurons from the very important lamina I projection neuron population (Todd, 2010).

Projection Neurons

Activity from the dorsal horn is relayed supraspinally via projection neurons, many of which have axons that cross the midline and project rostrally through the spinal white matter to key brain areas involved in pain processing. The majority of lamina I projection neurons respond to noxious stimulation, although a few respond to innocuous cooling (Andrew, 2009; Bester et al., 2000; Han et al., 1998; Willis et al., 1974; Zhang and Giesler, 2005).

Lamina I contains a higher density of projection neurons than that observed in any other laminae of the spinal cord expressing projection neurons (III-VI) (Todd, 2010). However, to gain perspective of this limited population of neurons, it has been shown that only 5% of all lamina I neurons are projection neurons (Bice & Beal 1997b; Bice & Beal 1997a; Spike et al. 2003; Al-Khater & Todd 2009).

Neurochemically, the neurokinin-1 receptor (NK1) has long been of interest due to the fact that 80% of lamina I projection neurons are known to express this receptor. NK1 however, is not restricted to projection neurons and is found to also label excitatory interneurons albeit to a lesser degree than projection neurons (Al Ghamdi et al., 2009; Littlewood et al., 1995). Importantly, toxin-conjugated ablation techniques have revealed that NK1 positive projection neurons are known to play a key role in neuropathic and inflammatory pain processing (further discussed in Chapter 4) (Mantyh et al., 1997; Nichols et al., 1999). NK1 projection neurons are also present in laminae III-IV, and have dendrites that project superficially into lamina I (Naim et al., 1997; Todd et al., 2000)

A class of NK1 negative projection neurons, called giant cells, are sparse in existence (1-2% of lamina I projection neurons) and are predominantly spinothalamic (Polgár et al., 2008; Puskár et al., 2001). Supraspinal targets of projection neurons will be discussed below in 1.2.2.2.

Inputs to Projection Neurons

Studies have found that lamina I NK1 positive projection neurons receive monosynaptic glutamatergic C and Aδ-fibre input (Torsney and MacDermott, 2006) from peptidergic primary afferents, the majority of which contain SP (Lawson et al., 1997; Naim et al., 1997; Todd et al., 2002) (Figure 1.4). Importantly, after peripheral inflammation, monosynaptic Aδ-fibre input is increased, contributing to C-fibre-induced heterosynaptic facilitation at lamina I NK1 positive neurons, and potentially underlying states of hypersensitivity (Torsney, 2011).

NK1 negative lamina I giant cell projection neurons do not receive much direct input from peptidergic primary afferents. They do however, have a high density of both glutamatergic and GABAergic synapses, labelled by the vesicular glutamate transporter 2 (VGLUT2) and nNOS/GABA, respectively (Polgár et al., 2008), supporting the finding that lamina I neurons are under permanent GABAergic interneuron control (Dahlhaus et al., 2005; Seagrove et al., 2004).

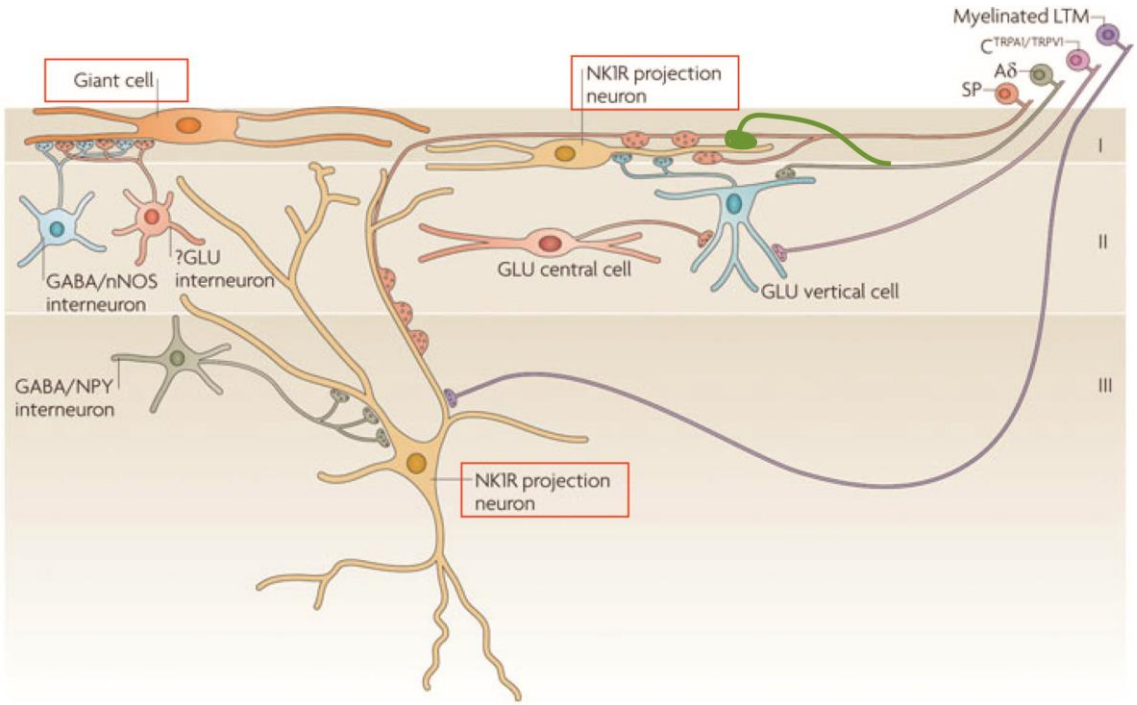


Figure 1.4 Inputs to projection neurons. Although describing only a limited picture of dorsal horn circuitry, the figure above indicates the major inputs to lamina I and III NK1 projection neurons (~80% of lamina I projection neurons are NK1 positive). The great majority of input to non-NK1 projection neurons is from polysynaptic Aδ- and Aβ-fibres (not pictured) (Torsney and MacDermott, 2006). Red boxes indicate projection neurons. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Vol 11, Todd, A.J., Neuronal circuitry for pain processing in the dorsal horn, pp. 823–836, copyright (2010).

1.2.2.2 Ascending projections/pathways and supraspinal centres

Projection neurons can be identified using the injection of retrograde tracers into supraspinal targets, a technique first utilised by Trevino and Carstens (1975) and currently along with anterograde tracing, the most reliable way to confirm a neuronal projection neuron identity. Importantly, experiments identifying lamina I projection neurons using the retrograde tracer technique have shown that there are roughly 400 projection neurons on each side of the spinal cord within the lumbar L4 segment (Al-Khater and Todd, 2009). In general, the ascending circuits originating in lamina I projection neurons differ from those originating in deeper laminae III-V (Gauriau and Bernard, 2002).

A major target of lamina I projection neurons is the lateral parabrachial area (LPb), which contains neurons that subsequently project to the amygdala and hypothalamus of the limbic system. In the rat, 95% of lamina I projection neurons in the lumbar L4 region terminate in the LPb (Al-Khater et al., 2008; Spike et al., 2003; Todd et al., 2000). Additionally, the other main targets of lamina I projection neurons, include the caudal ventrolateral medulla (CVLM), nucleus of the solitary tract (NTS), the periaqueductal grey matter (PAG), and lateral thalamus/thalamic nuclei including the ventral posterior nuclei (including VPL, VPM), which project to the somatosensory and insular cortices (Al-Khater et al., 2008; Gauriau and Bernard, 2004) (Figure 1.5). Projection neurons in deeper laminae III-IV have also been found to project to the thalamus, primarily to the central lateral nucleus in the medial (anterior) thalamus which subsequently sends projections to the anterior cingulate cortex (ACC) (Gauriau and Bernard, 2004; Marshall et al., 1996; Naim et al., 1997). The majority (>90%) of lamina III projection neurons in L4 have been found to project to the contralateral reticular nuclei, and a little more than half to the LPb (Todd et al., 2000). The range of supraspinal projection neuron targets indicates the diversity of structures involved in the pain response, including those responsible for emotional/affective processing (limbic structures amygdala, hypothalamus, insular cortex) and sensory/discriminative processing (somatosensory cortex). Moreover, tracer studies of projection neurons have indicated that ascending projections to the brain exhibit extensive collateralisation, meaning that each fibre projects to more than one target (Al-Khater and Todd, 2009). The majority of these projections project contralaterally, although some do project bilaterally (Spike et al., 2003).

Figure 1.5 Supraspinal pathways to and from the dorsal horn.

Supraspinal centres

Dorsal horn projection neurons allow activity generated by nociceptive transmission to reach the brain, where perception of the pain experience occurs. Studies utilising human functional neuroimaging techniques have greatly enhanced our knowledge of brain structures activated during noxious stimulation. In particular, nociceptive stimulation has been found to elicit activity from a network of structures, including the primary and secondary somatosensory (S1/S2), insular, anterior cingulate (ACC), and prefrontal (PFC) cortices, and thalamus (Apkarian et al., 2005; Tracey and Mantyh, 2007; Treede et al., 1999) (Figure 1.5). Many studies have implicated the existence of a 'pain matrix,' a group or signature of structures specific to processing of noxious stimuli (Tracey and Mantyh, 2007). However, it has been shown that these structures demonstrate activity in response to a variety of sensory modalities rather than exclusively to nociceptive somatosensory stimulation (Mouraux et al., 2011). Thus, a more recent theory has proposed that the cortical areas activated during noxious stimuli represent the recognition of a general threat occurrence to 'any behaviourally relevant or salient stimulus,' including important responses such as nociception, rather than a network specifically attributed for a nociceptive specific response alone (Iannetti and Mouraux, 2010; Legrain et al., 2011; Mouraux et al., 2011). Moreover, evidence also indicates that the increased activity of brain areas observed during a nociceptive event is contextually dependent, and that perceived pain intensity can be dissociated

from the magnitude of brain response activity, which further supports the role of brain responses that are less tuned to sensory modality but to the response generated in the presence of potential harm (Iannetti and Mouraux, 2010); for example, stress and arousal.

1.2.3 The descending pathway and pain modulation

The existence of an endogenous inhibitory pain modulation system has long been observed, with some of the earliest studies indicating that affective components such as stress or anxiety had an effect on the degree of pain reported, necessity for pain relief, or latency to report pain following injury (Beecher, 1946; Melzack et al., 1982). It is now generally accepted that a top-down modulation of pain exists in the form of a number of descending circuits from the brain to the spinal cord, which has provided a mechanism through which the brain can enhance or suppress dorsal horn activity and the overall pain response (Figure 1.5). In particular, one example that has provided evidence for the top-down modulation of the pain response is the activation of cortical structures during placebo (Petrovic et al., 2002). The inhibitory system in particular is activated by situations inducing stress, primarily through the endogenous opioidergic system (Siegfried et al., 1990; Terman et al., 1984).

The majority of the descending circuits in the brain converge upon the periaqueductal grey matter (PAG) (Figure 1.5). In particular, the PAG receives input from the ACC, amygdala, and hypothalamus. Rather than sending direct projections to the spinal cord, the majority of output from the PAG projects to the rostral ventromedial medulla (RVM) (Hunt and Mantyh, 2001). Historically, the role of the PAG in pain modulation was first identified through the specific injection of opiates [morphine] into the PAG, an action that produced antinociceptive effects in the rabbit (Tsou and Jang, 1964). Further studies indicated that direct electrical stimulation of the PAG produced profound analgesia in the rat (Reynolds, 1969), and importantly, the application of naloxone reversed the effect of stimulation-produced analgesia which indicated the presence of an endogenous inhibitory system mediated by opioids (Hosobuchi et al., 1977). Not surprisingly, the PAG is an important pharmacological target for opiate-mediated analgesia (Herz et al., 1970; Yaksh et al., 1976).

The RVM is inclusive of the nucleus raphe magnus (NRM) and the nucleus reticularis gigantocellularis (GiA), and projects a descending modulatory influence upon the spinal cord (Basbaum and Fields, 1979; Fields et al., 1977). The delivery of lidocaine in the RVM temporarily reverses allodynia and hyperalgesia induced as a result of nerve injury, and has been implicated in the maintenance but not induction of neuropathic pain (Burgess et al., 2002; Vera-Portocarrero et al., 2006). Importantly however, the

RVM can have bidirectional inhibitory and facilitatory descending influences on spinal nociception (Urban and Gebhart, 1999), activity which has been observed during direct electrical stimulation applied to the RVM. In general, low intensities of electrical stimulation have been shown to facilitate spinal nociception, while high intensities have an inhibitory effect (Urban and Gebhart, 1997; Urban and Smith, 1993; Zhuo and Gebhart, 1997, 1992).

Crucially, the importance of the spino-bulbo-spinal circuit has been highlighted in nociception through studies indicating that projection neurons are essential to the full activation of molecular pathways in the superficial dorsal horn, via activation of descending regulatory controls (Géranton et al., 2008; Suzuki et al., 2002). Furthermore, more recently, the activation of descending inhibition has been shown to be engaged following nerve injury, and may be an explanation for understanding why nerve injury develops into chronic pain in certain subjects (De Felice et al., 2011). Descending modulation will also be discussed in Chapter 5.

1.3 States of sensitisation

The nervous system is extremely plastic and can undergo many functional changes as a result of persistent activity. These activity-induced changes can contribute to a range of processes, including cognitive processes such as memory formation and abnormal psychiatric states such as depression, schizophrenia, as well as neural function related to sensory processes such as visual cortical development (Espinosa and Stryker, 2012; Kandel et al., 2014; Tsankova et al., 2007). Activity-induced plasticity of neural function also occurs in nociceptive processing (Kuner, 2010; Latremoliere and Woolf, 2009; Scholz and Woolf, 2002; Woolf and Ma, 2007; Woolf and Salter, 2000).

Importantly, the protection provided by nociceptive processing is enhanced by a process called sensitisation. Specifically, plasticity of the nociceptive system manifests in peripheral and central components, which occurs following repeated or intense stimulation, and leads to a reduction in nociceptive thresholds, amplified neural signal, and subsequent behavioural hypersensitivity (Latremoliere and Woolf, 2009; Woolf and Salter, 2000). Sensitisation is thus an adaptive response and also a key hallmark of the nociceptive process; in normal nociceptive processing it can be long-lasting but only through to the point of injury healing. Importantly, sensitisation also underlies diseased or pathological pain states, such as chronic inflammatory or neuropathic pain, and is also responsible for pain states persisting following injury healing or without any apparent injury, such as fibromyalgia.

1.3.1 Peripheral sensitisation

Nociceptor plasticity occurs as a result of activity resulting from peripheral inflammation or tissue damage. This phenomenon is called peripheral sensitisation, associated with a reduction in nociceptor activation threshold which leads to increased excitability and enhanced firing to a stimulus of the same intensity, typically in C-fibres. Importantly, peripheral sensitisation contributes to the development of both inflammatory and neuropathic pain (Hucho and Levine, 2007; Reichling et al., 2013; Reichling and Levine, 2009); it primarily plays a key role in heat hyperalgesia, not mechanical hyperalgesia, the latter of which is a key hallmark of chronic pain conditions.

A number of inflammatory mediators are released by damaged tissue and immune cells, which bind to respective receptors at the nociceptor peripheral terminals and act to lower the threshold of the nociceptor and enhance the magnitude of nociceptive response. In particular, the 'inflammatory soup' released by damaged tissue includes peptides (bradykinin), lipids (prostaglandins), neurotransmitters (ATP, 5-HT) and trophic factors (NGF, GDNF), as well as protons released due to tissue acidosis (Julius and Basbaum, 2001; Woolf and Ma, 2007). Furthermore, the activation of immune system leukocytes includes the production of the cytokines tumour necrosis factor α (TNFα), interleukins (IL-1, IL-6), and chemokines, as well as the enzyme cyclooxygenase-2 (COX-2).

The binding of inflammatory mediators activates intracellular signalling cascades which play an important role in nociceptor hyperexcitability by allowing a cell to respond quickly to various extracellular stimuli (Reichling and Levine, 2009; Woolf and Ma, 2007). Signalling kinases are capable of directly modulating transducer receptor subunits on peripheral terminals, activity which can lead to the enhancement of sensory stimuli signal transduction. For example, a key mechanism is NGF-induced TRPV1 phosphorylation mediated by kinase activity, which can lead to increased receptor subunit trafficking and changes in channel kinetics (Chuang et al., 2001; Ji et al., 2002b; Zhang et al., 2005). Importantly, it has been shown that the molecular events leading to persistent chronic hyperalgesia are due to a switch in intracellular kinase signalling, from protein kinase A (PKA) signalling in acute nociception, to both PKA and protein kinase Cε (PKCε) signalling in states of chronicity. Joint signalling activity thus maintains a 'primed' state of the nociceptor which underlies an enhancement of the hyperalgesic response during subsequent injury (Reichling and Levine, 2009)

Peripheral sensitisation can also result in long-term changes due to gene transcription at the DRG cell body. Such changes involve the synthesis of neurotransmitters to

either enhance central transmission (i.e. BDNF) (Mannion et al., 1999), or the novel synthesis of neurotransmitters normally not expressed, for example, a phenotypic switch involving the synthesis of SP (Neumann et al., 1996). Alterations in ion channel synthesis can also lead to hyperexcitability, such as the increase in voltage gated calcium channels, which play a crucial role in regulating the release of neurotransmitters at the central terminal (Bourinet et al., 2014; Dolmetsch et al., 2001; Ghosh and Greenberg, 1995), or BDNF-induced decrease in potassium channel transcription post-nerve injury (Waxman and Zamponi, 2014).

The reduced threshold and hyperexcitable state of the nociceptor means that normally non-noxious stimuli are able to activate what are normally high threshold nociceptors. Increased sensitivity for activation can manifest as increases in spontaneous pain, and in rats, increased spontaneous lifting of the affected limb (Djouhri et al., 2006). Interestingly, spontaneous lifting has been attributed mechanistically to peripheral processes as lifting correlates with C-fibre activity, but not with allodynia, which is associated with central sensitisation, discussed below. The importance of peripheral activity cannot be ignored, and studies have crucially indicated that local anaesthesia is capable of attenuating hyperalgesia associated with central sensitisation in various pain models (Reichling and Levine, 2009).

1.3.2 Central sensitisation

Central sensitisation was first identified by Woolf (1983), and can be understood as “increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input” (Sandkühler, 2009). While peripheral sensitisation contributes to the initiation of central pathway sensitisation, it is restricted to fibres innervating the injured area, and maintenance typically requires ongoing peripheral input or injury. Certain aspects of pain such as secondary hyperalgesia, allodynia, and pain that is uncoupled to stimulus presence, intensity, or duration (including pain without apparent injury) cannot be accounted for by peripheral mechanisms alone (Latremoliere and Woolf, 2009). Central sensitisation recruits novel, normally non-nociceptive input to contribute to hypersensitivity associated with pain states. Central sensitisation occurs following repeated and sustained intense stimulation, and can be maintained with or without apparent injury/cause, leading to an abnormal state of responsiveness of the nociceptive system (Woolf, 1983; Woolf and Salter, 2000).

The cellular processes underlying central sensitisation can be identified as increased membrane excitability, facilitation of synaptic strength, and disinhibition of nociceptive circuits (Latremoliere and Woolf, 2009).

1.3.2.1 Induction of central sensitisation

Central sensitisation via post-synaptic mechanisms occurs as a result of noxious C-fibre stimulation that leads to the activity of a number of cell-surface receptors in dorsal horn neurons, including ionotropic AMPAR, NMDAR, and kainate receptors, as well as metabotropic glutamate receptors (mGlu), all of which are activated by glutamate which is released at central terminals of nociceptors. One of the most important consequences of postsynaptic activation is the influx of Ca^{2+}, a crucial step to the induction of intracellular signalling events.

Activation of the NMDA receptor is vital to the induction of central sensitisation (Woolf and Thompson, 1991). NMDAR in the dorsal horn are composed of mainly NR1 and NR2A/B subunits (Nagy et al., 2004). Upon sufficient membrane depolarisation resulting from central neurotransmitter-mediated activation of post-synaptic receptors, the voltage-sensitive Mg^{2+} ion normally blocking the NMDAR pore is displaced and allows for the influx of Ca^{2+} into the neuron, which subsequently leads to the activation of a number of intracellular signalling pathways that mediate the development of the sensitised state (see 1.3.2.3) (Mayer et al., 1984). Notably, intrathecal (i.t.) delivery of NMDAR antagonists (Ma and Woolf, 1995a; Woolf and Thompson, 1991), and the deletion of NR1 have been shown to prevent the development of acute (formalin) central sensitisation (South et al., 2003). Other sources of synaptic activity-induced Ca^{2+} include L-type voltage-dependent calcium channels and mGluR-IP_3 mediated release of Ca^{2+} stores (Fagni et al., 2000; Hagenston and Bading, 2011). Additionally, SP-NK1 activity has been shown to play a major role in central sensitisation, and has been shown to have sustained effects in dorsal horn activity (Henry, 1976; Khasabov et al., 2002; Mantyh et al., 1997; Ma and Woolf, 1995b). The ablation of NK1 expressing dorsal horn neurons has been shown to prevent the development of central sensitisation induced by capsaicin as observed using behavioural and electrophysiological methods (Khasabov et al., 2002; Mantyh et al., 1997; Suzuki et al., 2002).

It should be noted that dorsal horn central sensitisation can also occur as a result of presynaptic mechanisms. Luo et al. (2012) genetically targeted presynaptically localised protein kinase G-1 (PKG-1), a type I cGMP kinase crucial to intracellular signalling, which prevented the development of sensitisation in spino-PAG dorsal horn neurons. Notably, the presynaptic deletion of PKG-1 also significantly attenuated CFA-induced mechanical and thermal hypersensitivity, as well as formalin nocifensive behaviours, most likely due to regulation of the presynaptic neurotransmitter release from nociceptor central terminals.

1.3.2.2 LTP and Central Sensitisation

Similarities have been highlighted between the induction of central sensitisation- the neural correlate of plasticity underlying the generation of persistent pain states- and early long-term potentiation (LTP), a lasting increase in synaptic strength and a substrate for learning and memory processes. Both LTP and central sensitisation are key mechanisms of neural plasticity.

There is an ongoing debate regarding the use of nociceptive spinal LTP vs. central sensitisation terminology (Latremoliere and Woolf, 2009; Sandkühler, 2010). This is particularly due to the generally accepted homosynaptic nature of LTP observed in the brain (Bliss and Collingridge, 1993; Lee et al., 2009), whereas heterosynaptic facilitation has been cited as one of the distinguishing features of spinal cord LTP (Figure 1.6). Recently, both homo and heterosynaptic potentiation have been suggested to occur at GABAergic and C-fibre synapses in the dorsal horn (Fenselau et al., 2011), as well as in lamina I NK1 neurons (Torsney, 2011). Importantly, Torsney (2011) has shown that inflammation can induce heterosynaptic facilitation of silent Aδ-fibre monosynaptic input to these key lamina I NK1 neurons, many of which project supraspinally (Torsney, 2011). Indeed, homosynaptic potentiation in the dorsal horn could contribute to primary hyperalgesia (Ikeda et al., 2006, 2003), but two major features of the behavioural pain response include secondary hyperalgesia and allodynia, both of which require heterosynaptic facilitatory mechanisms. It should be noted that both homo and heterosynaptic potentiation of cortical synapses has also been observed, and it has been suggested that LTP can be induced at most, if not all synapses in the CNS (Sandkühler and Gruber-Schoffnegger, 2012). Interestingly, although dendritic spine remodelling associated with inflammation-induced hypersensitivity has been observed in the dorsal horn (Simonetti et al., 2013), it does not appear to occur to the same extent as the high density of dendritic spines often promoted by hippocampal LTP (Bosch and Hayashi, 2012; Cingolani and Goda, 2008), which could perhaps influence the nature of synaptic potentiation.

While there are indeed differences in the location, and nature (synaptic-i.e. excitatory/inhibitory; potentiation-i.e. homo or heterosynaptic) of LTP observed in the CNS, the long-term persistence of increased synaptic strength is a common feature.

Importantly, studies have indicated that one major similarity between central sensitisation and early-LTP are the post-synaptic signalling events involved in their induction and maintenance. In particular, central sensitisation and LTP share the same post-translational regulation of AMPA and NMDA receptors and intracellular signalling cascades such as the p44/42 extracellular signal-regulated kinase/mitogen-activated

protein kinase (ERK/MAPK) pathway leading to transcription of immediate early gene programmes such as *Zif268* and *CREB* (Ji et al., 2003). Moreover, it has been suggested that the molecular events underlying the synaptic plasticity observed in nociception and memory formation are evolutionarily conserved (Rahn et al., 2013).

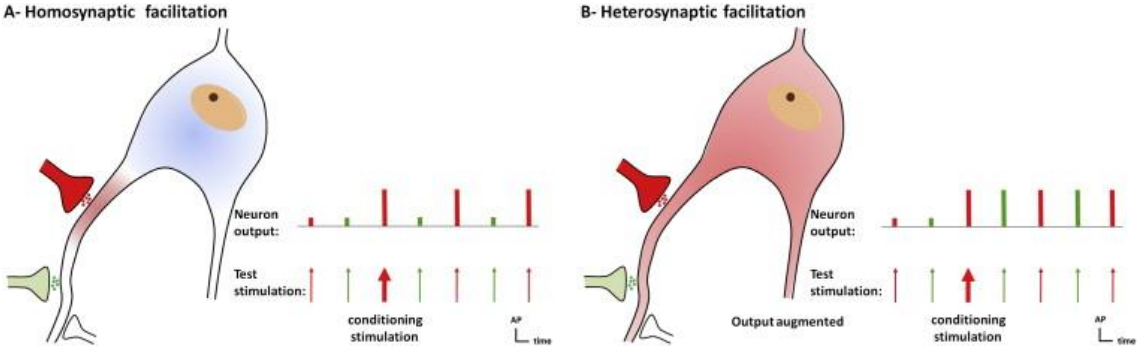


Figure 1.6 Homo vs. heterosynaptic facilitation. Central sensitisation in the dorsal horn is primarily due to heterosynaptic facilitation (right); following activity-dependent activation (red synapse), facilitation can spread to nearby synapses (green) which can also demonstrate enhanced response. Homosynaptic facilitation (left) prevails in hippocampal LTP. Reprinted from The Journal of Pain, Vol 10, Latremoliere A. and Woolf C.J., Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity, pp. 895-926, Copyright (2009), with permission from Elsevier.

1.3.2.3 Molecular mechanisms of central sensitisation

A number of post-synaptic molecular events are involved in developing the key attributes of neural activity in central sensitisation, which can be distinguished temporally by short-term and long-term changes. Similarly to peripheral sensitisation, short-term immediate effects involve largely post-translational alterations, whereas evidence of gene transcription is indicative of long-term changes.

Intracellular signalling

Ca^{2+} influx is particularly important for the activation of cytoplasmic kinase signalling events involved in central sensitisation, such as the activation of calcium calmodulin dependent kinase II (CaMKII), PKA, PKC, and extracellular signal-regulated kinase (ERK) (Fagni et al., 2000; Pezet et al., 2008).

Activation of kinase signalling molecules leads to the post-translational modification of membrane receptors which increase the excitability of the post-synaptic neuron. Specifically, PKA phosphorylates the GluR1 subunit of the α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), and PKC phosphorylates residues of the GluR1, GluR2, and NR1 subunits (Carvalho et al., 2000; Chen and Roche, 2007) which can lead to subunit turnover, trafficking of new subunits, and ultimately structural and functional changes in the receptor (Carvalho et al., 2000; Lau and Zukin, 2007).

Studies have shown that after acute inflammatory injury, a switch occurs in the dorsal horn for the preferential expression of AMPAR GluR1 subunits in place of GluR2, to permit the influx of Ca^{2+} which is not possible in the presence of the Ca^{2+} impermeable GluR2 subunit (L. Fang et al., 2003; Li Fang et al., 2003; Larsson and Broman, 2008; Vikman et al., 2008). Moreover, the AMPAR GluR1 subunit is also targeted post-translationally by CaMKII. PKA, PKC, and CaMKII signalling are known to converge upon ERK, which causes increased excitability of dorsal horn neurons (Hu et al., 2003; Ji et al., 2009). ERK activity is required for the phosphorylation of the NR1 subunit of NMDAR following capsaicin or direct application of BDNF (Slack et al., 2004; Zou et al., 2000). ERK has also been shown to mediate the phosphorylation of the potassium channel Kv4.2 in the dorsal horn, which leads to increased excitability by inhibiting A-type K+ currents and decreasing K+ efflux which typically regulates action potential firing (Hu et al., 2007).

ERK/MAPK signalling for longer-term changes in nuclear activity

The p44/42 extracellular signal-regulated kinase (ERK) is one of three members of the mitogen-activated protein kinase (MAPK) family, and regulates a multitude of cellular functions including growth, differentiation, apoptosis, and very importantly, gene expression (Widmann et al., 1999). ERK comes in two forms: ERK1 (p44) and ERK2 (p42), which are similarly activated and are often described together as ERK. Canonical ERK signalling occurs as a result of extracellular growth factor activation of Ras G-protein-coupled receptor tyrosine kinases. Downstream of the Ras, Raf, and MEK kinases, ERK is activated (phosphorylated) at threonine 202 and tyrosine 204 residues (Figure 1.7).

PERK regulates downstream signalling that can result in nuclear activity leading to gene transcription and the synthesis of new protein. Translocation of PERK into the nucleus is essential to the activity of nuclear proteins including the phosphorylation of the nuclear mitogen and stress-activated protein kinase 1 (PMSK1) (Deak et al., 1998), and cAMP response element-binding protein (PCREB) in dorsal horn neurons following noxious stimulation, particularly in NK1 positive neurons (Anderson and Seybold, 2000; Ji and Rupp, 1997; Kawasaki et al., 2004). CREB is a well-known transcription factor that binds to regulatory CRE-binding sites embedded within specific genes, and is a key regulator of long-term neural plasticity (Impey et al., 1996; Lonze and Ginty, 2002). In the case of nociception, CREB regulates the activity-induced expression of immediate early genes (IEG) such as *Zif268*, *Arc*, and *c-Fos*, as well as late-response genes key to nociception and maintenance of central sensitisation, such as those encoding NK1, prodynorphin, or TrkB (Ji et al., 2002a). Importantly, PERK is widely used as a marker of neuronal activation by noxious stimuli in the spinal cord and brain

(Gao and Ji, 2009; Ji et al., 2009, 1999), and its role in nociception will be discussed in Chapter 3.

Notably, activation of the canonical ERK/MAPK cascade is not the only means of transducing synaptic activity to the nuclear compartment. Elevated Ca^{2+} can alternatively activate CaMKI or CaMKIV kinases, which are capable of CREB phosphorylation. It has been demonstrated that CREB-dependent transcription (inclusive of canonical ERK-CREB transcription) requires activation of its co-transcription factor, CREB binding protein (CBP). This can only be accomplished via generation of nuclear Ca^{2+} signalling, which is mediated by CaMKIV (Chawla et al., 1998; Hardingham et al., 1999; Impey et al., 2002). Notably, nuclear Ca^{2+} activity has been shown to regulate inflammation-induced gene transcription in the dorsal horn (Simonetti et al., 2013).

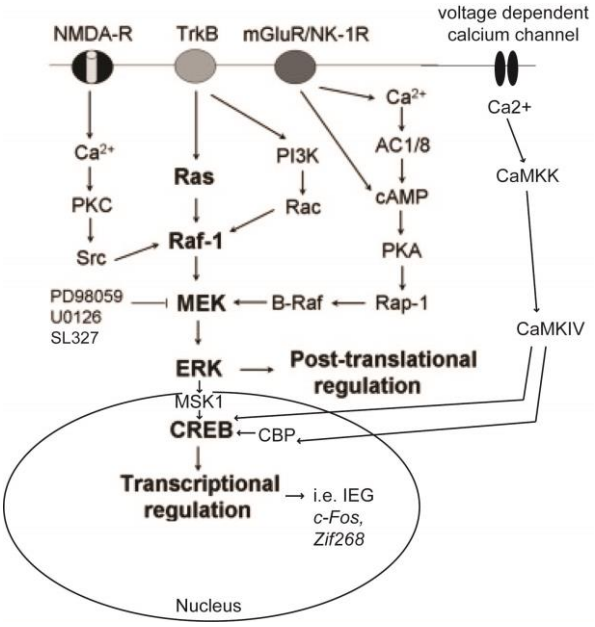


Figure 1.7 Diagram of signalling events leading to CREB-mediated gene transcription, including ERK/MAPK. Adapted from Brain Research Reviews, Vol 60, Ji R.R., Gereau R.W., Malcangio M., Strichartz G.R., MAP kinase and pain, pp. 135-148, Copyright (2009), with permission from Elsevier.

ERK/MAPK and neuronal plasticity

It is well established that ERK signalling plays an important role in neural plasticity, and PERK is used as a marker of neural plasticity and activation in the CNS (Impey et al., 1999). Physiology and behavioural studies investigating the role of ERK in neuronal signalling have made great advances due to the synthesis of the MEK inhibitor compounds PD98059, U0126 and SL327. English and Sweatt were the first to implicate a role for ERK/MAPK in neural plasticity through the use of PD980589 to

block NMDA-activated hippocampal long-term potentiation (LTP) (English and Sweatt, 1997). The group were subsequently the first to establish a behavioural role for ERK/MAPK in long-term memory formation using both cued and contextual fear conditioning paradigms (Atkins et al., 1998). Perhaps not surprisingly, studies have subsequently highlighted the importance of ERK/MAPK signalling in nociceptive plasticity, and the aforementioned MEK inhibitors have also been used toward the attenuation of injury-induced nociceptive hypersensitivity (Ji et al., 2009). For further information on the role of ERK/MAPK in nociceptive plasticity, please refer to 3.1.2.

1.4 Animal models of pain

Measuring pain in humans- particularly chronic pain- is incredibly complex because it is not simply limited to alterations in sensory thresholds, but also characterised by spontaneous pain and includes a substantial emotional-affective component (Tappe-Theodor and Kuner, 2014). Moreover, understanding human pain is often most reliable through personal communication and self-reporting, a measurement which is largely subjective and obviously, unattainable in animals (Mogil, 2009). The translational applicability of animal studies to better understanding human pain has been debated (Langley et al., 2008); in particular, considerations of ethics, and the applicability of both assay (i.e. applicability to human aetiology of pain) and behavioural measurements utilised have been highlighted (Mogil, 2009). However, due to the broad similarities in nociceptive processing in human and rodents (Mogil, 2009), animal models represent a crucial first step toward basic understanding of the underlying mechanisms of human pain. Specifically, animal models allow for investigation into the causal or correlative links between neurobiology and measures of nociceptive behaviour. Although there are nociceptive, inflammatory, neuropathic, and idiopathic pain assays, only inflammatory assays were utilised in this thesis.

1.4.1 Inflammatory pain assays

The generation of inflammation is used to study inflammatory pain, typically by injecting agents into the cutaneous tissue of the animal hindpaw which activate primary afferent fibres that project to the lumbar region of the dorsal spinal cord, in particular L4-L6 (Le Bars et al., 2001).

Commonly injected compounds include agents that activate the immune response such as Complete Freund's Adjuvant (CFA) and carrageenan (Honoré et al., 1995; Stein et al., 1988), which produce longer-lasting inflammatory hypersensitivity that can persist for days. Alternatively, some short-term cutaneous inflammatory models include direct injection of endogenous inflammatory molecules such as prostaglandin, bradykinin, or cytokines (Dina et al., 2008; Ferreira et al., 1978). Other less clinically relevant but

widely used models to generate hindpaw inflammation includes injection of a dilute solution of formalin, a cross-linking fixative (Dubuisson and Dennis, 1977), or capsaicin (Gilchrist et al., 1996), to directly activate TRPV1 receptors. Although a variety of inflammatory agents exists, they are chosen primarily because they generate thermal and mechanical hypersensitivity, as well as other measurable nocifensive behaviours.

1.4.2 Formalin

The formalin stimulus is described as 'moderate, continuous pain generated by injured tissue' that induces 'tonic' pain which manifests a set formalin response-related behaviours (Tjølsen et al., 1992). Moreover, formalin-induced injury involves components of peripheral inflammatory as well as spinal dorsal horn central nervous system mechanisms (Tjølsen et al., 1992). However, considering its working function as a tissue fixative, formalin as a noxious stimulus is probably not mediated by a specific receptor, although it does act as an agonist for TRPA1 (McNamara et al., 2007).

For the great majority of experiments in this thesis (with the exception of *Experiment 2*, Chapter 3) I chose to use the rat formalin model of acute inflammation, for a number of reasons. At higher concentrations (2.5% vs. 0.5%) formalin is known to cause broad activation of all primary afferents including Aβ, Aδ and C-fibres in the first phase response (Puig and Sorkin, 1996). This aspect could be considered advantageous compared to the capsaicin model which selectively activates C-nociceptors. Moreover, the time course of the formalin model is short in duration (1h) and ideal for quick screening of therapeutic compounds. One final main advantage of the formalin model is that it generates a biphasic response of nocifensive behaviours which include spontaneous behaviours licking, flinching, and lifting (Dubuisson and Dennis, 1977). While the exact mechanisms of formalin-induced behaviour and molecular targets are still not entirely known, it is of general agreement that the first phase (first 10min) of the response is a result of peripheral nociceptor activation, while the second phase (20-60min) is indicative of central sensitisation in spinal cord circuits following on from peripheral nociceptor sensitisation (Coderre and Melzack, 1992; Dickenson and Sullivan, 1987) maintained by sustained Aδ and C-fibre activity (Dubuisson and Dennis, 1977; Puig and Sorkin, 1996; Tjølsen et al., 1992). Chapters 3 and 6 will address the formalin assay in-depth.

1.4.3 Measuring nociceptive behaviour

Importantly, a number of pain-related nociceptive animal behaviours can be measured, including evoked reflexive measures, spontaneous, operant, and complex affective behaviours (i.e. stress, anxiety, social interaction) (Mogil, 2009).

Importantly, considering that human pain is not limited to the testing of evoked sensory thresholds, it has been suggested that measurement of voluntary and spontaneous behaviours should be investigated in addition to stimulus-dependent evoked behaviour (i.e. traditional mechanical or thermal threshold testing such as using von Frey hairs or Hargreaves method). A focus on spontaneous behaviours is particularly important due to the fact that human chronic pain conditions are often characterised by ongoing pain which can manifest in spontaneous episodes. In particular, spontaneous measurements such as gait analysis/ weight bearing, flinching, guarding, licking, and lifting are considered to be simple reflexes that, although laborious, are the most reliable and objective to quantify (Mogil, 2009). Furthermore, slightly more complex operant measures such as conditioned place preference/avoidance can be utilised to measure ongoing states of pain (Vierck et al., 2008).

Finally, animal behaviours that reflect quality of life have increasingly received attention, and include measures related to pain-related affective states such as anxiety, depression or social interaction (Blackburn-Munro, 2004). These tests can include measurements in activity, such as home cage behaviours or paradigms such as open field, wheel running, burrowing, and measures of social interaction (Tappe-Theodor and Kuner, 2014). Although it is not often realistic to measure all aspects of pain-related behaviours, observation across a range of relevant animal behaviours, particularly in addition to the most often utilised evoked measurement of hypersensitivity, is ideal.

1.5 Neuroepigenetics

The term epigenetics literally refers to molecular events occurring above or upon (‘epi’ in greek) the DNA sequence. Conrad Waddington was the first to introduce the ‘epigenetic landscape’ to illustrate the genome-environment interactions leading to phenotypic variation. Specifically, epigenetics proposed a solution to the conundrum of how a diversity of cell-types (i.e. muscle cell vs neuron) could arise from a single cell during development (Waddington, 1942). Today, the word epigenetics is commonly used to describe phenotypic differences between individuals that are independent from their genetic code. A very good example of this concept is the case of genetically identical twins that over a lifetime can show different vulnerability to diseases.

At the molecular level, epigenetic mechanisms are responsible for a set of modifications that regulate the structure of chromatin- the combination of DNA, histones, and other proteins that make up chromosomes (Figure 1.8 A). These modifications lead to the dynamic remodelling of chromatin, which affects the differential expression of genes without altering the DNA sequence itself. Epigenetic

mechanisms manifest as chemical marks (i.e. DNA methylation, histone modification), the sum of which are referred to as the epigenome. Although originally discovered as mechanisms regulating cell division and development, it is now widely accepted that epigenetic mechanisms can be engaged by environmental experience, such as stress, learning, or addiction (Borrelli et al., 2008; Renthal and Nestler, 2008; Weaver et al., 2004), indicating that activity-dependent experiences can be imprinted onto the fixed DNA sequence in the form of the epigenome (Day and Sweatt, 2011; Lester et al., 2011; Sweatt, 2013). Consequently, the emerging field of 'neuroepigenetics'- also called 'behavioural epigenetics', refers to epigenetic mechanisms and processes that allow experience-dependent regulation of the epigenome specifically in neurons (Day and Sweatt, 2011; Sweatt, 2013) and has important implications for post-mitotic neural function in the central nervous system. Importantly, epigenetics is a means of refining neural activity according to experience, and allows for life-long biological changes in both gene expression and behaviour as a result of environmental influence.

1.5.1 Epigenetic mechanisms

Epigenetic regulation involves a variety of mechanisms including histone modification, DNA methylation, non-coding RNA activity (i.e. microRNAs) and retrotransposable elements (Landry et al., 2013; Rajasethupathy et al., 2012; Sweatt, 2013). This thesis is only focused on histone modifications, which are described below. Histone modifications and DNA methylation are well-known to interact, and therefore DNA methylation will be briefly introduced.

1.5.1.1 Histone modifications

The nucleosome is the basic building block of chromatin, and is composed of 147 base pairs of DNA wrapped around an octamer of histone proteins, which contains two copies each of H2A, H2B, H3 and H4 (Figure 1.8 B). The N-terminal tails of histones protrude from the nucleosome, and are highly accessible and subject to post-translational modifications, such as lysine acetylation (Ac), serine/threonine phosphorylation (P), lysine/arginine methylation (single methyl = Me, dimethyl = Me2, trimethyl = Me3), histone ubiquitination and ADP-ribosylation. These modifications are particularly abundant at sites on histones H3 and H4 (Figure 1.8). Histone modifications and their catalysing enzymes have been the subject of many reviews (Cheung et al., 2000; Kouzarides, 2007; Strahl and Allis, 2000; Zhou et al., 2011).

Histone modifications regulate a significant amount of control over chromatin dynamics, in particular due to the presence of electronegative acetyl or phospho groups, which decrease the affinity between histone proteins and negatively charged DNA. This activity contributes to the overall relaxation of chromatin and thus increased access of

transcriptional machinery leading to active transcription (Figure 1.8 B). In general, phospho and acetyl modifications serve as indicators of active gene transcription, while many methylated histone marks indicate transcriptional silencing (Figure 1.9). However, histone methylation is not entirely straightforward as it can also indicate active gene expression, for example Me3H3K4 is a marker of gene activation, whereas Me2H3K9 and Me3H3K9 are markers of silencing. Furthermore, the interpretation of histone marks is not straightforward since the presence of both active and repressive marks can leave certain parts of DNA in a 'poised' state, susceptible to subsequent activity-induced changes. Finally, some histone marks are known to participate in 'crosstalk' (Molina‐Serrano et al., 2013) and the combinatorial sum of DNA methylation and histone modifications can jointly affect recruitment and binding of chromatin binding complexes (such as RNA Pol II) (Jenuwein and Allis, 2001; Meaney and Ferguson-Smith, 2010).

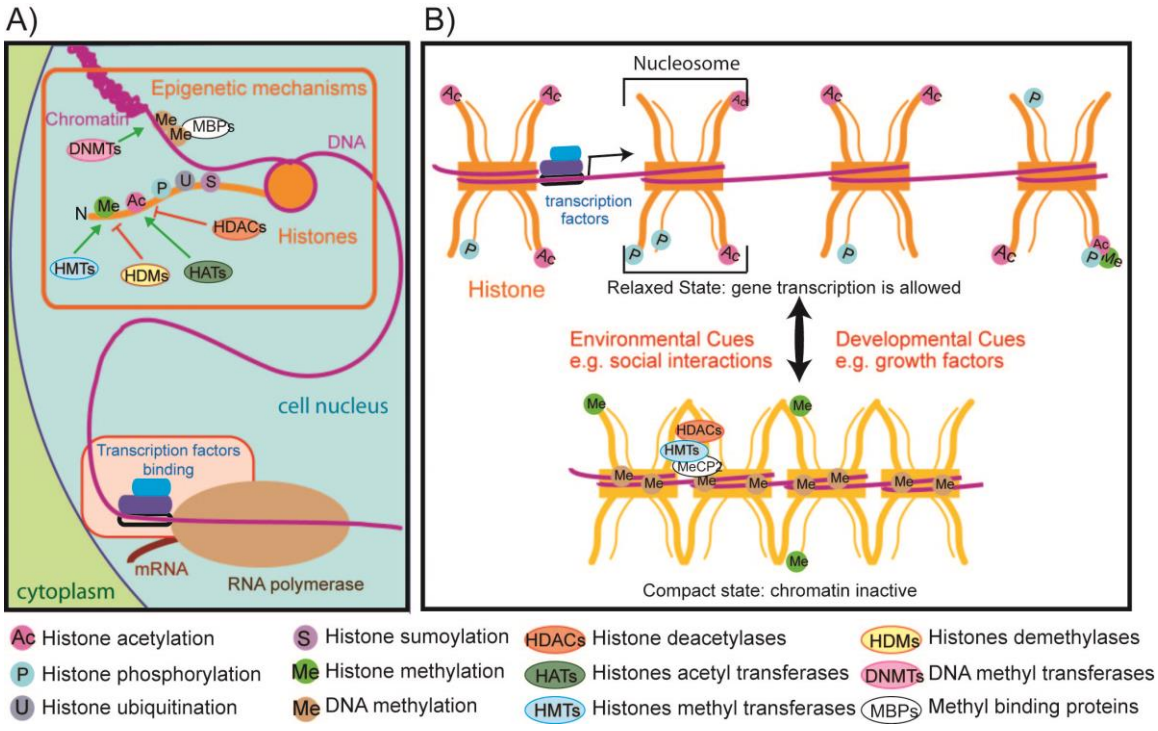


Figure 1.8 A diagram of epigenetic mechanism involved in the regulation of chromatin structure. A) N-terminal histone tails are subject to covalent modification which plays a key role in epigenetic regulation of chromatin remodelling. Histone modifications also participate in cross-talk with DNA methylation. B) The sum of epigenetic marks, particularly histone modification, aid in the relaxation and condensation of chromatin. Image from Géranton and Tochiki (In press).

Histone modifying enzymes

There are specific enzymes dedicated to imparting and removing histone modifications (Figure 1.9). Histone acetyltransferases (HATs) are responsible for the addition of

histone acetyl marks, a process of transferring an acetyl group from acetyl coenzyme A to histone tail lysine residues (Lee and Workman, 2007). Histone acetylation is negatively regulated by histone deacetylases (HDACs), of which there are four classes, including zinc-dependent classes I, II, and IV, and the nicotinamide adenine dinucleotide (NAD)- dependent class III (also called sirtuins) (Haberland et al., 2009). HDACs have gained much attention as pharmacological targets of inhibition, particularly due to the fact that deregulation of epigenetic processes, including histone modifications, has been associated with a broad spectrum of neurological disorders (Akbarian, 2010; Deutsch et al., 2008; Qureshi and Mehler, 2010). Although HDAC inhibition is largely non-specific to histones, the overall aim of HDAC inhibitors (HDACis) is to increase the transcription of 'favourable' genes to battle disease. Furthermore, deregulation of histone acetylation is a hallmark of cancer and HDACis have received significant attention in cancer drug development (Wee et al., 2014). Traditionally, HDACis have been used to hyper-acetylate histones and favour transcription of tumour suppressor genes.

Signalling to histone modifications

Modulation of histone modifications occurs via the activation of surface-to-nucleus signalling cascades such as MAPK and NFkB (Riccio, 2010; Suganuma and Workman, 2012; Zhou et al., 2013), which specifically target the enzymatic machinery responsible for chromatin modification, by either inducing phosphorylation of specific regulators or by modulating their interactions (i.e. transcription factor complexes). For example, activation of MSK1 via ERK/MAPK leads to histone H3 phosphorylation, a mechanism important for synaptic activity-induced chromatin remodelling in neurons (Arthur et al., 2004; Soloaga et al., 2003).

Understanding histone modifications and histone modifying enzymes are only a single aspect of the complex interaction that occurs across all epigenetic mechanisms (i.e. histone-DNA methylation interaction, transcription factor binding complexes) upon the genomic sequence. It is therefore important to not over-interpret the meaning of histone modifications (Meaney and Ferguson-Smith, 2010).

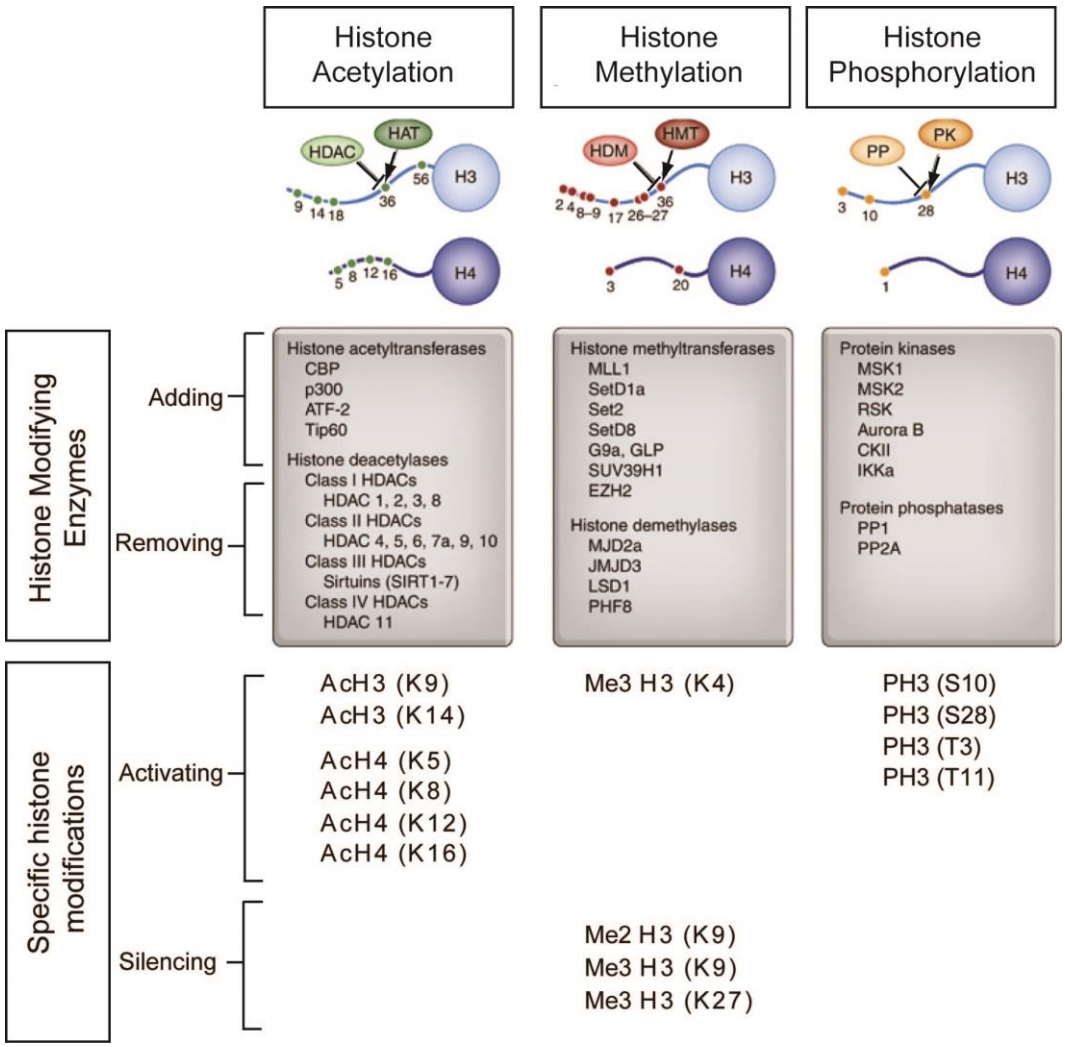


Figure 1.9 A few of the most common histone marks and their enzymes. Histone tails on H3 and H4 are the most extensively modified. The numbers on the histone tails indicate the residue position; acetylation and methylation most often occur at lysine (K) residues, phosphorylation occurs at serine (S) residues. Histone modifying enzymes are listed in grey boxes. The majority of acetylated and phosphorylated residues are markers of transcriptional activation, whereas histone methylation, which can occur in mono, di, or tri methylated states, can indicate both silencing and activation. Adapted by permission from Macmillan Publishers Ltd: Neuropsychopharmacology, Vol 37, Day J.J. and Sweatt J.D., Epigenetic Treatments for Cognitive Impairments, pp. 247-260, copyright (2012).

1.5.2 Epigenetic Mechanisms: Learning and Memory studies

Substrates for lifelong memories have long been investigated, and understandably include histone modifications and the enzymes responsible for these experience-dependent marks upon the epigenome. Although the rationale for epigenetic regulation of pain states is strong, the current pool of studies is very limited. It is therefore essential to draw from extensive evidence showing that epigenetic processes are engaged in and required for memory formation (Barrett and Wood, 2008; Borrelli et al., 2008; Day and Sweatt, 2011, 2010; Gräff and Tsai, 2013; Peixoto and Abel, 2013).

Given the similarities of central sensitisation and LTP (see 1.3.2.2), key studies identifying the epigenetic mechanisms underlying the plasticity of memory formation are particularly relevant, and are presented below.

1.5.2.1 Histone modifications in memory formation

It is well established that the expression of histone modifications is altered during memory formation, but it is important to note that these marks vary spatiotemporally and by learning paradigm (Gräff and Tsai, 2013).

David Sweatt's group were the first to identify upregulation of transcriptionally permissive histone modifications on histone H3 (PH3S10, AcH3K14, and phospho-acetyl S10/K14) in the hippocampus during the consolidation phase of contextual fear conditioning (Chwang et al., 2006; Levenson et al., 2004) (Figure 1.10).

Subsequent experiments identified that these same marks (PH3S10, AcH3K14) in addition to others (AcH4K5, Me3H3K36 active transcription mark) were again upregulated in the hippocampus 10min-24h following a different paradigm, novel-object recognition learning (Gräff et al., 2012b). Additionally, AcH3K14, AcH4K5 and Me3H3K36 were also increased with delayed expression in the prefrontal cortex, and persisting for up to 7d, implicating an important role for these marks in remote memory formation (Gräff et al., 2012b). Notably, acetylation of histone H3 (AcH3K9) has also been shown to act as a cortical 'tag' for remote olfactory memories (Lesburgueres et al., 2011).

Furthermore, studies have used chromatin immunoprecipitation (ChIP, whereby a DNA-associated protein of interest is selected and its associated gene sequence probed) and ChIP-sequencing to identify promoter-specific increases of transcriptionally active histone marks at pro-memory formation genes such as *Bdnf, Zif268,* and *Homer1* following memory formation paradigms (Gupta et al., 2010, 2010; Lubin et al., 2008; Mahan et al., 2012).

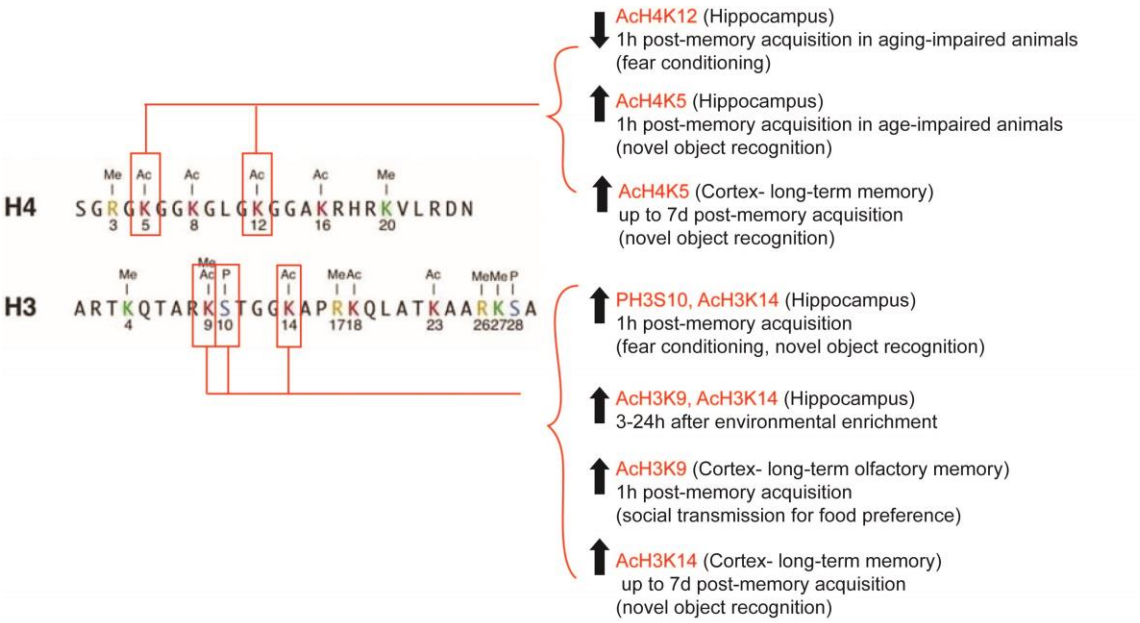


Figure 1.10 Examples of cognitive behavioural correlates of histone H3/H4 modifications (rodent models).

1.5.2.2 Antagonising histone modifications

Crucially, the correlation of histone marks with learning behaviour does not imply that these marks are necessary to memory formation. In order to understand the role of histone modifications in such processes, it is necessary to target these marks and observe the resulting effect on animal behaviour. While largely unspecific, targeting the enzymes responsible for imparting or removing key histone modifications (HATs or HDACs) has been the main method of approach. In particular, many studies have targeted HDAC activity with the aim to improve cognitive function by overexpression of pro-memory forming genes (Gräff and Tsai, 2013). As highlighted in 1.5.2.1, histone acetylation has been observed in various brain areas during memory formation, and inhibiting HDAC activity, or genetic manipulation of HDAC expression has been the focus of many of these studies.

Genetic modification: HDACs 2 and 3

The role of Class I HDACs 2 and 3 have been the most extensively studied, using either genetic or pharmacological approaches. The experimental use of genetically modified knockout mice has identified HDAC2 and HDAC3 as negative regulators of memory (Guan et al., 2009; McQuown et al., 2011).

HDAC2 was upregulated in the hippocampi of mice exhibiting memory deficits due to aging, or induced neurodegeneration (CK-P25 mice) when compared with control animals (Castellano et al., 2012; Gräff et al., 2012a). In the latter case, HDAC2 was

associated with promoter regions of genes crucial to memory formation, indicating that they were silenced (Gräff et al., 2012a). Finally, knockdown of HDAC2 with virally transfected shRNA in the hippocampus and PFC has also been shown to improve memory deficits (Gräff et al., 2012a).

Use of pharmacological HDAC inhibitors

In general, the systemic or localised (i.e. hippocampal infusion) delivery of HDACis such as sodium butyrate (NaB), trichostatin A (TSA) and valproate (VPA) have been shown to enhance normal cognitive function when given during the memory consolidation phase shortly after training (Bredy and Barad, 2008; Fischer et al., 2007; Guan et al., 2009; Gupta et al., 2010; Levenson et al., 2004; Lubin et al., 2008; Mahan et al., 2012; Roozendaal et al., 2010; Stefanko et al., 2009; Vecsey et al., 2007). These inhibitors have been shown to act predominantly by increasing histone acetylation globally, as well as at gene-specific promoter regions.

Moreover, HDACis have also been shown to reversibly recover deficits in memory formation. For example, chronic intraperitoneal (i.p.) NaB re-established access to long-term memory in memory-impaired neurodegenerating mice, which also increased AcH3K14 and AcH4K5 levels, as well as the expression of synaptic proteins (Fischer et al., 2007). Furthermore, hippocampal infusion of suberoyl anilide hydroxamic acid (SAHA) reinstated AcH4K12 expression and reversed cognitive deficits in aged (16 month old) mice (Peleg et al., 2010).

While the use of HDACis could be expected to affect the expression of a very large number of genes, a gene array study has found that non-selective HDACis *in vitro* upregulated only 10-15% of all genes analysed (Tabuchi et al., 2006). As this value might be expected to be higher, a 10-15% increase potentially indicates some specificity of genes targeted by HDACis.

1.5.3 Pain and neuroepigenetics studies

Investigation into the epigenetic regulation of pain states is a relatively new field; however, the number of studies produced around the subject has increased rapidly in the past few years. Pain studies have focused on injury-induced changes to the epigenome, and have also targeted the marks on the epigenome, particularly with the goal of regulating nociception by pre-emptively treating or reversing existing pain states (Bai et al., 2014; Denk and McMahon, 2012; Géranton, 2011; Géranton and Tochiki, In press; Rahn et al., 2013b; Stone and Szyf, 2013).

1.5.3.1 Injury induced changes in epigenetic modifications and modifying enzymes

A number of studies have investigated the presence of marks comprising the epigenome at different locations along the pain pathways (Géranton and Tochiki, In press). Thus far changes have been reported in the sciatic nerve, DRGs, dorsal horn and brain (the nucleus raphe magnus- NRM- and the frontal cortex). Many studies have focused on histone acetylation levels, either globally, or at gene-specific promoter areas, including genes relevant to nociception *MeCP2, Bdnf, Gad2, Hdac1,* and *CXCR2* (Denk et al., 2013; Kiguchi et al., 2013, 2012; Liang et al., 2013; Matsushita et al., 2013; Sun et al., 2013; Zhang et al., 2011; Zhu et al., 2014). Although the majority of reports have shown increased levels in histone acetylation following injury, indicating activation of gene transcription, this information is far from conclusive as both increases and decreases in histone acetylation have been reported in the promoter area of pro-nociceptive genes. Such variation is not entirely surprising and likely to differ by the nature of injury (i.e. inflammatory, neuropathic) (Sun et al., 2013); however, it is an important consideration when targeting epigenetic mechanisms for pain relief. Additionally, examining global histone acetylation levels of a particular anatomical region may not necessarily be reflective of locus-specific gene changes. In particular, global H3 acetylation was increased in the NRM following CFA stimulation, but in fact H3 acetylation was decreased specifically at the promoter area of the *Gad2* gene (Zhang et al., 2011).

The expression of a number of epigenome modifying enzymes are also altered in the dorsal horn and DRGs in pain states, including MeCP2 (Tochiki et al., 2012; Wang et al., 2011), the HAT, p300 (Zhu et al., 2014, 2012), and several of the DNA-methyltransferases (DNMTs) and HDACs (Bai et al., 2010; Qi et al., 2013; Tochiki et al., 2012; Zhang et al., 2013). The studies, however, looked at different pain models and there was no obvious agreement in terms of time scale or direction of the changes. Please see Figures 7.1 and 7.2 for a detailed summarisation of the aforementioned studies.

1.5.3.2 Antagonising histone modifications to improve injury- induced hypersensitivity: HDACs and HATs

Pharmacological inhibition of HDACs to alter nociception in animal models has so far been the most frequently utilised approach in pain studies. The administration of HDACis MS-275 and SAHA has generally had overall positive outcomes in attenuating pain behaviours, both inflammatory and neuropathic.

The administration of the HDACi SAHA has been shown to attenuate both the second phase of formalin response and intraplantar CFA -induced hypersensitivity (Bai et al., 2010; Chiechio et al., 2009; Zhang et al., 2011), albeit via different proposed mechanisms. Chiechio et al. (2009) attributed the effect of the HDACis on formalin behaviour to an upregulation of mGlu2 metabotropic glutamate receptors in the DRG and spinal cord, a receptor known to produce analgesic effects in inflammatory and neuropathic pain states. Due to the non-specificity of HDACi, the authors found hyperacetylation of the p65 transcription factor, a regulator of NFκB genes including *mGlu2*. However, no injury-induced changes in acetylation were reported in this study. Zhang et al. (2011) showed that NRM infusions of TSA and SAHA could improve injury-induced reduction of tail flick latency by targeting impaired presynaptic GABAergic synaptic function (Zhang et al., 2011). HDACi attenuation of nociceptive behaviour was found to be associated with increased histone H3 acetylation at the *Gad2* promoter.

HDACis have also been used to attenuate hypersensitivity in neuropathic pain models. Intrathecal infusion of MS-275 reduced thermal and mechanical hyperalgesia by 50% in including nerve injury models, as well as neuropathy induced by the antiretroviral drug, d4T (Stavudine) (Denk et al., 2013). The authors concluded that MS-275 acted specifically via spinal HDAC1 and caused increase in spinal cord AcH3K9 levels. However, when given after injury, MS-275 was unable to reverse hypersensitivity.

Moreover, C-fibre hypoesthesia induced by neuropathic injury was also prevented by systemic TSA and valproate, and can be reversed by SAHA (Matsushita et al., 2013). The underlying mechanism of neuropathic C-fibre hypoesthesia- silencing of *Nav1.8* due to the binding of transcription factor NRSF/REST- is prevented by HDACi due to hyperacetylation of histone H3/H4 at the NRSE (binding sequence for NRSF) within the *Nav1.8* gene in the DRG (Matsushita et al., 2013; Uchida et al., 2010). The NRSF/REST silencing mechanism also occurs within the Kv4.3 gene following nerve injury (Uchida et al., 2010). Another study by the same group used chromatin immunoprecipitation to reveal hyperacetylation of histone H3 and H4 at the exon I transcript of the *Bdnf* gene in primary afferent nociceptors, which might be responsible for the pronociceptive role of BDNF in the development of neuropathic pain states. Crucially however, the authors never fully investigated the behavioural consequences of targeting these epigenetic targets (Uchida et al., 2013).

Further highlighting the complexity of the epigenetic contribution to pain behaviours, HDACis have been found to exacerbate hypersensitivity induced by the skin incision model (Sun et al., 2013) and chronic opioid use (Liang et al., 2013). As expected, HAT

inhibitors (anacardic acid (AA) and curcumin, respectively) significantly attenuated the hypersensitivity associated with these models.

A detailed breakdown of the main studies investigating epigenetic mechanisms in pain processing can be found in the General Discussion (Figure 7.1, Figure 7.2).

1.6 Hypothesis and aim of this thesis

It is well-established that histone modifications play an important role in neural plasticity and changes in gene expression (Borrelli et al., 2008). Plasticity-related changes in gene expresion are known to occur following both noxious stimulation (Géranton et al., 2007) and memory acquisition (Day and Sweatt, 2011). The initial conception of experiments in this thesis follows on primarily from learning and memory studies (see 1.5.2) identifying increases in hippocampal acetylation and phosphorylation at specific histone residues 30-60 minutes following memory acquisition (Chandramohan et al., 2008; Fischer et al., 2007; Peleg et al., 2010). These studies have clearly identified histone modification as a key molecular event regulating learning and memory by altering changes in gene expression through use of pharmacological or animal knockout methods (Day and Sweatt, 2011; Gräff and Tsai, 2013; Peixoto and Abel, 2013).The similarity of molecular events involved in the induction and maintenance of LTP, the proposed underlying neural correlate of memory formation, and central sensitisation (Ji et al., 2003), indicate that these similarities may also be inclusive of epigenetic modifications such as histone modification.

More recently, studies have emerged that have directly investigated a role for spinal histone acetylation in the full expression of neuropathic behaviour (Bai et al., 2010; Denk et al., 2013). While injury-induced histone phosphorylation has not yet been investigated in the dorsal horn, it is of particular interest as it is known to occur downstream of ERK signalling, a key event in the development of nociceptive plasticity.

The global aim of this thesis was to use a model of inflammatory pain to investigate the role of spinal dorsal horn histone modification in the development and regulation of central sensitisation and injury-induced nociceptive behaviour. Specifically, the aims of this thesis were to:

1. Identify specific histone modifications displaying altered expression in the spinal dorsal horn following peripheral noxious stimulation (PH3S10) (Chapter 3).

2. Use the formalin model to investigate the overlap/expression of the formalin-induced PH3S10 population with markers of the pain pathways (Chapter 4).

3. Investigate the role of descending serotonergic activity on the expression of formalin-induced PH3S10 (Chapter 5).

4. Elucidate the molecular signalling events upstream of PH3S10 (ERK, MSK1) using pharmacological inhibitors, to determine if preventing PH3S10 by targeting MSK1 can regulate formalin-induced nocifensive behaviour and central sensitisation (Chapter 6).

Chapter 2 Materials and Methods

This section describes general methods used throughout this thesis. Experimental design (i.e. n numbers) as well as the specifics of some methods is addressed in-detail within the Methods section of each Results chapter. For solution recipes, please refer to the Appendix.

2.1 Animals

All procedures were licensed under the *Animals (Scientific Procedures) Act 1986.* Experiments were performed using adult male Sprague-Dawley rats at 200-230g at the beginning of the experiment and bred by the UCL Biological Services Unit. For experiments where animals required intrathecal surgery (Chapter 5, Chapter 6 *Experiment 2*) all subjects were between 170-185g. Animals were housed four maximum to a cage, with a 12h light-dark cycle (lights on beginning at 08:00) and access to food and water provided *ad libitum*.

2.2 Model of acute pain

2.2.1 Injections

Acute inflammation was induced with an intraplantar hindpaw injection of 50µl, 2% formalin under isoflurane anaesthesia (5% for induction and 2.5% for maintenance under nose cone combined with 100% O_2, 2L/min) or 20µl, 5% formalin without anaesthesia using a 1ml syringe and disposable 30G ½ inch needle. For the latter, the animal was lightly restrained in a leather glove during injection. Animals injected under anaesthesia were returned to a recovery box until regaining consciousness. Sham-anaesthetised animals were subject to the same anaesthesia as the formalin injected animals but received no injection, while sham-non-anaesthetised animals had handling/restraint and no injection.

In this thesis, with the exception of Chapter 6, *Experiment 2*, all animals were injected with formalin (50µl, 2%) under isoflurane anaesthesia. The requirements of Chapter 6 *Experiment 2, Does inhibition of MSK1 activity block formalin-induced PH3S10 and c-Fos expression, and attenuate formalin behaviour?* necessitated the injection of formalin without anaesthesia in order to monitor behaviour immediately following injection. Due to Home Office licence limitations, rats injected without anaesthesia had to be given a smaller volume but higher concentration of formalin (20µl, 5%). Importantly, it should be noted that the two formalin doses deliver the same amount of formalin but in different volumes. The purpose of Chapter 6, *Experiment 3*: Are there any differences in spinal PERK expression following two different conditions of i.pl. formalin injection? was to serve as a control experiment to investigate any differences

in PERK activation between the slightly different conditions of formalin injections used in this thesis. Please also refer to 6.2.1.3.

The survival time following formalin or sham stimulation ranged from 5min to 4h depending on the experiment.

2.2.2 Scoring of formalin behaviour

Animals were recorded for 60min in an 11 x 17 cm perspex box with a Panasonic SDR-H100 video camera following injection. Formalin behaviour was scored and quantified as the length of time spent licking and flinching, or the number of total flinches in 5min bins across the 60min following injection. All scoring was done with the experimenter blind to treatment.

2.3 Drugs and method of delivery

Table 2.1 Drugs. DMSO, dimethyl sulfoxide; EtOH, ethanol.

Drug	Company Cat #	Delivery	Solvent	Amount delivered	Vehicle equivalent
Desipramine (Chapter 5)	Sigma D3900	i.p.	0.9% Saline	25mg/kg	-
5,7-DHT (Chapter 5)	Sigma 37970	i.t.	0.9% Saline	60µg in 10µl	0.9% Saline
SL327 (Chapter 6)	Tocris 1969	i.p.	DMSO	50mg/kg	100% DMSO
SB747651A (Chapter 6)	Tocris 4630	i.t.	10mg/200 µl EtOH + 2ml 0.9% saline (10.49mM)	433ng (10µM,10µl) or 4.33ng (1µM, 10µl)	0.0086% EtOH/saline

2.3.1 Intrathecal surgery

Intrathecal surgery was performed under isoflurane anaesthesia (5% for induction and 2.5% for maintenance under nose cone combined with 100% O_2, 2L/min). The head of the animal was shaved and the animal secured in a stereotaxic frame. Following sterilisation of the shaved area, an incision was made using a no. 22 blade from the top of the head to the base of the skull/first vertebra. The muscle was parted to reveal the atlanto-occipital membrane, which was carefully pierced and a small opening cleared to allow insertion of a polyethene tube (diameter 0.28mm) attached to a 50µl Hamilton syringe containing either drug or vehicle solution (see Table 2.1). The tubing was

carefully inserted into the subarachnoid space and guided to a predetermined location identifying the L4/L5 spinal cord region and 10µl of solution delivered. The tube was inserted without resistance and care was taken not to damage the spinal cord or dorsal roots. Once the tube was removed, the overlying muscle was sutured once using 5-0 mersilk, and the wound subsequently closed with three 11x2mm suture clips. The animal was then placed into a temperature controlled recovery chamber and monitored closely for signs of locomotor impairment or distress once awake. Animals receiving 5,7-DHT were also monitored over the 6 day period for any weight loss (Chapter 5).

2.4 Tissue collection

Following formalin stimulation, animals were sacrificed and spinal cords dissected out either fresh or fixed with paraformaldehyde for western blot or immunohistochemistry, respectively.

2.4.1 Fresh Tissue

Animals were sacrificed using carbon dioxide (CO_2) asphyxiation by steady flow of CO_2 into a chamber until unconscious and not breathing. A cervical dislocation was subsequently performed to ensure death. The spinal cord was quickly dissected on ice. The ipsilateral and contralateral (to the injected hindpaw) dorsal horn of L4-L6 (weighing roughly 10mg each) were cut out and placed in separate tubes and immediately frozen on dry ice. Tissue was stored at -80˚C until processing.

2.4.2 Perfusion

Animals were deeply anaesthetised first with isoflurane and subsequently with pentobarbital (Euthatal, typically 0.8ml/animal, i.p.). After breathing stopped and the animal failed to exhibit eyelid reflex, a transcardial perfusion was performed first with heparinsed saline (5000IU/litre 0.9% saline) followed by 4% paraformaldehyde (PFA)/0.1MPB for 7min, using a pump and 18 gauge cannula delivering the fixative at 30ml/min. The PFA solution contained 0.2% (w/v) sodium fluoride (NaF), a phosphatase inhibitor.

The spinal cord was then post-fixed for 2.5-3h in the same PFA solution and subsequently stored at 4˚C in 30% sucrose/0.1M PB/0.02% (w/v) azide for cryoprotection for at least 48h before further processing.

2.5 Immunohistochemistry

Immunohistochemistry was used to visualise antigens of interest within spinal cord tissue sections using antigen-specific generated antibodies. The antibodies were visualised with either fluorescent or chromogenic methods and typically the signal was

amplified using principles of avidin-biotin affinity (see 2.5.4.1 below). The specifics of antibody combinations used for colocalisation are located in the Methods section of relevant Results chapters. For solution recipes please see the Appendix.

2.5.1 Tissue Sectioning

The L4 to L6 region of the perfused spinal cord was identified using L4 and L5 roots, and isolated for sectioning on a Leica SMR2000R freezing microtome. 40µm coronal sections were taken serially in six sets and stored in 5% sucrose/0.1M PB/0.02%(w/v) azide until staining.

2.5.2 Blocking of non-specific antigens

All staining of tissue was done free floating in volumes of 1ml solutions with gentle rocking agitation unless otherwise stated. 3 x 10min washes in 0.1M PB were performed after incubation with all solutions except after incubation with blocking solution. Washes were done in excess of 1ml.

In some cases, an antigen retrieval step was required (see asterisk, Table 2.2). Sections were acid pretreated with 2N hydrochloric acid (HCl) for 30min followed directly by a neutralisation step of 0.1M Boric acid for 10min prior to serum block.

Tissue sections were blocked for 1h in 3% normal goat or horse serum depending on the host of the secondary antibody (Vector, UK,

Table 2.3) and 0.3% triton X-100 in 0.1M PB to minimise non-specific binding of secondary antibodies. For chromogenic staining, 2% H_2O_2 was added to the blocking solution to quench endogenous peroxidase activity and minimise background. Following removal of the blocking solution, primary antibody was added either overnight (O/N) at room temperature (RT) or over 3d at 4˚C in Triton-Tris-buffered saline (TTBS). See Table 2.2 for further antibody information.

2.5.3 Antibodies- Immunohistochemistry

Table 2.2 Primary antibodies (immunohistochemical experiments) Asterisk indicates acid pretreatment antigen retrieval was required prior to the blocking step (see 2.5.2). Rb, rabbit; Ms, mouse.

Antibody	Host	Company	Cat. No	Concentration	Incubation time and temperature	Detection or amplification method
5-HT	Rat	Chemicon	MAB352	1:75	3d, 4˚C	Biotin Amplification
AcH3K9	Rb	Upstate	06-942	1:1000	O/N, RT	Direct Alexa
AcH3K14	Rb	Cell signalling	4318	1:1000	O/N, RT	TSA*
AcH4K5	Rb	Millipore	17-211 06-759	-	-	-
AcH4K8	Rb	Millipore	17-211 06-760	1:1000	O/N, RT	Direct Alexa*
AcH4K12	Rb	Millipore	17-211 06-761	1:1000	O/N, RT	Direct Alexa
AcH4K16	Rb	Millipore	17-211 06-762	1:1000	O/N, RT	Direct Alexa
APC CC1	Ms	Calbiochem	OP-80	1:100	O/N, RT	Direct Alexa
Calbindin	Ms	Sigma	C8666	1:1000	O/N, RT	Direct Alexa
c-Fos	Rb	Calbiochem	PC38	1:10,000 1:20,000	O/N, RT O/N, RT	DAB TSA
GFAP	Rb	Dako	Z0334	1:4000	O/N, RT	Direct Alexa
NK1	Rb	Millipore	AB5060	1:20,000	3d, 4˚C	TSA
NeuN	Ms	Chemicon	MAB377	1:1000	O/N, RT	Direct Alexa
nNOS	Rb	Cell signalling	4231S	1:1000	O/N, RT	TSA
Parvalbumin	Rb	Swant	PV28	1:1000	O/N, RT	Direct Alexa
PERK (Thr202/ Tyr204)	Rb	Cell signalling	9101	1:250 1:500	O/N, RT O/N, RT	TSA Direct Alexa and DAB
PH3S10	Ms	Abcam	ab14955	1:10,000 1:5,000 1:500	3d, 4˚C O/N, RT O/N, RT	TSA DAB Direct Alexa*

PH3S10/ AcH3K14	Rb	Millipore	07-081	1:20,000	3d, 4˚C	TSA
PMSK1 (Thr581)	Rb	Cell signalling	9595	1:500	3d, 4˚C	TSA
Zif268 (Egr-1)	Rb	Santa Cruz	sc-189	1:10,000	O/N, RT	TSA

Table 2.3 Secondary antibodies (immunohistochemical experiments). Ch, chicken; Gt, goat; Hs horse.

Secondary antibody	Host	Company	Cat. No	Concentration	Incubation time (all RT)
Biotinylated anti-Rb IgG	Gt	Vector	BA-1000	1:400 1:500	1.5h, TSA 2h, DAB
Biotinylated anti-Ms IgG	Hs	Vector	BA-2001	1:400 1:500	1.5h, TSA 2h, DAB
Biotinylated anti-Rat IgG	Gt	Vector	BA-9400	1:400 1:500	1.5h, TSA 2h, DAB
Alexa 594 anti-Ms IgG	Gt	Invitrogen	A11072	1:500	O/N
Alexa 594 anti-Rb IgG	Gt	Invitrogen	A11037	1:500	O/N
Alexa 488 anti-Ms IgG	Gt	Invitrogen	A11001	1:500	O/N
Alexa 488 anti-Rb IgG	Ch	Invitrogen	A21441	1:500	O/N

2.5.4 Fluorescent immunohistochemical methods

After incubation with primary antibody, typically two fluorescent immunohistochemical methods were used to visualise primary antibodies, either:

1. Tyramide signal amplification (using fluorescein isothiocyanate FITC avidin), or
2. Direct detection (using Alexa fluorophore-conjugated secondary antibody)

These methods were either used singly or combined in sequence if a colocalisation study was conducted. Under a fluorescent microscope beam, FITC avidin and Alexa 488 fluoresce green when excited at a wavelength of 488nm, and Cy3 Streptavidin and Alexa 594 fluoresce red when excited at 594nm. Using two fluorochromes that have

non-overlapping excitation/emission wavelengths is particularly useful when visualising two different antigens on the same tissue (Figure 2.4) (Figure 2.6).

2.5.4.1 Tyramide signal amplification (TSA)

Amplification of antibody signal using TSA allows for the antibody to be used at a low concentration. This is particularly useful if the antibody is in limited supply, or if a double stain is desired with an antibody of the same host species. Biotinylated-tyramide is activated by the enzyme horseradish peroxidase (HRP) and deposited at electron-rich regions at HRP binding sites. In this case HRP is conjugated to an avidin-biotin complex (ABC) aggregate and maximises the number of sites for binding with avidin-conjugated fluorochrome (FITC avidin). Avidin and biotin have an extremely high affinity for one another, with every avidin glycoprotein having four identical subunits that bind one biotin each. (Figure 2.1).

Following incubation with primary antibody, a respective biotinylated secondary antibody (Vector, UK) was subsequently applied at a dilution of 1:400 in TTBS for 1.5h, followed by 30min in ABC solution diluted to 1:250 of each Vectastain A and Vectastain B solutions (Vector, UK). A further amplification step was completed using a dilution of 1:75 biotinylated tyramide in amplification diluent in 0.25ml only (Perkin Elmer, UK) for 7min. Sections were then incubated with FITC avidin (Vector, UK), 1:600 in TTBS for 2h in a dark box.

If colocalisation with another antigen was desired, the tissue was subsequently incubated with a second primary antibody of interest in TTBS and left to incubate O/N in the dark at RT (see Table 2.2 Primary antibodies (immunohistochemical experiments) Asterisk indicates acid pretreatment antigen retrieval was required prior to the blocking step (see 2.5.2) The following day, the antibody was detected with an Alexa-conjugated secondary as described below (see 2.5.4.3). If no colocalisation was desired, sections were mounted from 0.01M PB onto gelatinised slides. It should be noted that where a double stain was completed with two antibodies of the same host, a negative control eliminating the second primary confirmed no cross reactivity of secondary antibodies. Once dry, slides were coverslipped with Fluoromount (Sigma, UK) to minimise fluorescent fading.

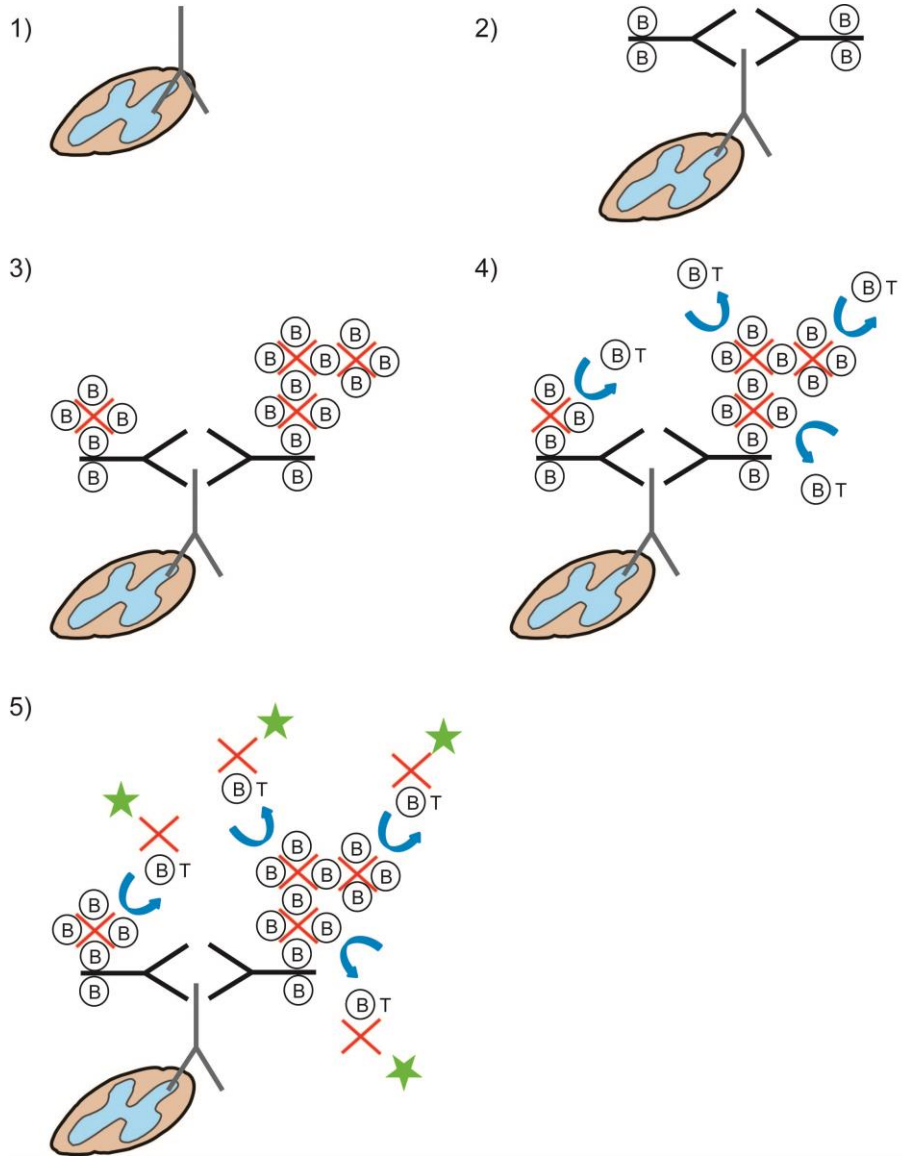


Figure 2.1 Tyramide signal amplification (TSA). 1) The tissue is incubated with primary antibody overnight or over 3d; the primary antibody binds to the antigen of interest. 2) The primary antibody is detected by a secondary antibody conjugated to biotin (B). 3) The high affinity avidin (red X)-biotin (B) aggregate binds to the biotinylated secondary antibody. 4) The avidin-biotin aggregate is conjugated to HRP (not shown), which catalyses (blue arrow) the deposition of biotinylated tyramide (BT). 5) FITC (green star) conjugated avidin (red X) binds to biotinylated tyramide, completing the amplification signal of the primary antibody. FITC is visualised under the fluorescent microscope.

2.5.4.2 Biotin amplification

A biotin amplification does not amplify signal strength to the same extent as TSA but more so than direct detection with an Alexa-conjugated secondary (see 2.5.4.3). Biotin amplification was used for the antibody 5-HT only and cannot be combined with TSA. Following incubation with the 5-HT antibody, a goat anti-rat biotinylated secondary antibody (Vector, UK) was subsequently applied at a dilution of 1:500 in TTBS for 1.5h,

followed by 45min in Cy3 streptavidin 1:4000 in TTBS (Jackson Immunoresearch, Europe). Tissue was mounted and once dry, slides were coverslipped with Fluoromount (Figure 2.2)

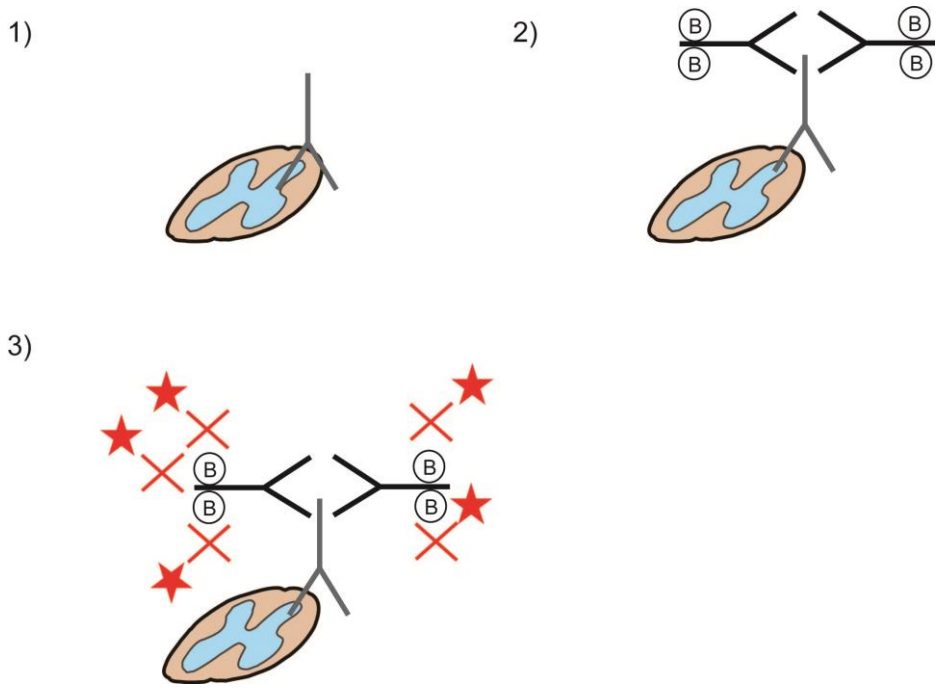


Figure 2.2 Biotin amplification. 1) The tissue is incubated with primary antibody overnight or over 3d; the primary antibody binds to the antigen of interest. 2) The primary antibody is detected by a secondary antibody conjugated to biotin (B). 3) Cy3 (red star)-conjugated streptavidin (red X) binds to the biotinylated secondary antibody. Cy3 is visualised under the fluorescent microscope.

2.5.4.3 Direct detection with Alexa

Sections were incubated in a dark box O/N in Alexa Fluor conjugated secondary antibody at 1:500 (see

Table 2.3 Secondary antibodies (immunohistochemical experiments). The next day they were mounted from 0.01M PB onto gelatinsed slides and coverslipped with Fluoromount (Figure 2.3)

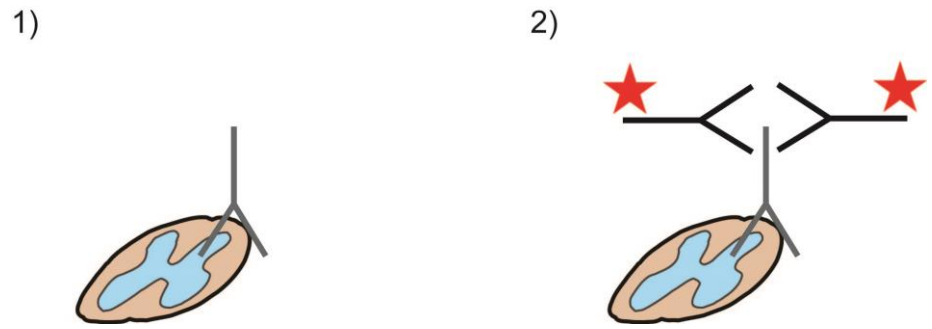


Figure 2.3 Direct Alexa detection of primary antibody. 1) The tissue is incubated with primary antibody overnight; the primary antibody binds to the antigen of interest. 2) The primary antibody is detected by an Alexa fluor (488 or 594)-conjugated secondary antibody. Alexa 488 or 594 is visualised under the fluorescent microscope.

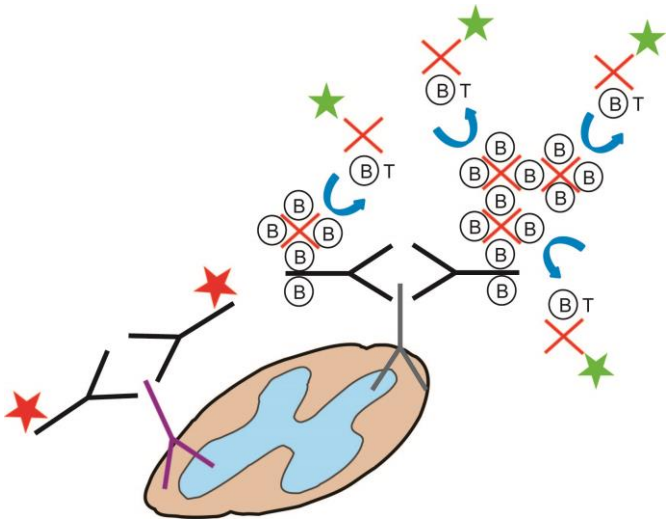


Figure 2.4 Combining TSA and Direct Alexa methods to 'double stain' for two different antigens. Combination of protocols in **Figure 2.1** and **Figure 2.3**.

2.5.5 Chromogenic immunohistochemical methods: 3,3'-Diaminobenzidine (DAB) reaction

In some cases, particularly for quantification of cell counts, a 3,3'-diaminobezidine (DAB) reaction was employed. Amplification of antibody signal using DAB is similar to TSA, again utilising the affinity of the ABC complex. DAB is a chromogenic electron donor which deposits a brown polymer in the presence of H_2O_2 and HRP.

Following incubation for 1h in blocking solution (see 2.5.2) respective biotinylated secondary antibody was subsequently applied at a dilution of 1:500 in TTBS for 2h, followed by 1h in ABC solution diluted to 1:1000 of each Vectastain A and Vectastain B solutions. Sections were then developed using a DAB kit (Vector, UK) for 5-7min under constant visualisation using a stereoscopic microscope. The reaction was terminated by transferring the sections into dH_20 followed by 0.1M PB (5min each).

Sections were mounted from 0.01M PB and left to dry O/N before dehydration in EtOH of increasing concentration (10s in dH_20 followed by 2 x 1min in each 70% EtOH, 95% EtOH, 100% EtOH, Histoclear (National Diagnostics, UK). Slides were coverslipped directly from histoclear with DPX mountant (VWR, UK) (Figure 2.5).

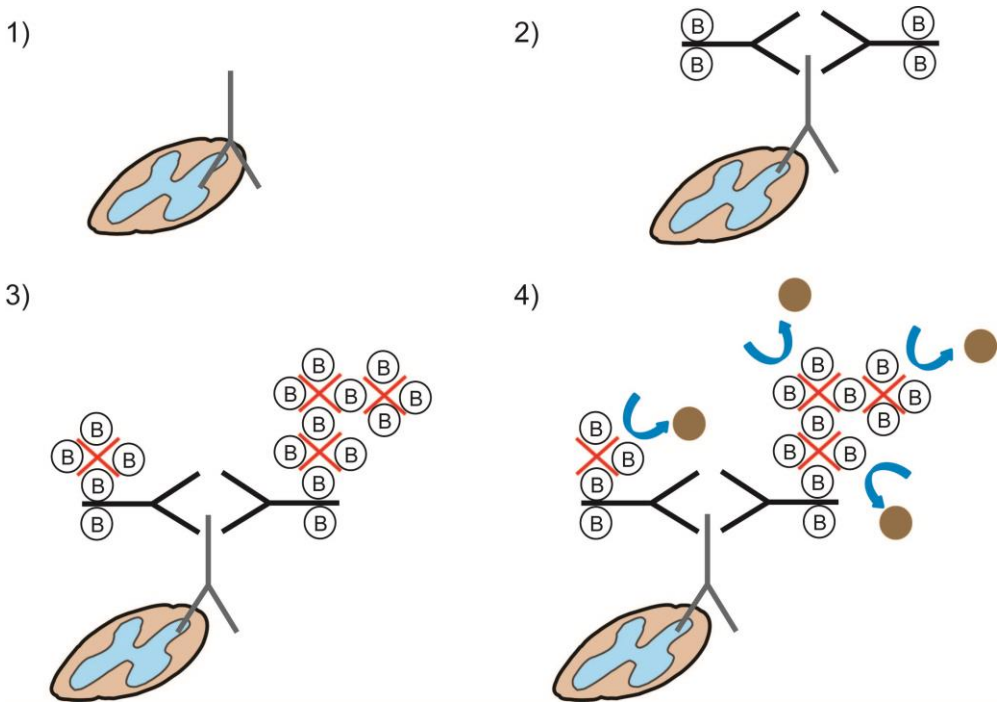


Figure 2.5 3,3' Diaminobenzidine (DAB) amplification. 1) The tissue is incubated with primary antibody overnight or over 3d; the primary antibody binds to the antigen of interest. 2) The primary antibody is detected by a secondary antibody conjugated to biotin (B). 3) The high affinity avidin (red X)-biotin (B) aggregate binds to the biotinylated secondary antibody. 4) The avidin-biotin aggregate is conjugated to HRP (not shown), which catalyses (blue arrow) the deposition of DAB polymer (brown circle). The DAB signal is visualised under the light microscope.

Figure 2.6 Immunohistochemical methods used in this thesis: degrees of amplification. TSA and DAB are the protocols yielding the highest amount of amplification of primary antibody; Direct Alexa is the lowest degree of amplification.

2.5.6 Imaging

Images of DAB staining were captured using a Nikon Coolpix E4500 digital camera attached to a Nikon Eclipse E800 microscope. Images of fluorescent stains and some DAB stains (Chapter 6, Figure 6.13) were captured using a Hamamatsu CCD C4742 digital camera attached to a Leica DMR microscope using the software Volocity (PerkinElmer, Waltham MA, USA). In some images of fluorescent staining, confocal microscopy was employed using a Leica TCS SPE Laser scanning microscope. A single focal plane was used for any colocalisation images presented using confocal microscopy as well as when determining colocalisation under the Leica DMR microscope.

2.5.7 Quantification of immunohistochemistry

Immunopositive cells for all sections were counted manually with experimenter blind to treatment using a Leica DMR fluorescent microscope. The immunopositive cells were separated by laminae I-II and III-V. Once all sections were counted, per animal, five sections with the maximum response (ipsilateral) were used and the average taken on the ipsilateral and, if necessary, contralateral side of the dorsal horn. The average of the treatment group was then calculated along with the standard error of each group mean (SEM).

2.6 Western Blot

For recipes of Western Blot solutions, please see the Appendix.

2.6.1 Tissue homogenisation and histone extraction (TX Buffer or CHAPS extraction)

Two extraction methods were used: a whole-cell lysate extraction (CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) and a nuclear lysate which is designed specifically for the extraction of histones (TX Buffer). The CHAPS extraction worked well for only one antigen of interest, AcH4K8. All other Western Blots used a specific nuclear fraction lysate obtained with TX Buffer extraction.

2.6.1.1 TX Buffer extraction

Fresh tissue samples (see 2.4.1) were kept on dry ice until the last possible moment, and homogenised using a FastPrep Biopulverisier (MP Biomedicals, France), twice at a speed of 9.5 for 10 seconds each, in 200µl of TX Buffer with 12-15 beads of lysing matrix D (MP Biomedicals, France) per tube. The lysate was briefly spun down and transferred to a 1.5ml Eppendorf on ice for 15min. Samples were then centrifuged at 2,000rpm, 4˚C for 10min. The supernatant was discarded and the pellet washed in TX Buffer. The pellet was resuspended in 50µl TX Buffer + 0.2M HCl and left on ice for 30min to lyse the nuclei. Samples were centrifuged at 10,000rpm, 4˚C for 10min. The supernatant was recovered and stored at -20˚C until bicinchoninic acid (BCA) or immunoblot.

2.6.1.2 CHAPS extraction

In the AcH4K8 blot, the CHAPS protein extraction was used. Tissue samples were homogenised using the FastPrep Biopulveriser method as mentioned above, but instead with 200µl of CHAPS buffer. Following homogenisation, the lysate was transferred to a 1.5ml Eppendorf on ice and left for 2h. The samples were centrifuged at 12,000rpm, 4˚C for 15min. The supernatant was kept and stored at -20˚C until BCA Assay and immunoblot.

2.6.2 BCA Assay

Total protein concentration was measured using a BCA protein assay kit (Pierce, UK). Six protein standards of known concentration were loaded into a 96-well plate in duplicate and experimental samples also added in duplicate to the plate. BCA and copper sulphate was added to all wells and left to incubate at 37 ˚C for 30min. The samples were then measured using a spectrophotometer at 570nm.

2.6.3 Gel run and transfer

Samples were prepared with 4x NuPage LDS loading sample buffer (Life Technologies, UK) to a volume of 18µl and 8 or 10µg of protein. After boiling for 5min, 15µl of prepared sample was loaded into a 12% gel for sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, UK) set in a chamber containing 3-(N-Morpholino)propanesulfonic acid, 4-Morpholinepropanesulfonic acid (MOPS) running buffer. 5µl of Kaleidoscope Precision Plus Protein ladder was also loaded in the gel (Bio-Rad, UK). The gel was run at 160V for 1h. Proteins were subsequently electrophoretically transferred from the gel to a polyvinylidine difluoride (PVDF) membrane in a chamber containing transfer buffer and an ice pack to prevent overheating at 100V for 1h (Bio-Rad, UK). The PVDF membrane had been activated prior to transfer by soaking for 10min in 100% methanol. After the transfer, the membrane was removed and blocked in 0.24% iBlock (Tropix/Applied Biosystems, UK) for 1h and left in a tube on a roller O/N at 4˚C in primary antibody in iBlock (see Table 2.4 Primary Antibodies (Western Blot)).

2.6.4 Immunoblotting

All washes were done 6 x 5min with PBS-Tween on a rocker. The PVDF membrane was washed and incubated in the respective HRP-conjugated secondary antibody (see Table 2.5 Secondary Antibodies (Western Blot) for 50 min at 1:2000/iBlock. HRP activity was reacted with SuperSignal West Pico, a chemiluminescent substrate (Pierce, UK) and visualised either a Chemi Doc XRS or Chemi Doc MP (Bio-Rad, UK). Signal intensity was measured using Quantity One software or Image Lab (Bio-Rad, UK).

2.6.4.1 Housekeeping genes and normalisation

After probing the membrane for the protein of interest the membrane was washed, and subsequently incubated O/N in the nuclear housekeeping gene product calnexin or histone H4, both at 1:1000/iBlock. For the AcH4K8 immunoblot which utilised whole-cell CHAPS extracted lysate, the cytoplasmic housekeeping gene product glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used at 1:2000/iBlock. The following day, detection and visualisation of calnexin was completed the using the same protocol mentioned above (see 2.6.4)

2.6.4.2 Antibodies- Western Blot

Table 2.4 Primary Antibodies (Western Blot)

Antibody	M.W.	Host	Company	Cat. No	Concentration
AcH3K9	17kDa	Rb	Upstate	06-942	1:500
AcH3K14	17kDa	Rb	Cell signalling	4318	1:1000
AcH4K5	14kDa	Rb	Millipore	17-211 06-759	-
AcH4K8	10kDa	Rb	Millipore	17-211 06-760	1:1000
AcH4K12	14kDa	Rb	Millipore	17-211 06-761	1:1000
AcH4K16	10- 14kDa	Rb	Millipore	17-211 06-762	1:500
Calnexin	90kDa	Rb	BioVision	3811-100	1:1000
GAPDH	38kDa	Ms	Millipore	MAB374	1:2000
H3	17kDa	Rb	Millipore	06-755	-
H4	10kDa	Rb	Millipore	07-108	1:1000
PH3S10	17kDa	Ms	Abcam	ab14955	1:1000

Table 2.5 Secondary Antibodies (Western Blot)

Antibody	Host	Company	Cat. No	Concentration
HRP anti-Mouse IgG	Gt	Santa Cruz	sc2302	1:2000
HRP anti-Rabbit IgG	Gt	Santa Cruz	sc2301	1:2000

Antibody Controls- specificity of phospho-site for PH3S10 antibody

It should be noted that the antibody for phospho-histone H3 serine 10 (PH3S10) in future will require a control experiment to confirm the specificity of the antibody for the phosphorylated epitope beyond the use of a blocking peptide. To confirm the antibody specificity for a phosphorylated epitope, tissue sections or protein homogenate should be treated with phosphatases prior to IHC or Western blot. Subsequent completion of the IHC or Western blot protocol using the PH3S10 antibody should indicate no detection of antigen.

Additionally, it is worth noting that PH3S10 is not the only phosphorylated histone residue on H3 (Figure 1.9), which also includes phosphorylation at serine 28 (PH3S28). Notably, PH3S10 and PH3S28 have the same amino acid sequence preceding the serine phospho-site (arginine, lysine, serine), which may cause some concerns over antibody specificity for PH3S10. Although lack of specificity would seem unlikely in this case as the antibody was purchased from a reputable source (ChIP-grade mouse monoclonal, Abcam, UK), it would be interesting to see if IHC detection using PH3S28 would reveal a different pattern of staining in the dorsal horn following formalin stimulation.

2.7 Statistical analysis

All statistical analyses were performed using IBM SPSS PC+ (Chicago IL, USA) or Microsoft Excel (Redmond WA, USA). Analyses were performed using independent t-tests or univariate Analysis of Variance (ANOVA). Data was checked for normal distribution using the Shapiro-Wilk analysis, and homogeneity of variance using Levene's test prior to performing parametric analyses. If the data violated the assumptions required to perform parametric analyses, a $\log_{10}$ transformation was done to achieve a normal distribution (Chapter 6 Behaviour, Figure 6.11). Data was checked for sphericity using Mauchley's test of sphericity and where violated, the Greenhouse-Geisser correction was used. For all experiments a statistical significance of $p<0.05$ was used. For more details regarding analysis please see the relevant Methods section of each chapter.

2.7.1 Quantification and analysis of IHC

In Chapters 5 and 6, cell counts were compared between treatment groups. Comparison was analysed using independent t-test or univariate ANOVA, with *'treatment'* as the between-subjects factor, and *'side'* (ipsilateral vs. contralateral) as the within-subjects factor.

2.7.2 Quantification and analysis of western blots

The signal from the protein of interest was normalised with the intensity of the respective housekeeping protein signal from the same blot. This provided a protein/housekeeping gene ratio which was expressed as a percentage change from the mean control group (sham anaesthesia only) value. The mean of the value for control animals was arbitrarily set to 100%. Normalised signals were averaged for each treatment group and expressed as mean ± SEM, and were compared in sham (control), treated-ipsilateral, and treated-contralateral animals by univariate ANOVA in IBM SPSS PC+ (Chicago IL, USA).

2.7.3 Quantification and analysis of behaviour

In Chapter 6, Formalin-induced nociceptive behaviour was compared in animals receiving Vehicle and animals receiving drug. Data was analysed using a repeated measures ANOVA, with *'treatment'* as the between-subjects factor, and *'time'* as the within-subjects factor. A significant main effect of treatment or significant interaction led to post-hoc analysis by least significant difference (LSD) and Bonferroni tests.

To determine the degree of drug treatment effect, percentage reductions in formalin-induced nocifensive behaviour were calculated using the area under the curve (AUC) for the two formalin phases (first phase 0-10 minutes, second phase 15-60 minutes).

Chapter 3 Peripheral noxious stimulation induces histone phosphorylation in the spinal cord

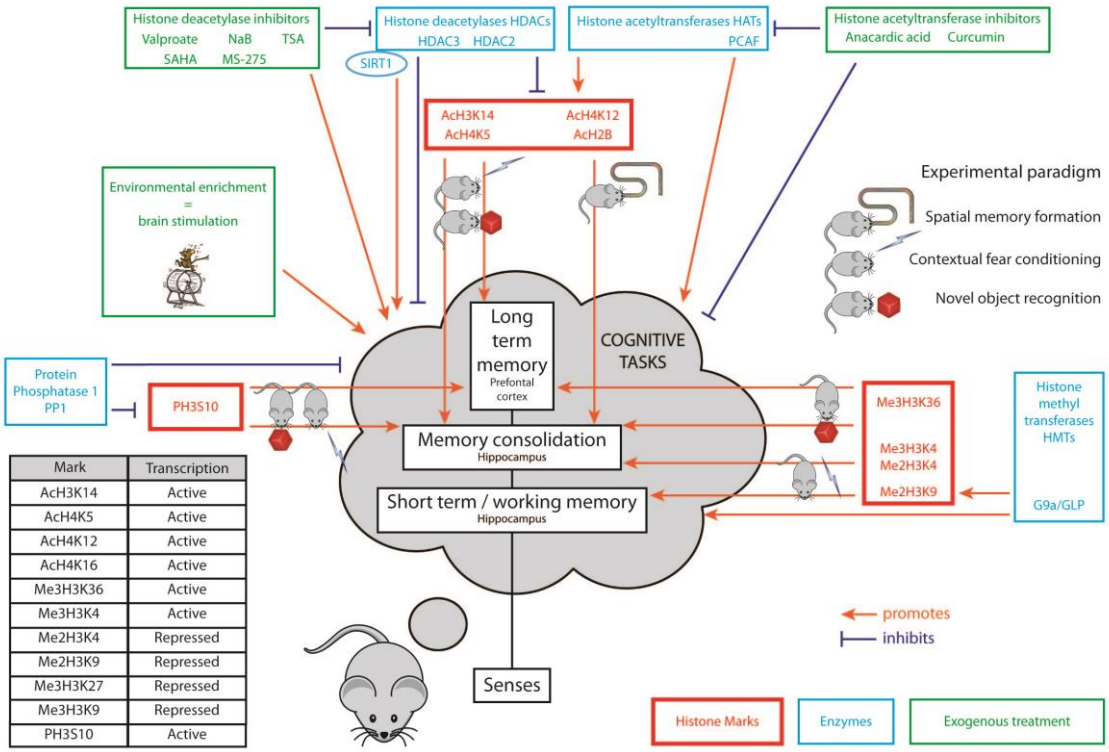
3.1 Introduction

Studies have shown that regulation of gene expression occurs following induction of persistent inflammatory pain states in both DRGs and spinal cord (Géranton et al., 2007; Griffin et al., 2007; Lacroix-Fralish et al., 2006; Valder et al., 2003; Vega-Avelaira et al., 2009; Xiao et al., 2002) and these changes in gene expression are believed to be key to the development and maintenance of central sensitisation. Crucially, it is well known that epigenetic mechanisms, inclusive of dynamic histone modifications, are required for long-term synaptic plasticity and gene regulation (Borrelli et al., 2008; Zocchi and Sassone-Corsi, 2010). This chapter describes experiments investigating expression of histone modifications in the spinal cord after noxious hindpaw stimulation.

3.1.1 The role of histone modification in memory formation

There are many neurobiological similarities regarding the induction of central sensitisation and early- LTP, the respective neural correlates underlying the development of pain states and learning processes (see General Introduction 1.3.2.2 and 1.5.2). An abundance of evidence exists to indicate the involvement of epigenetic mechanisms in learning and memory formation (Day and Sweatt, 2011; Gräff and Tsai, 2013; Roth and Sweatt, 2009). As there is limited albeit growing evidence for the role of epigenetic mechanisms in pain states, it is understandably worthwhile to look at studies investigating histone modifications involved in learning and memory studies.

Importantly, the majority of studies investigating memory consolidation show that alteration of histone modifications, primarily upregulation of histone acetylation, occurs quickly and within hours of memory acquisition. In particular, hippocampal and cortical histone H3 and H4 acetylation associated with activity-induced gene regulation in learning and memory paradigms have been extensively investigated (Figure 3.1). The studies indicate that the expression of histone modifications varies spatiotemporally and is dependent on learning paradigm (Gräff and Tsai, 2013).



Mark	Transcription
AcH3K14	Active
AcH4K5	Active
AcH4K12	Active
AcH4K16	Active
Me3H3K36	Active
Me3H3K4	Active
Me2H3K4	Repressed
Me2H3K9	Repressed
Me3H3K27	Repressed
Me3H3K9	Repressed
PH3S10	Active

Figure 3.1 Histone modifications involved in learning and memory. From Géranton and Tochiki (In press).

3.1.2 Extracellular signal-regulated kinase (ERK) in neural plasticity

A very important and common feature between the induction of central sensitisation and early- LTP is ERK signalling. ERK/MAPK signalling was introduced in Chapter 1 General Introduction (see 1.3.2.3), and plays a crucial role in neuronal plasticity (Impey et al., 1999).

3.1.2.1 Nociceptive activation of ERK/MAPK in the spinal cord

In the pain field, PERK is a widely used marker of neuronal activation by nociceptive stimuli in the spinal cord and brain (Gao and Ji, 2009; Ji et al., 2009). The first evidence for the role of ERK/MAPK in nociceptive processing came from (Ji et al., 1999) who showed the nociceptive-specific, intensity-dependent activation of PERK in the superficial dorsal horn of the spinal cord. Importantly, Ji et al. showed that pre-emptive blocking of PERK with i.t. delivery of PD98059 regulated pain behaviour by attenuating the second phase of nocifensive behaviours associated with hindpaw formalin stimulation by 70%.

It is now known that persistent inflammation induced by various types of stimuli, such as hindpaw inflammation with formalin, CFA, capsaicin, scorpion venom, as well as CFA ankle joint inflammation and visceral pain hyperalgesia caused by bladder distention induce PERK expression in the spinal cord (Cruz et al., 2005; Ji et al., 2002,

1999; Lai et al., 2011). Under normal conditions, PERK requires a stimulus duration significant enough to induce persistent neural activity, and can be activated by high threshold mechanical stimulation mediated by C and Aδ-fibre activity (Gao and Ji, 2010; Hao et al., 2005; Ji et al., 1999). Under pathological conditions however (i.e. inflammation, nerve injury), tactile stimulation is sufficient to induce PERK, and stimulation of Aβ fibres is sufficient to induce PERK following disinhibition by $GABA_A$ receptor antagonists (Baba et al., 2003; Hao et al., 2005). Generally, spinal PERK expression peaks within five minutes and persists for no longer than an hour in the dorsal horn following noxious stimulation.

Activation of PERK is known to be NMDA-dependent (Ji et al., 1999; Lever et al., 2003; Wei et al., 2006), and metabotropic glutamate receptor (mGlu) 1 and 5 are also required for PERK activation in models of inflammation (Karim et al., 2001; Kawasaki et al., 2004). Furthermore the activated neurokinin 1 (NK1) receptor is known to colocalise with PERK, although some controversy exists regarding NK1 activity contributing to PERK expression (Ji et al., 2002; Kawasaki et al., 2004; Lever et al., 2003; Polgár et al., 2007; Wei et al., 2006). Not surprisingly, BDNF, the neurotrophin that binds to the TrkB receptor tyrosine kinase is also known to be a major contributing factor in PERK activation (Pezet et al., 2002).

3.1.3 Aims

A key study by Peleg et al. (2010) found increased expression of five of six investigated acetylated histone residues in the hippocampus 1h hour post- fear conditioning training, a time point associated with the early induction of synaptic plasticity associated with memory acquisition. The modifications investigated included AcH3K9, AcH3K14, AcH4K5, AcH4K8, AcH4K12 and AcH4K16 which was the only acetylated residue with unaltered expression 1h post-fear conditioning. Furthermore, a study by Chwang et al. (2006) found that AcH3K14, PH3S10, and joint phosphoacetylation (PH3S10-AcH3K14) were upregulated in the hippocampus 1h after fear conditioning training.

The similarity of molecular events involved in the induction and maintenance of LTP, the proposed underlying neural correlate of memory formation, and central sensitisation (Ji et al., 2003), indicate that changes in histone modification may also be observed at early time points following injury-induced sensitisation. Furthermore, changes in gene expression, known to occur in the dorsal horn following noxious stimulation (Géranton et al., 2007), are preceded by and require histone modifying events.

The experiments in this chapter investigate eight specific histone modifications implicated in the studies by Peleg et al. (2010) and Chwang et al. (2006). Specifically, the aim of this chapter was to:

1. Use Western blot and IHC to screen for any alterations in histone modification levels in the spinal cord following noxious formalin stimulation at early time points (1h) known to be key to the induction of central sensitisation.

2. Investigate the effect of selective activation of C-fibre nociceptors on histone modification in the dorsal horn following i.pl. capsaicin.

3.2 Materials and Methods

Please also see Chapter 2 for General Materials and Methods.

3.2.1 Experimental Protocol

There are 2 separate experiments in this chapter:

3.2.1.1 ***Experiment 1:*** *The preliminary analysis of various histone modifications in the spinal cord following noxious formalin stimulation*

There were 2 treatment groups:

1. Formalin with anaesthesia
2. Sham anaesthesia only

All animals were sacrificed for fresh tissue (n=6 each group) or perfusion (n=1 each group) 1h following stimulation for western blot or immunohistochemistry (IHC) analysis, respectively.

3.2.1.2 ***Experiment 2:*** *Histone modification in the spinal cord following noxious capsaicin stimulation*

There was 1 group:

1. Capsaicin with anaesthesia

All animals were sacrificed for perfusion 1h following stimulation for IHC (n=4). There was no separate control group as the previous experiment established a very low level of PH3S10 nuclei in sham anaesthesia animals. Expression of PH3S10 in the contralateral dorsal horn corresponding to the non-stimulated hindpaw was so low that it was used as an internal control but not quantified here.

3.2.2 Formalin stimulation

Under isoflurane anaesthesia animals were given intraplantar formalin, 50µl 2% (see 2.2). Sham animals were given general anaesthesia only.

3.2.3 Capsaicin stimulation

Intraplantar capsaicin, 25µl, 1% was given under isoflurane anaesthesia (see 2.2).

3.2.4 Immunohistochemistry

All staining done in this chapter were single stains done using either fluorescent IHC with TSA or Direct Alexa (see 2.5). Note that for this chapter PERK and PH3S10 were detected with TSA. See Table 2.2 and 2.3 for primary and secondary antibody concentrations.

Table 3.1 IHC experiments completed in Chapter 3. All investigations 1h post-stimulation. Asterisk indicates use of acid pretreatment antigen retrieval step (see 2.5.2).

Immunostain	Method of detection
PERK	TSA
PH3S10	TSA
PH3S10-AcH3K14	TSA
AcH3K9	Direct Alexa
AcH3K14	TSA*
AcH4K8	Direct Alexa*
AcH4K12	Direct Alexa
AcH4K16	Direct Alexa

3.2.4.1 Immunohistochemistry- Image acquisition

Images of fluorescent IHC in this chapter were taken on the Leica TCS SPE Laser scanning confocal microscope using an air 10x optic lens, or a Hamamatsu CCD C4742 digital camera attached to a Leica DMR microscope using the software Volocity (PerkinElmer, Waltham MA, USA). Images of DAB staining were captured using a Nikon Coolpix E4500 digital camera attached to a Nikon Eclipse E800 microscope

3.2.5 Western blot

Ipsilateral and contralateral (to the injected hindpaw) dorsal horn tissue of L4-L6 (see 2.4.1) was taken fresh and processed for histone extraction and western blot (see 2.6) (n=6 each group).

3.2.5.1 Tissue homogenisation and histone extraction

The nuclear-lysate TX Buffer extraction method (see 2.6) was used for the AcH3K9, AcH3K14, AcH412, AcH4K16, PH3S10 immunoblots. The lysate used for the AcH4K8 immunoblot was homogenised and extracted using whole-cell lysate CHAPS extraction (see 2.6). See Tables 2.4 and 2.5 for primary and secondary antibody concentrations.

3.2.5.2 Housekeeping gene products used for normalisation

Due to the nature of the CHAPS extraction, on the AcH4K8 immunoblot, GAPDH was used as the internal housekeeping gene. For the AcH3K9, AcH3K14, AcH412, and AcH4K16 blots, Calnexin was used. For the PH3S10 blot, H4 was used. Calnexin and H4 are nuclear housekeeping gene products, suitable for use with the nuclear lysate-

specific TX-buffer extraction, while GAPDH is a whole cell-lysate housekeeping gene product suitable for CHAPS extracted tissue samples.

3.2.5.3 Western blot- Image acquisition

Immunoblots were visualised using either a Chemi Doc XRS or Chemi Doc MP (Bio-Rad, UK). Signal intensity was measured using Quantity One software or Image Lab (Bio-Rad, UK).

3.2.5.4 Western blot- Quantification

For the western blots in *Experiment 1*, the signal from the protein of interest was normalised with the intensity of the respective housekeeping protein signal from the same blot. This provided a protein/housekeeping gene ratio which was expressed as a percentage change from the mean control group (sham-anaesthesia only) value. The mean of the value for control animals was arbitrarily set to 100%. Normalised signals were averaged for each treatment group and expressed as mean ± SEM.

3.2.6 Statistical analysis

Please also see 2.7 for full details of statistical analysis.

3.2.6.1 Western blots

Group means as calculated above (3.2.5.4) were analysed by univariate ANOVA with treatment (sham, treated- ipsilateral, and treated-contralateral) as the between-subjects factor.

3.3 Results

3.3.1 Quantification of 6 different histone modifications in the dorsal horn indicates no significant changes following formalin stimulation

Western blot was used to screen six different histone residues in ipsilateral and contralateral dorsal horn of formalin-stimulated and sham animals. Quantification of western blots did not show any significant formalin-induced changes in PH3S10, AcH3K9, AcH3K14, AcH4K8, AcH4K12, or AcH4K16 expression although a trend towards increased expression of PH3S10 was seen. (Figure 3.2). It should be noted that while the aim was to investigate the 8 aforementioned histone modifications by western blot, the experiment for AcH4K5 was unsuccessful as the antibody could not identify protein at the correct position on the gel. Moreover, PH3S10-AcH3K14 was not investigated by western blot.

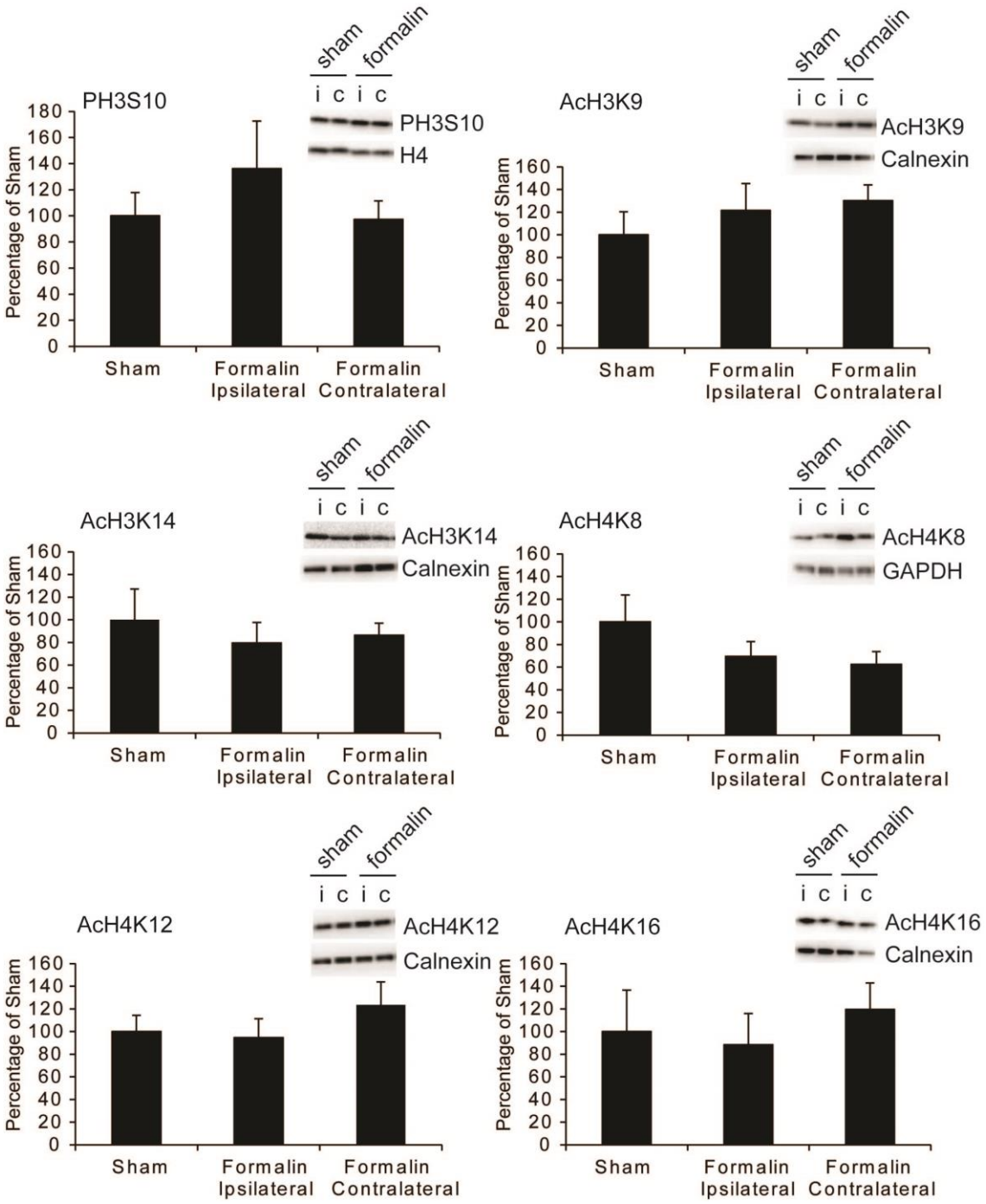


Figure 3.2 Western blot analysis indicates no significant change in histone acetylation or phosphorylation in the dorsal horn of the spinal cord 1h post-intraplantar formalin stimulation. (n=5/6)

3.3.2 Immunohistochemistry suggests that histone phosphorylation is upregulated in the superficial dorsal horn 1h following formalin stimulation

Although no significant formalin-induced changes in histone modification were observed in the dorsal horn with western blot analysis, additional preliminary screening was completed using IHC in order to observe the localisation of the histone modifications in dorsal horn tissue of formalin animals. There was no apparent difference between contralateral and sham expression as indicated by western blot, therefore only IHC from formalin stimulated tissue is shown.

The majority of stains indicated that histone acetylation (AcH3K9, AcH3K14, AcH4K8, AcH4K12, AcH4K16, and co-occuring PH3S10-AcH3K14) was nuclear in expression and very densely expressed throughout both the ipsilateral and contralateral dorsal horn of formalin stimulated and sham animals (Figure 3.3). There were no visible differences between ipsilateral and contralateral expression in stimulated animals, and manual quantification would have been difficult due to the ubiquitous nature of positive staining.

Remarkably, IHC suggested that histone phosphorylation at serine 10 (PH3S10) was the only histone residue to show a visible and obvious upregulation in the ipsilateral medial superficial dorsal horn following formalin stimulation and with low resting levels of activation. PH3S10 was upregulated in a small but distinct population of cells, and this upregulation was not observed in contralateral or sham ipsilateral tissue (Figure 3.3). The upregulation of PH3S10 in the ipsilateral dorsal horn displayed a distribution pattern similar to the expression of PERK at 30min post-formalin (Figure 3.3, shown for comparison).

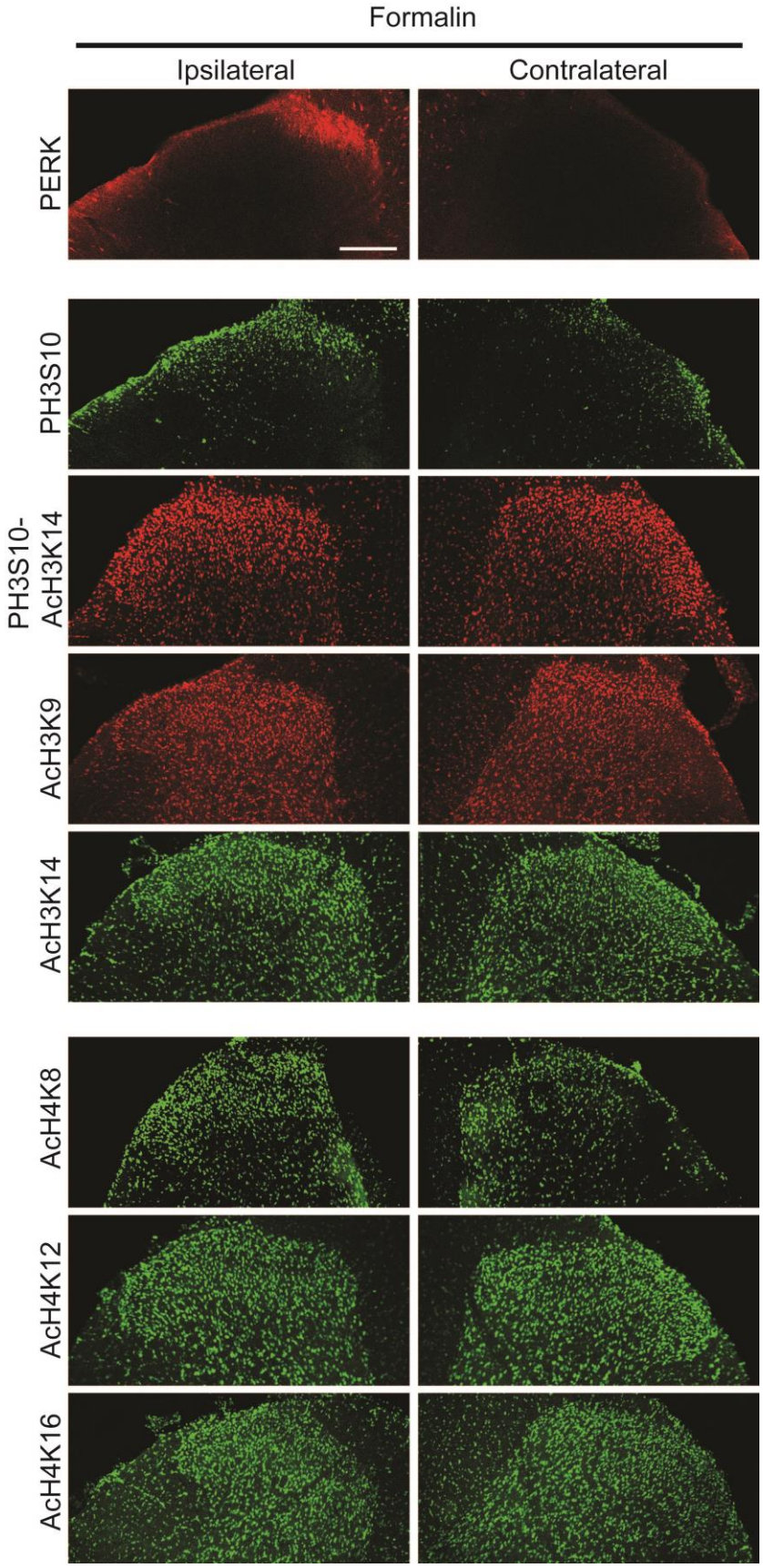


Figure 3.3 IHC screen of 7 histone modifications (at 1h) post-formalin. Preliminary screen suggests ipsilateral upregulation of histone H3 phosphorylation (PH3S10) but no change in histone H3 or H4 acetylation in the superficial dorsal horn 1h post-formalin. Scale bar 100µm.

3.3.3 PH3S10 is upregulated in the superficial dorsal horn following selective stimulation of C-fibres by capsaicin

After preliminary IHC screening suggested that PH3S10 was upregulated in the dorsal horn following formalin stimulation, a full investigation into noxious stimulation-induced upregulation of PH3S10 was completed using i.pl. stimulation with capsaicin. In contrast to formalin which is capable of activating all primary afferents (see Discussion 3.4), capsaicin selectively stimulates TRPV1- expressing C-fibre nociceptors. At 1h, capsaicin-induced PH3S10 was diffusely distributed across laminae I-II of the dorsal horn (10.0±1.0 nuclei/40µm section, n=4) (Figure 3.4).

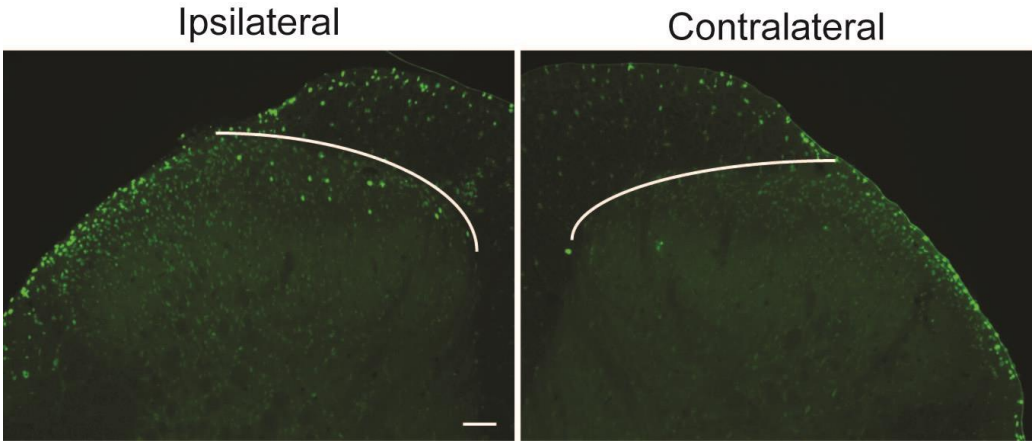


Figure 3.4 PH3S10 is upregulated in the ipsilateral superficial dorsal horn following intraplantar capsaicin stimulation. Scale bar 50µm. White line indicates border between lamina I and white matter.

3.4 Discussion

It is well-established that key histone modifications indicative of active gene transcription are involved in models of neural plasticity, specifically in the hippocampus following memory formation. It is also well-known that changes in gene expression are essential to the full development of pain states. The main aim of this chapter was to identify candidate histone modifications involved in nociceptive processing, by investigating the expression of histone modifications in the dorsal horn following noxious stimulation. A Western analysis of a number of candidate modifications failed to show obvious differences between stimulated and unstimulated sides of the dorsal horn of the spinal cord. However a follow up screen using immunohistochemistry confirmed high constitutive levels of most histone modifications examined but a specific and unique upregulation of PH3S10.

3.4.1 Using formalin to induce acute inflammation

Preliminary IHC experiments (*Experiment 1)* and results from the capsaicin study (*Experiment 2*) demonstrated that histone H3 is phosphorylated at the serine 10 residue (PH3S10) in the dorsal horn following acute noxious stimulation. Following on from these results, it was decided that the rest of the experiments in this thesis would continue to utilise the formalin model to further investigate the role of PH3S10 in pain processing.

There are many reasons why formalin is a relevant model for the purposes of this thesis. The formalin stimulus is described as 'moderate, continuous pain generated by injured tissue' that generates 'tonic' pain which is measured across 1h of the formalin response (Tjølsen et al., 1992). Moreover, formalin-induced injury involves components of peripheral inflammatory and spinal dorsal horn central nervous system mechanisms (Tjølsen et al., 1992). However, considering its working function as a tissue fixative, formalin as a noxious stimulus may not be mediated by a specific receptor, although recent reports may suggest otherwise (see 3.4.1.1). While this caveat makes it difficult to dissect out fibre-specific contributions to behaviour and molecular targets, formalin at higher concentrations (2.5%) is known to cause broad activation of all primary afferents including Aβ, Aδ and C fibres in the first phase response (Puig and Sorkin, 1996), and it has been reported that at 5% formalin induces upregulation of the nerve injury marker, ATF3, in both myelinated and unmyelinated fibres in the DRG (Bráz and Basbaum, 2010). Notably, *Experiment 2* confirmed that stimulation of nociceptive C-fibres alone, with i.pl. capsaicin was sufficient to induce PH3S10 in the dorsal horn (Caterina et al., 1999). However, when investigating whether novel epigenetic mechanisms are

engaged in nociceptive processing, broad activation of primary afferent activity by formalin is advantageous as it includes C, Aδ, and Aβ nociceptor activity, instead of selective C-fibre stimulation as is the case with capsaicin. Furthermore, the formalin response is an advantageous model behaviourally as it does not require stimulus-evoked measurements to calculate changes in nociceptive threshold, but rather is characterised by spontaneous pain behaviours, a dominant clinical feature of chronic pain conditions (Tappe-Theodor and Kuner, 2014). Finally, for the purposes of Chapter 6, the time course of the formalin model is short in duration (1h) and ideal for quick screening of therapeutic compounds.

3.4.1.1 Mechanisms and effects of formalin concentration

While the exact mechanisms of formalin-induced behaviour and molecular targets are still not entirely known, it is of general agreement that the first phase (first 10min) of the response is a result of peripheral nociceptor activation, while the second phase (20-60min) is partly mediated by central sensitisation in spinal cord circuits following on from peripheral nociceptor sensitisation maintained by sustained Aδ and C-fibre activity (Dubuisson and Dennis, 1977; Fischer et al., 2014; Puig and Sorkin, 1996; Tjølsen et al., 1992). These findings have been elucidated by modulating the first phase of activity with pharmacological agents and looking at the second phase response. Both local hindpaw anaesthetic as well as brief spinal anaesthesia during the first phase response are sufficient to attenuate behavioural and neuronal characteristics observed during the second phase response (Coderre et al., 1990; Dickenson and Sullivan, 1987a, 1987b; Haley et al., 1990).

More recently, it has been shown that at low concentration of formalin (0.5%), i.t. ablation of TRPV1 primary afferent central terminals using capsaicin attenuates the first 10 minutes of nociceptive behavioural response seen after formalin stimulation (Shields et al., 2010). TRPV1-expressing unmyelinated neurons contain a small subset of TRPA1-expressing fibres which may account for these observations (Kobayashi et al., 2005; Story et al., 2003) as it has been shown that low doses of formalin (0.5%) directly interact with the non-specific cation channel TRPA1 (Macpherson et al., 2007; McNamara et al., 2007). Interestingly, double ablation of the majority of C-fibres by targeting TRPV1-expressing nociceptors and Mrgprd, the G-protein coupled receptor that is expressed on the majority of non-peptidergic C-fibres, increased the latency to onset of formalin response (2min) but did not attenuate total nocifensive behaviour in either phase of formalin response generated with a higher dose of formalin (2%) (Shields et al., 2010). Although TRPV1/Mrgprd ablation did indeed lead to a reduction

in spinal cord c-Fos expression, Shields et al. suggest that the few remaining C-fibres after ablation as well as larger untouched myelinated Aδ afferents are sufficient to generate a full formalin response at the 2% formalin dose. However, the lack of behavioural differences seen may also be accounted for by different factors used in scoring of the formalin response (i.e. intensity vs. duration).

3.4.2 Histone acetylation, HDACs, and HDAC inhibitors in the spinal cord after noxious stimulation

It was somewhat surprising to find no global changes in formalin-induced histone acetylation in the spinal cord by western blot analysis (*Experiment 1*). Given that global HDAC levels in the dorsal horn are indeed altered during CFA-induced persistent pain states in mice and rats (Bai et al. 2010; Tochiki et al. 2012), it would seem logical to also expect changes in histone acetylation levels. Bai et al. showed an increase in dorsal horn expression of class IIa HDACs 4, 5, 7, and 9 up to 6 hours following intraplantar CFA inflammation. The same study also found that pre-emptive i.t. delivery of HDAC inhibitors suberoyl anilide hydroxamic acid (SAHA), valproic acid (VPA), trichostatin A (TSA), 4-phenyl butyrate (4-PB), and LAQ-824 delayed the onset of thermal hyperalgesia, which was shown to cause an increase in spinal cord AcH3K9 levels. Tochiki et al. (2012) reported bilateral upregulation of class I/IIa HDACs 1 and 5 mRNA in the dorsal horn 7d after CFA ankle joint injection. However, it is important to note that while Bai et al. showed attenuation of thermal hypersensitivity with Class I and Class IIa HDAC inhibitors (with the exception of MS-275), the authors never indicated whether injury lead to changes in histone acetylation per se. This is an important consideration due to the fact that HDAC inhibitors can act on substrates other than histones.

Moreover, although western blot analysis in *Experiment 1* showed no global changes in formalin-induced acetylation levels, i.p. delivery of the class I HDAC inhibitor, MS-275, as well as class II inhibitor SAHA have been previously shown to attenuate the second phase of the formalin response (Chiechio et al., 2009). Due to the non-specificity of HDAC inhibitors, Chiechio et al. found hyperacetylation of the p65 transcription factor, a regulator of NFκB genes including the mGlu2 receptor, which increased in DRG and spinal cord after HDAC inhibition. However, again, no injury-induced changes in acetylation were reported in this study.

It is worth mentioning that changes in global acetylation can easily overlook locus-specific changes in acetylation levels. Indeed, the majority of pain studies concerning histone modification have investigated acetylation levels linked to specific transcription

factor or gene targets such as transcription factors p65 and REST, *Bdnf* and *Nav1.8* in the DRG, *KC* (ligand for CXCR2) and *CXCR2* in the spinal cord, and *Gad2* in the NRM (Chiechio et al., 2009; Matsushita et al., 2013; Sun et al., 2013; Uchida et al., 2010; Uchida et al., 2010; Uchida et al., 2013; Zhang et al., 2011). Furthermore, results in this chapter indicated that basal levels of histone acetylation in sham animals were much higher and more ubiquitously expressed in comparison to that of PH3S10, which may have made it difficult to detect any significant changes in expression after stimulation. It also suggests that the obvious increase in spinal cord PH3S10 may indicate that histone phosphorylation is more specific to nociception than histone acetylation.

The initial studies in the pain epigenetics field were quick to investigate the effects of HDAC inhibitors on behavioural hypersensitivity in inflammatory and neuropathic pain models. While the studies showed that global or locus-specific histone acetylation levels expectedly increased *after* delivery of HDACi (Bai et al., 2010; Chiechio et al., 2009; Denk et al., 2013; Matsushita et al., 2013; Sun et al., 2013; Zhang et al., 2011), only Matsushita et al., Sun et al., and Zhang et al. rightly established first that acetylation is in fact deregulated following induction of a pain state (Nerve injury-induced hypoacetylation of H3/H4 at *Nav1.8* in the DRG, incision-induced AcH3K9 in the spinal cord, and hypoacetylation of H3 at *Gad2* in the NRM, respectively).

The literature conclusively indicates that histone acetylation plays a role in various types of pain states, and IHC results in this chapter also indicate that histone phosphorylation is engaged and upregulated after noxious stimulation.

3.4.3 Technical limitations

PH3S10 western blot vs. immunohistochemistry

Although the IHC screen of histone modifications completed in *Experiment 1* (Figure 3.3*)* was not exhaustive (i.e. insufficient number of animals), preliminary results revealed a ubiquitous distribution of acetylated histone residues in the spinal cord. There were no obvious visible formalin-induced differences in histone acetylation, which was confirmed by western blot. It should be noted that distribution of histone acetylation as indicated by IHC was ubiquitous and extremely variable (i.e. patchy), which would have made manual quantification difficult. Thus, no further attempts were made toward the investigation of these residues.

Interestingly, the upregulation of PH3S10 nuclei in the ipsilateral dorsal horn visible by IHC was not confirmed by results from the western blot. While this is disappointing, it is not surprising as the quantified PH3S10 population was roughly 20 labelled nuclei at

the 1h time point. It is likely that a western blot might not be sensitive enough of a technique to detect such a limited population size. Furthermore, the spinal cord dissection used here included the entire dorsal half of the spinal cord rather than a laminae I-II specific sampling and therefore may have resulted in a dilution of any changes in expression. However, it should be noted here that Denk et al. (2013) were able to detect differences in histone acetylation (AcH3K9) in dorsal ipsilateral vs. contralateral segments in the d4T (stavudine, anti-retroviral drug known to cause neuropathy) and spinal nerve transection models of neuropathic pain. The IHC results from this *Experiment 1* and *Experiment 2* suggested that IHC was the best method forward for investigating the PH3S10 population.

Western blot housekeeping gene products

Total H3 was an additional candidate as a housekeeping gene product for normalisation of histone modifications investigated, but the antibody did not detect protein at the correct position on the gel and thus could not be used.

3.5 Conclusions

In summary, preliminary IHC results indicated that noxious stimulation by both formalin and capsaicin caused an increase in histone modification in the ipsilateral superficial dorsal horn. Upregulation following noxious stimulation was specific to histone phosphorylation (PH3S10), as no changes in histone acetylation were observed. Furthermore, expression of PH3S10 was highly specific and restricted to a small population of neurons, and upregulation at the 1h time point correlated with the induction phase of pain states.

Following on from these results, the next aim was to use IHC to further investigate the PH3S10 population after noxious formalin stimulation, and in particular, examine the expression of PH3S10 within the pain pathway.

Chapter 4 Cellular characterisation of PH3S10 in the dorsal horn following noxious stimulation

4.1 Introduction

Findings in Chapter 3 indicated that histone phosphorylation (specifically PH3S10) was upregulated in the superficial dorsal horn following hindpaw formalin and capsaicin stimulation. Studies have indicated that PH3S10 is a marker of active gene transcription (Crosio et al., 2003; Mahadevan et al., 1991; Thomson et al., 1999), and is induced after both pharmacological and experience-dependent activity in the brain, specifically the hippocampus (Borrelli et al., 2008; Riccio, 2010; Sweatt, 2009; Zocchi and Sassone-Corsi, 2010). However, PH3S10 has never been studied in the spinal cord. This chapter describes experiments aimed at further investigating the expression and cellular profile of PH3S10 in the dorsal horn after noxious stimulation.

4.1.1 PH3S10: A marker of gene transcription

Mahadevan et al. were the first to correlate activity-induced PH3S10 expression with the IEGs *c-Fos* and *c-jun*, subsequently setting the scene for PH3S10 as a marker of active gene transcription in both non-neural and neural cells (Crosio et al., 2003; Mahadevan et al., 1991; Thomson et al., 1999). Along with other histone modifications, PH3S10 plays an important role in chromatin remodelling, in particular the decondensation of tightly-wrapped chromatin to allow transcription factor access to DNA. Levels of PH3S10 are known to correlate with synaptic activity-induced increases in nuclear infolding, with high infolding of the nucleus being indicative of increased gene transcription (Wittmann et al., 2009). Furthermore, histone phosphorylation is engaged in various forms of activity-induced neuronal activity, namely memory formation, drug addiction, and visual processing (Chandramohan et al., 2007; Chwang et al., 2006; Ciccarelli et al., 2013; Crosio et al., 2000; Kumar et al., 2005; Putignano et al., 2007; Rotllant and Armario, 2011). Interestingly, PH3S10 is also widely used as a marker of mitotic cell division, and studies conversely indicate that PH3S10 is correlated with chromatin condensation during this event (Prigent and Dimitrov, 2003). The seemingly contradicting roles for PH3S10 in chromatin remodelling indicate that the same modification can have separate functions depending on context (Jenuwein and Allis, 2001; Nowak and Corces, 2004).

PH3S10 is regulated by ERK/MAPK signalling, primarily by the mitogen- and stress-activated protein kinases 1 and 2 (MSK1, MSK2), which lie downstream of ERK (Deak et al., 1998; Soloaga et al., 2003). The fact that ERK/MAPK signalling also plays a crucial role in pain processing provides a strong basis for investigating PH3S10 in nociception. Further information on MSK1 and MSK2 will be addressed in Chapter 6, and ERK regulation of S10 is discussed at the end of this chapter.

4.1.2 PERK and immediate early gene (IEG) proteins c-Fos and Zif268: Markers of neuronal activation following noxious stimulation

As previously highlighted, PERK is required for the full development of inflammatory pain states, and is widely used as a marker of nociceptive neuronal activation in the spinal cord and brain (Gao and Ji, 2009; Ji et al., 2009).

The IEG product c-Fos is another widely used classical marker of noxious stimulation, *c-Fos* being a proto-oncogene rapidly induced following neurotransmitter release (Greenberg et al., 1986). Hunt et al. were the first to identify upregulation of c-Fos protein in the ipsilateral dorsal horn following peripheral stimulation using noxious heat and mustard oil (Hunt et al., 1987), and it has subsequently been found that c-Fos is also induced by noxious mechanical and various chemical stimuli in an intensity-dependent manner. Similar to PERK, c-Fos can be induced by normally non-noxious stimuli following inflammation and nerve injury (Bester et al., 2000; Kosai et al., 2001; Ma and Woolf, 1996; Molander et al., 1994; Wei et al., 1999).

The majority of c-Fos expression occurs within laminae I-II, similar to that of PERK; however c-Fos is nuclear in expression and has a comparatively larger population size (Gao and Ji, 2009). As c-Fos protein expression requires gene transcription, it is difficult to target unless using RNA interference. In the rodent dorsal horn, peak protein levels of c-Fos occur at the 1-2 hour time point after noxious stimulation, although *c-Fos* mRNA is known to peak at 30 minutes (Figure 4.1). It has been shown that formalin-induced c-Fos occurs in both excitatory and inhibitory interneurons in the dorsal horn (Hossaini et al., 2010). Some controversy exists over the use of PERK vs. c-Fos as a marker of activation by nociceptive stimuli (Gao and Ji, 2009) due to the fact that certain repetitive non-noxious stimuli can induce c-Fos but not PERK, such as rotarod walking or brush stroking (Neumann et al., 2008). Moreover, the duration of noxious stimulation required to induce central sensitisation is also a factor as only longer lasting stimuli generating persistent lasting changes in spinal cord activity are capable of activating PERK (Gao and Ji, 2009). Please see Chapter 6 for discussion of c-Fos and PERK correlation with pain behaviour.

Zif268 (encoded by *Egr-1*) has also been established in the dorsal horn as a marker of nociceptive stimulation (Wisden et al., 1990), although it is not as frequently used as c-Fos. Zif268 is well characterised in the hippocampus as being necessary to the induction of LTP, and is generally recognised as a marker of neural plasticity (Beckmann and Wilce, 1997; Hughes and Dragunow, 1995; Lanahan and Worley, 1998). Rapidly activated *c-Fos* and *Zif268* appear to regulate a multitude of

downstream gene targets and the protein products themselves are inducible transcription factors. In nociception, hindpaw CFA- induced Zif268 peaks at 1-2 hours (Géranton et al., 2008) (Figure 4.1), and antisense knockdown of Zif268 upregulation has been shown to attenuate mechanical hyperalgesia (Rygh et al. 2006). Furthermore, Zif268 expression is regulated by descending serotonergic activity from the RVM, and ablation of these fibres leads to a reduction of both spinal c-Fos and Zif268 (Géranton et al., 2008).

A final transcription factor and epigenetic "reader" that is also expressed in the superficial dorsal horn following noxious stimulation is phospho-methyl-CpG-binding protein 2 (PMeCP2) (Géranton et al., 2008, 2007). PMeCP2 is a global regulator of gene transcription and is known to peak in the dorsal horn 1h following noxious peripheral stimulation. Silencing the transcription of one of the downstream targets of PMeCP2, the serum and glucocorticoid kinase 1 (SGK1), delays the onset of CFA-induced mechanical hypersensitivity.

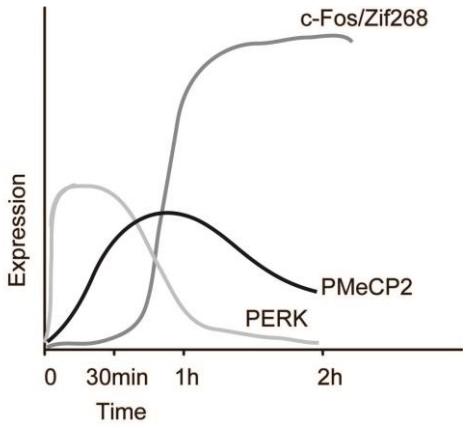


Figure 4.1 Time course of PERK, PMeCP2, and IEG product expression in the superficial dorsal horn following noxious peripheral stimulation. PERK is known to peak at 5 minutes and PMeCP2 at 1h, while IEG products c-Fos and Zif268 are not expressed before 1h.

4.1.3 The role of NK1 projection neurons in pain processing

The neurokinin-1 (NK1) receptor is the target for the neurotransmitter substance P (SP), and has long been of interest in nociception because it is selectively expressed on dorsal horn neurons responsive to noxious stimuli (De Koninck and Henry, 1991; Doyle and Hunt, 1999; Salter and Henry, 1991). In lamina I of the lumbar region, 45% of neurons are known to be immunoreactive for NK1 (Todd et al., 1998). While not all lamina I projection neurons are NK1 positive, the majority are (80%), and synapse in the lateral parabrachial area (95% of all projection neurons) (Al-Khater et al., 2008; Spike et al., 2003; Todd et al., 2000). NK1 positive projection neurons in particular are known to play a key role in neuropathic and inflammatory pain processing (Mantyh et

al., 1997; Nichols et al., 1999). Mantyh et al. were the first to demonstrate the importance of these neurons in nociceptive processing using SP conjugated to the ribosome inactivating toxin, saporin (SP-SAP). I.t. SP-SAP led to an 85% reduction of SP immunoreactivity 28 days following delivery, and attenuated 85% and 60% of capsaicin-induced mechanical and thermal hyperalgesia, respectively. Ablation of NK1 projection neurons is also known to decrease receptive field size in deep dorsal horn neurons, and activity of NK1 projection neurons is thought to act via descending pathways from the brainstem containing 5HT (Suzuki et al., 2002). Specifically, the ablation of NK1-positive projection neurons prevented inflammation-induced changes in hypersensitivity and spinal excitability to the same degree as the ablation of the descending 5HT facilitatory projections to the dorsal horn from the RVM. NK1 itself is upregulated in the superficial dorsal horn 48 hours after intraplantar CFA stimulation (Abbadie et al., 1997; Ji et al., 2002).

4.1.4 Aims

Results from Chapter 3 established that PH3S10 upregulation induced by noxious stimulation was coupled with the induction phase of pain states. It is well-established that PH3S10 is regulated by ERK/MAPK via MSK1 and MSK2 (Deak et al., 1998; Soloaga et al., 2003). Due to the fact that ERK/MAPK signalling is known to play a crucial role in the full expression of pain states (Ji et al., 1999), it was expected that PH3S10 expression would occur within cells activated by nociceptive signalling.

The aim of this chapter was to further investigate the expression of PH3S10 in the spinal cord after hindpaw formalin stimulation. Specifically, to use IHC to:

1. Investigate the time course of formalin-induced PH3S10 upregulation.
2. Identify the cellular specificity of PH3S10 (as identified by colocalisation with neuronal marker NeuN, inhibitory interneuron markers PV and nNOS, excitatory interneuron marker calbindin, and glial markers APC CC1, GFAP, and Iba-1)
3. Investigate the colocalisation of PH3S10 with markers indicating neurons key to pain processing (PERK, NK1, c-Fos, and Zif268)

4.2 Materials and Methods

Please also see Chapter 2 for General Materials and Methods.

4.2.1 Experimental design

All animals were stimulated in the hindpaw with formalin, sacrificed for perfusion, and spinal cord tissue used for immunohistochemistry (see 2.4.2).

For the PH3S10 Time Course experiment: Animals had a survival time of 5min, 30min, 1h, 2h, 4h or 6h (n=3 for all time points except 4h and 6h, n=1).

For PH3S10 Colocalisation Studies: The survival time post- formalin stimulation depended on the molecular marker of interest; a time point was chosen to maximise the overlap of PH3S10 and the second population of interest (see Figure 4.10). All colocalisation investigations of PH3S10 in this chapter used the 1h time point, except for PH3S10/ PERK which was investigated 30min (n=3/4 each double stain) (Table 4.1).

4.2.2 Formalin stimulation

All animals were given intraplantar formalin, 50µl 2% under isoflurane anaesthesia (see 2.2.1).

4.2.3 Immunohistochemistry

Please refer to section 2.5 for the full immunohistochemical protocol and antibody information. The majority of immunohistochemistry experiments done in this chapter were double label stains (Table 4.1).

Table 4.1 IHC experiments completed in this chapter

Immunostain	1st primary	Method of detection	2nd primary	Method of detection	Time point
PH3S10 time course	PH3S10	TSA	-	-	various
PH3S10/**NeuN**	PH3S10	TSA	NeuN	Direct Alexa 594	1h
PH3S10/**GFAP**	PH3S10	TSA	GFAP	Direct Alexa 594	1h
PH3S10/**APC CC1**	PH3S10	TSA	APC CC1	Direct Alexa 594	1h
PH3S10/**Iba-1**	PH3S10	TSA	Iba-1	Direct Alexa 594	1h
PH3S10/**nNOS**	nNOS	TSA	PH3S10	Direct Alexa 594	1h
PH3S10/**PV**	PH3S10	TSA	PV	Direct Alexa 594	1h
PH3S10/**Calbindin**	PH3S10	TSA	Calbindin	Direct Alexa 594	1h
PH3S10/**PERK**	PERK	TSA	PH3S10	Direct Alexa 594	30min
PH3S10/**NK1**	NK1	TSA	PH3S10	Direct Alexa 594	1h
PH3S10/**c-Fos**	c-Fos	TSA	PH3S10	Direct Alexa 594	1h
PH3S10/**Zif268**	Zif268	TSA	PH3S10	Direct Alexa 594	1h

Where PH3S10 was applied as a second primary, acid pretreatment was used for antigen retrieval prior to blocking (see 2.5.2).

4.2.4 Immunohistochemistry- Image acquisition

Images of fluorescent immunohistochemistry in this chapter were taken on the Leica TCS SPE laser scanning confocal microscope, or a Hamamatsu CCD C4742 digital camera attached to a Leica DMR microscope using the software Volocity (PerkinElmer, Waltham MA, USA). All images for colocalisation were taken in a single focal plane.

4.2.5 Immunohistochemistry- Quantification

PH3S10 Time Course

With the experimenter blind to treatment, immunopositive cell counts for all sections were quantified manually by laminae (I-II and III-V) on the ipsilateral and contralateral side of the dorsal horn using a Leica DMR fluorescent microscope.

Once all sections were counted, per animal, five sections with the maximum response (ipsilateral) were used and the average taken. The average of the treatment group was then calculated along with the standard error of each group mean (SEM).

Double Stains- population percentage overlap

The goal was to obtain a percentage overlap of the two labelled populations of interest. Firstly, cells were quantified using above protocol (PH3S10 Time Course) for the PH3S10 population. Then, using the same tissue sections, the second population of interest, as well as the population of double labelled cells were quantified. The percentage of double labelling was then calculated for each section of tissue, and averaged per animal. Finally, the average percentage of double labelling for the group was presented as mean ± SEM.

Because the maximal expression of two populations of interest may not occur in the same tissue sections, out of interest, the maximum the percentage overlap was also calculated by selecting the tissue sections with maximal response from the non-PH3S10 population. Five sections with maximum response for the non-PH3S10 population (ipsilateral) were selected. The PH3S10 counts and double labels from the same sections of tissue were counted. The percentage of double labelling was calculated for each section of tissue and averaged per animal. Finally, the average percentage of double labelling for the group was presented as mean ± SEM.

All means were calculated from a 40µm section of spinal cord tissue.

It was previously established in Chapter 3 that upregulation of PH3S10 nuclei were only observed in the ipsilateral dorsal horn and rarely seen in sham-only, or contralateral spinal cords of stimulated animals (~1 cell/40µm section in superficial laminae) (Figure 3.2). Thus, sham anaesthesia controls were not used in this chapter. Instead, the contralateral side of the spinal cord of stimulated animals was used as an internal control. Contralateral PH3S10 expression was only quantified in the PH3S10 Time Course experiment.

4.3 Results

4.3.1 PH3S10 time course: PH3S10 is upregulated in the spinal cord 5 minutes post-formalin and peaks at 30-60 minutes

PH3S10 serves as an indicator of active gene transcription *in vitro*, and we investigated its expression following noxious stimulation. There was a rapid expression of PH3S10 labeled nuclei in the medial superficial dorsal horn following intraplantar formalin injection. The number of PH3S10 neurons was greater on the ipsilateral side compared to the contralateral side, where PH3S10 expression levels were extremely low (Figure 4.2). Expression of PH3S10 in superficial laminae I-II peaked at the 30min time point (mean, 20.5 ± 3.2 cells per 40µm section). In deeper laminae III-V, expression of PH3S10 was maximal at 1h (mean, 13.3 ± 1.7 cells per 40µm section). By 4 hours, PH3S10 levels were comparable to the counts seen in the contralateral as well as sham animals (data not shown).

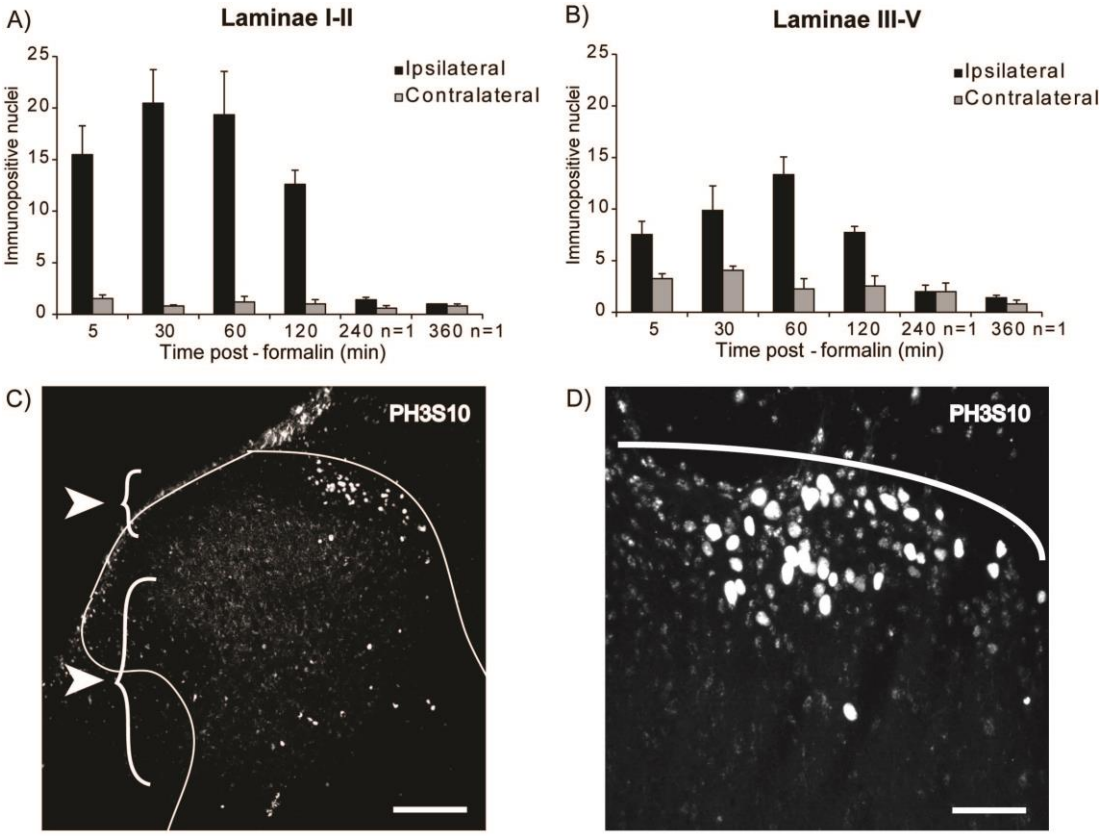
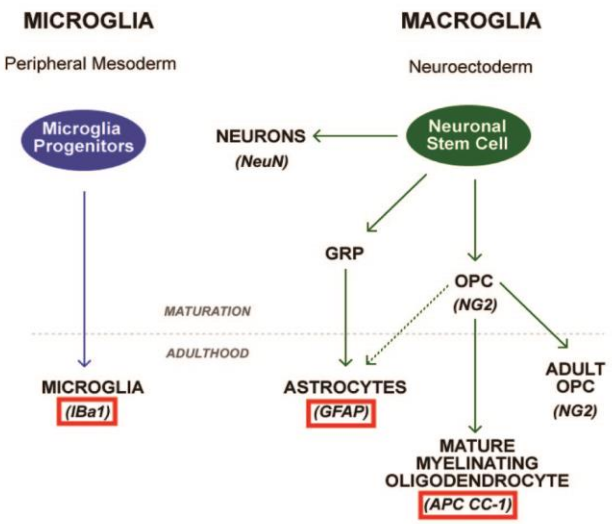


Figure 4.2 PH3S10 is expressed in the ipsilateral dorsal horn after hindpaw formalin inflammation. A) Ipsilateral and contralateral expression of PH3S10 in laminae I-II and B) laminae III-V (n=3 each time point, 5 sections per animal). Values presented as group mean ± SEM (per 40µm section). C) Low-power representative image of PH3S10 distribution within the L4 dorsal horn. Arrowheads indicate expression in medial superficial dorsal horn and deeper laminae, respectively, at 30min. Scale bar 50µm. D) High power image of PH3S10 indicates that it is nuclear in distribution. Scale bar, 30µm. White lines indicate border between grey and white matter.

4.3.2 Formalin-induced PH3S10 expression is mainly neuronal and rarely observed in glia

Colocalisation of PH3S10 and the neuronal marker NeuN indicated that the great majority of PH3S10 was upregulated in neurons. PH3S10 was rarely seen to colocalise with glial cells using the astrocyte marker, glial fibrillary acidic protein (GFAP), as well as the oligodendrocyte precursor marker APC CC1, and Iba-1, a marker of microglial activation. Some colocalisation, however, was observed although primarily between background levels of PH3S10 and respective glial marker (Figure 4.3).

A)



B)

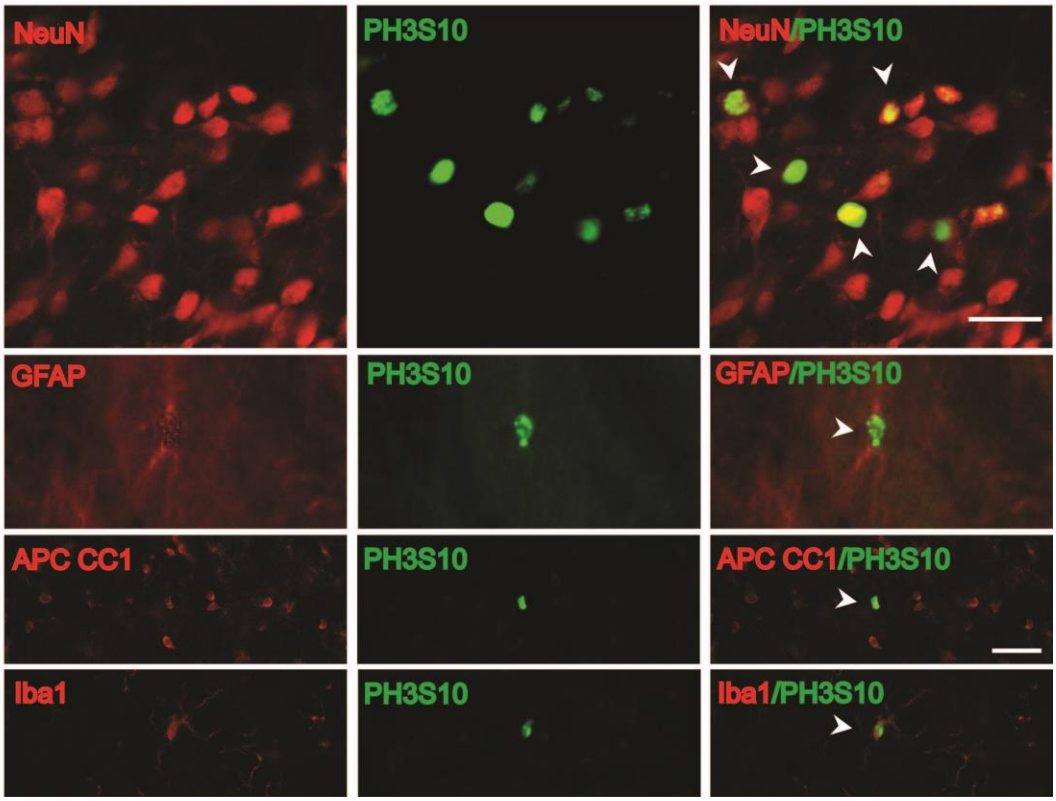


Figure 4.3 Formalin-induced PH3S10 is expressed in neurons and although rare, was found to colocalise with glial markers in the dorsal horn. A) Lineage and markers of glial subtypes. Image from Cunningham (unpublished). B) Double labelled stains taken in a single focal plane for PH3S10 with NeuN, GFAP, APC CC1, and Iba1 (Rows 1-4, respectively) in formalin-stimulated animals. First column images (red) indicate positive staining for cell type investigated (NeuN, GFAP, APC CC1, Iba1). Second column images (green) indicate PH3S10, and third column images are column 1 and 2 images merged; yellow indicates colocalisation. Arrowheads indicate examples of colocalisation and show that almost all PH3S10 nuclei were NeuN positive. In merged glial images, PH3S10 represents background staining. Scale bar top row, 25µm, lower scale bar 40µm. It should be noted that the above colocalisation shown of PH3S10 with glial markers GFAP, APC CC1, and Iba1 was rare and not representative of the extent of overlap.

4.3.3 Formalin-induced PH3S10 is not expressed in GABAergic inhibitory interneurons labelled with PV or nNOS

NPY, Galanin, PV, and nNOS are known to label GABAergic interneurons, and an attempt was made to colocalise PH3S10 to PV and nNOS. PH3S10 did not colocalise with the inhibitory interneuron markers nNOS or PV as the population distributions were in different areas of the superficial dorsal horn. Both nNOS and PV appeared to localise in deeper lamina Iii, with PV distributed more laterally (Figure 4.4). An attempt was made to colabel PH3S10 with the markers NPY and Galanin, but it was not possible to identify cell bodies and colocalisation could not be observed.

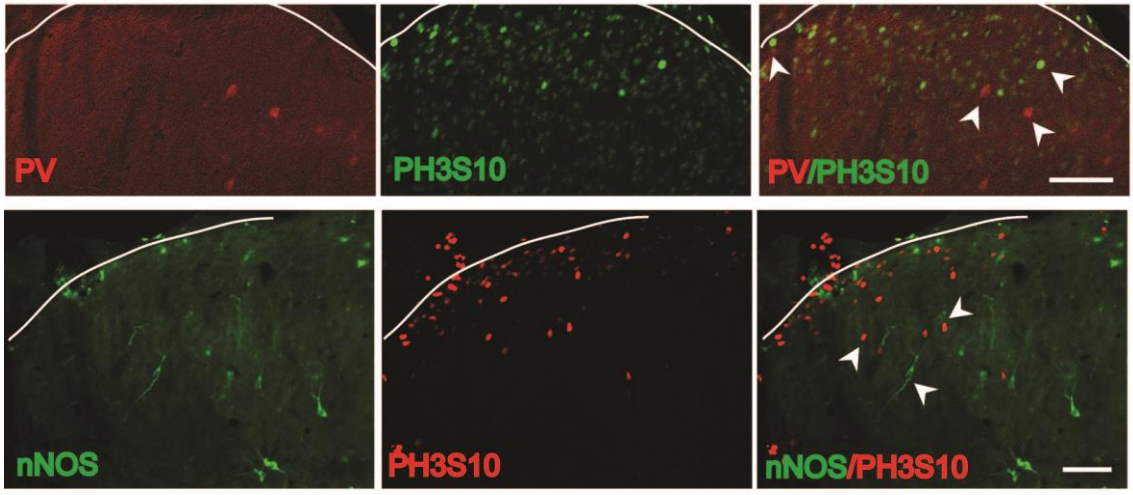


Figure 4.4 Formalin-induced PH3S10 does not colocalise with inhibitory interneurons labelled with PV and nNOS. Dorsal horn images of PV and PH3S10, and nNOS and PH3S10 double labelling taken in a single focal plane in a formalin-stimulated animal. White lines indicate border between lamina I and white matter; arrowheads indicate PH3S10 and PV or nNOS positive cells that are clearly not overlapped. Scale bars 50µm.

4.3.4 Formalin-induced PH3S10 does not appear to be expressed in glutamatergic calbindin positive neurons

The calcium-binding protein calbindin is a marker labelling glutamatergic cell bodies (Todd, 2010), and an attempt was made to colabel PH3S10 with calbindin. PH3S10 nuclei did not appear to colocalise with calbindin, although some colocalisation was observed between calbindin and background levels of PH3S10 (Figure 4.5).

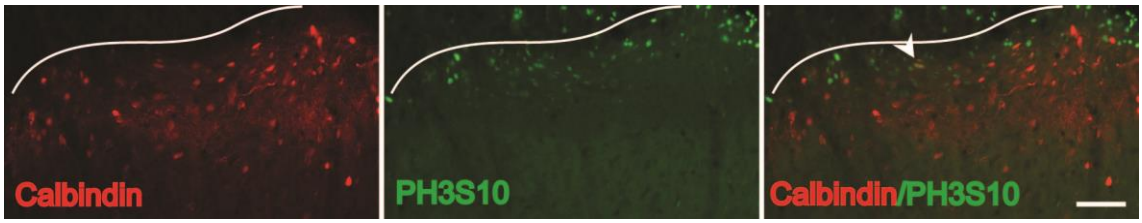


Figure 4.5 PH3S10 does not colocalise with a population of glutamatergic cells labelled with Calbindin. Dorsal horn images of Calbindin and PH3S10 double labelling taken in a single focal plane in a formalin-stimulated animal. White lines indicate border between lamina I and white matter; arrowhead indicates one example of background PH3S10 staining which appears to colocalise with calbindin, but this was considered background staining. Scale bar 50µm.

4.3.5 PH3S10 colocalises with NK1 neurons

Excitatory NK1 positive neurons are selectively activated by noxious stimuli and colocalisation of PH3S10 and NK1 was investigated. Analysis of PH3S10 colocalisation with lamina I NK1 neurons at 1h post-formalin indicated that 17% of lamina I NK1 expressing neurons also coexpressed PH3S10 (mean NK1 population 6.5 ± 1.5). Due to the small NK1 population size, only 5% of laminae I-II PH3S10 coexpressed NK1 (mean PH3S10 population 27.1 ± 3.8). For laminae III-V, 17% of NK1 positive cells colabelled with PH3S10 (mean NK1 population 6.1 ± 1.2), while only 4% of PH3S10 colabelled NK1 (mean PH3S10 population 18.2 ± 3.6) (Figure 4.6, Table 4.2). Importantly, there was no difference in percentage overlap values when using 5 sections with maximum response per animal from either the PH3S10 or NK1 population.

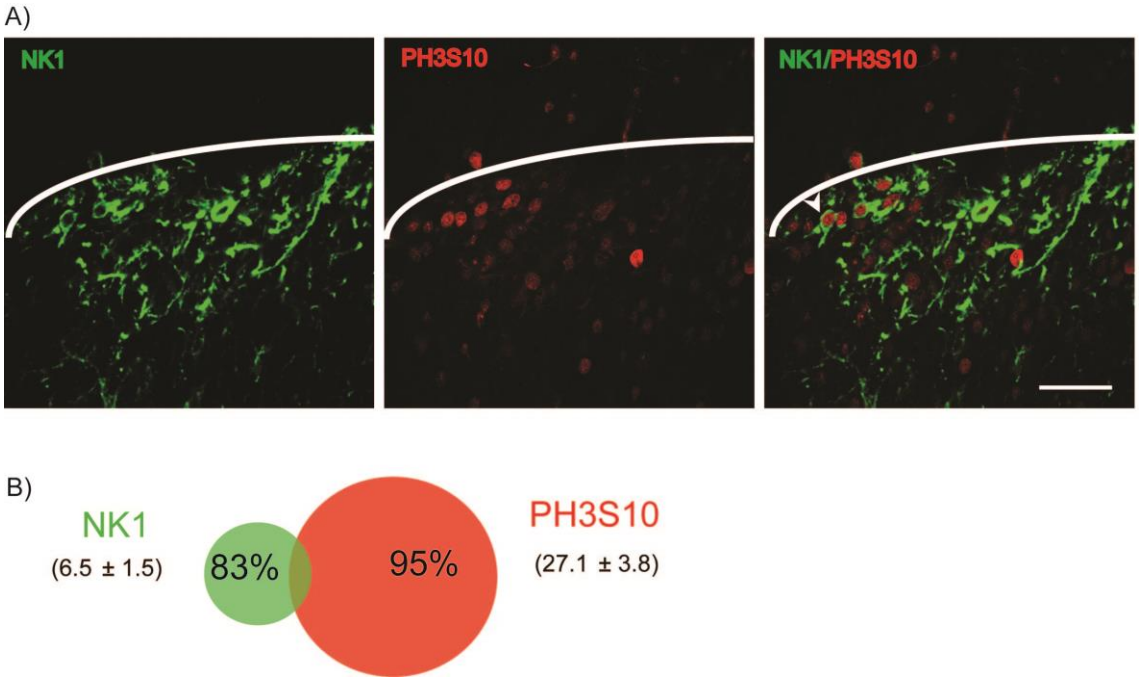


Figure 4.6 Formalin-induced PH3S10 is partially found in lamina I NK1 neurons. A) Dorsal horn images of NK1 (green) and PH3S10 (red) double labelling taken in a single focal plane in a formalin-stimulated animal. The final image shows a merge of the first two images; NK1 is a cell-surface receptor and thus colocalisation is shown as NK1 surrounding the PH3S10 labelled nucleus. White line indicates medial border between lamina I and white matter; arrowhead indicates example of colocalisation. Scale bar, 30µm. B) Venn diagram depicting overlap of PH3S10 and NK1 populations. Percentages indicate percentage of population not double labelled; values in parentheses indicate population size expressed as mean value (immunopositive cells per 40µm section) ± SEM (n=3, 5 sections per animal).

4.3.6 PH3S10 colocalises with PERK, a marker of nociceptive activation

PERK was used to label neurons in the dorsal horn receiving nociceptive-specific primary afferent input. As expected PERK showed a strong upregulation in laminae I-II in an area larger than where PH3S10 was expressed but still overlapping. Coexpression of PH3S10 and PERK populations was assessed at 30min, a time point used to obtain the maximum overlap of the two populations given their respective time course of expression following noxious stimulation (Figure 4.10). Indeed, PERK expression peaks 5 minutes post-formalin but is still strongly expressed at 30 minutes. 58% of all PH3S10-expressing cells colabelled with PERK (mean PH3S10 population 26.3 ± 1.2), while 42% of PERK-expressing cells also expressed PH3S10 (mean PERK population 30.7 ± 3.7). For deeper laminae III-V, similar percentages of colocalisation were observed. 53% of PH3S10-expressing cells colabelled PERK (mean PH3S10 population 10.3 ± 0.6) and 40% of PERK- expressing cells also coexpressed PH3S10 (mean PERK population 16.6 ± 5.5) (Figure 4.7, Table 4.2). Importantly, there was no

difference in percentage overlap values when using 5 sections with maximum response per animal from either the PH3S10 or PERK population.

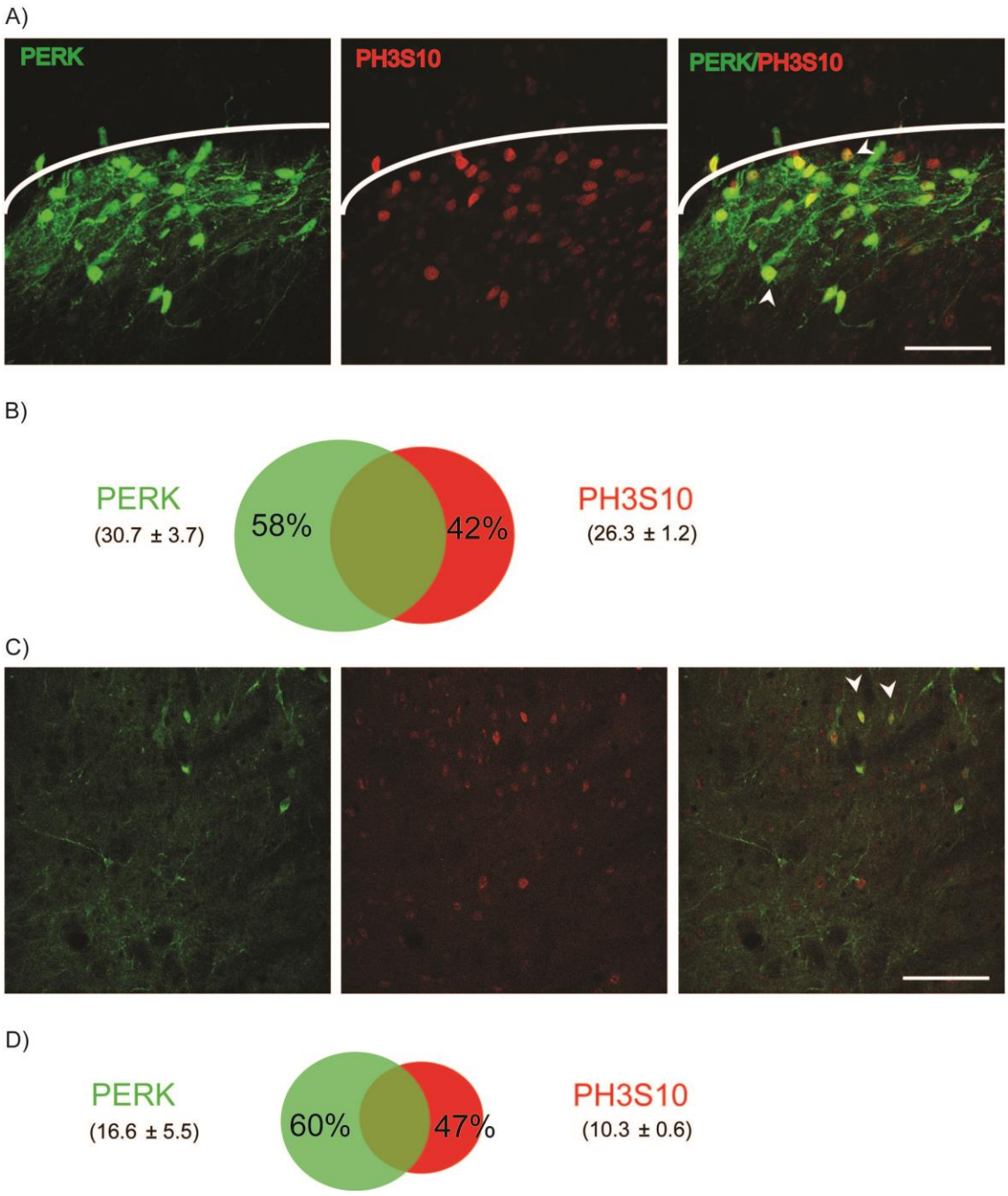


Figure 4.7 Formalin-induced PH3S10 is partially expressed in PERK labelled neurons in the dorsal horn. A, C) Dorsal horn images of PERK (green) and PH3S10 (red) double labelling taken in a single focal plane in a formalin-stimulated animal, in laminae I-II (A) and III-V (C). The final image shows a merge of the first two images; colocalisation appears yellow. White line indicates medial border between lamina I and white matter; arrowheads indicate examples of colocalisation. Scale bar 30µm (A), 50µm (C). B, D) Venn diagram depicting overlap of PH3S10/PERK populations in laminae I-II (B) and III-V (D). Percentages indicate percentage of population not double labelled; values in parentheses indicate population size expressed as group mean (immunopositive cells per 40µm section) ± SEM (n=3, 5 sections per animal).

4.3.7 PH3S10 colocalises with the immediate early gene products c-Fos and Zif268

c-Fos and Zif268 are IEG markers of neuronal plasticity known to have maximal expression in the ipsilateral dorsal horn 1-2h after noxious stimulation. Robust upregulation of both c-Fos and Zif268 was observed 1h post-formalin. Both populations had a larger and more anatomically widespread distribution compared to the PH3S10 population. 60% of PH3S10 coexpressed c-Fos (mean PH3S10 population 19.3 ± 4.2), while 17% of c-Fos coexpressed PH3S10 (mean c-Fos population 38.5 ± 2.4) (Figure 4.8, Table 4.2). 57% of the PH3S10 population of neurons coexpressed Zif268 (mean PH3S10 population 10.6 ± 1.1), while 12% of the Zif268 population coexpressed PH3S10 (mean Zif268 population 59.5 ± 6.6) (Figure 4.9, Table 4.2). Importantly, there were no differences in percentage overlap values when using 5 sections with maximum response per animal from either the PH3S10 or c-Fos/Zif268 population.

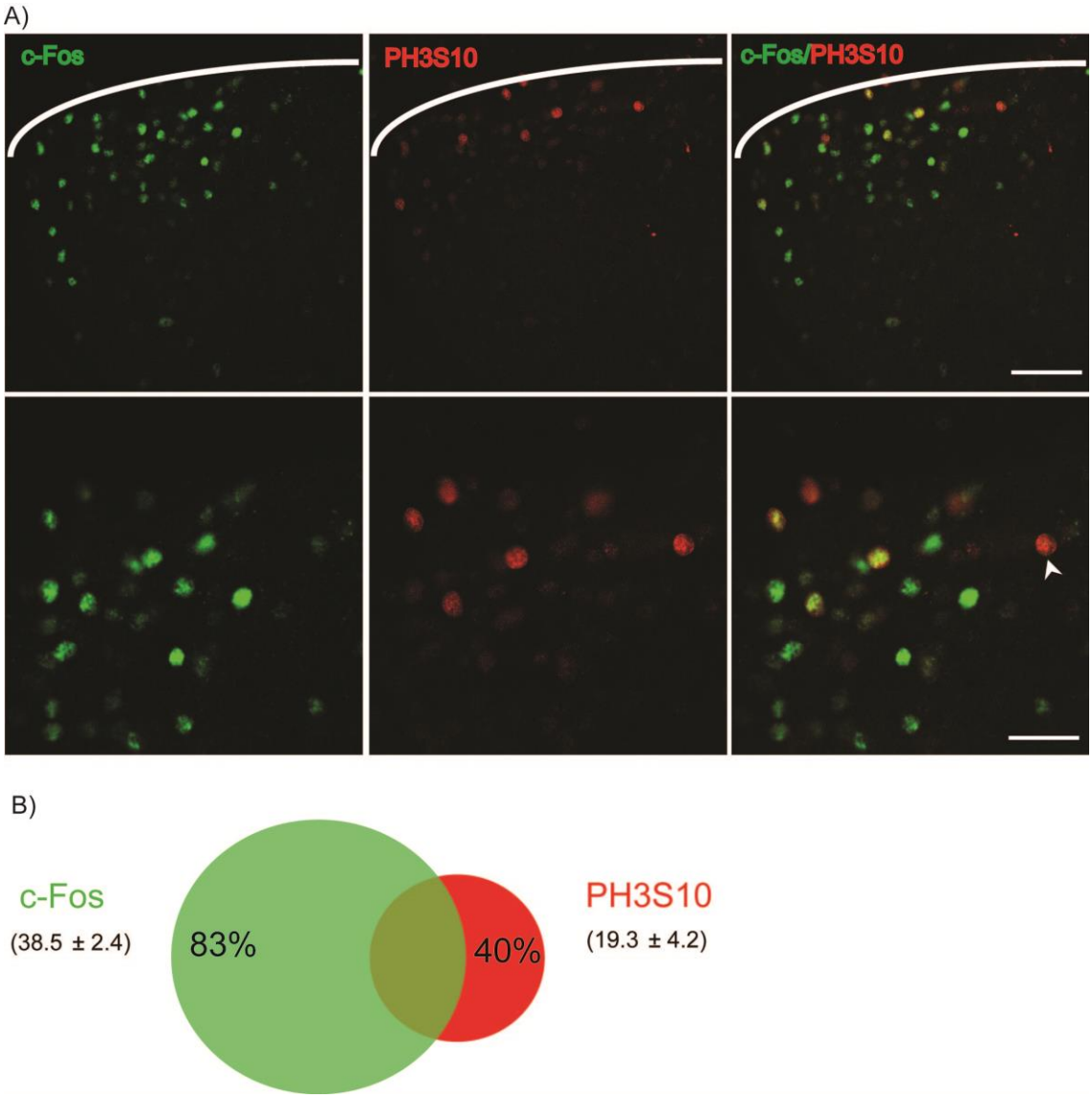


Figure 4.8 Formalin-induced PH3S10 colocalises with immediate early gene product and marker of nociceptive synaptic plasticity, c-Fos. Dorsal horn images of double labelling for PH3S10 and c-Fos in formalin-stimulated animals taken in a single focal plane. A) Top row contains low power images and bottom row higher power images of the same region. First column images (green) indicate positive staining for c-Fos. Second column images (red) indicate PH3S10, and third column images are column 1 and 2 images merged; colocalisation appears yellow. White line indicates medial border between lamina I and white matter; arrowhead indicates example of PH3S10 nuclei not double labelled with c-Fos. Scale bar upper, 50µm; lower, 30µm. B) Venn diagram depicting overlap of two populations. Percentages indicate percentage of population not double labelled; values in parentheses indicate population size expressed as mean value (immunopositive cells per 40µm section) ± SEM (n=4, 5 sections per animal).

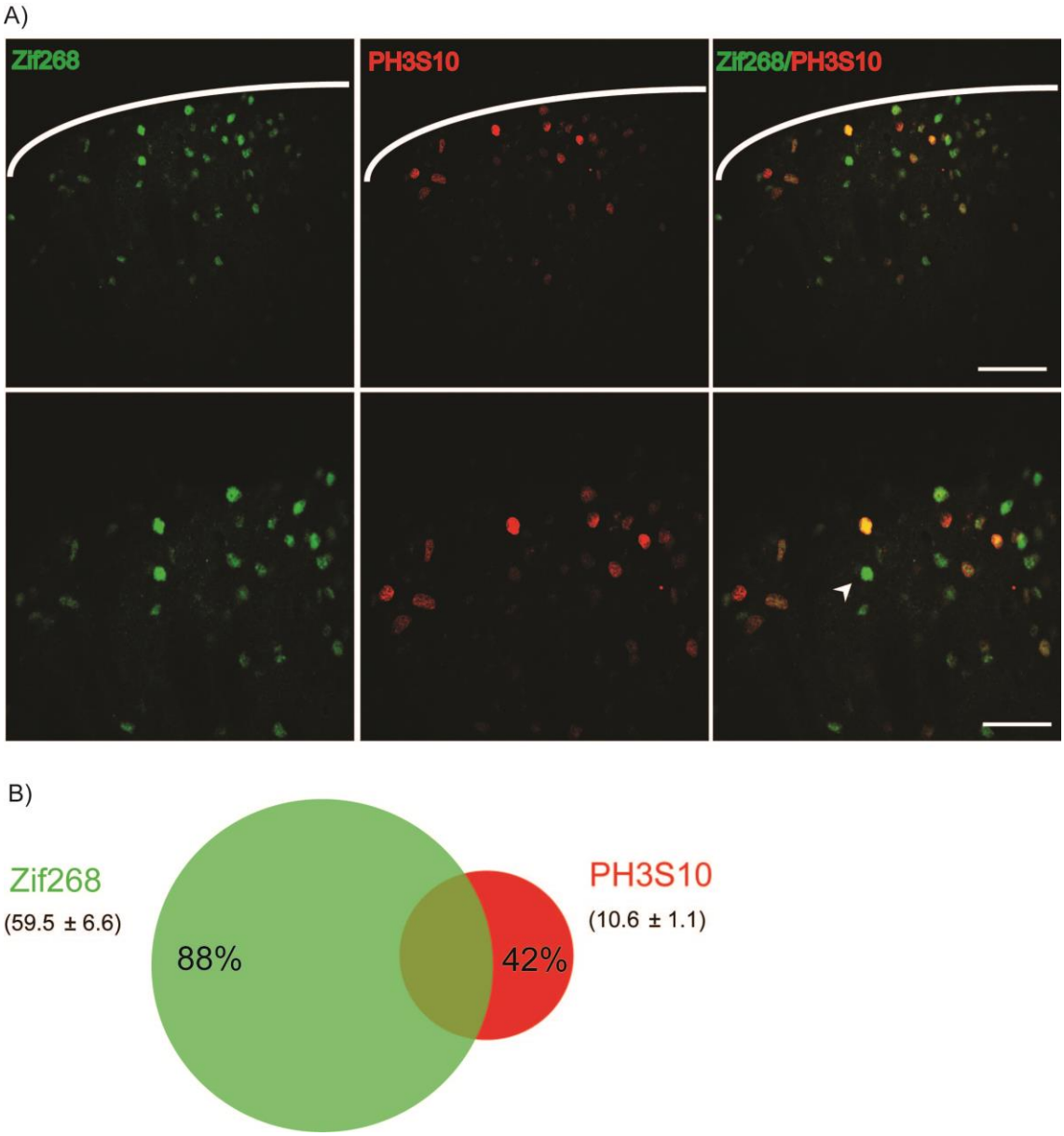


Figure 4.9 Formalin-induced PH3S10 colocalises with immediate early gene product Zif268. Dorsal horn images of double labelling for PH3S10 and Zif268 in formalin-stimulated animals taken in a single focal plane. A) Top row contains low power images and bottom row higher power images of the same region. First column images (green) indicate positive staining for c-Fos. Second column images (red) indicate PH3S10, and third column images are column 1 and 2 images merged; colocalisation appears yellow. White line indicates medial border between lamina I and white matter; arrowhead indicates example of Zif268 nucleus not double labelled with PH3S10. Scale bar upper, 50µm; lower, 30µm. B) Venn diagram depicting overlap of PH3S10 and Zif268 populations. Percentages indicate percentage of population not double labelled; values in parentheses indicate population size expressed as mean value (immunopositive cells per 40µm section) ± SEM (n=4, 5 sections per animal).

Table 4.2 Summary of population sizes and overlap of PH3S10 with markers of the pain pathway in the ipsilateral dorsal horn post-formalin. Values are presented as mean ± SEM per tissue slice, 40µm thickness (n=3/4 animals each colocalisation; 5 tissue sections per animal).

Marker	Population Size (# cells)	PH3S10 population size (# cells)	Double labelled (# cells)	% PH3S10 expressing *Marker*	% *Marker* expressing PH3S10	Time point investigated
PERK (Lam1-2)	30.7 ± 3.7	26.3 ± 1.2	8.9 ± 4.1	58%	42%	30 min
PERK (Lam3-5)	16.6 ± 5.5	10.3 ± 0.6	5.1 ± 1.1	53%	40%	
NK1 (Lam 1)	6.5 ± 1.5	27.1 ± 3.8	1.3 ± 0.7	5%	17%	1h
NK1 (Lam3-5)	6.1 ± 1.2	18.2 ± 3.6	0.8 ± 0.4	4%	17%	
c-Fos (Lam1-2)	38.5 ± 2.4	19.3 ± 4.2	6.2 ± 1.2	60%	17%	1h
Zif268 (Lam1-2)	59.5 ± 6.6	10.6 ± 1.1	6.3 ± 1.2	57%	12%	1h

4.4 Discussion

PH3S10 is a histone modification associated with transcriptional activation in neurons and is regulated by ERK/MAPK signalling in various forms of neuronal plasticity. In this chapter it was shown that PH3S10 is upregulated in the dorsal horn of spinal cord neurons immediately and transiently after noxious hindpaw stimulation. It was also shown that PH3S10 occurred within neurons expressing markers of the pain pathway, PERK, NK1, c-Fos, and Zif268.

4.4.1 Intracellular signalling: linking PERK, PH3S10, and IEG events in models of synaptic plasticity

PERK and c-Fos are classical markers of primary afferent nociceptive activation and central sensitisation (Gao and Ji, 2009). It is therefore of great interest that more than half of the PH3S10 population overlapped with both PERK and c-Fos populations at 58% (30 min) and 60% (1h), respectively. No other studies had investigated PH3S10 in the spinal cord following noxious stimulation, but strong coupling of PERK, PH3S10, and c-Fos expression has been confirmed in various models of activity-induced synaptic plasticity in the brain (Brami-Cherrier et al., 2009; Chwang et al., 2006; Ciccarelli and Giustetto, 2014; Crosio et al., 2003).

PERK and PH3S10

It was not surprising that the majority of PH3S10 colocalised with PERK at 30 minutes, as PH3S10 is known to occur downstream of ERK via the mitogen- and stress- related kinase 1/2, MSK1/2 (Soloaga et al., 2003). Following pharmacological activation of neuronal plasticity, 80% of PH3S10 nuclei colocalised with PERK in the dentate gyrus 1 hour after glutamatergic activation with kainic acid (Crosio et al., 2003). In the same study, virtually all of PH3S10 nuclei expressed *c-Fos* mRNA, and PH3S10 expression always preceded that of *c-Fos*. Moreover, in the hippocampus PH3S10 was found to be regulated by ERK following PKC and PKA pharmacological activation *in vitro,* and in synaptic plasticity induced by contextual fear conditioning *in vivo* (Chwang et al., 2006). ERK regulation of activity-upregulated PH3S10-AcH3K14 in the hippocampus was also observed in another stress-induced behavioural learning paradigm, the forced swim test (Chandramohan et al., 2008, 2007).

Interestingly, PH3S10 expression was found to be highly coexpressed (94%) with PERK in the lateral septum of the brain 1 hour following plasticity induced by naloxone-precipitated morphine withdrawal (Ciccarelli et al., 2013). Regulation of synaptic

activity-induced PH3S10 by ERK has also been observed in visual cortical plasticity and drug addiction (Brami-Cherrier et al., 2009; Putignano et al., 2007).

In this chapter, the percentage overlap of PH3S10/PERK, and PH3S10/c-Fos both occurred around 60%. A 60% overlap between the PH3S10 population and PERK, or c-Fos populations indicated that at least 20% of the PH3S10 population expressed both PERK and c-Fos. However, it should be noted that the percentage overlaps reported here may not be an exact reflection of the true co-existence between the two markers due to an issue of timing. PERK, PH3S10, and c-Fos are known to peak at different time points (Figure 4.10), and it is only feasible to examine the population overlap at a single time point optimal for the maximal expression of both populations when using IHC (Figure 4.10). Therefore, the reported percentages are reflective of a minimum overlap between the two populations. An alternate approach to this limitation would be to block PERK activity using an inhibitor and observe the subsequent expression of PH3S10 and c-Fos (see Chapter 6). Furthermore, it should also be taken into consideration that a 60% overlap could also indicate that a signalling cascade other than ERK/MAPK might be responsible for expression of formalin-induced PH3S10 or c-Fos.

PERK and c-Fos/ Zif268

It is difficult to deduce whether PERK and c-Fos always occurs in the same cells of the dorsal horn due to differences in peak expression after noxious stimulation (Figure 4.10); however, it has been shown that ERK has signification regulation over c-Fos expression following nociceptive activation (Kawasaki et al., 2004). In particular, following c-fibre activation by intraplantar capsaicin, the majority of PERK positive cells colocalised with c-Fos. Furthermore, PERK colocalised substantially (89%) with the transcription factor PCREB, which is upregulated more rapidly than c-Fos and is known to bind to CRE-regulatory sequences within genes, including *c-Fos* and *Zif268* (Kawasaki et al., 2004). Regulation of *c-Fos* by ERK has also been observed in the striatum and nucleus accumbens in acute amphetamine administration, and other models of drug addiction (Brami-Cherrier et al., 2009; Rotllant and Armario, 2011). Less is known about ERK regulation of Zif268 in the spinal cord, however ERK regulation of *Zif268* and *c-Fos* has been well established in striatal and dentate gyrus neurons exhibiting LTP (Davis et al., 2000; Sgambato et al., 1998).

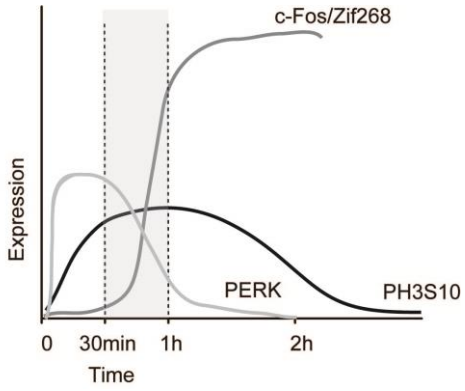


Figure 4.10 Time course of marker expression in the superficial dorsal horn of the spinal cord after peripheral noxious stimulation.

While the evidence linking PERK, PH3S10 and IEG expression in various models of neuronal plasticity is strong, care must be taken in interpreting results from other brain areas and plasticity-inducing paradigms. Indeed, the studies mentioned above clearly indicate that expression and interaction of these markers differs according to stimulus and region (Wisden et al., 1990). Nevertheless, other examples of neuronal plasticity support the results presented in this chapter indicating a strong relationship of ERK, PH3S10, and IEG events in the ERK/MAPK signalling cascade. A more in-depth discussion on the functional necessity of PERK and PMSK1/2 for PH3S10 expression following noxious stimulation will take place in Chapter 6.

4.4.2 The existence of PH3S10 in a small subset of NK1 positive lamina I projection neurons

In this chapter, the colocalisation of PH3S10 with NK1 was investigated because of the importance of NK1 projection neurons to the full development of inflammatory pain states (Mantyh et al., 1997; Nichols et al., 1999).

Analysis of staining revealed a small level of colocalisation between NK1 and PH3S10 populations. However, it cannot be said for certain whether colocalisation occurred in lamina I projection neurons, as retrograde labelling from main supraspinal targets (the CVLM and LPb) was not performed. In lamina I of the lumbar region, 45% of neurons are immunoreactive for NK1 (Todd et al., 1998), yet only 5% of all lamina I neurons are projection neurons (Bice & Beal 1997b; Bice & Beal 1997a; Spike et al. 2003; Al-Khater & Todd 2009). NK1 positive cells that are not projection neurons are thought to be excitatory interneurons, as those in lamina I are not immunoreactive for GABA (Al Ghamdi et al., 2009; Littlewood et al., 1995). Based on a value of 6.5 ± 1.5 NK1 cells/40µm section quantified in this chapter, the statistics indicate that roughly 2 cells/40µm section would have been NK1 positive projection neurons. As 17% of

lamina I NK1 positive neurons colabelled with PH3S10, there is a small possibility that PH3S10 occurred in lamina I NK1 projection neurons.

It should be noted however, that although only 17% of NK1 positive neurons colabelled with PH3S10, there is a basis for underestimation of this value. Formalin-induced PH3S10 is localised only within the medial superficial dorsal horn, while retrograde tracing has indicated that lamina I projection neurons are concentrated in the middle (mediolateral) of lamina I (Todd, 2010). Therefore, only 50% of the NK1 neurons quantified fall into the region of PH3S10 expression. Thus a value of 17% represents a diluted value and colocalisation should actually be higher (potentially 35%). If 35% of NK1 neurons expressed PH3S10, the possibility of PH3S10 in NK1 projection neurons would be substantially higher.

Another previously suggested possibility (Torsney, 2011) is that the average lamina I NK1 population size observed and quantified in this chapter (6.5 ± 1.5) is not reflective of the 45% of all neurons reported to be NK1 positive in lamina I (Todd et al., 1998). The NK1 immunostaining presented in this chapter is most likely labelling projection neurons, which are known to exhibit the strongest NK1 staining (i.e. compared to interneurons).

It has been more recently shown that NK1 projection and NK1 interneurons differ in terms of soma size and AMPA receptor subunit expression (Al Ghamdi et al., 2009; Polgár et al., 2010). 99% of NK1 cells with larger soma (>200μm^2) are known to colabel with retrograde tracer from the CVLM and LPb, conversely, only 10% of smaller (<200μm^2) soma colabelled with retrograde tracer suggesting an interneuron identity (Al Ghamdi et al., 2009). The two categories of morphology also have differential expression of AMPA subunits, which are known to influence forms of LTP, an event that is known to occur in lamina I NK1 neurons (Esteban et al., 2003; Ikeda et al., 2006, 2003; Polgár et al., 2010). The GluA4 subunit was found highly clustered on the large NK1 positive projection neurons, while the small area NK1 interneurons are positive for the GluA1 subunit (Polgár et al., 2010). The two groups also had no differences in ERK activation, suggesting that they are not activated by distinct types of noxious stimuli. In this chapter, the area of NK1 immunopositive cells was not measured and thus those that colocalised with PH3S10 cannot be classified by this method. However, it is possible that re-examination of staining using this classification would allow for categorisation of NK1 subtype expression PH3S10.

4.4.3 PH3S10 in NK1 laminae III-IV projection neurons

NK1 positive projection neurons also exist in deeper laminae III and IV cells with dendrites extending to lamina II. The great majority of these cells (>90%) project mainly to the LPb and lateral reticular nucleus (Todd et al., 2000), and selectively receive input from myelinated low threshold mechanoreceptors and local GABAergic NPY interneurons (Naim et al., 1998; Polgár et al., 1999). Lamina III-IV NK1 positive neurons also have dendrites extending dorsally into lamina I-II and receive dense innervation from C-fibre SP-containing primary afferents (Naim et al., 1997). 94% are activated by peripheral noxious stimulation (formalin, pinch, and heat) as indicated by colocalisation with PERK, and dendritic NK1 receptor internalisation (Polgár et al., 2007; Todd et al., 2000). Importantly, the high percentage of NK1 positive projection neurons (>90%) in laminae III-IV indicates statistically that at least 4% of the deeper laminae PH3S10 population did indeed occur in projection neurons (17% NK1 expressing PH3S10) in the deep dorsal horn. However, the laminae III-IV NK1 projection neuron population is small, at roughly only 20 on each side of the dorsal horn within L4 (Todd et al., 2000); therefore the number of PH3S10 labelled neurons is low.

Finally, it is worth noting that evidence of NK1 receptor internalisation was not investigated here. NK1 endosome-mediated internalisation is an indication of agonist binding and primary afferent input in both acute and persistent pain states (Honor et al., 1999; Mantyh et al., 1995). Lamina I NK1 internalisation peaks 8 minutes post-formalin injection, and the majority (55-60%) of NK1 immunopositive staining at 1h is still known to express internalised NK1 (Honor et al., 1999). It should be noted that the NK1 immunostaining presented in this chapter does not appear to exhibit receptor internalisation but is instead found on the cell surface. It is possible that during the quantification process there was a bias toward identification of NK1 staining that was cell surface in expression, and that some NK1 cells could have been over looked.

4.4.4 PH3S10: presence in inhibitory or excitatory interneurons?

Nearly all PH3S10 nuclei were seen to colocalise with the neuronal marker NeuN, and we endeavoured to find a distinctive neurochemical profile for PH3S10 indicative of neuronal function in the dorsal horn. 5% colocalisation of PH3S10 with NK1 indicated that at least some of the PH3S10 population occurred within excitatory neurons (Littlewood et al., 1995).

Subsequently, an additional attempt was made to determine the identity of PH3S10 neurons using four functionally and neurochemically distinct populations of non-overlapping GABAergic inhibitory interneurons: galanin, NPY, nNOS, and PV (Polgár et al., 2013). IHC staining for Galanin and NPY was unsuccessful; therefore no colocalisation was carried out with PH3S10. Furthermore, no colocalisation of PH3S10 with PV and nNOS was observed. This was primarily due to the fact that the populations did not occur in the same region of the dorsal horn with PV and nNOS occurring more laterally in deeper laminae IIi. A study by Polgar et al. (2013) indicated that 8% of nNOS and 0% PV cells overlapped with PERK in the dorsal horn post-formalin, it is not surprisingly that no colocalisation with nNOS or PV was observed in the experiments presented here. Moreover, the study by Polgar et al. indicated that galanin and NPY populations each had 68% and 66% overlap with PERK, respectively. Considering that experiments in this chapter reported 42% of PERK cells coexpressing PH3S10, it is possible that colocalisation of PH3S10 might have more likely been observed in galanin and NPY cells.

GABA is present in 25 and 30% of lamina I and II neurons, respectively (Polgár et al., 2003). However, achieving GABA or glycine immunolabelling of cell bodies is difficult and requires a glutaraldehyde fixation which would obscure the detection of other antigens (i.e. PH3S10). Admittedly, the investigation of PH3S10 with inhibitory interneuron markers in this chapter was not exhaustive, and therefore cannot be ruled out as a potential neuronal subtype. An additional technique to identify inhibitory interneurons could be to acquire genetically modified mice with a green fluorescent protein (GFP) reporter for *GAD* (GAD-GFP mice) to label GABAergic cells, and to double stain the spinal cord tissue with PH3S10.

While it is probable that non GABA/glycinergic neurons in the dorsal horn are glutamatergic (Todd, 2010), it is similarly difficult to achieve cell body staining of glutamatergic neurons as most markers (i.e. vesicular glutamate transporters-VGLUTs) label axon terminals. In addition to low coexpression with excitatory NK1 neurons, it is possible that PH3S10 is expressed in a larger percentage of excitatory glutamatergic interneurons; however, the only other investigation into this possibility was with glutamatergic neuronal marker, calbindin. Notably, it was low background levels of PH3S10 that were observed to colocalise with calbindin. Other neurochemical markers expressed in glutamatergic interneurons include calretinin, neurotensin, NKB, PKCɣ, substance P, and somatostatin (Todd, 2010). Calretinin is the next main candidate of interest as it is known to label whole cell bodies and is expressed in the

superficial dorsal horn. PKCγ is most likely precluded as a colocalisation target for PH3S10 as its expression is too deep, in lamina IIi and III (E. Polgár et al., 1999).

4.4.5 PH3S10 in glia

It was rare to find colocalisation of formalin-induced PH3S10 with glial markers GFAP, APC CC1 and Iba1, although some colocalisation of background PH3S10 staining was noted. This was somewhat surprising as PH3S10 was originally identified in non-neural cells and additionally serves as a marker of mitotic cell division (Prigent and Dimitrov, 2003). Notably, greater colocalisation of PH3S10 and glia may be observed following different types of noxious stimulation. Glial cells, particularly activated microglia labelled with Iba1 (Beggs and Salter, 2007), are involved in the spinal cord immune response to peripheral nerve injury. Thus, a different model might have been more appropriate to study colocalisation of PH3S10 with glial markers. However, it is not yet known if and when PH3S10 would be observed in the spinal cord following a neuropathic injury.

4.4.6 Technical limitations

The colocalisation data presented in this chapter are presented at time points chosen for maximal overlap of the populations of interest (see Discussion point 4.4.1 PERK and PH3S10, Figure 4.10). We were able to do this knowing the maximal expression for each population; however, the findings may not be indicative of the full overlap. Thus if anything, due to the constraints of analysing one point in time, the colocalisation values may indeed be higher than the values reported here (refer to Figure 4.10).

It should also be noted that the colocalisation percentages presented in Table 4.2 (columns 5 and 6) may not seem to reflect the presented values of respective population sizes (columns 2-4). A decision was made to present colocalisation values per each section, rather than across whole populations, as this was thought to be a more accurate representation of colocalisation (see Methods 4.2.5). The fact that the colocalisation percentages do not directly reflect colocalisation quantified across populations suggests that there is high variability of PH3S10 overlap with respective populations of interest between tissue regions/sections.

4.4.7 Conclusions

In summary, PH3S10 occurred in neurons of the dorsal horn that expressed markers of the pain pathway. More than half of the PH3S10 population colocalised with dorsal horn markers (PERK, c-Fos, and Zif268) indicating activation by noxious stimulation of

primary afferents. PH3S10 also colocalised partially with NK1 lamina III projection neurons, and potentially lamina I NK1 projection neurons.

A substantial percentage of PH3S10 cells did not colocalise with PERK, which indicated that PH3S10 may be regulated by another kinase. Thus, one of the next aims was to determine a functional link between ERK/MAPK signalling on PH3S10 expression by pharmacologically targeting PERK activity following inflammation (Chapter 6).

Furthermore, as dorsal horn activity is under the regulation of descending input from the brain, a second aim was to investigate the role of descending controls on formalin-induced PH3S10 expression (Chapter 5).

Chapter 5 Descending serotonergic regulation of PH3S10

5.1 Introduction

Top-down modulation of pain exists in the form of a number of descending circuits from the brain to the spinal cord, which have provided a pathway through which the brain can enhance or suppress dorsal horn activity and the overall pain response. Descending serotonergic release from fibres originating in the rostral ventromedial medulla (RVM) are known to regulate nociceptive activity in the spinal dorsal horn (Gebhart, 2004; Millan, 2002; Ossipov et al., 2010). Chapters 3 and 4 established the immediate upregulation of PH3S10 in the dorsal horn after intraplantar formalin stimulation, and this chapter describes experiments investigating the role of descending serotonin (5-HT) on PH3S10 expression.

5.1.1 The rostral ventromedial medulla (RVM)

The RVM receives input from the PAG and is the main origin of descending fibres projecting from the brain to the dorsal horn of the spinal cord. Importantly, descending activity from the RVM is known to have a modulatory influence upon spinal cord activity, which is overall inhibitory (Basbaum and Fields, 1979; Fields et al., 1977). In particular, the RVM has been implicated in the maintenance but not induction of neuropathic pain, and delivery of RVM lidocaine can temporarily reverse allodynia and hyperalgesia that results from nerve injury (Burgess et al., 2002; Pertovaara et al., 1996; Vera-Portocarrero et al., 2006). Furthermore, more recently, it has been demonstrated that activation of descending inhibition may be an explanation for why certain subjects fail to develop chronic pain after nerve injury (De Felice et al., 2011).

Crucially, however, the RVM can have bidirectional inhibitory and facilitatory descending influences on spinal nociception (Urban and Gebhart, 1999). Evidence suggests that the bidirectional effect of the RVM on descending activity is in part due to the role of serotonergic RVM neurons.

5.1.2 Serotonin (5-HT) as a neurotransmitter in nociceptive processing

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter that regulates a variety of CNS functions including sleep, mood, appetite, as well as cognitive processes. 5-HT also plays a significant role in pain processing; in particular, the regulation of nociceptive spinal cord excitability. Spinal 5-HT is derived mainly from supraspinal sources, specifically the RVM (Kwiat and Basbaum, 1992). Descending fibres containing 5-HT terminate most densely within dorsal horn laminae I-II and are known to synapse primarily onto projection neurons and interneurons, and less so onto primary afferent fibres (Li et al., 1997; Millan, 2002; Ruda, 1988; Wu and Wessendorf, 1992). There is a general acceptance that 5-HT can have bidirectional effects on

nociceptive activity, due to the fact that it can act on a diverse set of 5-HT receptor subtypes that are known to mediate either pro or anti-nociception (Millan, 2002).

5.1.3 5-HT receptors in the CNS

There are fifteen different 5-HT receptors that can be categorised into seven subtypes, 5-HT_1-5-HT_7. All seven 5-HT receptor subtypes are expressed in the spinal cord (Hamon and Bourgoin, 1999; Hannon and Hoyer, 2008) and function as G-protein coupled receptors with the exception of 5-HT_3, which is a ligand-gated ion channel. While the majority of the subtypes have been studied to some extent, dorsal horn 5-HT_1, 5-HT_2 and 5-HT_3 have been implicated in mediating descending facilitation, while 5-HT_1 and 5-HT_7 subtypes have been shown to have inhibitory effects on nociceptive processing (Millan, 2002). Specifically, i.t. administration of a 5-HT_{2A} antagonist is able to attenuate SNL and formalin-induced nociceptive behaviour (Nitanda et al., 2005; Obata et al., 2004), and 5-HT_3 antagonists are similarly effective in producing anti-nociceptive effects in hypersensitivity seen in inflammatory and neuropathic pain states (Dogrul et al., 2009; Lagraize et al., 2010; Oatway et al., 2004; Oyama et al., 1996; Silveira et al., 2010; Suzuki et al., 2002; Svensson et al., 2006; Zeitz et al., 2002). 5-HT_1 has also been shown to mediate descending facilitation of the nociceptive reflex, and is thought to act by inhibiting inhibitory interneurons (Wang et al., 2009). However, 5-HT_1 also has an overall inhibitory role in attenuating formalin-induced behaviour (Oyama et al., 1996) and it is thought that under these circumstances 5-HT_1 directly inhibits the activity of projection neurons (Millan, 2002). 5-HT_7 has been shown to mediate inhibitory effects, as spinal administration of 5-HT_7 antagonists has been shown to reverse antinociception produced by morphine injection into the RVM (Dogrul et al., 2009).

5.1.4 5-HT lesion studies in pain

Studies have used i.t. delivery of the neurotoxin 5,7-dihydroxytryptamine creatinine sulphate (5,7-DHT) to lesion 5-HT-containing descending fibres from the RVM in order to investigate the role of 5-HT in nociception. The majority of studies using 5,7-DHT depletion of spinal 5-HT have concluded an overall facilitatory role for 5-HT in nociception. Specifically, these studies have shown an attenuation of the second phase of the formalin response after spinal depletion of 5-HT (Svensson et al., 2006), as well as attenuation of mechanical and thermal hypersensitivity induced by nerve injury or persistent hindpaw or ankle joint inflammation following CFA injection (Carr et al., 2014; Géranton et al., 2008; Rahman et al., 2006). Furthermore, molecular depletion of descending 5-HT activity from the RVM using RNAi to silence tryptophan hydroxylase 2 (TPH, rate-limiting enzyme involved in synthesis of 5-HT) has also been

shown to attenuate formalin, CFA, and SNL-induced hypersensitivity (Wei et al., 2010) without altering acute baseline responses to thermal or mechanical sensitivity.

5.1.5 Aims

Previous studies have shown reductions in dorsal horn molecular markers of nociception after 5,7-DHT ablation of descending serotonergic fibres from the RVM. PERK, PMeCP2, and the IEG protein products c-Fos and Zif268, all markers coupled with neural activation following noxious stimuli, are significantly reduced after i.t. 5,7-DHT, indicating a net facilitatory role for descending 5-HT (Géranton et al., 2008; Svensson et al., 2006).

Chapters 3 and 4 indicated that formalin-induced PH3S10 colocalised with PERK, c-Fos, and Zif268, and provided a strong basis for the investigation of PH3S10 expression after 5,7-DHT ablation.

The aim of this chapter was to investigate the role of descending 5-HT activity from the RVM on spinal cord molecular activity, specifically the expression of formalin-upregulated PERK and PH3S10 following i.t. ablation with 5,7-DHT.

5.2 Materials and Methods

Please also see Chapter 2 for General Materials and Methods.

5.2.1 Experimental design

The aim of this Chapter was to investigate the role of descending serotonergic activity from the RVM on formalin-induced PH3S10 in the dorsal horn (Figure 5.1). An hour before i.t. surgery, all animals received i.p. desipramine (25mg/kg), a noradrenaline reuptake inhibitor, in order to prevent toxicity to noradrenergic neurons (Breese et al., 1978). Animals subsequently underwent a surgical procedure and received either i.t. 5,7-DHT to ablate serotonergic fibres or i.t. saline (n=8 each group). 7 days after i.t. surgery, all animals underwent hindpaw stimulation with formalin, and 30 minutes later were sacrificed for perfusion (Figure 5.2). Confirmation of the 5,7-DHT depletion was done using immunohistochemistry, and animals that did not show visible reductions in spinal 5-HT were discounted from further analysis (2 animals, leaving an n=7 each group for further molecular analysis).

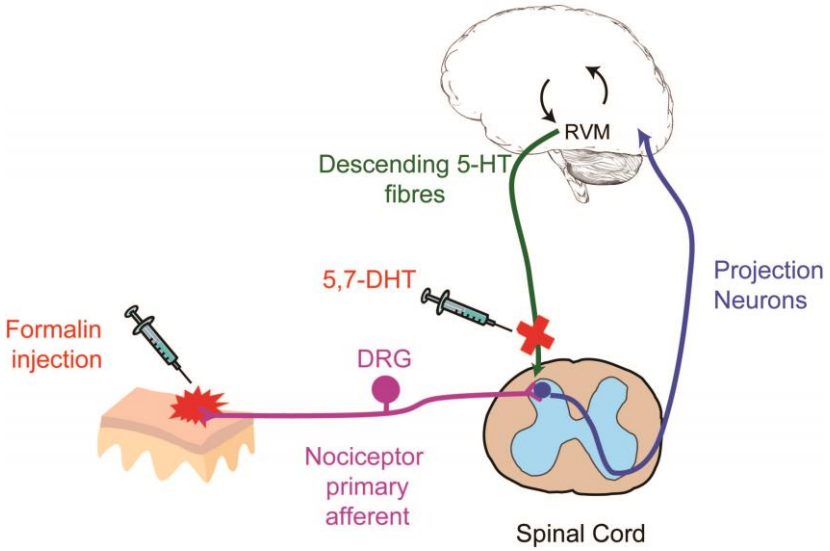


Figure 5.1 Intrathecal delivery of 5,7-DHT chemically damages 5-HT-containing descending fibres from the RVM. Red 'x' indicates site of lesion.

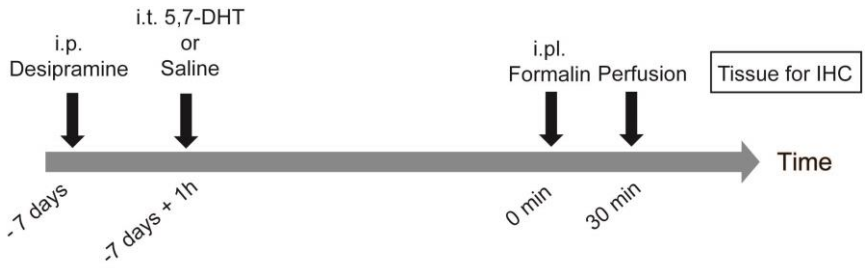


Figure 5.2 Timeline of experimental procedures.

5.2.2 Intrathecal surgery

Intrathecal surgery was performed to deliver either 5,7-DHT or saline. Please see 2.3.1 for full procedure details.

5.2.3 Drugs

5,7-DHT was given 60µg/10µl/0.9%saline (i.t.) and desipramine was given 25mg/kg/0.9% saline (i.p.) (see Table 2.1).

5.2.4 Formalin stimulation

All animals were given intraplantar formalin, 50µl 2% under isoflurane anaesthesia (see 2.2.1).

5.2.5 Immunohistochemistry

Please refer to section 2.5 for the full immunohistochemical protocol and antibody information. All stains done in this chapter were single stains for one antigen.

Table 5.1 IHC experiments completed in Chapter 5

Antibody	Method of detection
5-HT	Biotin amplification
GFAP	Direct Alexa 488
NeuN	Direct Alexa 594
PERK	TSA
PH3S10	DAB

5.2.6 Immunohistochemistry- Image acquisition

Images of fluorescent immunohistochemistry in this chapter were taken on a Hamamatsu CCD C4742 digital camera attached to a Leica DMR microscope using the OpenLab software (PerkinElmer, Waltham MA, USA). Images used for FIJI analysis were taken on the Leica TCS SPE laser scanning confocal microscope.

5.2.7 Immunohistochemistry- Quantification

For manual quantification of PERK (n=3 each group) and PH3S10 (n=7 each group) immunopositive cells, all sections were counted in laminae I-II with experimenter blind to treatment using a Leica DMR microscope. Once all sections were counted, per animal, five sections with the maximum response (ipsilateral) were used and the average taken on the ipsilateral dorsal horn. The average of each treatment group was then calculated along with the standard error of each group mean (SEM).

For PERK, an additional method was used to quantify immunostaining. FIJI (ImageJ) image analysis was performed on 3 tissue sections from each animal (n=4 each group). The sections were selected at random from five sections with the maximum response (ipsilateral). Two protocols were used:

1. FIJI particle analysis

   Particle analysis was used to quantify the number of cell bodies using automatic particle counting. The number of cell bodies was averaged across the 3 sections.

2. FIJI intensity analysis

   Intensity analysis was used to quantify immunofluorescence from cell bodies in addition to other immunopositive staining such as dendritic processes. A pixel threshold of 11 was used and the sum of the weighted intensity particles greater than 11 was averaged across the 3 sections.

Group data was presented as mean ± SEM.

Gross visual analysis was performed on 5-HT, GFAP, and NeuN immunostains for all animals to confirm 5-HT depletion and no change in glial activation or neuronal death.

5.2.8 Statistical analysis

Please also see 2.7 for full details of statistical analysis.

PH3S10 counts were compared in animals receiving i.t. saline and animals receiving i.t. 5,7-DHT. Comparison was analysed using a univariate ANOVA, with *'treatment'* (saline vs. 5,7-DHT) as the between-subjects factor, and *'side'* (ipsilateral vs. contralateral) as the within-subjects factor.

5.3 Results

5.3.1 Confirmation of 5-HT depletion

Ablation of descending serotonergic neurons 7 days after i.t. 5,7-DHT was confirmed using immunohistochemical analysis. There was a clear lack of 5-HT immunostaining in the dorsal horn of animals receiving i.t. 5,7-DHT compared to a dense superficial expression of 5-HT in i.t. saline treated animals (Figure 5.3 A).

5.3.2 No change in primary afferent activation of ERK in the dorsal horn following depletion of spinal 5-HT

It was subsequently investigated whether ablation of descending serotonergic fibres had any effect on formalin-induced nociceptive primary afferent activity upon the dorsal horn as indicated by PERK. PERK expression was analysed using three methods: manual quantification, FIJI particle analysis, and FIJI intensity analysis. Contrary to the findings of Svensson et al., who found that i.t. 5,7-DHT blocked upregulation of PERK at 30 minutes (Svensson et al., 2006), manual quantification revealed no significant reduction in PERK expression in the dorsal horn 30 minutes post-formalin in 5,7-DHT vs. saline treated groups. There was however, a trend towards a reduction in PERK expression in the 5,7-DHT group (Figure 5.3 B). Further quantification using FIJI particle analysis and FIJI intensity analysis (Figure 5.3 C, D) confirmed no difference between groups, and no further attempt was made to increase n numbers.

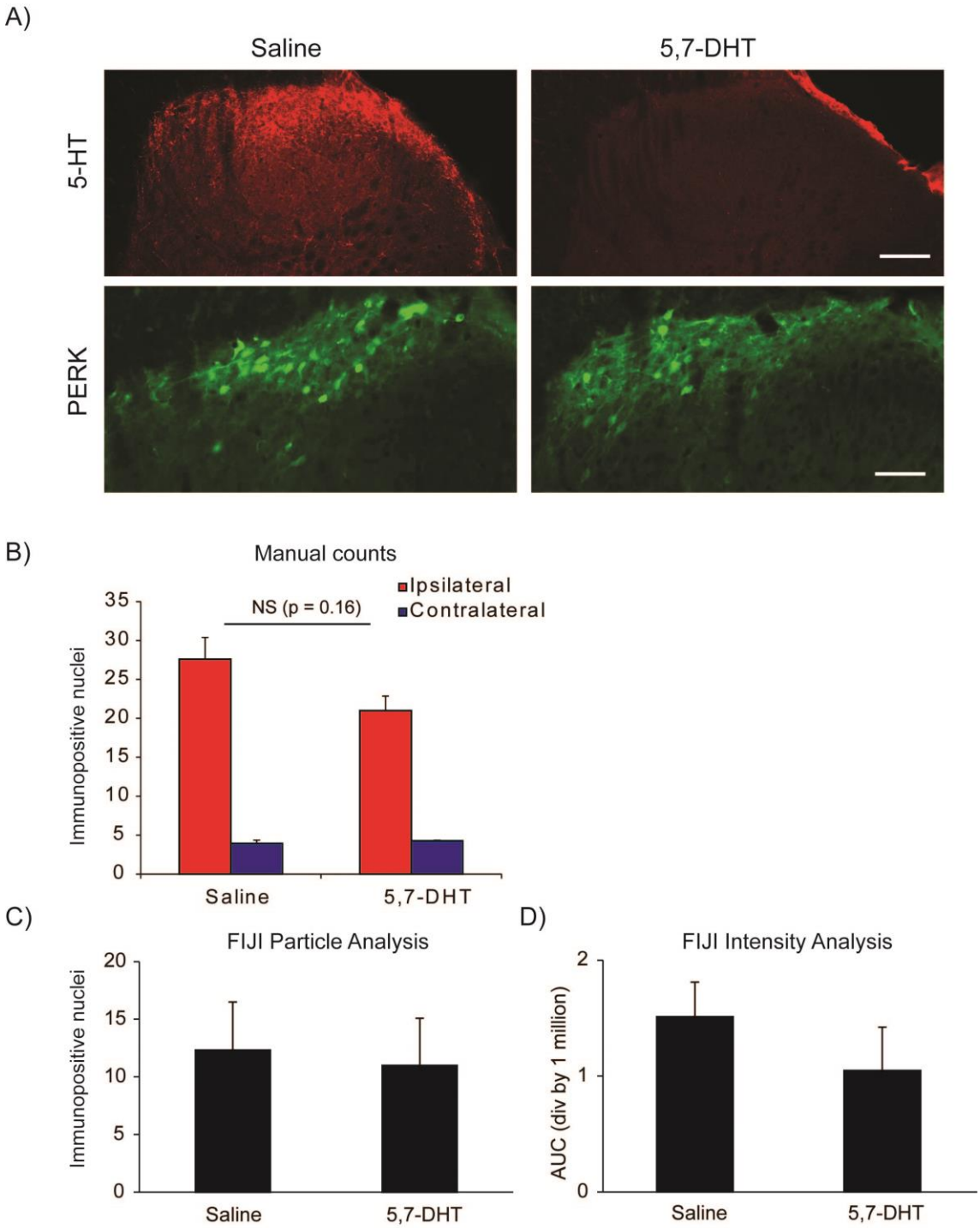


Figure 5.3 Ablation of serotonergic fibres with 5,7-DHT leads to a reduction in 5-HT but does not prevent formalin-induced ERK activation in the dorsal horn when quantified using three different methods. A) Typical dorsal horn images of 5-HT and PERK-fluorescent staining in animals receiving i.t. saline or i.t. 5,7-DHT at 30 min post-formalin stimulation (rows=antigen of interest; columns=i.t. treatment). Scale bar upper, 50µm; lower, 30µm. B) Quantification of PERK cell bodies in the ipsilateral and contralateral dorsal horn using manual counts (n=3, 5 sections per animal). C) Quantification of ipsilateral PERK cell body counts using FIJI particle analysis. D) Intensity quantification of ipsilateral PERK positive staining using FIJI intensity analysis (C and D, n=4, 3 sections per animal). Values presented as group mean (per 40µm section) ± SEM. All graphs- no statistically significant differences.

5.3.3 Depletion of spinal 5-HT prevents full expression of formalin-induced PH3S10

After confirmation of spinal 5-HT depletion, expression of PH3S10 was investigated. Although there was no difference in dorsal horn PERK expression between 5,7-DHT vs. saline treated groups, the same treatment revealed that there was nearly a 50% reduction in PH3S10 nuclei 30 minutes post-formalin. The data showed a main effect of treatment ($F_{(1,24)}$=9.44, P<0.01), and an interaction between side and treatment ($F_{(1,24)}$=9.14, P<0.01).

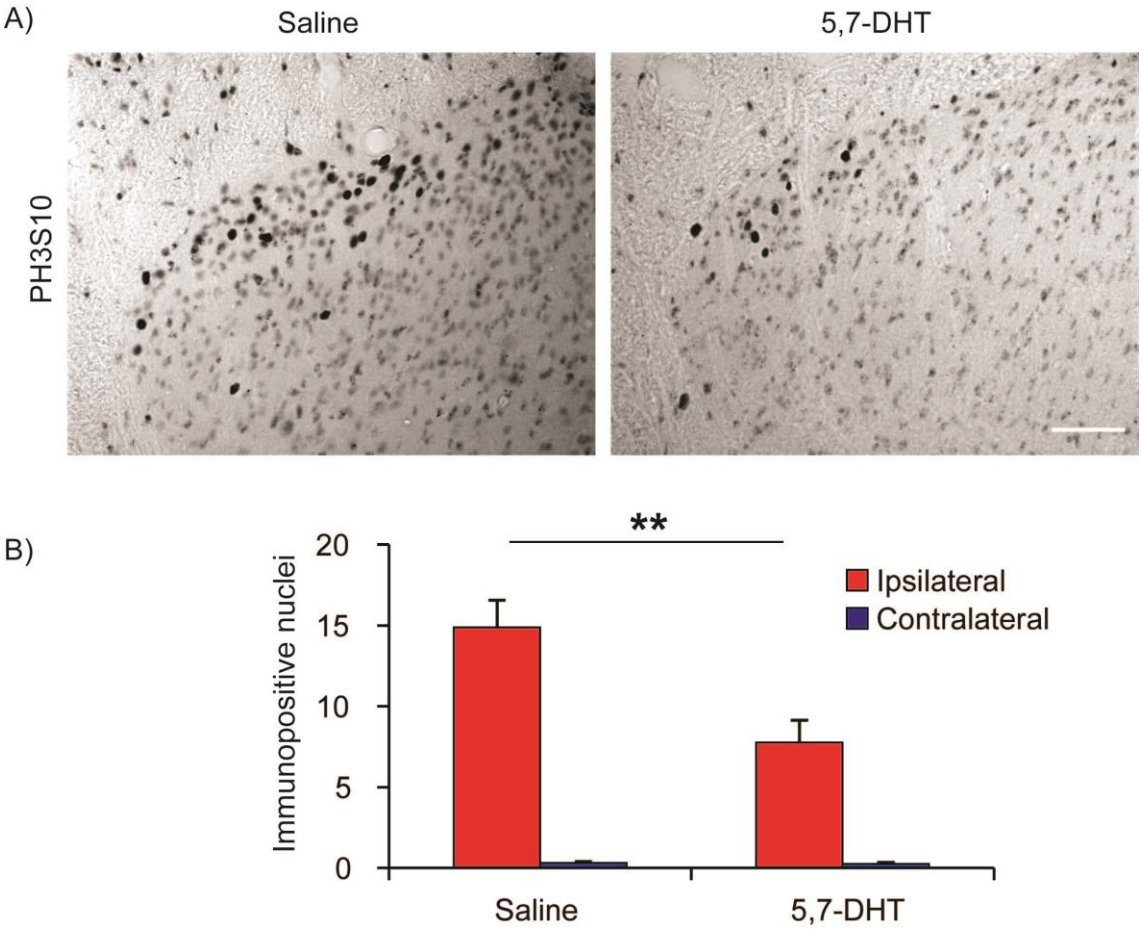


Figure 5.4 Depletion of spinal serotonin prevents the full expression of formalin-induced PH3S10. A) Dorsal horn image of PH3S10-DAB staining 30 min after formalin in animals receiving i.t. saline or i.t. 5,7-DHT. Scale bar 30µm. B) Manual quantification of PH3S10 nuclei in ipsilateral and contralateral dorsal horn (n=7 each group, 5 sections per animal). Values presented as group mean ± SEM (per 40µm section). **P<0.01

5.3.4 No gross indication of neuronal death or glial proliferation after depletion of descending 5-HT fibres caused by 5,7-DHT

5,7-DHT caused a specific depletion of 5-HT in the spinal cord, and there were no indications of neuronal death or gliosis as shown by NeuN or GFAP immunostaining at 30 minutes post-formalin in animals depleted of spinal 5-HT.

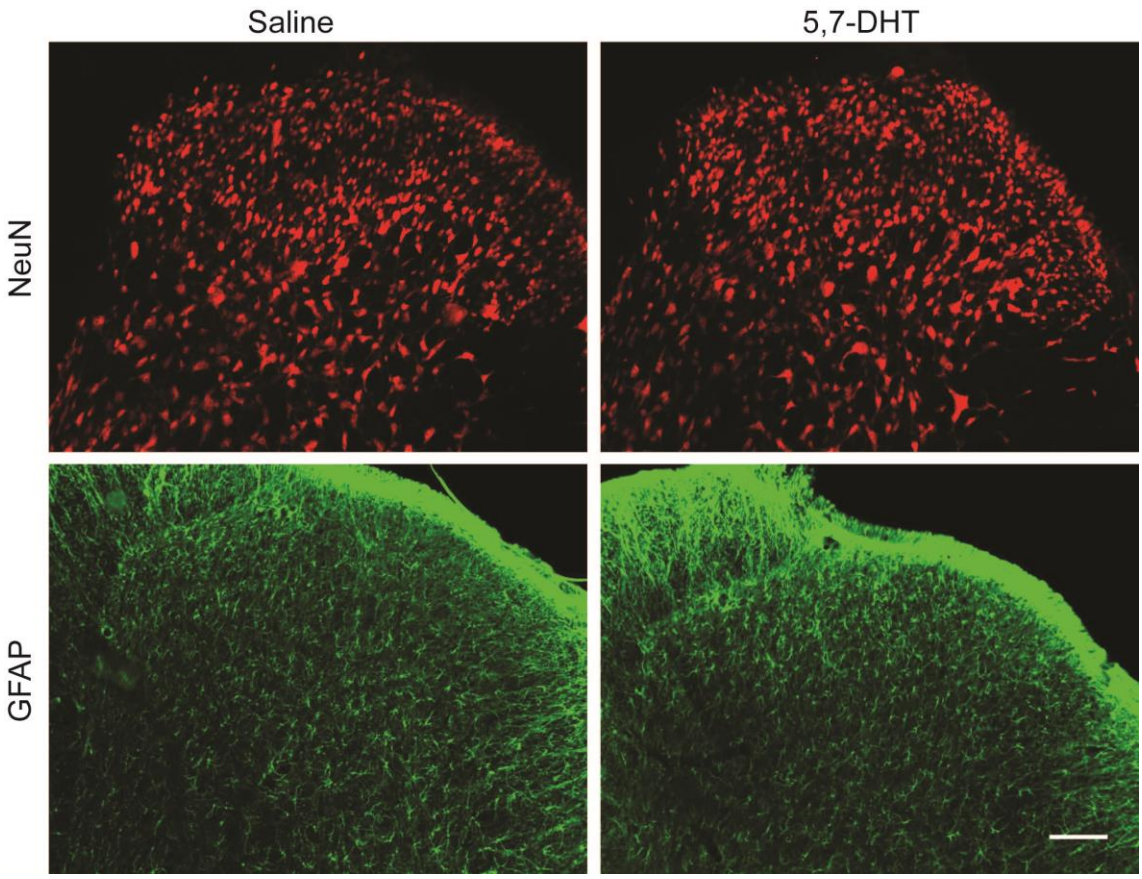


Figure 5.5 There were no changes in NeuN or GFAP expression after depletion of spinal 5-HT. Typical dorsal horn images of NeuN and GFAP in animals receiving i.t. saline or i.t. 5,7-DHT at 30 min post-formalin stimulation (rows=antigen of interest; columns=i.t. treatment). Scale bar 50µm.

5.4 Discussion

RVM descending fibre release of spinal 5-HT can have facilitatory and inhibitory effects on nociception depending on the receptor subtype mediating its actions (Bardin, 2011; Millan, 2002; Ossipov et al., 2010). The experiments in this chapter showed no reduction in formalin-induced PERK and a significant reduction in the expression of PH3S10 after i.t. 5,7-DHT-mediated depletion of spinal 5-HT.

5.4.1 No changes in PERK after i.t. 5,7-DHT

Svensson et al. showed that loss of spinal 5-HT caused by i.t. 5,7-DHT attenuates formalin-induced nociceptive behaviour and PERK expression in the spinal cord (Svensson et al., 2006). In this chapter, although there was a trend toward a reduction in formalin-induced PERK expression after i.t. 5,7-DHT, it was not significant. The same 5,7-DHT induced depletion however, caused a significant reduction in dorsal horn PH3S10 expression. PH3S10 is a well-known downstream target of PERK in neurons (Crosio et al., 2003), and results from Chapter 4 indicated that at least 42% of formalin-induced PERK neurons colocalised with PH3S10. PERK and PH3S10 also share a similar time course of expression with PERK and PH3S10 levels, peaking at 5 minutes and 5-60 minutes, respectively (Ji et al., 1999). It is therefore quite surprising that no reduction in PERK activation was observed despite attempts at various methods of quantification.

The study by Svensson et al. (2006) and the experiment presented in this chapter both studies examined spinal PERK expression 30 minutes after i.pl. formalin stimulation; however, there were 2 apparent differences in protocol. Firstly, the investigators induced hindpaw inflammation with formalin 2 days after 5,7-DHT while the experiments here waited until day 7. It is possible that 2d was not long enough for a complete 5-HT depletion; however, the authors reported a 78-84% reduction in spinal 5-HT levels, so this discrepancy cannot explain the difference in result. Secondly, Svensson et al. used western blot to analyse levels of PERK1 and PERK2 isoforms separately. Taking a similar approach, the FIJI intensity analysis was employed specifically to quantify all immunopositive PERK staining, including that observed in processes as well as cell bodies, which appeared to contribute to a dense plexus of positive staining in the superficial dorsal horn. All three methods of quantification shared the same result: a non-significant trend toward the reduction in dorsal horn PERK after 5,7-DHT. Thus, it does not appear that there are any major experimental differences between that of Svensson et al. and the experiments in this chapter.

Much of the evidence suggests that spinal cord activity is in fact reinforced by descending regulation from supraspinal structures (Ossipov et al., 2010; Svensson et al., 2006). Furthermore, ERK activation is seen in the RVM as early at 30 minutes after CFA-induced peripheral stimulation (Imbe et al., 2005). Imbe et al (2005) found that roughly 20% of the PERK positive cells in the RVM were serotonergic, thus suggesting that the RVM is engaged at early time points after noxious stimulation. And finally, attenuation of behavioural hypersensitivity due to 5,7-DHT has been observed as early as 1 hour after the induction of short-term inflammatory models such as CFA (i.pl.) or 20 minutes post-formalin (i.pl.) (Géranton et al., 2008; Svensson et al., 2006). It should be noted however, that temporal correlations between behaviour and expression of molecular markers should be investigated with caution (discussed also in Chapter 6).

Results from this chapter suggest that there may, in fact, be no effect of 5,7-DHT on ERK activation. One possible explanation for the lack of PERK reduction after 5,7-DHT is that the descending 5-HT system is not engaged until after the induction of PERK. PERK is largely indicative of primary afferent activity and peaks almost immediately within 5 minutes of noxious stimulation, primarily due to primary afferent activity and release of neurotransmitters SP and glutamate onto the spinal cord (Ji et al., 1999). Furthermore, when considering the effect of the 5,7-DHT ablation on nociceptive behaviour, significant attenuation of hypersensitivity is not observed until 1-2 days after the induction of hyperalgesia caused by persistent pain models such as SNI or CFA-ankle joint inflammation (Carr et al., 2014; Rahman et al., 2006).

5.4.2 Facilitatory role of 5-HT on PH3S10 expression

The role of descending 5-HT is controversial, with earlier studies implicating mainly an inhibitory role for 5-HT on thermal and mechanical hypersensitivity in naïve animals (Bardin et al., 1997; Crisp et al., 1991; Xu et al., 1994; Yaksh and Wilson, 1979). However, an early study by Zhuo and Gebhart showed that bidirectional descending facilitation or inhibition occurred when the RVM was stimulated at low and high frequencies, respectively, and that the low frequency- induced pronociceptive activity was also mediated by 5-HT (Zhuo and Gebhart, 1992, 1991). Subsequent studies have produced strong evidence to support descending facilitation of 5-HT from the hindbrain, and the majority of recent studies using the 5,7-DHT ablation have supported a facilitatory role for descending 5-HT. Depletion of spinal 5-HT using the i.t. 5,7-DHT ablation has been previously shown to attenuate the second phase of the formalin response (Okamoto et al., 2004; Svensson et al., 2006), as well as mechanical and thermal hypersensitivity induced by nerve injury or persistent hindpaw or ankle joint inflammation with CFA (Carr et al., 2014; Géranton et al., 2008; Rahman et al.,

2006). Furthermore, RNAi silencing of TPH, and thus reduction in 5-HT in the RVM has also been shown to attenuate formalin, CFA, and SNL-induced hypersensitivity (Wei et al., 2010) without altering acute baseline responses to thermal or mechanical sensitivity.

The reduction in formalin-induced PH3S10 after i.t. 5,7-DHT seen here indicates that 5-HT is required for the full expression of PH3S10 and is reminiscent of other studies showing reductions in dorsal horn molecular markers of nociception after 5,7-DHT. Specifically, PERK, PMeCP2, and the IEGs c-Fos and Zif268 (Géranton et al., 2008), markers indicative of neural activation following noxious stimuli, are all significantly reduced after i.t. 5,7-DHT thus corroborating a net facilitatory role for descending 5-HT; although as previously noted no change in PERK was observed here (Svensson et al., 2006). The role of PH3S10 in nociception is yet to be investigated (see Chapter 6), however, results from Chapter 4 indicated that PH3S10 is rapidly and robustly upregulated in the dorsal horn as early as 5 minutes after formalin stimulation and may contribute to spinal processing of nociceptive behaviour.

5.4.3 A possible role for the 5-HT_3 receptor?

Numerous studies have implicated 5-HT_3 in mediating descending facilitation and spinal cord excitability. 5-HT_3 is highly expressed on axons of primary afferents, dorsal horn excitatory interneurons, and importantly contributes to regulation of spinal excitability of NK1-expressing projection neurons (Conte et al., 2005; Huang et al., 2004; Maxwell et al., 2003; Suzuki et al., 2002; Zeitz et al., 2002). 5-HT_3 is unique from the other 5-HT receptors in that it is a ligand-gated ion channel that, along with 5-HT_2, enhances intracellular signalling via phospholipase C (PLC) after being activated (Maxwell et al., 2003). i.t. delivery of the 5-HT_3 antagonist, ondansetron, has been shown to attenuate hypersensitivity generated by formalin, nerve injury, intraplantar CFA, CFA ankle joint inflammation, and post-operative pain (Dogrul et al., 2009; Lagraize et al., 2010; Oatway et al., 2004; Okamoto et al., 2004; Oyama et al., 1996; Silveira et al., 2010; Svensson et al., 2006; Zeitz et al., 2002) and when injected into the RVM produces analgesia and a conditioned place preference in SNL-ondansetron treated animals (Wang et al., 2013). Furthermore, 5-HT_3A knockout mice also display attenuation in the second phase of the formalin response, without alterations in acute mechanical and thermal responses (Kayser et al., 2007). The expression of formalin-induced PH3S10 after targeting of 5-HT_3 was not investigated here. However, elimination of 5-HT_3 activity using genetic targeting or i.t. ondansenton has been shown to lead to a reduction in noxious stimuli-induced c-Fos expression in the spinal cord and thus it is possible that PH3S10 expression would also be decreased under such

circumstances (Green et al., 2000; Suzuki et al., 2002; Zeitz et al., 2002). While the evidence seems to overwhelmingly implicate 5-HT3 in descending facilitation, some studies have reported anti-nociceptive roles, specifically utilising 5-HT_3 agonists (Alhaider et al., 1991; Glaum et al., 1990; Paul et al., 2001). Although 5-HT_3 is the most heavily investigated receptor underlying descending facilitation, other 5-HT receptors associated with facilitatory effects include 5-$HT1_a$ (Zhuo and Gebhart, 1991) and 5-HT_2 (Bardin 2011).

5.4.4 Controversy over the 5,7-DHT depletion

Utilisation of 5,7-DHT to investigate the role of descending 5-HT activity has attracted some criticism. The 5,7-DHT ablation targets and chemically damages 5-HT containing fibres which also consequently eliminates the activity of other functional neurotransmitters (such as GABA, SP, Dynorphin, Enkephalin) in the same fibres. Although the experiments in this chapter and others have reported no apparent change in neuronal death after 5,7-DHT, the complete elimination of fibre function leaves some to argue that 5,7-DHT is not a direct way of investigating the role of 5-HT function itself. Furthermore, it should be noted that experiments using the 5,7-DHT ablation have yielded contradicting effects on nociception, with some groups reporting attenuation of CFA-induced mechanical hyperalgesia after i.t. 5,7-DHT (Carr et al., 2014; Géranton et al., 2008; Rahman et al., 2006) and nerve injury-induced thermal hyperalgesia (Rahman et al., 2006), while other studies found that the treatment exacerbated thermal hypersensitivity after inflammation (Millan et al., 1997; Pertovaara et al., 2001; Wei et al., 1999; Zhao et al., 2007).

A recent study by Wei et al. used a novel method to selectively deplete 5-HT from descending fibres originating in the RVM without chemically damaging 5-HT containing fibres. The method utilised plasmid-mediated RNAi (shRNA) silencing of TPH in the RVM, which kept descending fibres fully intact and preserved the contribution of non-5-HT neurotransmitter activity thereby selectively isolating the effects of 5-HT in neurotransmission itself (Wei et al., 2010). Importantly, Wei et al. also found 5-HT to have a net facilitatory role upon spinal cord activity, with reported attenuation of inflammatory and neuropathic hypersensitivity but no changes to acute nociception following depletion. Interestingly, deletion of descending 5-HT did not affect descending inhibition produced by opioid analgesia using the synthetic mu receptor agonist, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), which supports studies indicating that 5-HT signalling in the RVM is not necessary for RVM mu opioid-mediated analgesia.

It is worth noting that another recent study has used leading-edge optogenetic activation of tryptophan hydroxylase 2 (TPH2)- channelrhodopsin 2 (ChR2) transgenic mice to investigate the behavioural effects of activating 5-HT-expressing RVM neurons. The authors found that activation of 5-HT RVM neurons had an overall descending facilitatory pain effect. Activation of 5-HT RVM neurons in TPH2-ChR2 mice induced c-Fos expression in TPH positive RVM neurons, and decreased both mechanical and thermal pain thresholds in an intensity-dependent manner, an effect not seen in wild-type mice (Cai et al., 2014).

5.4.5 Technical considerations

It is worth mentioning that the significant reduction of spinal PH3S10 following 5,7-DHT utilised an n=7, while the non-significant trend toward a decrease in PERK expression used only an n=3 (both experiments, quantification by manual counts). Considering the difference in n numbers between the two experiments, it is possible that an increase in experimental subjects in the PERK study may have led to a significant reduction in PERK (i.e. using also n=7), but this approach was not utilised. Instead, the decision was made to employ two additional methods of immunohistochemical quantification to quantify PERK (FIJI particle analysis and FIJI Intensity analysis, n=4 each analysis), but both methods showed no reduction in PERK after 5,7-DHT.

5.4.6 Conclusions

The results from this chapter indicate that spinal depletion of 5-HT with i.t. 5,7-DHT after 7 days prevents the full expression of formalin-induced PH3S10. This result is further evidence that PH3S10 may be involved in nociceptive processing and that descending 5-HT has a net facilitatory effect on spinal activity, in particular PH3S10 expression following noxious stimulation. Subsequently, the next aims were to investigate the specific contribution of PH3S10 in nociception.

Chapter 6 Intracellular regulation of PH3S10 in nociceptive processing

6.1 Introduction

Chapter 4 results indicated that 58% of formalin-induced PH3S10 in the dorsal horn colocalised with PERK, a marker of nociceptive activity. As previously mentioned in Chapter 4, ERK/MAPK regulation of PH3S10 has been demonstrated in various models of activity-induced synaptic plasticity such as memory formation (Chandramohan et al., 2008, 2007; Chwang et al., 2006). In the field of pain, numerous studies have used MEK inhibitors to show that PERK is essential to the full expression of pain states (Ji et al., 2009). The effect of MEK inhibition on PH3S10 expression, however, has never been investigated. The experiments in this chapter investigate the specific contribution of PH3S10 and its kinase, MSK1, in nociceptive processing and behaviour.

6.1.1 ERK/MAPK signalling: MSK1 and PH3S10, downstream targets of PERK

The role of ERK/MAPK signalling in nociceptive processing was discussed in 3.1.2.1. In addition, *in vitro* studies have shown that PERK is capable of translocation into the nuclear compartment where it phosphorylates the nuclear mitogen and stress-activated protein kinase (MSK). MSK is an intermediate kinase known to regulate gene transcription via downstream phosphorylation of histone H3, CREB, and other transcription factors (Schuck et al. 2003; Soloaga et al. 2003; Wiggin et al. 2002) (Figure 6.1), events which have been observed in the nervous system (Brami-Cherrier et al., 2005; Chandramohan et al., 2008; Chwang et al., 2007, 2006; Ciccarelli et al., 2013; Correa et al., 2012; Crosio et al., 2003; Gräff et al., 2012; Gutièrrez-Mecinas et al., 2011; Karelina et al., 2012; Putignano et al., 2007; Rotllant and Armario, 2011). However, neither histone phosphorylation on H3 nor MSK have been investigated in nociception; a surprising finding considering the importance of ERK/MAPK signalling in nociceptive processing (Ji et al., 1999).

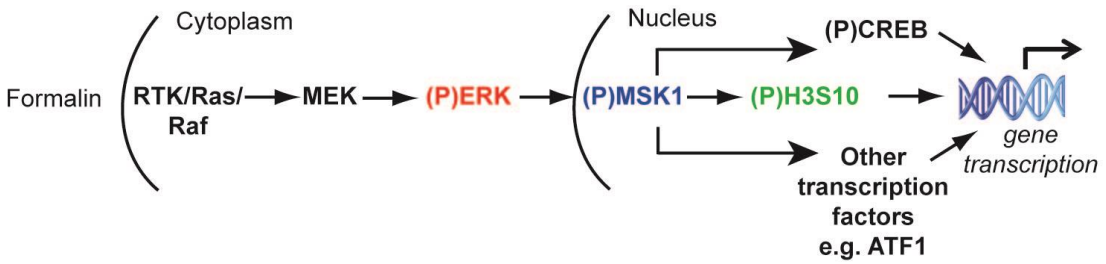


Figure 6.1 The proposed signalling of ERK/MAPK following noxious stimulation involves activity of nuclear kinase MSK1 and PH3S10. It is known that PCREB is upregulated in the dorsal horn following noxious stimulation (Ji and Rupp, 1997) and is regulated by MSK1 in neurons (Arthur et al., 2004). MSK1 also phosphorylates ATF1, but this has only been shown in fibroblasts (Wiggin et al., 2002).

6.1.2 The mitogen and stress-activated protein kinase (MSK)

The mitogen and stress-activated protein kinases (MSKs) are serine/threonine protein kinases, of which there are two isoforms: MSK1 and MSK2 (64% homology). MSK1 is the predominant isoform in the mammalian brain (Arthur et al., 2004; Frenguelli and Correa, 2013), and will therefore be referred to as MSK1 for the remainder of this thesis. A wide range of stimuli are known to activate MSK1, including neurotrophins BDNF/NGF/NT-3 and glutamate (Arthur et al., 2004; Brami-Cherrier et al., 2007; Frenguelli and Correa, 2013; Rakhit et al., 2005). MSKs are directly regulated by ERK/MAPK and p38 signalling. Specifically, PERK directly regulates MSK1 C-terminal kinase domain activity by phosphorylating S360, T581 and T700 (McCoy et al., 2005), and as already mentioned, MSK1 itself regulates phosphorylation of downstream targets implicated in neural plasticity: H3S10 and CREB (Arthur et al., 2004; Deak et al., 1998; Soloaga et al., 2003; Wiggin et al., 2002). Indeed, MSK -/- striatal neurons display a lack of glutamate-induced expression of PH3S10 and show reduced expression of *c-Fos* (Brami-Cherrier et al., 2007).

MSK1 itself is a novel protein of interest in nociception, and during this study it became increasingly apparent that MSK1 would be the best target for specifically investigating the role of PH3S10 in nociception particularly due to the availability of pharmacological inhibitors (Naqvi et al., 2012).

6.1.3 Aims

PERK is a widely used marker of neuronal activation by nociceptive stimuli in the spinal cord and brain, and pharmacological inhibition of its upstream kinase MEK has shown that PERK signalling is required for the full expression of pain states (Gao and Ji, 2009; Ji et al., 2009). Given the widely accepted importance of PERK in pain processing, it is somewhat surprising that neither MSK1 nor PH3S10, known downstream components of ERK signalling (Soloaga et al., 2003), have been investigated following noxious stimulation. Chapter 4 results showed that 58% of PH3S10 colocalise with PERK. Therefore, evidence of PH3S10 regulation by ERK (Chwang et al., 2006) and MSK1 (Brami-Cherrier et al., 2009; Chwang et al., 2007) in other areas of the CNS suggest that inhibition of MEK and MSK1 kinases would reduce formalin-induced spinal PH3S10 expression and attenuate formalin-induced nocifensive behaviours.

The aim of this chapter was to investigate the intracellular regulation of PH3S10 in nociceptive processing in order to determine the role of histone phosphorylation (PH3S10) on nociceptive behaviour. Specifically, to

1. Determine whether ERK/MAPK regulates formalin-induced PMSK1 (the kinase responsible for PH3S10) and PH3S10 by inhibition of MEK activity with i.p. SL327

2. Determine whether PMSK1 regulates expression of formalin-induced PH3S10 and c-Fos by inhibition of MSK1 with i.t. SB747651A

3. Determine whether PMSK1 regulates nociceptive behaviour by inhibiting MSK1 with i.t. SB747651A

Finally, for the majority of the experiments in this thesis, 2%, 50µl formalin was injected under general isoflurane anaesthesia. However, experiments in Chapter 6 required 5%, 20µl formalin injections without general anaesthesia in order to assess nocifensive behaviour immediately following injection. Thus one final aim of this chapter was

4. To determine whether there were any differences in spinal PERK expression between the two conditions of formalin injection (2%, 50µl injected under anaesthesia vs. 5%, 20µl injected without anaesthesia)

6.2 Materials and Methods

6.2.1 Experimental design

There are 3 main experiments in this chapter:

6.2.1.1 *Experiment 1:* Does MEK inhibition block formalin-induced PMSK1 and PH3S10 expression? (Aim 1)

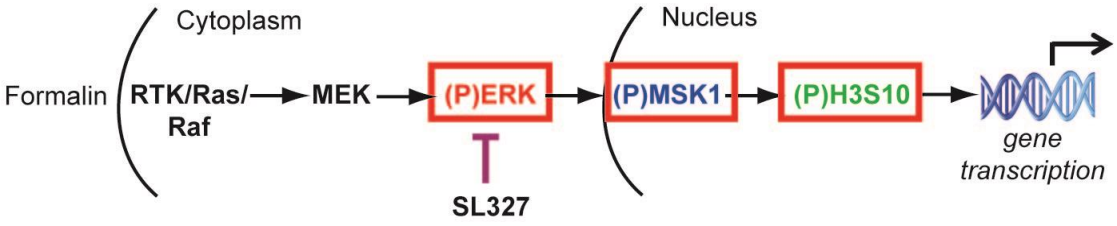


Figure 6.2 Pharmacological blockade of MEK with SL327. Red boxes indicate main targets of interest.

There were 2 treatment groups:

1. SL327
2. Vehicle (100% DMSO)

The MEK inhibitor SL327 crosses the blood brain barrier and has been previously shown to block ERK activation and attenuate the first and second phase of the formalin response (Alter et al., 2010). SL327 or DMSO (n = 4 each group) was delivered i.p. 30 minutes prior to i.pl. formalin stimulation and 30 minutes later animals were sacrificed for perfusion to investigate spinal expression of PERK, PMSK1, and PH3S10 using IHC (Figure 6.2, Figure 6.3).

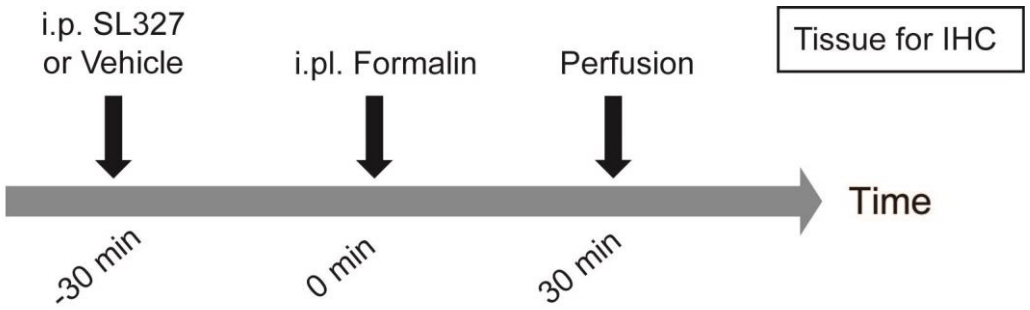


Figure 6.3 Timeline of experimental procedures (Experiment 1).

6.2.1.2 Experiment 2: Does inhibition of MSK1 activity block formalin-induced PH3S10 and c-Fos expression, and attenuate formalin behaviour? (Aims 2 and 3)

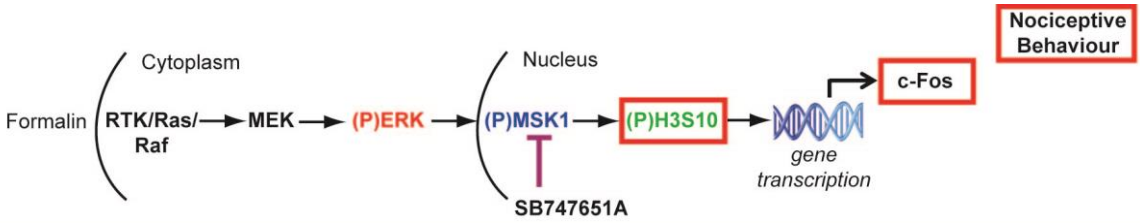


Figure 6.4 Pharmacological blockade of MSK1 with SB747651A. Red boxes indicate main targets of interest.

There were 3 treatment groups:

1. Vehicle (0.0086% EtOH/0.9% Saline)
2. SB747651A (10µM)
3. SB747651A (1µM)

The MSK1 inhibitor SB747651A or Vehicle was delivered i.t (see 2.3.1 for description of surgical procedure) 30 minutes prior to i.pl. formalin stimulation. Animals were then perfused 30 minutes (investigation of PERK and PMSK1 for control measures) (n = 4) or 1h (investigation of PH3S10 and c-Fos) later for IHC (n =14/15/8; Vehicle/10µM/1µM groups) (Figure 6.4, Figure 6.5). For the 1h survival, formalin behaviour was also recorded after stimulation (n = 11/16/6; Vehicle/10µM/1µM groups) (see 2.2.2).

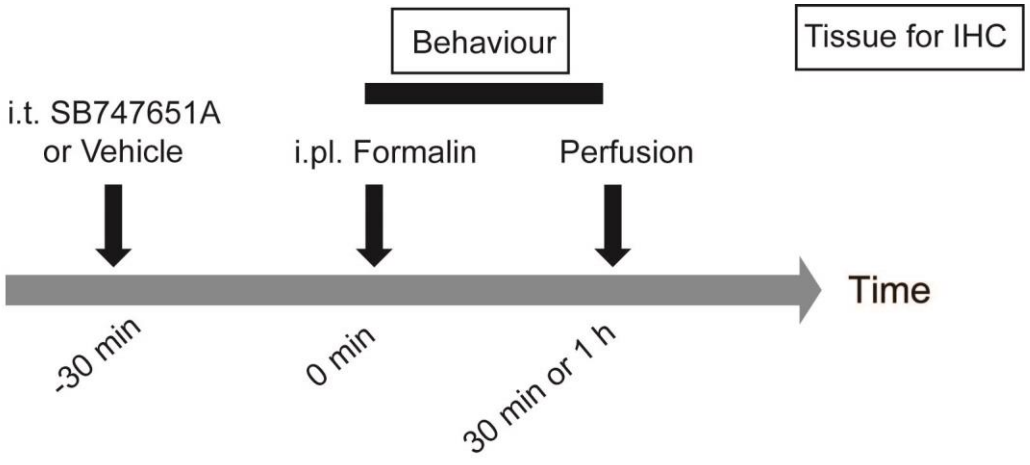


Figure 6.5 Timeline of experimental procedures (Experiment 2).

6.2.1.3 *Experiment 3:* Are there any differences in spinal PERK expression following two different conditions of i.pl. formalin injection? (Aim 4)

In this thesis, with the exception of Chapter 6, *Experiment 2*, all animals were injected with i.pl. formalin (50µl, 2%) under isoflurane anaesthesia. Chapter 6, *Experiment 2, Does inhibition of MSK1 activity block formalin-induced PH3S10 and c-Fos expression, and attenuate formalin behaviour?* necessitated the injection of formalin without anaesthesia in order to investigate nocifensive behaviour. However, due to Home Office licence limitations, rats injected without anaesthesia could only receive a maximum i.pl. injection of 20µl. In order to deliver the same total amount of formalin as the 50µl, 2% dose given under isoflurane anaesthesia, a higher concentration was given (20µl, 5%).

Thus, the aim of *Experiment 3* was to serve as a control experiment to identify whether the two conditions of formalin injection induced the same amount of PERK activation in the dorsal horn.

Sham animal groups were either given general anaesthesia only or light restraint only and no injection.

There were 4 treatment groups:

1. Formalin (50µl, 2%) with anaesthesia (Form-A)
2. Formalin (20µl, 5%) without anaesthesia (Form-NA)
3. Anaesthesia only (Sham-A)
4. Handling/Restraint only (Sham-NA)

All animals (n=3 each group) were sacrificed for perfusion 30min following stimulation and spinal cords processed for immunohistochemistry.

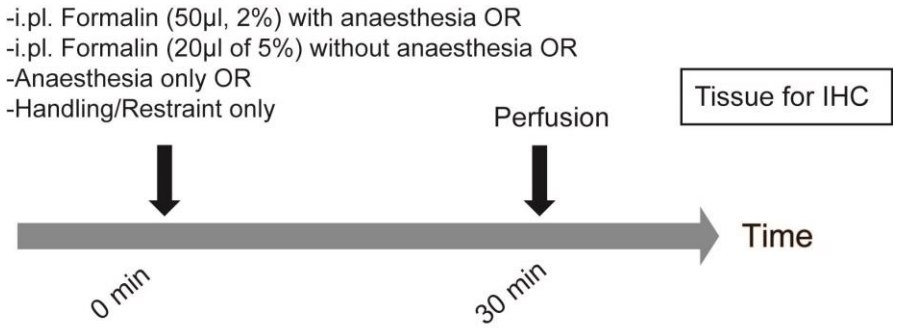


Figure 6.6 Timeline of experimental procedures (Experiment 3)

6.2.2 Drugs

For *Experiment 1*: SL327 (50mg/kg/DMSO) or Vehicle (100% DMSO) was given i.p. in 0.5ml without anaesthesia.

For *Experiment 2:* SB747651A (1 or 10µM/0.0086% EtOH in 0.9% saline) or Vehicle (0.0086% EtOH in 0.9% saline) was given i.t. in 10µl (see Table 2.1 for more drug information; see 2.3.1 for description of surgical procedure).

6.2.3 Formalin stimulation

For *Experiment 1*: All animals were given intraplantar formalin, 50µl, 2% under isoflurane anaesthesia (see 2.2.1).

For *Experiment 2:* All animals were given intraplantar formalin, 20µl, 5% without anaesthesia, in order to immediately observe nociceptive behaviour after stimulation (see 2.2.1).

For *Experiment 3:* One group of formalin stimulated animals was given intraplantar formalin, 50µl, 2% under isoflurane anaesthesia, while the other group was given intraplantar formalin, 20µl, 5% without anaesthesia. See 6.2.1.3 for an explanation behind the use of two different doses.

6.2.4 Immunohistochemistry

Please refer to section 2.5 for the full immunohistochemical protocol and antibody information.

Table 6.1 Table of IHC experiments completed in *Experiment 1* Where PH3S10 was applied as a second primary, acid pretreatment was used for antigen retrieval prior to blocking (see 2.5.2).

Immunostain	1st primary	Method of detection	2nd primary	Method of detection	Time point
PH3S10/PERK	PH3S10	TSA	PERK	Direct Alexa 594	30min
PMSK1/PH3S10	PMSK1	TSA	PH3S10	Direct Alexa 594	30min
PMSK1/PERK	PMSK1	TSA	PERK	Direct Alexa 594	30min

Table 6.2 Table of IHC experiments completed in *Experiment 2* Please note that PERK staining in *Experiment 3* also used DAB detection, at 30min time point.

Immunostain	Method of detection	Time point
PERK	DAB	30min
PMSK1	TSA	30min
PH3S10	TSA	1h
c-Fos	DAB	1h

6.2.5 Formalin behaviour- Scoring and quantification

Formalin behaviours were scored and quantified in 5min bins across the 1h following injection. All scoring was done with the experimenter blind to treatment.

1. Length of time spent licking and flinching
2. Number of flinches

6.2.6 Immunohistochemistry- Quantification

Immunopositive cells for all sections were counted manually with experimenter blind to treatment using a Leica DMR microscope. Immunopositive cells bodies (PERK) or nuclei (PMSK1, PH3S10, c-Fos) were counted in laminae I-II in the ipsilateral dorsal horn of all tissue sections. Once all sections were counted, per animal, five sections with the maximum response (ipsilateral) were used and the average taken on the ipsilateral side of the dorsal horn. The average of the treatment group was then calculated along with the standard error of each group mean (SEM). Data from Chapter 3 showed that PH3S10 nuclei in sham and contralateral tissue was virtually inexistent and showed such low expression that only ipsilateral PH3S10 expression was quantified for the experiments done in this chapter.

6.2.7 Immunohistochemistry- Image acquisition

Images of fluorescent immunohistochemistry in this chapter were taken on the Leica TCS SPE laser scanning confocal microscope, or a Hamamatsu CCD C4742 digital camera attached to a Leica DMR microscope using the software Volocity (PerkinElmer, Waltham MA, USA). All images for colocalisation were taken in a single focal plane.

6.2.8 Statistical analysis

Please also see 2.7 for full details of statistical analysis.

6.2.8.1 Formalin behaviour

Formalin-induced nociceptive behaviour (see 6.2.5) in *Experiment 2* was compared in animals receiving Vehicle and animals receiving Drug (SB747651A; 2 doses 10µM or 1µM in 10µl). Data was analysed using a repeated measures ANOVA, with *'treatment'* as the between-subjects factor, and *'time'* as the within-subjects factor. A significant main effect of treatment or significant treatment-time interaction led to post-hoc analysis by LSD or Bonferroni tests. To determine the effectiveness of drug treatment, percentage reductions in formalin-induced nocifensive behaviour were calculated using the area under the curve (AUC) for the two formalin phases (first phase 0-10 minutes, second phase 15-60 minutes).

6.2.8.2 Immunohistochemistry

For *Experiment 1,* group means were analysed by univariate ANOVA, with *treatment* (vehicle or SL327, see 6.2.1.1) as a between-subjects factor.

For *Experiment 2,* group means (vehicle and 10µM dose) were analysed by independent t-test.

For *Experiment 3*, group means for PERK counts were analysed by univariate ANOVA, with *treatment* (4 groups- see 6.2.1.3) as a between-subjects factor, and *side* (ipsilateral or contralateral) as a within-subjects factor. A significant main effect of the independent variable or interaction of factors led to post-hoc analysis of treatment.

6.3 Results

6.3.1 PMSK1, the activated kinase responsible for PH3S10, is expressed 30 minutes post-formalin and requires ERK activation

The role of MEK inhibitors, specifically SL327, in nociceptive formalin behaviour has already been investigated. Specifically, it has been shown that SL327 attenuates both first and second phase of the formalin response (Alter et al., 2010). In this chapter, SL327 was utilised to assess the functional role of novel molecular targets downstream of ERK. Results from Chapter 4 indicated that 58% of PH3S10 in the dorsal horn colocalised with PERK after formalin, and this information suggested that PH3S10 was at least partially dependent on ERK/MAPK signalling. PMSK1 is part of the ERK/MAPK pathway and an intermediate kinase linking PERK and PH3S10 activity, and was therefore also a target of interest.

Both PERK and PMSK1 populations were quantified from a double stain of PERK/PMSK1. Specifically, the number of both double-labelled and single-labelled cells was noted. Formalin stimulation induced expression of PERK and PMSK1 in laminae I-II of the ipsilateral dorsal horn at 30 minutes (Figure 6.7 A, DMSO-control, first row). To quantify the magnitude of MEK inhibition, the number of PERK immunopositive cells in laminae I-II was compared in DMSO and SL327 groups. There was a 68% reduction of downstream target PERK after systemic SL327, which was significant ($F_{(1, 7)} = 13.39$, $P<0.05$) (Figure 6.7 B).

Although no time course investigation into PMSK1 expression was completed, it is worth referring back to results from Chapter 4 which indicated that PH3S10 peaked 30 minutes post-formalin. PMSK1 lies downstream of PERK (5 minute peak post-formalin) and upstream of PH3S10, suggesting that it is likely to have a peak expression between 5 and 30 minutes; thus, an investigation into PERK/PMSK1 colocalisation at 30 minutes seemed appropriate. Even when considering that the 30 minute time point for PMSK1 expression might not be optimal, the great majority of PMSK1 colocalised with PERK in DMSO-control animals (87%) (Figure 6.7 B).

SL327 caused a significant reduction (77%) in total PMSK1 nuclei in laminae I-II of the ipsilateral dorsal horn ($F_{(1, 7)} = 13.19$, $P<0.05$) (Figure 6.7 B). Specifically, there were significant reductions post-SL327 in *PMSK1 Only* cells and double labelled cells (*PERK + PMSK1*) ($F_{(1, 7)} = 6.42$, $P<0.05$ and $F_{(1, 7)} = 12.24$, $P<0.05$) (Figure 6.7 C, D). Following SL327, the majority of PMSK1 nuclei (63%) still colocalised with PERK (Figure 6.7 B). It is of note that the formalin-induced upregulation of PERK in DMSO-controls was similar to the magnitude of PERK upregulation seen in Chapter 4 at the same 30

minute time point (23.1 ± 7.3 cells vs. 30.7 ± 3.7 cells, respectively), therefore indicating that there was no presumed effect of DMSO on nociceptive activation in the dorsal horn (Figure 6.7 B).

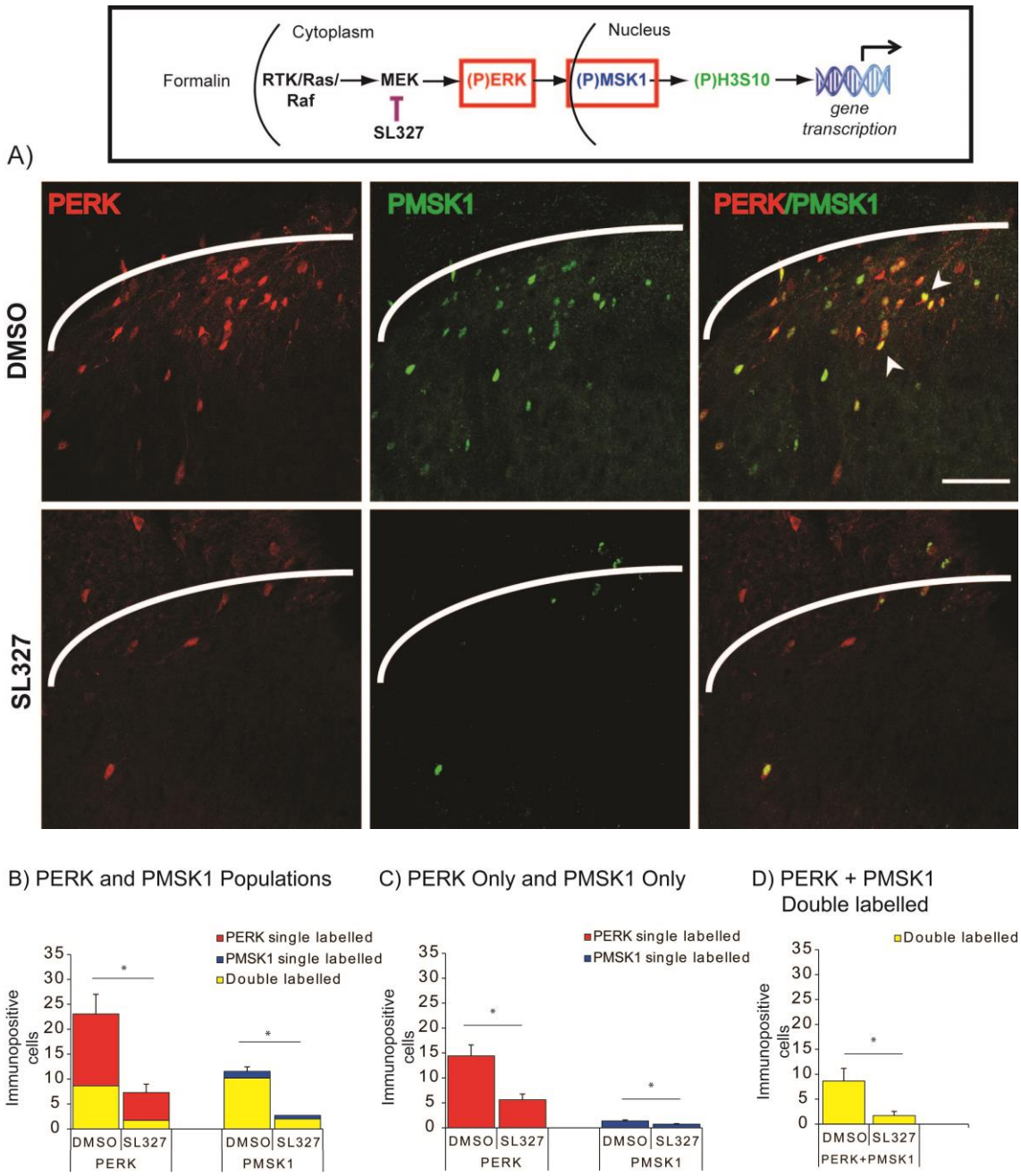


Figure 6.7 Formalin-induced PMSK1 expression in the dorsal horn is prevented by MEK inhibitor SL327. A) Images are examples of double labelling of PERK and PMSK1 taken from a single focal plane 30 min after formalin stimulation in animals receiving i.p. DMSO or SL327. Images in the final column show a merge of first two images; colocalisation appears yellow; arrowheads indicate examples of colocalisation. White line indicates medial border between lamina I and white matter. Scale bar, 50µm. B) Quantification of PERK and PMSK1 populations (yellow = double labelled; red = PERK only, blue = PMSK1 only, n = 4 each treatment group, 5 sections per animal). C) Single- labelled populations (PERK Only or PMSK1 Only) taken from graph B for ease of visualisation and further comparison. D) Population of double labelled (PERK + PMSK1) cells. All values presented as group mean ± SEM (per 40µm section). *P < 0.05, versus DMSO control.

6.3.2 The majority of formalin-induced PH3S10 nuclei are downstream of ERK signalling

The next step was to determine the functional link between ERK activation and PH3S10 expression investigating a double stain of PERK/ PH3S10. Again, inhibition of MEK activity with SL327 was confirmed with a 63% reduction of PERK immunopositive cells, which was significant ($F_{(1, 7)} = 22.46$, $P < 0.01$) (Figure 6.8 B). [NB: The reduction in PERK is different from that in 6.3.1 because the counts were obtained from a different set of tissue.]

Importantly, it was found that 73% of PH3S10 neurons colabelled with PERK in DMSO-control animals (Figure 6.8 B). SL327 caused a 69% reduction in total PH3S10 nuclei in laminae I-II of the ipsilateral dorsal horn, which was significant ($F_{(1,7)} = 36.39$, $P<0.001$) (Figure 6.8 B). Specifically, there was a significant reduction post-SL327 in double labelled cells (*PERK+ PH3S10*) ($F_{(1, 7)} = 138.22$, $P<0.0001$), but not *PH3S10 Only* cells (Figure 6.8 D, C). 46% of the PH3S10 population still colocalised with PERK after SL327 (Figure 6.8 B).

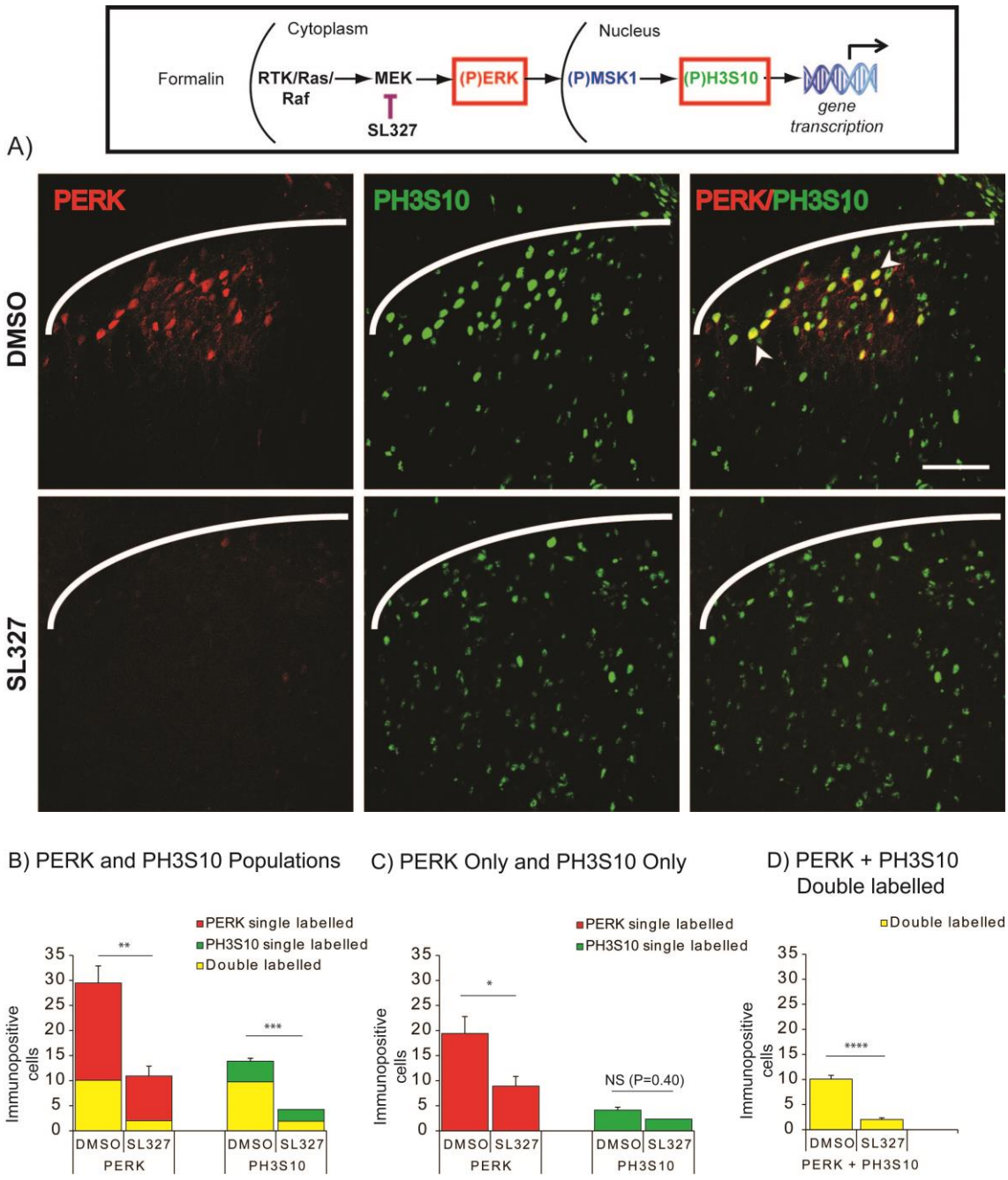


Figure 6.8 Formalin-induced PH3S10 expression in the dorsal horn is prevented by MEK inhibitor SL327. A) Images are examples of double labelling of PERK and PH3S10 taken from a single focal plane 30 min after formalin stimulation in animals receiving i.p. DMSO or SL327. Images in the final column show a merge of first two images; colocalisation appears yellow; arrowheads indicate examples of colocalisation. White line indicates medial border between lamina I and white matter. Scale bar, 50µm. B) Quantification of PERK and PH3S10 populations (yellow = double labelled; red = PERK only, green = PH3S10 only, n = 4 each treatment group, 5 sections per animal). C) Single- labelled populations (PERK Only or PH3S10 Only) taken from graph B for ease of visualisation and further comparison. D) Population of double labelled (PERK + PH3S10) cells. All values presented as group mean ± SEM (per 40µm section). *P < 0.05, **P < 0.01, ***P< 0.001, ****P< 0.0001 versus DMSO control.

6.3.3 The majority of PH3S10 nuclei colocalise with PMSK1

The overlap of PH3S10 and PMSK1 after SL327 was then investigated using a double stain directly comparing the two populations. The results indicated that the total number of PMSK1 cells was similar to, if not slightly larger than that of PH3S10 (10.8 ± 2.5 vs. 6.9 ± 0.8 nuclei/40µm section, DMSO-control treated tissue). The total number of PMSK1 and PH3S10 cells were more similar in number with each other than to that of PERK (which was consistently >20 cells in DMSO-controls) (Figure 6.9 B). 87% of PH3S10 nuclei coexpressed PMSK1 in contrast to 56% of the PMSK1 population coexpressing PH3S10 (Figure 6.9 B). SL327 produced a 69% reduction in PMSK1 expression and a 49% reduction in PH3S10 ($F_{(1, 7)}$ = 8.09, P<0.05 and $F_{(1,7)}$ = 9.16, P<0.05) (Figure 6.9 B). There was a significant reduction in double labelled cells (*PMSK1* + *PH3S10*) post-SL327 ($F_{(1,7)}$ = 11.28, P<0.05) (Figure 6.8 D). Interestingly, there were no reductions in both *PMSK1 Only* and *PH3S10 Only* cells, after SL327 (Figure 6.9 C) (see also PH3S10, Figure 6.8 C).

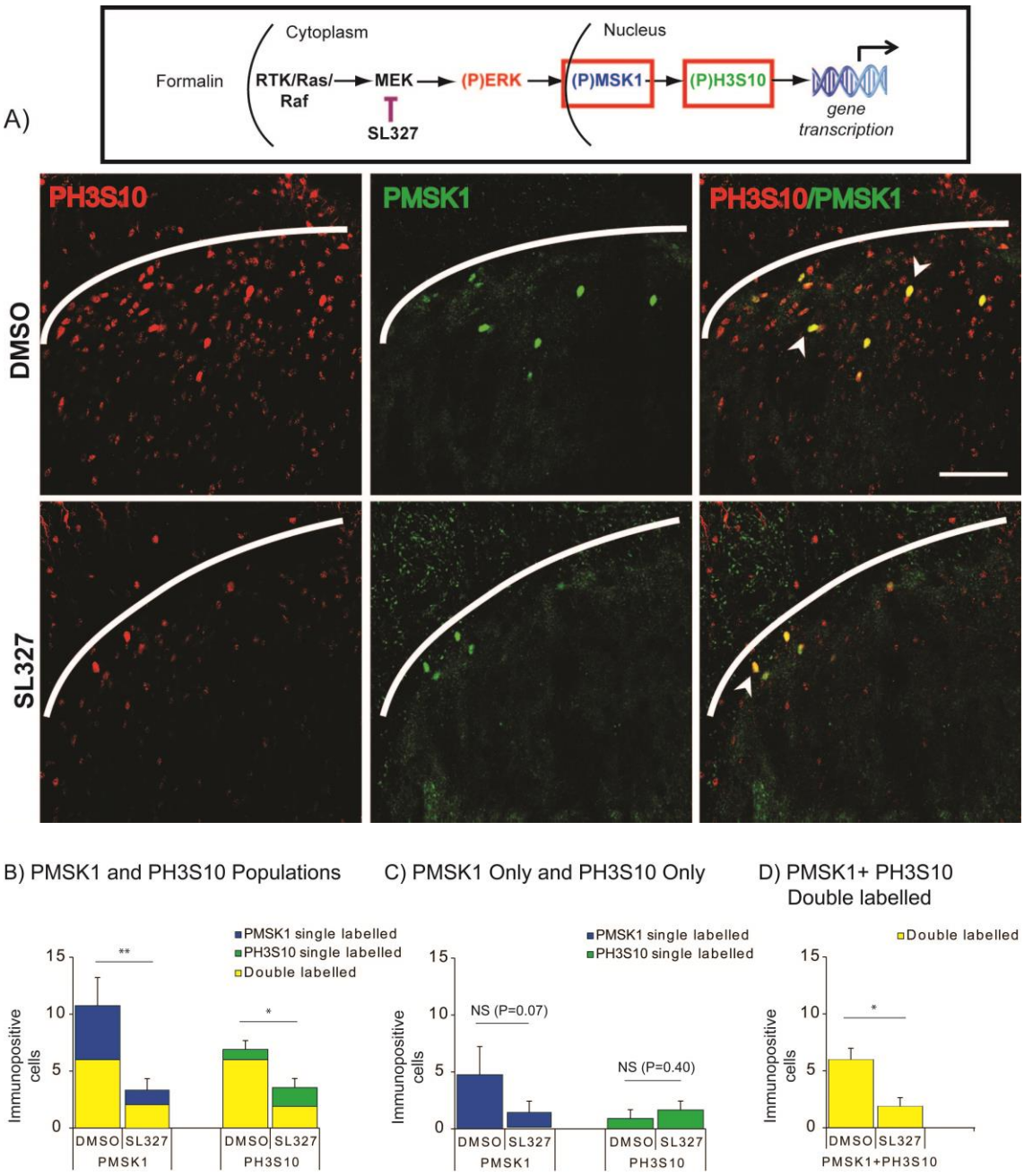


Figure 6.9 The majority of PH3S10 nuclei coexpress PMSK1 A) Typical images of PMSK1 and PH3S10 double labelling taken from a single focal plane 30 min after formalin stimulation in animals receiving i.p. DMSO or SL327. Images in the final column show a merge of first two images; colocalisation appears yellow; arrowheads indicate examples of colocalisation. White line indicates medial border between lamina I and white matter. Scale bar, 50µm. B) Quantification of PMSK1 and PH3S10 populations (yellow = double labelled; blue = PMSK1 only, green = PH3S10 only, n = 4 each treatment group, 5 sections per animal). C) Single-labelled populations (PMSK1 Only or PH3S10 Only) taken from graph B for ease of visualisation and further comparison. D) Population of double labelled (PMSK1 + PH3S10) cells. All values presented as group mean ± SEM (per 40µm section). *P< 0.05, **P<0.01, versus DMSO control.

6.3.4 Inhibition of MSK1 with SB727651A reduces formalin-induced PH3S10 expression but has no effect on c-Fos in the dorsal horn

The previous experiments indicated that the majority the PH3S10 population colabelled with the PMSK1 population (86%) (Figure 6.9 B), and that PMSK1 and PH3S10 populations were of similar size. Therefore, PMSK1 seemed an ideal pharmacological target to manipulate PH3S10 expression. The novel MSK1 inhibitor, SB747651A, was delivered i.t. at two different doses 30 minutes prior to formalin stimulation in order to investigate the role of PH3S10 in nociceptive behaviour.

Expectedly, treatment with i.t. SB747651A (10µM dose) yielded a significant reduction (42%) in superficial dorsal horn expression of PH3S10 1h post-formalin (independent t-test, $P<0.05$). There was no effect of i.t. SB747651A (1µM) on spinal PH3S10 expression.

Furthermore, no reductions were observed in spinal expression of IEG product c-Fos 1h post-formalin following i.t. SB747651A (10µM dose) (Figure 6.10).

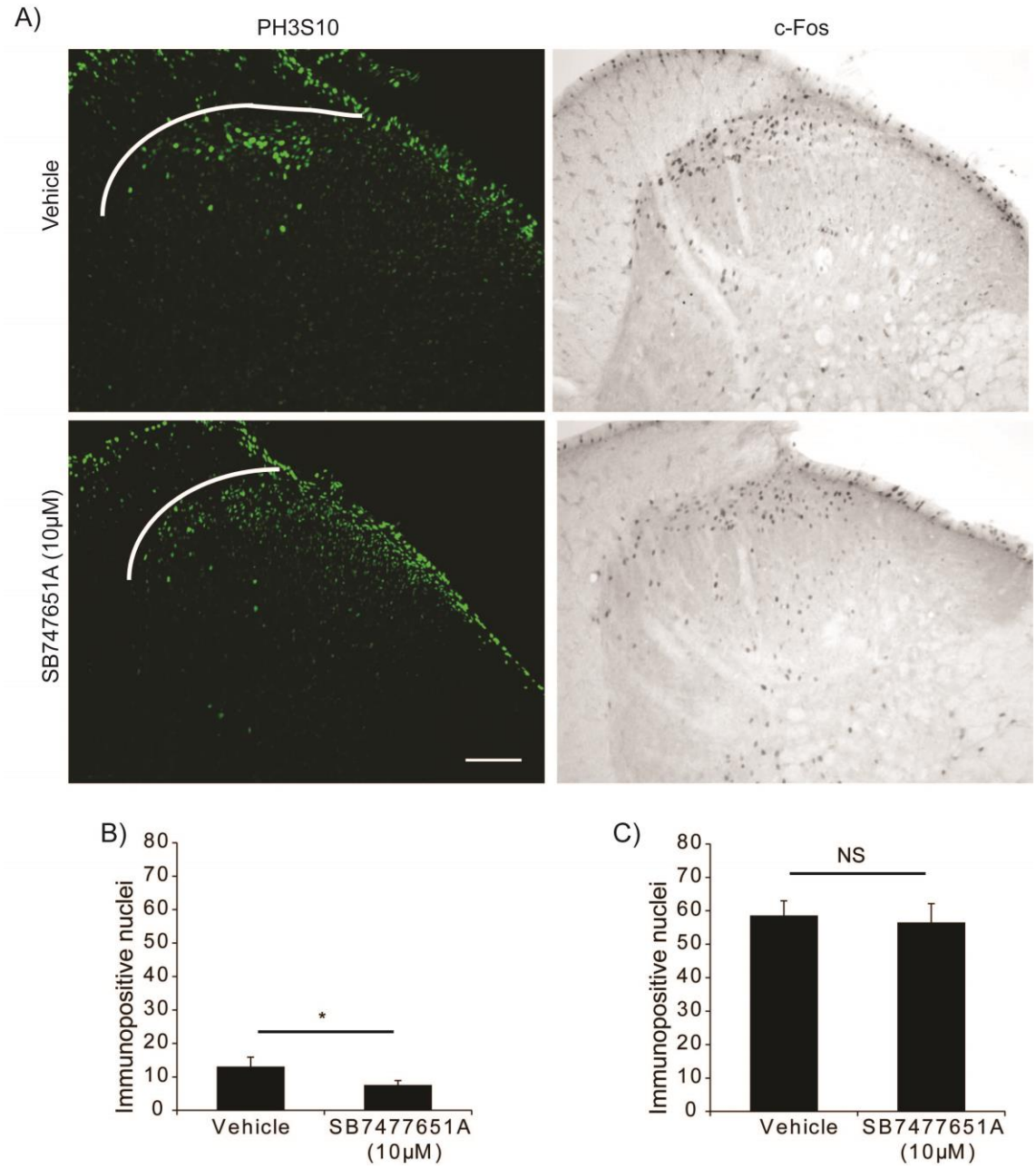


Figure 6.10 SB747651A causes a significant reduction in formalin-induced PH3S10 but not c-Fos. A) Typical images of PH3S10 and c-Fos in the dorsal horn in Vehicle and SB747651A (10µM) treated animals. Scale bar, 100µm; white line indicates medial border of lamina I and white matter. B) Quantification of PH3S10 (n = 11/13 each group, 5 sections per animal) and C) c-Fos (n = 13/15, 5 sections per animal). There was a significant reduction in formalin-induced PH3S10 but not c-Fos after i.t. SB747651A (10µM). Values presented as group mean ± SEM (per 40µm section). *P<0.05

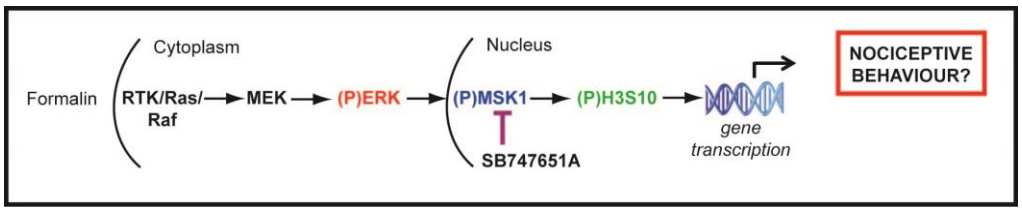
6.3.5 Inhibition of MSK1 activity using SB747651A prevents the full expression of formalin-induced nocifensive behaviour without affecting PERK or PMSK1 expression in the dorsal horn

The final aim was to determine whether inhibition of MSK1 could modify nociceptive behaviour. Two different formalin response behaviours were scored for 1h following stimulation in all animals. There was a significant main effect of treatment at 35-60 minutes on the time spent licking and flinching ($F_{(2,30)}$ = 5.13, P<0.01). SB747651A (10µM dose) attenuated the formalin response compared to the Vehicle-treated group (Bonferroni *post-hoc* test, P<0.01). Calculation of area under the curve (AUC) indicated a 30% reduction of time spent licking/flinching in SB747651A (10µM) treated animals during the second phase (15-60 minutes) of the formalin response. There was no difference between the Vehicle and 1µM dose groups (Figure 6.11 A).

Interestingly, SB747651A significantly attenuated both the first and second phase of the formalin response when quantified by total number of flinches. There was a main effect of treatment ($F_{(2,21)}$ = 4.939, P<0.05), specifically a difference between Vehicle and SB747651A (10µM dose) groups (Bonferroni *post-hoc* test, P<0.01). AUC calculation indicated a 43% reduction of flinch behaviour during the first phase (0-10 minutes) and 37% reduction during the second phase (15-60 minutes) in SB747651A (10µM) treated animals.

Analysis across the 15-30 minute time points also indicated a main effect of treatment ($F_{(2,21)}$ = 4.263, P<0.05) and differences between Vehicle and SB747651A (10µM dose) groups, as well as Vehicle and SB747651A (1µM dose) groups (LSD *post-hoc* test, P<0.05, each comparison) (Figure 6.11 B).

Importantly, specificity of SB747651A as an MSK1 inhibitor was demonstrated by no change in the activation of upstream PERK expression between Vehicle and SB747651A (10µM dose) groups 30 minutes post- formalin (Figure 6.12). There was also no difference in PMSK1 expression between the two groups.



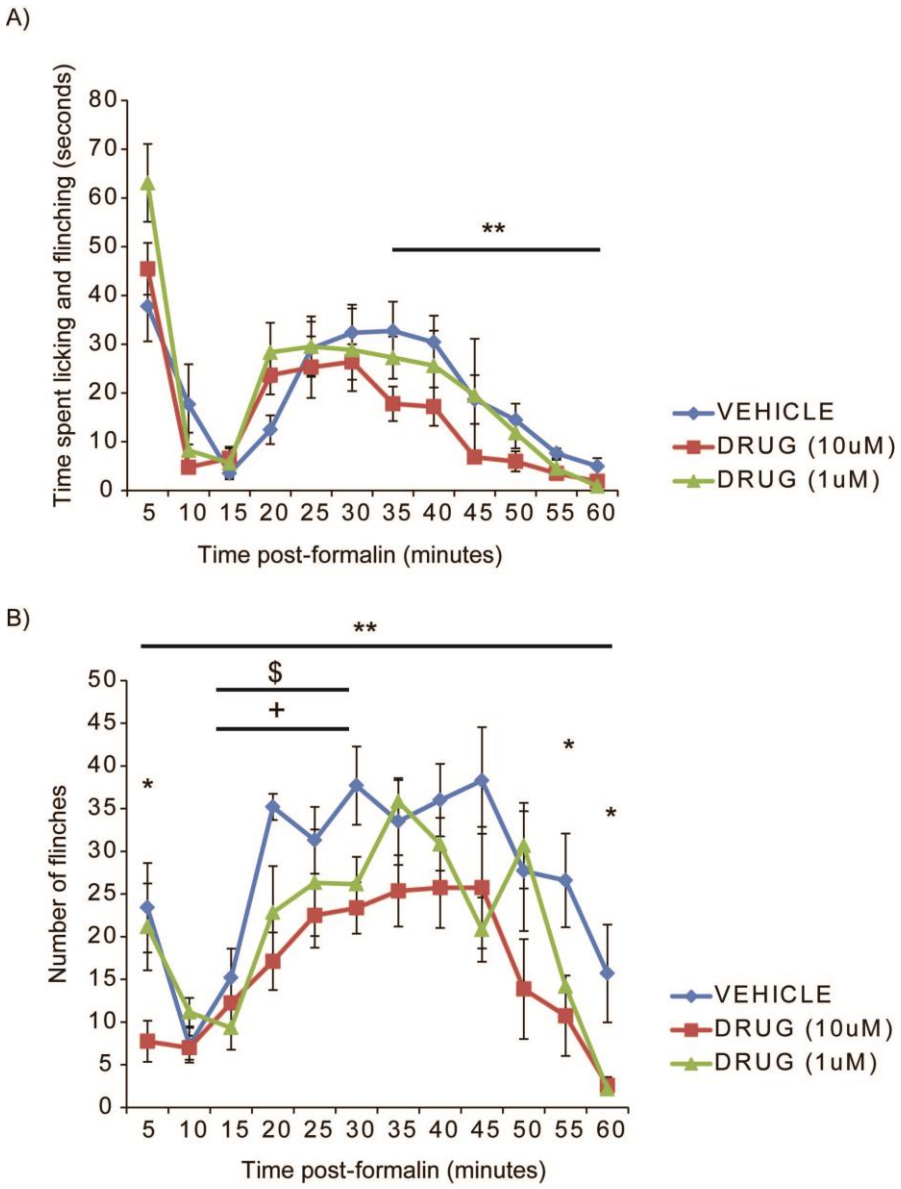


Figure 6.11 Inhibition of MSK1 reduces nociceptive behaviour. A) SB747651A attenuated the second phase of the formalin response 35-60 minutes post-formalin **P<0.01, between Vehicle and 10µM groups (n = 11/16/6; Vehicle/10µM/1µM) B) SB747651A reduces formalin-induced flinching during the first and second phase of the formalin response. **P<0.01, between Vehicle and 10 µM; +P<0.05, between Vehicle and 10 µM; $P<0.05 between Vehicle and 1µM; *P<0.05, between Vehicle and 10µM (n = 10/8/6; Vehicle/10µM/1µM).

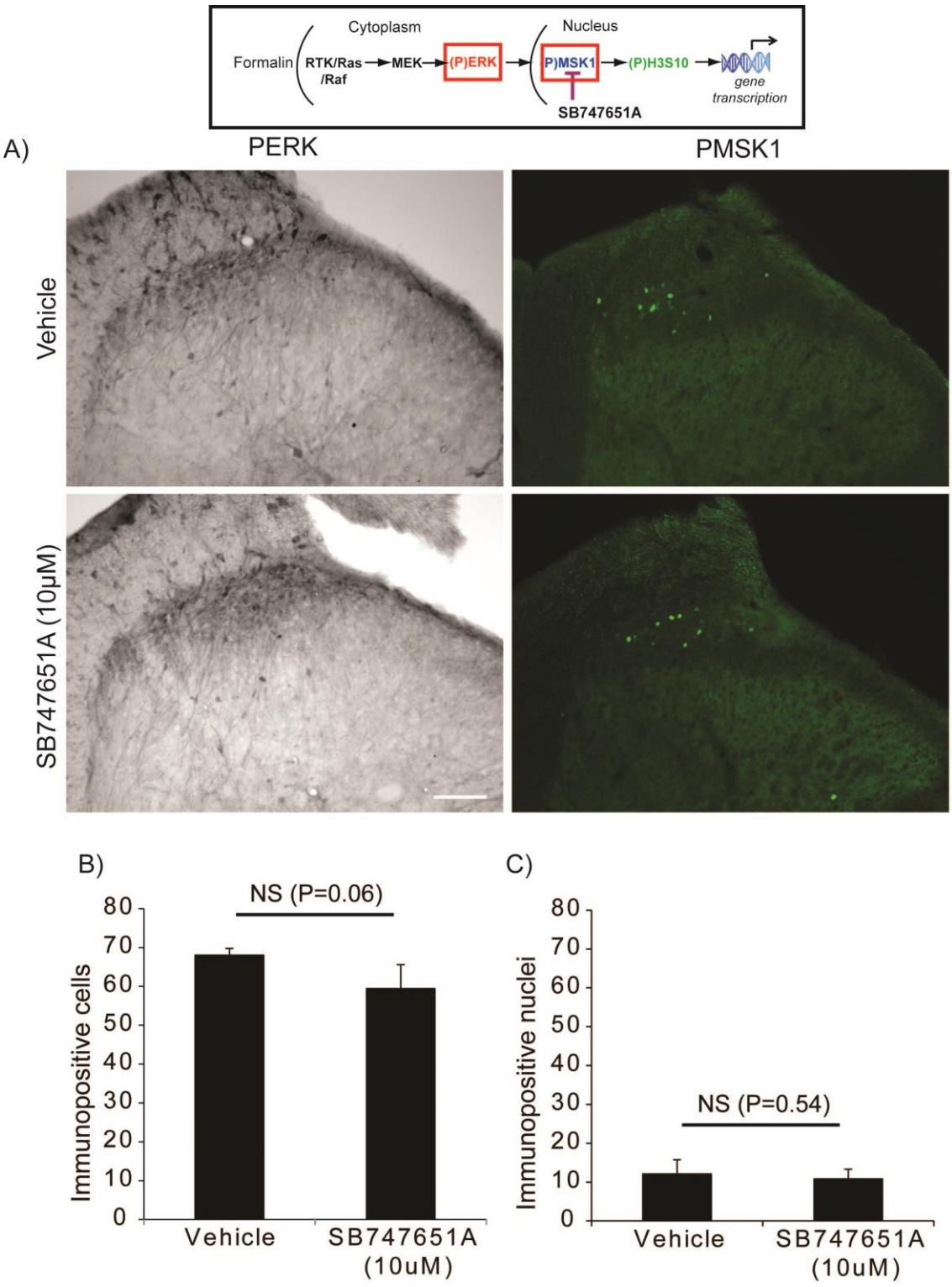


Figure 6.12 i.t. delivery of SB747651A does not alter nociceptive primary afferent input to the dorsal horn as indicated by PERK expression. A) Typical images of PERK and PMSK1 in the dorsal horn in Vehicle and SB747651A (10µM) treated animals. Scale bar, 100µm. B) Quantification of PERK and C) PMSK1 (n = 4 each group, 5 sections per animal). There was no change in PERK or PMSK1 expression 30 minutes after formalin stimulation in Vehicle or SB747651A (10µM) treated groups. Values presented as group mean ± SEM (per 40µm section).

6.3.6 50µl, 2% formalin injected under general isoflurane anaesthesia and 20µl, 5% formalin injected without anaesthesia caused the same degree of spinal PERK activation at 30 minutes

Finally, the investigation of MSK1 inhibition with SB747651A on formalin behaviour (see 6.3.5 and Figure 6.11) in this chapter required the injection of formalin without anaesthesia. Due to Home Office regulations, this meant that the formalin injection had to be given in a smaller volume at a higher concentration (20µl, 5% formalin). This dose importantly delivered the same total amount of formalin into the hindpaw but in a different volume. Therefore, as the rest of the experiments in this thesis delivered formalin under anaesthesia at 50µl, 2% as a stimulus, we decided to investigate the effect of the two formalin treatments on nociceptive primary afferent activity as indicated by PERK expression in the dorsal horn.

There was a main effect of treatment (ANOVA $F_{(3,8)}=9.92$, $p<0.01$); however, importantly there was no difference in PERK activation between the two different doses and conditions of formalin stimulation (Form-A and Form-NA groups, NS) (Figure 6.13 A, B). There was also no difference between sham groups (Sham-A and Sham-NA, NS).

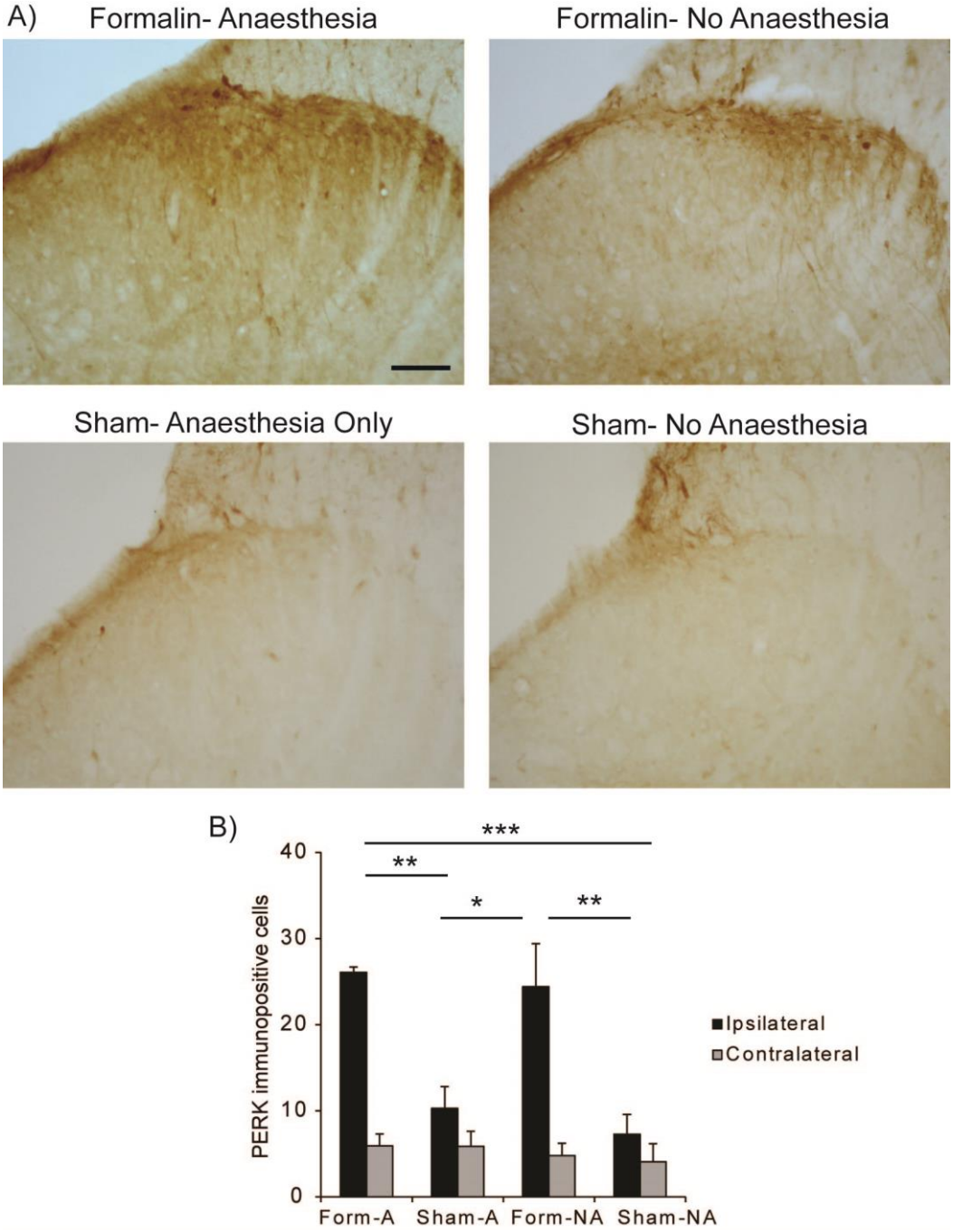


Figure 6.13 No difference in formalin-induced spinal PERK between two formalin treatments at 30min (50µl, 2% formalin injected under general isoflurane anaesthesia vs. 20µl, 5% formalin injected without anaesthesia) A) Typical PERK expression in the ipsilateral superficial dorsal horn for each treatment group. Scale bar 50µm. B) No difference between Form-A and Form-NA, LSD post-hoc p=0.71, NS. *P<0.05, **P<0.01, ***P<0.001, Formalin vs. Sham groups. Data presented as means ± SEM (n=3 each group, 5 sections per animal).

6.4 Discussion

PERK is required for the full expression of pain states and is used as a marker of neuronal activation by nociceptive stimulation in the spinal cord (Gao and Ji, 2009; Ji et al., 2009, 1999). It is widely known that PERK regulates nociceptive behaviour, but less is known about the specific molecular events downstream of PERK that lead to gene regulation. The results from this chapter are the first to indicate that PERK signalling engages downstream targets MSK1 and PH3S10 to regulate nociceptive behaviour.

6.4.1 ERK/MAPK regulation of PMSK1 and PH3S10 expression

Chapter 4 results indicated that the majority of PH3S10 nuclei colocalised with PERK 30 minutes post-formalin, and results from this chapter (*Experiment 1: Does MEK inhibition block formalin-induced PMSK1 and PH3S10 expression?*) found that 73% of the PH3S10 population and 87% of the PMSK1 population colabelled with PERK (DMSO-control treated animals) (Figure 6.8 B, Figure 6.9 B). It should be noted that no time course was completed for PMSK1 expression, although knowing the peak expression times of PERK and PH3S10 in the dorsal horn (5 and 30 minutes, respectively, post-formalin) would suggest that PMSK1 would be somewhere in between, considering its position in ERK/MAPK pathway. It is worth noting that the induction of PMSK1 observed at 30 minutes post-stimulation is in agreement with a previous study which showed neuronal induction of PMSK1 after 15 and 30 minutes of glutamate stimulation *in vitro* (Brami-Cherrier et al., 2007).

In animals given SL327, 46% of PH3S10 and 63% of PMSK1 colocalised with PERK. Within each population, the reduction in colocalisation percentage with PERK (73%/46% DMSO/SL327 for PH3S10; 87%/63% DMSO/SL327 for PMSK1) indicated a requirement of upstream ERK signalling on the activity of PMSK1 and PH3S10 (Figure 6.7 C, PMSK1; Figure 6.8 B, PH3S10). Interestingly, the *PH3S10 Only* population was the only subpopulation that never exhibited a reduction after SL327 treatment (Figure 6.8 C, PH3S10; Figure 6.9 C, PH3S10), which indicates that there could be a population of PH3S10 occurring independently from ERK/MAPK signalling.

If indeed there is an ERK/MAPK-independent kinase leading to PH3S10 expression, this population would be small in number (1-2 per 40µm section). The possibility of a separately occurring PH3S10 population seems highly likely, and therefore a key question would be to identify another kinase which might be responsible for PH3S10. One example could be a non-ERK/MAPK kinase or cascade that converges onto MSK1, such as p38 MAPK. The only other kinase which has been suggested to activate PH3S10 is p70 ribosomal s6 kinase (RSK2), which also lies downstream of

ERK (Ciccarelli and Giustetto, 2014). No other candidates responsible for PH3S10 have been suggested (Davie, 2003).

Importantly, the technical limitation of choosing one time point to investigate the maximum overlap of two populations in Chapter 4 was eliminated in *Experiment 1* of this chapter. Specifically, blocking PERK with SL327 allowed us to focus on the optimal time point to investigate the downstream PH3S10 reduction. However, to conclusively establish whether an independent, non-ERK/MAPK PH3S10 population existed would have required the complete elimination of PERK expression- a condition which was not met in *Experiment 1*. The dose of SL327 (50mg/kg) was chosen because it has been shown to significantly attenuate the second phase of the formalin response, although it does not prevent it completely (Alter et al., 2010). Furthermore, *Experiment 1* (Figure 6.7 – 6.9, B) and the study by Alter et al. (2010) indicated that PERK was significantly reduced following SL327 (50mg/kg), but not prevented entirely at this particular dose. It is likely that higher doses causing the entire elimination of PERK activity might have led to undesired behavioural side effects.

Despite similar issues regarding experimental limitations regarding the complete blockade of ERK activation, other models of neural plasticity have also been able to show that PH3S10 is partially dependent on PERK signalling. In particular, the MEK inhibitor U0126 blocked PKC and PKA pharmacological activation of hippocampal PH3S10 *in vitro* (Chwang et al., 2006). Furthermore, pre-emptive delivery of i.p. SL327 caused reduced expression of PH3S10 1h after memory acquisition (memory formation by contextual fear conditioning), and a reduction in freezing behaviour 24h after memory acquisition (Chwang et al., 2006). The use of i.p. SL327 also demonstrated that inhibition of MEK regulated the activity-induced joint histone mark PH3S10-AcH3K14 in the hippocampus 2h after the forced swim test (Chandramohan et al., 2008, 2007); it has also shown regulation of synaptic activity-induced PH3S10 by ERK in visual cortical plasticity, striatal plasticity induced by drug addiction, and naloxone-precipitated morphine withdrawal (Brami-Cherrier et al., 2009; Ciccarelli et al., 2013; Putignano et al., 2007).

6.4.2 Targeting PH3S10 via MSK1- the best option

The investigation into MSK1 was interesting in itself as a novel target never before explored within the context of nociception. Additionally, being upstream of PH3S10, MSK1 was an ideal target for interfering with PH3S10 expression in *Experiment 2: Does inhibition of MSK1 activity attenuate formalin behaviour and block formalin-induced PH3S10 and c-Fos expression?*

In general, studies investigating the role of histone modifications in neuronal function and behaviour have pharmacologically targeted the enzymes responsible for either imparting or removing the histone marks. In particular, these studies have used HAT or HDAC inhibitors to examine the effects of manipulating histone acetylation levels in a relatively non-specific manner (Barrett and Wood, 2008; Gräff and Tsai, 2013; Peixoto and Abel, 2013). In order to manipulate PH3S10, there were two options: MSK1, the kinase responsible for PH3S10, or protein serine/threonine phosphatase 1 (PP1) the kinase responsible for H3S10 dephosphorylation (for discussion of PP1 refer to 6.4.3.5).

In order to choose an appropriate target, we investigated the distribution of PMSK1 in the dorsal horn after noxious stimulation. The results in this chapter (*Experiment 1*) indicated that PMSK1 and PH3S10 populations had similar distributions within the superficial dorsal horn. The majority of PH3S10 colabelled with PMSK1 (86%), and conversely the majority of PMSK1 colabelled with PH3S10 (59%) (Figure 6.9 B). While the number of PH3S10 nuclei coexpressing PMSK1 was very high, the reverse figure for the PMSK1 population indicated a substantial but not complete amount of population overlap (59%). However, this again could have been an issue of timing as 30 minutes may not have been optimal for PMSK1 expression. Additionally, PH3S10 is not the only downstream target of MSK1, as MSK1 is also known to phosphorylate transcription factors CREB and activating transcription factor 1 (ATF1) *in vitro* (Arthur et al., 2004; Soloaga et al., 2003; Wiggin et al., 2002), so perhaps a complete colocalisation between PMSK1 and PH3S10 should not be expected. Nonetheless, the substantial overlap between PH3S10 and PMSK1 populations suggested that MSK1 would be a good target for interfering with PH3S10 expression.

Many studies have investigated the role of MSK1 using genetically modified mice (Brami-Cherrier et al., 2005; Chandramohan et al., 2008; Chwang et al., 2007; Correa et al., 2012; Karelina et al., 2012). Recently however, a new MSK1 inhibitor, SB747651A has become available (Tocris, UK). Prior to the release of SB747651A, studies attempting to pharmacologically target MSK either utilised upstream inhibition of MEK (using inhibitors PD98059, SL327, U0126), Ro 31-8220 or H89- the latter two being non-selective MSK1 inhibitors also known to inhibit PKC and PKA, respectively, as well as other kinases. In contrast, using SB747651A causes less interference with other cell signalling activity, and allows for a refinement of MSK1 inhibition. Currently, no published work has used SB747651A *in vivo*, but recent evidence has conclusively shown that it has improved selectivity of MSK1 compared to Ro 31-8220 and H89 *in vitro* (Naqvi et al., 2012).

The use of global MSK1-/- mice has shown that MSK1 itself regulates a variety of forms of neural plasticity. In particular, MSK1 regulates memory formation (as shown by contextual fear conditioning, the forced swim test, and spatial and object-recognition tasks), environmental enrichment-induced cognitive enhancement, visual cortical plasticity, cocaine addiction, and neurodegeneration (Brami-Cherrier et al., 2005; Chandramohan et al., 2008; Chwang et al., 2007; Karelina et al., 2012; Putignano et al., 2007; Roze et al., 2007). MSK1 has also been shown to modulate dendritic morphology and synaptic plasticity involved in hippocampal environmental enrichment and homeostatic synaptic scaling, and is thought to do so by regulation of AMPA subunit GluR1 expression via the IEG *arc/arg3.1*, which is also known to be upregulated in the spinal dorsal horn by noxious stimulation (Correa et al., 2012; Hossaini et al., 2010).

Control experiments in this chapter indicated there were no upstream changes in PERK activation after i.t. delivery of SB747651A (10µM), which would support the targeted specificity of MSK1 inhibition within L4/5 of the superficial dorsal horn (Figure 6.12). However, it should be carefully noted that there was a trend toward a PERK reduction in the SB747651A-treated group, with only an n=4 (Figure 6.12, P=0.06). The significant reduction in formalin-induced PH3S10 observed following i.t. SB747651A (10µM) required an n=11/13 (Figure 6.10), a value much higher than the n=4 used in the control PERK experiment. Furthermore, i.t. SB747651A (10µM) caused a significant attenuation of flinching behaviour in the first phase of the formalin response (Figure 6.11); thus, if spinal PERK was directly indicative of nociceptor activity it would not be surprising to observe a significant reduction in PERK in SB747651A-treated animals with a higher n number.

Quantification of IHC indicated that there were also no changes in PMSK1 expression following SB747651A. However, this was expected- SB747651A is a specific inhibitor of the MSK1 N-terminal kinase, the domain which is reportedly essential for the phosphorylation of downstream MSK1 substrates (McCoy et al., 2005). The antibody used to detect PMSK1 was specific to phosphorylation at Threonine 581 in the C-terminal kinase domain, activity which precedes N-terminal activity. It is worth noting that SB747651A can inhibit ribosomal s6-kinase (RSK) and PKA at low levels (Naqvi et al., 2012). Non-specific inhibition of PKA could have potentially had an effect on the expression of PH3S10, as MEK is known to integrate upstream PKA and PKC activity in dorsal horn signalling (Hu et al., 2003). However, the fact that no changes in PERK were observed eliminates this potential confounding factor. RSK2 occurs downstream of ERK and therefore may be a factor requiring consideration.

6.4.3 MSK1 contributes to PH3S10 and nociceptive behaviour

6.4.3.1 The formalin response

I.t. inhibition of MSK1 significantly attenuated formalin-induced nociceptive behaviour when analysed by two criteria: 1) Time spent licking and flinching and 2) Total number of flinches; attenuation of nociceptive behaviour was accompanied by a reduction in PH3S10 nuclei at 30 minutes. Although opinion remains controversial (Abbott et al., 1995), it has been reported that scoring formalin behaviour using spontaneous behaviours such as flinch is a more robust measure than measuring time spent paw-licking in rats, whereas in mice it is the opposite (Bannon and Malmberg, 2007; Mogil, 2009). Indeed, there seems to be a variety of behavioural measures used to report formalin behaviour which include the aforementioned criteria, as well as weighting of behaviours to produce a formalin response 'score' (Dubuisson and Dennis, 1977). Results from this chapter showed that SB747651A (10μM) produced a significant reduction in time spent licking/flinching and total number of flinches during the second phase (15-60 minutes), which is attributed to central sensitisation (Fischer et al., 2014; Puig and Sorkin, 1996; Tjølsen et al., 1992). There was also a significant reduction in the quantification of flinch behaviour during the first phase. A significant effect was also seen for the 1μM dose with the flinch response, perhaps due to the fact that flinch was a more robust measure of quantification.

It has been reported that the formalin test is an ideal behavioural paradigm to understand the mechanisms generating persistent nociception (Dubuisson and Dennis, 1977; Ren and Dubner, 1999). Hindpaw formalin stimulation is known to induce a biphasic response across nociceptive behaviour, cardiac activity, and underlying dorsal horn electrical discharge (Dickenson and Sullivan, 1987a, 1987b; Taylor et al., 1995; Tjølsen et al., 1992). It is of general agreement that the first phase (first 5-10min) of the response results from chemical activation of all peripheral nociceptors (Puig and Sorkin, 1996). This phase is followed by a short period (5-10min) of quiescence, followed by a second phase response (20-60min) mediated by central sensitisation in spinal cord circuits following on from peripheral nociceptor sensitisation maintained by sustained Aδ and C-fibre activity (Dubuisson and Dennis, 1977; Puig and Sorkin, 1996; Tjølsen et al., 1992). However, it has been suggested that Aδ activity is sufficient for the full expression of the formalin response, as ablation of the majority of C-fibres only changed latency to onset but not total nocifensive behaviour (Shields et al., 2010). Furthermore, studies have indicated the role of the NMDA and NK1 receptors in the second phase of the formalin response (Haley et al., 1990; Henry et al., 1999).

The majority of the formalin studies have utilised the delivery of pharmacological agents before or after the first phase response and investigated the outcome of the second phase responses. Crucially, i.t. delivery of local anaesthetics or opioid agonists given prior to formalin injection have been shown to reduce or prevent nociceptive behaviour and neuronal activity accompanying the second phase of the response, indicating that dorsal horn sensitisation during the first phase is required for the full expression of the second phase (Abram and Yaksh, 1993; Coderre et al., 1990; Dickenson and Sullivan, 1987a; O'Connor and Abram, 1994; Sullivan et al., 1989; Yashpal et al., 1998). Other evidence however, suggests that the second phase response necessitates ongoing primary afferent activity, as local anaesthetic (QX-314) given at the site of injection after the first phase was sufficient to eliminate the second phase response (Taylor et al., 1997, 1995). The behavioural results in *Experiment 2* indicated that when analysed by *Time spent licking and scratching*, SB747651A had no effect on the first phase formalin response, but caused a reduction in the second phase response, suggesting that MSK1 plays a role in the development of central sensitisation.

6.4.3.2 Linking underlying molecular targets to formalin behaviour

Referring to colocalisation studies performed in this chapter and Chapter 4, it was expected that inhibition of MSK1 would reduce expression of downstream targets PH3S10 and c-Fos, as well as attenuate formalin-induced behaviour. Indeed SB747651A was found to have a significant effect on PH3S10 reduction, however, no change was observed with c-Fos. The reduction of PH3S10 expression suggests that MSK1 may act via PH3S10 to promote the full expression of formalin-induced nociceptive behaviour.

No change in c-Fos

Despite any controversy linking c-Fos expression and nociceptive behaviour (Gao and Ji, 2009), it was still surprising to find no reduction in c-Fos expression after MSK1 inhibition. MSK1 is required for *c-Fos* induction by lysophosphatidic acid in embryonic stem cells *in vitro* (Schuck et al., 2003), via phosphorylation of CREB (PCREB) (Shimada et al., 2010; Wiggin et al., 2002). MSK1 -/- striatal neurons showed no PH3S10 in response to glutamate, and *c-Fos* expression was reduced (Brami-Cherrier et al., 2007). Furthermore, *in vivo* models of synaptic plasticity show that the upregulation of c-Fos in the dentate gyrus caused by the forced swim test (a learning paradigm measuring behavioural immobility) was entirely prevented in MSK-/- mice (Chandramohan et al., 2008). This effect was specific to the hippocampus as no changes in c-Fos expression were seen in other brain regions.

Results from Chapter 4 indicated that 17% (only 6.2 ± 1.2 nuclei) of the formalin-induced c-Fos population colocalised with PH3S10 at 1h. Considering the size of the c-Fos population quantified in this chapter (>50 nuclei/40µm section), the reduction of a maximum of 6.2 ± 1.2 nuclei would be very difficult to detect using IHC. Other studies have successfully used a more sensitive technique, chromatin immunoprecipitation (ChIP), to identify links between histone modifications and transcription of specific genes. In particular, ChIP has revealed enrichment of the joint histone mark PH3S10-AcH3K14 at the *c-Fos* promoter in striatal tissue following chronic cocaine administration in rats, indicating that PH3S10-AcH3K14 modulated activity-induced c-*Fos* (Kumar et al., 2005). ChIP would have been a more sensitive and thus better method for the purposes of quantifying small changes in spinal cord *c-Fos* expression, and would have allowed for the quantification of PH3S10-associated *c-Fos* expression that was undetectable with IHC. This method would also have expedited the probing of additional candidate genes associated with PH3S10 regulation.

Correlating molecular targets and behaviour: c-Fos

It is tempting to correlate molecular targets involved in pain processing with nociceptive behaviour; however, the picture is not always straightforward. For example, studies investigating c-Fos expression within the context of formalin behaviour have suggested that c-Fos is indicative of the second phase of the formalin response (Abbadie et al., 1997). Abbadie et al (1997) used remifentanil, a short acting opioid agonist administered during the first phase of the formalin response to show that reduction in c-Fos expression (laminae I-II) occurred to the same degree as when lidocaine was administered during the second phase alone (27% reduction). Interestingly, utilisation of both remifentanil and lidocaine during first and second phases, respectively, reduced c-Fos to a greater degree (51% reduction), indicating that the second phase of the formalin response was necessary for the full expression of c-Fos in the dorsal horn.

Contrastingly, other studies have implicated that c-Fos expression is not closely linked with nociceptive behaviour, in particular because of its delayed expression (Gao and Ji, 2009). While nocifensive behaviour is immediately observed following formalin injection and other noxious stimuli, the c-Fos protein product does not appear until at least one hour after noxious stimulation, with expression peaking 2h after formalin stimulation (Ji and Rupp, 1997). Delayed upregulation c-Fos expression makes it difficult to interpret its correlation within the 60 minute biphasic behavioural response period to formalin. Moreover, it has been reported that i.t. lidocaine given after formalin stimulation can cause a reduction in c-Fos nuclei, but have no effect on formalin-induced nociceptive behaviour, which suggests that c-Fos does not correlate with nociceptive behaviour

(Yashpal et al., 1998). Furthermore, c-Fos can still be present in the superficial dorsal horn despite a complete opioid-induced blockade of second phase behaviour (Gogas et al., 1991; Jasmin et al., 1994; Presley et al., 1990). It is difficult to investigate the role of c-Fos in nociceptive behaviour by pharmacological intervention studies, and antisense mRNA knockdown of c-Fos has proven controversial. A few studies have shown reductions in c-Fos expression and increased formalin behaviour and hypersensitivity (Hunter et al., 1995; Ibrahim et al., 2001), although attenuation of formalin behaviour has been documented (Hou et al., 1997).

Correlating molecular targets and behaviour: PERK

It has been suggested that PERK could be a better molecular correlate of nociceptive behaviour, and its role in nociception has been investigated with pharmacological agents (Gao and Ji, 2009). Following formalin injection, PERK appears in the superficial dorsal horn within 3 minutes of injection and peaks at 5 minutes, which appears to correlate well with the onset of the behavioural response (Ji et al., 1999).

Interestingly, Ji et al (1999) showed that pre-emptive i.t. inhibition of MEK did not alter the first phase of the formalin response, and it is therefore surprising to have seen a reduction in first phase flinching after inhibition of MSK1. It is possible that the diffusion drug (i.t. SB747651A) to peripheral DRG sites could explain the reduction in first phase flinch data, although previous experiments in this lab suggest this is unlikely within the time frame of the experiment (Géranton et al., 2008). As motor control experiments are yet to be carried out, a first phase reduction caused by motor deficit cannot be ruled out. It is worth stressing however, that no differences in licking and flinching duration were observed between Vehicle and Drug treated groups during the same period of observation (Figure 6.11 A, B).

6.4.3.3 Inhibiting a population of limited size: Behavioural effects

Perhaps one surprising feature of attenuating nociceptive behaviour following MSK1 inhibition is the effect of inhibiting such a small population of cells within the dorsal horn. Indeed, the PH3S10 population rarely exceeded 20 cells/ 40µm section at peak expression, which is limited in comparison to classical markers PERK (roughly 30 cells/40µm section) and c-Fos (roughly 50 cells/40µm section). However, previous work has shown that another epigenetic regulator implicated in nociceptive processing, phospho-MeCP2 (PMeCP2), is similar in quantity and distribution within the superficial dorsal horn, and plays a key role in nociceptive behaviour (Géranton et al., 2008). In particular, Géranton et al. (2008) found that RNAi knockdown of a downstream target of MeCP2 activity, the serum- and glucocorticoid-regulated kinase-1 (SGK1), delayed

the onset of hypersensitivity caused by CFA ankle joint injection. In this chapter, i.t. inhibition of MSK1 by SB747651A (10μM) led to a 42% reduction in formalin-induced PH3S10 at 60 minutes, which related to a 30% reduction of time spent licking/flinching during the second phase (15-60 minutes) of the formalin response, and a 43% and 37% reduction of total flinches during the first (0-10 minutes) and second phases (15-60 minutes) of the response, respectively. Importantly, it cannot be said whether the behavioural effects of the MSK1 inhibitor SB747651A are strictly due to the reduction in formalin-induced PH3S10. MSK1 has other downstream targets that could also be affected following MSK1 inhibition (see below 6.4.3.4).

The wider implications of the restricted pattern of PH3S10 will be addressed in the General Discussion.

6.4.3.4 Other targets of MSK1 and PH3S10 signalling in nociceptive behaviour

Due to its importance in nociception (Hunt et al., 1987), c-Fos was an obvious target to investigate as a gene under the regulation of PH3S10. However, there could be other gene targets under the regulation of PH3S10. Furthermore, there are known targets of MSK1 other than PH3S10.

Candidate genes under the regulation of PH3S10

Candidate genes under the regulation of PH3S10 activity are most likely plasticity-related genes, and potentially genes related to nociceptive processing. In particular, *Zif268*, is known to regulate nociceptive processing (Rygh et al., 2006). Chapter 4 results showed that 12% of the Zif268 population coexpressed PH3S10. Indeed, ChIP experiments have shown that *Zif268*, under the regulation of PH3S10, is upregulated in the hippocampus during recent memory consolidation (1d), as well as in the prefrontal cortex during remote memory formation (7d) in an object recognition task (Gräff et al., 2012). Notably, a number of known genes under the regulation of the transcription factor, CREB, could also be under the regulation of PH3S10 (see below). As the general function of PH3S10 is to aid in the relaxation of chromatin, it is possible that PH3S10 and CREB (both via phosphorylation by MSK1) could act together to permit transcription.

Downstream targets of MSK1

MSK1 additionally phosphorylates cAMP response element-binding protein (PCREB), a transcriptional regulator which binds to regulatory CRE-binding sites embedded within specific genes. It is therefore possible that changes may have been observed in the expression of PCREB had it been investigated.

CREB is known to be activated by PKA, PKC, ERK/MAPK, and p38 MAPK, and plays a major role in neuronal signalling in response to neurotrophic factors such as NGF and BDNF (Finkbeiner et al., 1997; Ying et al., 2002), and PCREB is known to mediate NGF-induced *c-Fos* expression (Ginty et al., 1994). Furthermore, CREB activity regulates gene expression accompanying hippocampal LTP (Impey et al., 1996). It has been reported that PCREB activity does not always lead to activation of IEG transcription, which may also provide an explanation as to why no changes were observed in c-Fos expression after MSK1 inhibition, if indeed this is a downstream mechanism of MSK1 in nociceptive processing (Bonni et al., 1995; Tao et al., 1998). Other relevant genes under the regulation of CREB include *Cox-2, Zif268, NK1, prodynorphin, TrkB* and *nNOS; genes which have been reported to play a role in pain processing* (Lonze and Ginty, 2002).

MSK1 is also known to regulate the IEG *arc/arg3.1* via CREB in models of plasticity, specifically homeostatic synaptic scaling in dissociated hippocampal cultures (Correa et al., 2012). Arc/arg3.1 itself is known to regulate LTP, LTD, and homeostatic synaptic scaling (Bramham et al. 2008) and does so by regulating trafficking and endocytosis of AMPAR subunits at the synapse (Correa et al., 2012; Shepherd et al., 2006). Furthermore, *arc/arg3.1* is essential for memory consolidation and while knockout mice have normal short-term memory, they fail to exhibit long-term memory formation (Plath et al., 2006). In nociception, intensity dependent Arc/Arg3.1 upregulation in the superficial dorsal horn occurs after peripheral stimulation by capsaicin, CFA, or mustard oil, or i.t. delivery of BDNF (Hossaini et al., 2010). The authors found that 90% of Arc/Arg3.1 colocalised with c-Fos, and 68% colocalised with preproenkephalin. Arc/Arg3.1 knockout mice showed no deficits in nociceptive behaviour as demonstrated by the formalin response or mechanical and thermal response after CFA hindpaw inflammation. The behavioural results combined with high expression of Arc/Arg3.1 in preproenkephalin-expressing neurons led the authors to conclude that Arc/Arg3.1 plays a role in the anti-nociceptive response to noxious stimuli, which is particularly interesting considering the results of Correa et al, who propose upstream regulator of Arc/Arg3.1, MSK1, as a key homeostat in activity dependent synaptic plasticity.

It seems likely that MSK1 is actively contributing to the regulation of gene expression via direct manipulation of PH3S10 following noxious stimulation. Dynamic geometric remodelling of the nucleus, an indicator of active gene transcription and chromatin remodelling, has been shown to increase with neuronal activity (Wittmann et al., 2009). This is specifically caused by NMDA-mediated action potential bursting, which leads to

a high degree of nuclear infolding and small compartment formations- a process which required ERK/MAPK signalling and correlated with the degree of PMSK1 and PH3S10 expression. These correlations suggest a functional link between nuclear geometry and transcriptional regulation, in part dynamically regulated by histone modifications (such as PH3S10) which serve to make signalling-induced transcription gene more efficient. This could also be the case for the events occurring in the spinal cord following noxious stimulation.

6.4.3.5 PP1

Finally, we cannot eliminate protein phosphatase 1 (PP1) as an alternative regulator of PH3S10. PP1 is well-known to regulate synaptic plasticity and molecular mechanisms of learning and memory (Genoux et al., 2002; Munton et al., 2004), and use of PP1 genetically modified mice has demonstrated that PP1 is a negative regulator of memory formation (Koshibu et al., 2009). Inhibition of PP1 has been found to enhance long-term memory formation as shown by fear conditioning, object recognition learning, and spatial learning using the Morris Water Maze (Koshibu et al., 2011, 2009). Somewhat surprisingly, PP1 has been shown to have specificity for PH3S10 in the hippocampus. In particular, the use of doxycycline-inducible PP1 null mice indicated that PP1 was specific to histone proteins as phosphorylation of other nuclear enzymes MSK1, CREB, HDAC1, and MeCP2 did not occur (Koshibu et al., 2009). Remarkably, PP1 null mice displayed increases in PH3S10 without affecting phosphorylation at residues serine 28, threonine 3 or 11 (Koshibu et al., 2009). Furthermore, phosphorylation of non-nuclear targets GluR1 and GluR2 subunits were also unaffected in PP1 null mice. However, the picture becomes more complicated as Koshibu and colleagues showed that PP1 additionally regulates hippocampal histone acetylation and methylation at specific genes, in particular NFκB and CREB. These events act via an enzyme complex with Jumonji domain 2 demethylase (JMJD2A) and HDAC1. These additional histone modifying effects make PP1 a slightly less desirable enzyme candidate for targeting PH3S10.

While it would have indeed been interesting to explore the effects of targeting PP1, the availability of enhanced specificity with SB747651A and the ability to specifically inhibit MSK1 in the superficial dorsal horn (versus a global knockout), targeting MSK1 with SB747651A was the most appropriate and economical option for investigating the role of PH3S10 in nociceptive processing and behaviour.

6.4.4 No difference in spinal cord activation of PERK between two conditions of i.pl. formalin injection

Experiment 3: Are there any differences in spinal PERK expression following two different conditions of i.pl. formalin injection?, was a control experiment to identify whether two formalin injections of a slightly different nature (50µl, 2% with general anaesthesia vs. 20µl, 5% no anaesthesia) used in this thesis induced the same amount of PERK activation in the dorsal horn.

Importantly, the two conditions of formalin stimulation led to the same degree of PERK induction in the dorsal horn. It is difficult to unequivocally make conclusions about the effect of anaesthesia on spinal ERK activation due to the variability of formalin dose (i.e. formalin concentration, volume of injection). It is important to note however, that the two doses of formalin delivered the same *total amount* of formalin.

Previous studies have shown the ability of general anaesthetics to alter spinal dorsal horn neural activity and output as a result of noxious stimuli (Antognini et al., 2000a, 2000b). Although the effect of anaesthesia cannot be directly deduced from this experiment, isoflurane is thought to have an inhibitory effect on neural activity (Grasshoff and Antkowiak, 2006; Patel et al., 1999; Winegar and MacIver, 2006; Zaugg et al., 2003). While no study has directly investigated the effect of isoflurane anaesthesia on nociceptive spinal PERK activity, numerous studies have investigated the effect of general anaesthetic on spinal nociceptive c-Fos expression. The studies indicate that injectable anaesthetics such as propofol, fentanyl, and pentobarbital (Gilron et al., 1999; Sommers et al., 2008; Takasusuki et al., 2013) as well as inhalational anaesthetics such as nitrous oxide and isoflurane (Jinks et al., 2002) reduce c-Fos expression in the spinal cord. Furthermore, brief halothane has been shown to reduce expression of spinal PCREB and c-Fos induced by formalin, but these levels are still significantly higher than non-anaesthetised controls (Ji and Rupp, 1997). The anaesthetic used in our experiments, isoflurane, is known to enhance activity of two-P-domain potassium channels, TASK and TREK-1, leading to membrane hyperpolarisation (Patel et al., 1999). While isoflurane reduces c-Fos expression, it does not alter primary afferent peptide release into the spinal cord as investigated by internalisation of the NK1 receptor for SP (Takasusuki et al., 2013). Takasusuki et al. suggested that while this result could implicate a role for non-peptidergic primary afferent activity accounting for the effects of general anaesthesia, it is more likely that general anaesthetics play a role in post-synaptic suppression of molecular nociceptive targets. Indeed, PERK is more rapidly activated than *c-Fos* mRNA and protein, peaking within 3 minutes following noxious stimulation in contrast to a 30min and 2h peak for *c-*

Fos mRNA and protein respectively (Ji et al., 1999), a consideration which may be a factor for susceptibility to anaesthetic modulation.

A study investigating the duration of isoflurane anaesthesia in the cerebral cortex showed significant differences in PERK activity in rats treated with short (2-5min) vs. long (>10min) anaesthesia (Takamura et al., 2008). Animals under anaesthetic for more than 10 minutes exhibited a reduced expression of PERK compared to those subject to the shorter duration. It is therefore of crucial note that in our experiments animals receiving formalin with anaesthesia were never subject to anaesthetic for longer than 10 minutes. The experiments by Takasusuki et al. (2013) indicate that rats received formalin stimulation after 10 minutes of inhalation anaesthetic which may explain the reduction of c-Fos nuclei that we did not see with PERK, implicating factors of depth and duration of anaesthesia on c-Fos expression which have been previously investigated (Sommers et al., 2008). However, if isoflurane anaesthesia did have an inhibitory effect on spinal PERK in *Experiment 3*, it would suggest that the 50µl, 2% dose of formalin caused a greater induction of PERK than the 20µl, 5% dose injected without anaesthesia, which would be a logical explanation for the equal activation of spinal PERK caused by the two conditions.

Additional evidence from work in this lab has indicated that the two conditions of formalin stimulation presented in this chapter, while causing equal activation of spinal PERK, cause differential activation of PERK in the rostral anterior cingulate cortex (rACC) (Tochiki KK, unpublished observations).

6.4.5 Future experiments required

In *Experiment* 2, the inhibition of MSK1 on formalin behaviour requires a control study confirm that SB747651A does not impair motor function which could have interfered with the nocifensive behaviours measured (1. *Time spent licking and flinching*, and 2. *Number of flinches*). A few possible protocols are being considered, including the measurement of locomotor activity (i.e. open field experiment or home cage activity monitoring), motor coordination (i.e. rotarod), or motor function (i.e. suspension/gripping, stepping, or pulling tests).

6.4.6 Conclusion

In summary, the results in this chapter are the first to elucidate, at least in part, the key downstream players contributing to ERK/MAPK regulation of the nociceptive behavioural response to injury, PMSK1 and PH3S10.

Specifically, results from this chapter indicate that:

- Formalin-induced PMSK1 and PH3S10 are downstream of PERK, and can be reduced by the MEK inhibitor, SL327

- MSK1 activity blocked by SB747651A contributes to the development of central sensitisation and full expression of formalin-induced nocifensive behaviour, possibly via direct manipulation of PH3S10 and other downstream targets

- The administration of formalin in two varying conditions (50µl, 2% with general anaesthesia vs. 20µl, 5% no anaesthesia) shows no difference in spinal cord activation of molecular targets, specifically PERK expression

Chapter 7 General Discussion

7.1 Introduction

Chronic pain remains a major clinical problem, with nearly 1 in 5 adults in Europe having experienced moderate to severe chronic pain in their lifetime, and a further 40% reporting inadequate pain management (Breivik et al., 2006). The statistics undoubtedly illustrate that identification of novel pharmacological targets for the treatment of chronic pain remains essential, and raises the possibility of targeting epigenetic mechanisms as a valid option.

The aim of this thesis was to investigate the role of histone modifications, specifically histone phosphorylation, in nociceptive processing. Phosphorylation of histone H3 at serine 10 (PH3S10), is known to be specifically engaged by ERK/MAPK signalling during memory formation, a process which requires similar cellular activity to that seen in sensitised states following injury (Ji et al., 2003). PH3S10 was therefore a particular modification of interest given the importance of ERK/MAPK signalling in nociceptive processing (Ji et al., 1999). Using a combination of molecular and behavioural techniques, in addition to pharmacological blocking of signalling pathway components, I have demonstrated that MSK1 regulates the nociceptive response, and may do so through chromatin remodelling induced by PH3S10.

Deregulation of histone acetylation in human disease states such as cancer cells has long been known, and therapeutic treatment with HDAC inhibitors has attracted much attention in clinical practice (Wee et al., 2014). Deregulation of epigenetic processes are now associated with a broad spectrum of neurological disorders and activity induced-changes in neural plasticity, including schizophrenia, depression, addiction, neurodevelopmental disorders, and neurodegenerative disease (Deutsch et al., 2008; Qureshi and Mehler, 2010). Moreover, epigenetic processes are engaged in and essential to normal cognitive functions including the formation of short and long-term memories, and can even be targeted to enhance such processes (Barrett and Wood, 2008; Day and Sweatt, 2011, 2010; Gräff and Tsai, 2013; Peixoto and Abel, 2013). Furthermore, evidence from identical twins studies has clearly demonstrated that epigenetic mechanisms are responsible for activity or experience-dependent responses of the nervous system, as individuals with identical sets of DNA can have vastly different phenotypical outcomes with regard to disease (Nielsen et al., 2012). Consequently, it seems highly likely that environmental experience (such as prior injury, stress) in addition to genetic vulnerability, would contribute to lasting alterations in sensory processing and more specifically susceptibility to the development of chronic pain (Géranton and Tochiki, In press).

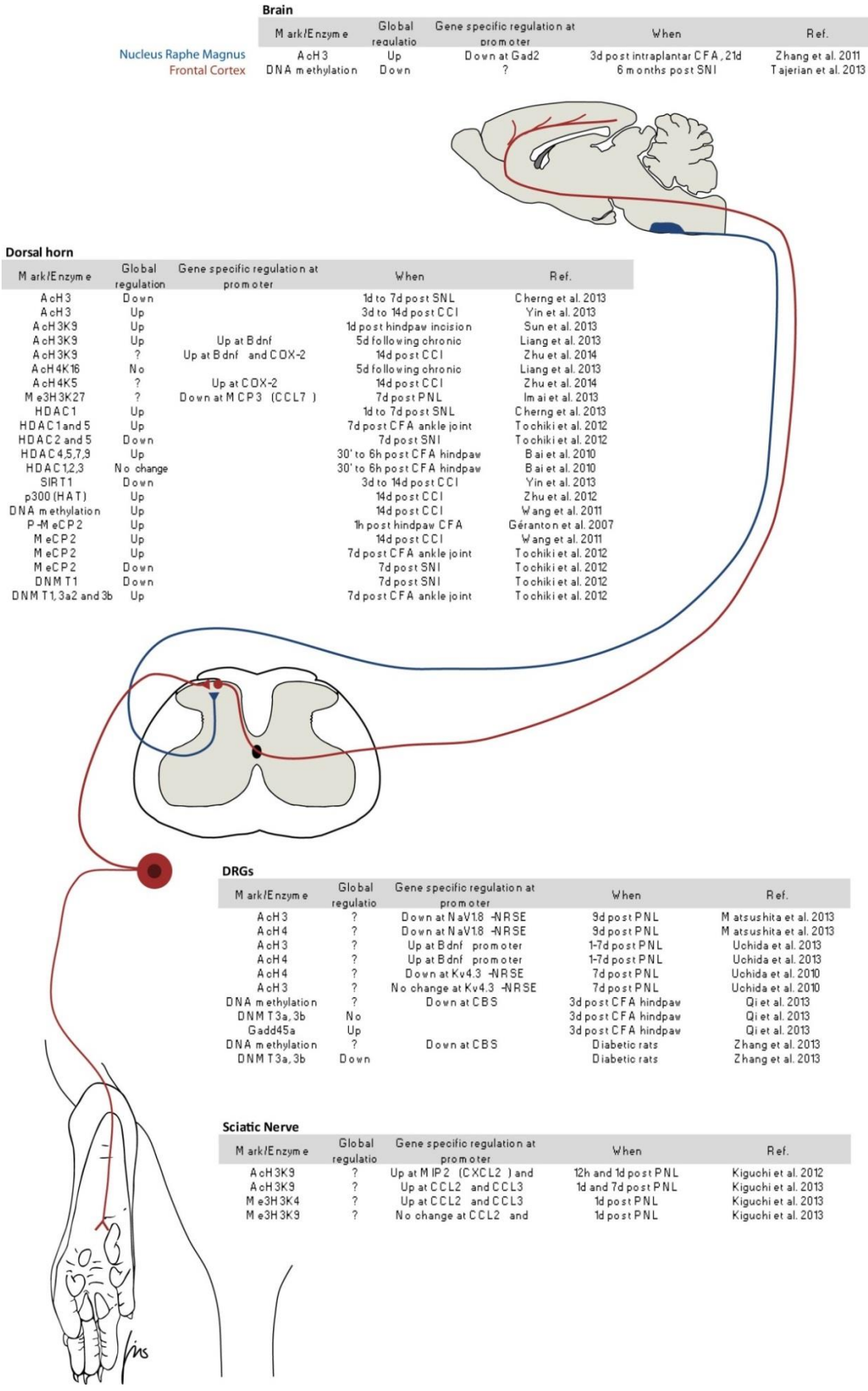
To date, only a handful of pain studies have shown that nociceptive processing engages epigenetic processes such as histone modification and DNA methylation in various structures along the pain pathways including the DRG, dorsal horn, and RVM, primarily using animal models (Figure 7.1, Figure 7.2). However, recent work in human subjects has demonstrated that altered responses to thermal stimuli correlated with gene-specific DNA methylation levels of *TRPA1* in the blood (Bell et al., 2014). The growing body of evidence for a key role of epigenetic mechanisms in nociceptive processing has pointed to the possibility of therapeutically targeting these mechanisms for clinical pain management (Doehring et al., 2011). Furthermore, the necessity of fully functioning epigenetic mechanisms for long-term synaptic plasticity and modulation of gene expression indicates a potential key role for epigenetic mechanisms underlying long-term pain states.

The few studies investigating histone modifications in nociceptive processing are similar to studies of learning and memory in that they have largely focused on histone acetylation due to the wide availability of HDAC inhibitors. The literature indicates strongly through the use of HDAC inhibitors that histone acetylation plays a functional role in nociception, but outcomes are not entirely straightforward and vary according to pain model and method of drug administration (Figure 7.1). Generally, when given pre-emptively, HDAC inhibitors have successfully attenuated hypersensitivity associated with both inflammatory and neuropathic pain; however, reversibility of hypersensitivity remains limited (Bai et al., 2010; Denk et al., 2013). The hindpaw plantar incision model is one exception not under the same influence of HDAC inhibitors, as administration has no effect on thermal hypersensitivity, and has actually been found to exacerbate mechanical hypersensitivity (Sun et al., 2013). It is important to note that the aforementioned studies tested the behavioural effects of HDAC inhibition in inflammatory and neuropathic pain models without first identifying injury-induced changes to the epigenome. While these studies showed that global or locus-specific histone acetylation levels expectedly increased *after* delivery of HDACi (Bai et al., 2010; Chiechio et al., 2009; Denk et al., 2013; Matsushita et al., 2013; Sun et al., 2013; Zhang et al., 2011), other studies have initially focused on injury-induced deregulation of the epigenome itself by looking at histone modification and DNA methylation pattern changes post-injury, or altered levels of enzymatic machinery (Figure 7.2). Indeed, histone acetylation levels have been found to be deregulated after plantar incision, CCI, and chronic morphine-induced sensitivity (Liang et al., 2013; Sun et al., 2013; Zhu et al., 2014), and studies have also found that HDAC and HAT expression levels are altered following SNI, CFA-ankle joint inflammation, and CCI (Tochiki et al., 2012; Zhu et al., 2012). In some studies, pharmacological targeting of these marks was only attempted

after alterations in the epigenome were identified, and this was the particular approach taken in this thesis.

	Pain Model(s)	Drug Treatment	Behaviour	Improved?	Proposed molecular mechanisms	Reference
Targeting histone modifications						
	HDAC inhibitors					
INFLAMMATORY	Formalin intraplantar	5x preemptive subcutaneous injection; MS-275 3mg/kg, SAHA 50mg/kg	Formalin behaviour attenuated with both drugs	Yes	Upregulation of p65/RelA acetylation in DRGs and spinal cord following injection of HDACis which correlated with mGlu2 upregulation. N.B. changes after injury not investigated.	Chiechio et al. 2009
	CFA intraplantar	1preemptive intrathecal injection; SAHA, TSA, LAQ, Valproate, 4PB, MS-275; SAHA 25ug	CFA induced thermal hypersensitivity attenuated with all except MS-275 (Class I)	Yes	Upregulation of AcH3K9/K18 in the spinal cord 30' after IT SAHA, MS-275. N.B. changes after injury not investigated.	Bai et al. 2010
	CFA intraplantar	4x daily NRM infusion or 4x daily intraperitoneal injection TSA 4mg/kg, SAHA 40mg/kg	NRM Infusion attenuated thermal tail flick latency following CFA	Yes	Injury induced a global upregulation of AcH3 in the NRM and downregulation of AcH3 at Gad2 promoter. Drug treatment upregulated AcH3 at Gad2 promoter in the NRM which correlated with GAD65 upregulation.	Zhang et al. 2011
NEUROPATHIC	d4T, L5 SNT, SNL	Intrathecal administration via osmotic minipump; MS-275, MGCD; 30 or 60nmol/d	40-50% mechanical and thermal attenuation with MS-275	Yes	Upregulation of AcH3K9 (global and at specific gene promoters: Cacna2d1, Hdac1, MeCP2) in the spinal cord after HDACis administration. There were no changes in the DRGs. N.B. changes after injury not investigated.	Denk et al. 2013
	PNL	3x to 9x daily intraperitoneal injection; TSA 1mg/kg, SAHA 5mg/kg, VPA 200mg/kg.	TSA/VPA blocked C-fibre hypoesthesia (mech/therm all/squeeze), SAHA reversed hypoesthesia	Yes	Downregulation of AcH3/H4 at Nav1.8 NRSE promoter in DRGs following PNL was reversed by HDACis, which reversed Nav1.8 mRNA downregulation.	Matsushita et al. 2013
	L5/6 SNL	VPA, oral, daily for 21 days, 300 mg/5 mL/kg	Valproate attenuated SNL induced mechanical hypersensitivity	Yes	Valproate prevented the SNL induced downregulation of the glutamate transporters GLT-1in the spinal cord. N.B. epigenetic changes were not investigated.	Yoshizumi et al. 2013
OTHERS	Incision and incisional priming	1x preemptive or 4x daily intraperitoneal injection; SAHA; 50mg/kg	SAHA exacerbated the mechanical hypersensitivity seen post incision but no effect on thermal hypersensitivity.	No	Global upregulation of AcH3K9 in the spinal cord post incision was exacerbated by SAHA and specific AcH3K9 at CXCR2/CXCL1 promoter was upregulated by SAHA. N.B.: Effect of incision on gene specific acetylation changes not evaluated.	Sun et al. 2013
	Opioid induced hyperalgesia	chronic administration; intraperitoneal injection; SAHA; 50mg/kg	SAHA exacerbated morphine induced mechanical, thermal hypersensitivity and physical dependence.	No	Increase in global and at BDNF promoter AcH3K9 in the spinal cord (no change in AcH3K16), reduced HDAC activity and HAT activity unchanged after chronic morphine. N.B: molecular changes after drug treatment not investigated.	Liang et al. 2013
	HAT inhibitors					
NEUROPATHIC	PNL	30-100umol/kg intraperitoneal injection AA30 min prior to surgery of AA	AA administered 30 min prior to PNL reduced tactile allodynia and thermal hyperalgesia at day 7 post PNL	Yes	AA reduced the PNL induced upregulation of AcH3K9 at MIP2 and CXCR2 promoter in the injured sciatic nerve, in neutrophils and macrophages.	Kiguchi et al. 2012
	PNL	30-100umol/kg intraperitoneal injection 30 min prior to surgery of AA	N.B. Behaviour was not investigated.	Yes	AA reduced the PNL induced upregulation of AcH3K9 and Me3H3K4 at CCL2 and CCL3 promoter in the injured sciatic nerve, in macrophages.	Kiguchi et al. 2013
	CCI	Curcumin; 7x daily intraperitoneal injection post CCI; 40-60mg/kg.	curcumin delivered 7days post CCI for 7 days reduced CCI-induced thermal hyperalgesia and mechanical allodynia.	Yes	Curcumin reduced CCI-induced AcH3K9 at BDNF promoter and both AcH3K9 and AcH4K5 at Cox-2 promoter in the spinal cord.	Zhu et al. 2014
OTHERS	Incision and incisional priming	1x preemptive or 4x daily intraperitoneal injection; AA; 5mg/kg intraperitoneally	AA 2 days before and 4 days after attenuated mechanical sensitivity post incision and incisional priming. No effect on thermal.	Yes	Global upregulation of AcH3K9 in the spinal cord post incision. N.B. Effect of AA not investigated at molecular level.	Sun et al. 2013
	Opioid induced hyperalgesia	chronic administration; intraperitoneal injection curcumin; 50mg/kg	Curcumin reduced morphine induced mechanical, thermal hypersensitivity and physical dependence.	Yes	Increase in global and at BDNF promoter AcH3K9 in the spinal cord (no change in AcH3K16), reduced HDAC activity and HAT activity unchanged after chronic morphine. N.B: molecular changes after drug treatment not investigated.	Liang et al. 2013
Targeting DNA methylation						
	CCI	5-azacytadine; IT catheter 10umol/d	5-aza attenuated CCI induced mechanical and thermal hypersensitivity	Yes	CCI induced spinal increase in global DNA Me was reduced by 5-aza.	Wang et al. 2011

Figure 7.1 Pharmacological targeting of histone modifications to modify pain states. Various studies have investigated the effects of drugs targeting HDACs, HATs and DNMTs in the treatment of pain states. Source: Géranton and Tochiki (In press)**.**



Brain

	Mark/Enzyme	Global regulatio	Gene specific regulation at promoter	When	Ref.
Nucleus Raphe Magnus	AcH3	Up	Down at Gad2	3d post intraplantar CFA, 21d	Zhang et al. 2011
Frontal Cortex	DNA methylation	Down	?	6 months post SNI	Tajerian et al. 2013

Dorsal horn

Mark/Enzyme	Global regulation	Gene specific regulation at promoter	When	Ref.
AcH3	Down		1d to 7d post SNL	Cherng et al. 2013
AcH3	Up		3d to 14d post CCI	Yin et al. 2013
AcH3K9	Up		1d post hindpaw incision	Sun et al. 2013
AcH3K9	Up	Up at Bdnf	5d following chronic	Liang et al. 2013
AcH3K9	?	Up at Bdnf and COX-2	14d post CCI	Zhu et al. 2014
AcH4K16	No		5d following chronic	Liang et al. 2013
AcH4K5	?	Up at COX-2	14d post CCI	Zhu et al. 2014
Me3H3K27	?	Down at MCP3 (CCL7)	7d post PNL	Imai et al. 2013
HDAC1	Up		1d to 7d post SNL	Cherng et al. 2013
HDAC1 and 5	Up		7d post CFA ankle joint	Tochiki et al. 2012
HDAC2 and 5	Down		7d post SNI	Tochiki et al. 2012
HDAC4,5,7,9	Up		30' to 6h post CFA hindpaw	Bai et al. 2010
HDAC1,2,3	No change		30' to 6h post CFA hindpaw	Bai et al. 2010
SIRT1	Down		3d to 14d post CCI	Yin et al. 2013
p300 (HAT)	Up		14d post CCI	Zhu et al. 2012
DNA methylation	Up		14d post CCI	Wang et al. 2011
P-MeCP2	Up		1h post hindpaw CFA	Géranton et al. 2007
MeCP2	Up		14d post CCI	Wang et al. 2011
MeCP2	Up		7d post CFA ankle joint	Tochiki et al. 2012
MeCP2	Down		7d post SNI	Tochiki et al. 2012
DNMT1	Down		7d post SNI	Tochiki et al. 2012
DNMT1,3a2 and 3b	Up		7d post CFA ankle joint	Tochiki et al. 2012

DRGs

Mark/Enzyme	Global regulatio	Gene specific regulation at promoter	When	Ref.
AcH3	?	Down at NaV1.8 -NRSE	9d post PNL	Matsushita et al. 2013
AcH4	?	Down at NaV1.8 -NRSE	9d post PNL	Matsushita et al. 2013
AcH3	?	Up at Bdnf promoter	1-7d post PNL	Uchida et al. 2013
AcH4	?	Up at Bdnf promoter	1-7d post PNL	Uchida et al. 2013
AcH4	?	Down at Kv4.3 -NRSE	7d post PNL	Uchida et al. 2010
AcH3	?	No change at Kv4.3 -NRSE	7d post PNL	Uchida et al. 2010
DNA methylation	?	Down at CBS	3d post CFA hindpaw	Qi et al. 2013
DNMT3a, 3b	No		3d post CFA hindpaw	Qi et al. 2013
Gadd45a	Up		3d post CFA hindpaw	Qi et al. 2013
DNA methylation	?	Down at CBS	Diabetic rats	Zhang et al. 2013
DNMT3a, 3b	Down		Diabetic rats	Zhang et al. 2013

Sciatic Nerve

Mark/Enzyme	Global regulatio	Gene specific regulation at promoter	When	Ref.
AcH3K9	?	Up at MIP2 (CXCL2) and	12h and 1d post PNL	Kiguchi et al. 2012
AcH3K9	?	Up at CCL2 and CCL3	1d and 7d post PNL	Kiguchi et al. 2013
Me3H3K4	?	Up at CCL2 and CCL3	1d post PNL	Kiguchi et al. 2013
Me3H3K9	?	No change at CCL2 and	1d post PNL	Kiguchi et al. 2013

Figure 7.2 Epigenetic marks and machinery modified after injury. A handful of studies have investigated changes in the epigenome and epigenome modifying enzymes following injury. Source: Géranton and Tochiki (In press).

7.2 Summary of Findings

For a concise summary of the main findings in this thesis, please see Figure 7.3.

7.2.1 Peripheral noxious stimulation induces histone phosphorylation in the spinal cord

Previous studies have indicated aberrant histone acetylation in the dorsal horn following injury (Figure 7.2). Profiling of the dorsal horn for alterations in histone modifications in Chapter 3 indicated that formalin stimulation did not change histone acetylation levels in the dorsal horn at 1h in contrast to a clear ipsilateral upregulation of a small number of cells expressing histone phosphorylation (PH3S10). Interestingly, expression of histone acetylation (various residues on H3, H4) appeared to be more ubiquitous in spinal cord distribution compared to histone phosphorylation which followed a very restricted pattern of expression. Positive staining of PH3S10 nuclei was upregulated in the ipsilateral superficial dorsal horn following formalin stimulation. Because PH3S10 is a globally expressed histone mark which presumably contributes to the regulation of chromatin structure at many loci, there were indeed low levels of PH3S10 immunolabelling within dorsal horn nuclei which were not quantified and considered here as background staining. It should be noted that this does not mean that background levels of PH3S10 should be ignored; in fact, techniques such as ChIP would enable the detection of PH3S10 associated genes (for example, *c-Fos*) that might not have been visible using IHC colabelling with candidate targets.

Notably, the experiments in this thesis are not the first to implicate the importance of histone phosphorylation in acute activity-generated CNS responses. A study by Kumar et al. (2005) found that acute cocaine administration induced increases in H4 acetylation as well as H3 phospho-acetylation (PH3S10-AcH3K14) at the *c-Fos* promoter in striatum. When H3 acetylation (K14) was investigated alone without the presence of H3 phosphorylation (S10), acute cocaine-induced increases at *c-Fos* were not observed. Interestingly however, a switch in histone upregulation occurred during chronic cocaine administration, which was associated with an upregulation of H3, and not H4 acetylation (at *FosB, BDNF,* and *CdK5*). Acute upregulation of H4 acetylation and H3 phospho-acetylation, and chronic upregulation of H3 acetylation has similarly been observed in acute and chronic seizure (Tsankova et al., 2004).

Importantly, the ipsilateral upregulation of PH3S10 following injury is reminiscent of other molecular markers crucial to nociceptive processing that are unilaterally upregulated post-injury, such as PERK (Ji et al., 1999), c-Fos (Hunt et al., 1987), and PMeCP2 (Géranton et al., 2007). Similar to MeCP2, PH3S10 contributes to global

regulation of gene transcription (Crosio et al., 2003), and therefore the results described in Chapter 3 suggested that PH3S10 might be contributing to the regulation of a set of gene programmes essential to the development of pain states.

7.2.2 Cellular characterisation of PH3S10 in the dorsal horn following noxious stimulation

Chapter 4 experiments confirmed the presence of PH3S10 in neurons of the pain pathways as shown by colocalisation with PERK, NK1, c-Fos and Zif268, results which further implicated a role for PH3S10 in nociceptive processing. The immediate expression of PH3S10 within 5-10 minutes following formalin injection was very similar to the induction of PERK which is immediately present after noxious stimulation. However, in contrast to the maximal expression of PERK at 5 minutes (Ji et al., 1999), PH3S10 levels were found to peak around 30 minutes after injury. As it is well-known that PH3S10 lies downstream of PERK (Soloaga et al., 2003), the peak expression of PH3S10 fits in well with this sequence of signalling events. Furthermore, expression of c-Fos protein is known to peak at 2 hours post-injury (Hunt et al., 1987), again evidence suggesting that PH3S10 could regulate downstream gene programmes crucial to the nociceptive response, including *c-Fos*.

The time course of formalin-induced PH3S10 showed the sustained expression of PH3S10 after injury. This prolonged upregulation indicated that PH3S10 has a unique role in nociceptive processing. While PERK expression largely disappears by 1h, PH3S10 levels are robust for at least 2h. The main function of PERK is presumably signal integration and relay of extracellular events (Kelleher et al., 2004; Kelleher et al., 2004), while downstream PH3S10 expression may be more indicative of long-lasting post-synaptic changes (changes in gene expression). In the nucleus, histone modifications are known to play a pivotal role in the long-term effects of neural plasticity by modifying chromatin structure and enhancing or obscuring the accessibility of transcriptional machinery to DNA, acting together with other epigenetic factors to promote or silence transcription at key gene loci (Borrelli et al., 2008; Zocchi and Sassone-Corsi, 2010). These epigenetic factors can include other histone modifications, DNA methylation, and epigenetic readers such as bromodomains, which bind to monoacetylated lysine residues on histone tails, and DNA binding complexes such as MeCP2.

Importantly, experiments in Chapter 4 allowed for the direct comparison of the PH3S10 population size relative to PERK, c-Fos, and Zif268 populations after injury. The PH3S10 population was notably smaller and had a more limited distribution than PERK,

c-Fos, and Zif268 populations. Similar observations have been drawn by Rotllant and Armario (2011), who found that amphetamine administration induced more restricted patterns of PH3S10 expression in areas of the brain key to addiction (nucleus accumbens, dorsal striatum) compared to c-Fos, which was more widely expressed throughout additional brain structures. The authors suggest that a restricted population of PH3S10 labelled neurons may indicate a unique and more specific role for PH3S10 positive cells in amphetamine-induced neural activity. It is possible that this may also be the case for PH3S10 in the dorsal horn, and that the restricted pattern of PH3S10 expression following injury may label neurons important in the response to noxious stimulation.

Finally, Chapter 4 results also indicated that while noxious stimuli-induced upregulation of PH3S10 nuclei indeed appeared to be entirely neuronal, background levels of PH3S10 did colocalise minimally with glial markers APC CC1 and Iba1. This is not surprising as PH3S10 is also a well-known marker of mitotic cell division (Hsu et al., 2000). Furthermore, it is entirely possible that injury-induced PH3S10 may be found to colocalise with glial markers if investigated using different pain models known to induce activation of the glial population, such as microglia in neuropathic injury (Tsuda et al., 2013). Interestingly, formalin has been shown to cause an ipsilateral activation of phospho-p38 MAPK (pp38) in the dorsal horn as early as 30 minutes, and pp38 largely occurs in microglia (Li et al., 2010). However, no unilateral increase of Iba1 expression was seen, and colocalisation of PH3S10 with pp38 was not investigated.

7.2.3 Descending serotonergic regulation of PH3S10 in nociceptive processing

It was found in Chapter 5 that PH3S10 in the dorsal horn was reduced after depletion of descending serotonergic fibres from the RVM. 5,7-DHT-induced depletion of descending 5-HT has been shown to lead to a reduction in spinal formalin-induced PERK, Zif268, and PMeCP2 (Géranton et al., 2008; Svensson et al., 2006) and has implicated descending 5-HT as having an overall pronociceptive role onto dorsal horn activity. Taking these results into consideration, a reduction in PH3S10 further suggested a key role in nociception.

7.2.4 Intracellular regulation of PH3S10 in nociceptive processing

Crucially, experiments from Chapter 6 proved a role for MSK1 in nociception, and suggested that MSK1 may act via PH3S10 to modulate the development of central sensitisation, reflected by attenuation of nocifensive behaviour in the second phase of the formalin response (Fischer et al., 2014), following i.t. inhibition by SB747651A. The experiments firstly confirmed that both formalin-induced PMSK1 and PH3S10 were

downstream of PERK, and secondly showed that inhibition of MSK1 led to a reduction in both PH3S10 expression and nociceptive behaviour caused by formalin.

Although it is a well-known fact that ERK regulates nociceptive behaviour, ERK is known to have multiple downstream targets. Importantly, the experiments in Chapter 6 managed to dissect out one of the downstream ERK/MAPK signalling cascades which significantly contribute to nociception, particularly via inhibition of MSK1.

Similar to PH3S10 (and as mentioned in 7.2.2), the PMSK1 population was limited in size compared to PERK when investigated at 30 minutes. The PERK population was considerably larger than PMSK1, and there were many PERK positive cells that did not coexpress PMSK1 (Chapter 6, *Experiment 1)* suggesting a refinement of the signalling cascade at this particular step. However, experimental limitations regarding the time point investigating overlap should be noted, and other downstream targets of ERK not investigated here obviously cannot be ignored (i.e. Elk, Myc, RSK2).

The expression of PERK in dorsal horn neurons does not necessarily indicate outcomes of activity-induced gene transcription, as PERK is also involved in many early activity-induced intracellular events such as post-translational modification of receptors (Slack et al., 2004; Zou et al., 2000). It is not known if there are specific factors determining whether extracellular stimulation is sufficient to induce lasting changes in gene transcription and protein synthesis post-synaptically. However, MSK1 is the only kinase in the ERK/MAPK cascade that appears solely in the nucleus, and its positioning downstream of ERK could indicate that PMSK1 expression represents primary afferent activation of intracellular signalling sufficient to drive long-term nuclear changes contributing to nociceptive behaviour, and therefore potentially the development of chronic pain. The suggestion that PMSK1 may indicate a lasting outcome/consequence of extracellular activity is a striking one, as other studies have implicated MSK1 as having a key role in BDNF/MAPK signaling. Specifically, Correa et al (2012) suggest that MSK1 is the ‘apex’ of BDNF/MAPK signalling which is a molecular control determining the direction of neuronal output, and that MSK1 is a key homeostatic regulator of neural activity, implications which will be discussed in 7.4.

Surprisingly, Chapter 6 results showed that MSK1 inhibition did not lead to a reduction in c-Fos expression in the dorsal horn. However, only a small reduction was expected, and it is entirely possible that IHC was insufficient at detecting a small reduction in c-Fos following MSK1 inhibition with SB747651A. A more sensitive technique such as ChIP might have indicated a change in PH3S10 mediated *c-Fos* transcription after formalin stimulation. ChIP experiments in other studies have proven that there is a

functional link between PH3S10 and the *c-Fos* expression, in particular cocaine-induced enrichment of PH3S10 at the *c-Fos* promoter has been observed in the brain (Brami-Cherrier et al., 2009). Investigating PH3S10-*c-Fos* associations using ChIP would be an important study contributing to the results in Chapter 6; it would also provide an important insight into other gene targets partially mediated by PH3S10 activity. Please see 7.5 Future directions.

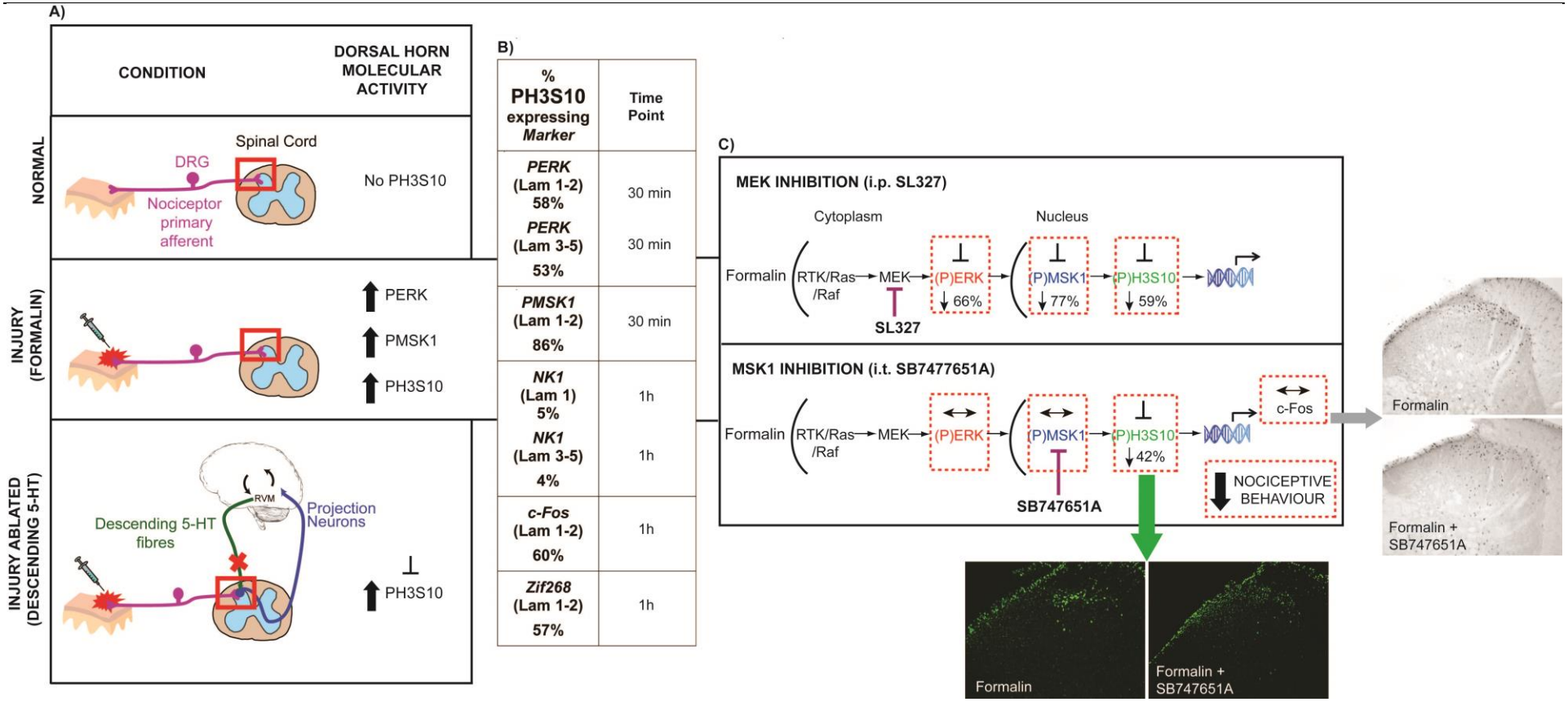


Figure 7.3 Summary of main findings in this thesis Box A) Expression of molecular targets investigated in the dorsal horn under naïve, injured, and injured-5-HT-ablated conditions. Red box indicates area of investigation. Box B) Percentage colocalisation of formalin-induced PH3S10 population with markers of the pain pathways. Box C) Pharmacological inhibition of ERK/MAPK signalling by SL327 led to a reduction in formalin-induced PERK, PMSK1, and PH3S10. In a separate experiment, inhibition of MSK1 by SB747651A reduced PH3S10 and prevented the full expression of pain states. Horizontal arrows indicate no change in injury-induced expression of molecular targets following drug treatment. Black inhibitory symbols indicate reduction in injury- induced expression of target following 5,7-DHT ablation or drug treatment.

7.3 MSK1: a key signalling molecule governing the post- synaptic structural plasticity underlying hyperalgesia and allodynia

The induction and maintenance of central sensitisation requires a number of structural and functional changes post-synaptically, which can be separated into short-term post-translational events, such as receptor subunit phosphorylation or trafficking (Carvalho et al., 2000; Chen and Roche, 2007; Lau and Zukin, 2007), as well as longer-term events which require gene transcription and synthesis of new protein, the same way that LTP can be distinguished by early- and late- LTP phases. Considering the importance of intracellular signalling in activity-dependent neural function, there appears to be a clear functional distinction between early signalling events responsible for immediate post-translational consequences of Ca^{2+} entry, such as PKA, PKC, and CaMKII signalling, which are known to converge upon ERK (Hu et al., 2003; Ji et al., 2009), and further downstream signalling inclusive of ERK and MSK1. ERK and MSK1 therefore integrate a number of signalling events, regulating the transduction of cell surface-to-nucleus communication and thus long-term translational consequences of plasticity (Kelleher et al., 2004; Kelleher et al., 2004).

The findings in this thesis suggest that MSK1 could be a regulator of the long-term events involved in central sensitisation. Studies have shown that at early time points after acute inflammation (Fang et al., 2002; Fang et al., 2003; Fang et al., 2003; Larsson and Broman, 2008), phosphorylation of the AMPAR GluR1 subunit is increased, indicating a preferential switch from GluR2 to GluR1 subunit expression, which results in Ca^{2+} permeability of AMPAR. The preferential expression for calcium permeable GluR1 AMPAR subunits has also been observed at later time points (3d) of longer-term persistent pain states induced by i.pl. CFA (Vikman et al., 2008) which suggests a role for MSK1 regulating gene expression and translation of the GluR1 subunit. As previously discussed (Chapter 6), MSK1 has been shown to regulate the cell surface expression of AMPAR GluR1 and dendritic spine volume in the hippocampus, which has been suggested to occur via regulation of downstream target *Arc/Arg3.1*, a CREB-mediated IEG (Bramham et al., 2008; Chowdhury et al., 2006; Correa et al., 2012; Shepherd and Bear, 2011). Notably, it has been shown that Arc/Arg3.1 is acutely upregulated in the dorsal horn following inflammation or i.t. delivery of BDNF, and 70% of these cells were found to express enkephalin. Furthermore, Arc/Arg3.1 was observed in a small percentage of NK1 positive neurons (Hossaini et al., 2010).

Finally, the literature indicates that MSK1 regulates nuclear epigenetic signalling events such as PH3S10 and CREB phosphorylation in other models of plasticity (Chwang et al.

2007 OTHERS). Studies have shown that CREB is, in fact, an effector of central sensitisation (Latremoliere and Woolf, 2009). CREB is phosphorylated by a variety of signalling events in the dorsal horn post-injury, and leads to the transcription of several genes including *c-Fos*, *NK1,* and *TrkB* (Ji et al., 2009; Kawasaki et al., 2004). Although the interpretations for the role of MSK1 are speculative, the evidence likely points to the fact that MSK1 is a key signalling molecule mediating the events occurring at the cell surface down to the nuclear level to regulate gene expression.

7.4 MSK1: a key role in experience-dependent synaptic scaling?

A few studies have implicated MSK1 in the long-term synaptic adaptation to prolonged neural activity caused by exposure to environmental enrichment (EE) (Correa et al., 2012; Karelina et al., 2012). MSK1-/- mice fail to exhibit the upregulation of synaptic strength in response to EE. Specifically, MSK1-/- mice fail to demonstrate enhancements in EE-induced spine density, a process which is also known to occur in the dorsal horn following persistent activity induced by intraplantar CFA injury and is associated with hypersensitivity, albeit via a different signalling mechanism (Simonetti et al., 2013). The role of MSK1 as an adaptive response in the face of prevailing activity has important implications for MSK1 signalling in long-term pain states and chronic pain, although without conclusively testing this function in an appropriate model such as a model of persistent or neuropathic pain, is merely speculative (Figure 7.4).

Others have also shown that MSK1 is necessary for adjustments in synaptic strength in models of homeostatic synaptic plasticity. Correa et al. (2012) used cultures from MSK1-/- mice to elucidate a role for MSK1 in a paradigm which demonstrates the ability of neurons to regulate firing activity either via network activity (circuit activity), or intrinsically, such as via insertion of ion channels that would cause changes in neural excitability (Correa et al., 2012; Turrigiano, 2011). Specifically, the role of homeostatic synaptic plasticity is to prevent runaway excitation or enhance neural activity in prevailing Hebbian synaptic activity such as LTP or LTD (Turrigiano, 2011). The experiments in this thesis focused on the induction phase of central sensitisation, and MSK1 was not investigated during the maintenance phase. Therefore, the role of MSK1 as a key homeostat requires further investigation in inflammatory pain states.

However, it might be interesting to consider the role of MSK1 as a homeostatic regulator within dorsal horn circuits during the recovery phase from inflammatory responses, or in long-term pain states. Homeostatic compensatory activity has usually been demonstrated as occurring several hours or longer after activity enhancement or

deprivation, and MSK1 may have wider implications in the long- term response to chronic pain states within dorsal horn circuits.

Finally, although we could not confirm that *c-Fos* was under the control of MSK1 signalling following injury, if the main function of MSK1 in dorsal horn signaling is as a homeostatic regulator (albeit not in the induction phase of inflammation), it is also possible that MSK1 could also control late response gene programmes crucial to the homeostatic response at the circuit-wide level. A recent publication by Spiegel et al. (2014) has identified an immediate early gene, *Npas4*, which is upregulated in both excitatory and inhibitory cortical neurons following neuronal activity. Crucially however, Npas4 differentially activates non-overlapping late-response gene programmes in inhibitory and excitatory neurons such that excitatory synapses are expressed on a specific set of inhibitory neurons (SST positive), thereby promoting GABAergic release, while regulating inhibitory synapses on excitatory neurons (Lin et al., 2008). Overall, the differential activation of late-response gene programmes work to balance overall network activity. Interestingly, Spiegel et al. (2014) suggest that epigenetic mechanisms, including histone modifications, could be responsible for the cell-type specific distinct activation of gene programmes downstream of global Npas4 upregulation, which may have interesting implications for PH3S10. Preliminary experiments indicate that Npas4 is colocalised within dorsal horn superficial laminae following noxious stimulation (Hunt SP and Lammertse H, personal communication).

Figure 7.4 MSK1 could play a role in short and long-term pain states.

7.5 Future directions

7.5.1 Investigation into MSK1 targets and PH3S10-associated genes

Experiments in this thesis indicated that MSK1 regulates nociceptive behaviour, potentially via PH3S10. Chapter 6 results showed that reducing PH3S10 by MSK1 inhibition did not lead to a reduction in c-Fos nuclei, as was expected following a 60% colocalisation of PH3S10 with c-Fos (Chapter 4). The lack of significant change in c-Fos expression in this case does not necessarily eliminate the possibility that c-Fos is regulated by MSK1, and could have been a limitation of the IHC technique used for this analysis to detect the expected small changes in c-Fos expression. Furthermore, IHC could have only indicated correlative results for the relationship of c-Fos to that of PH3S10. In order to determine whether PH3S10 may functionally contribute to *c-Fos* gene regulation, it would be necessary to investigate locus specific enrichment of the *c-Fos* gene with PH3S10 following peripheral formalin stimulation using a chromatin immunoprecipitation experiment (ChIP).

A ChIP experiment could reveal PH3S10/*c-Fos* associations that would have gone unseen with the limited analysis provided by IHC. Incidentally, if PH3S10 was specific to the regulation of *c-Fos*, this would possibly remain unidentified using IHC as PH3S10 would be upregulated only at the *c-Fos* locus. As there is only one copy of the

c-Fos gene within each neuronal nucleus, PH3S10 might not appear strongly enough to label the entire nucleus, as was the appearance of the nuclei quantified/counted in this thesis. In fact, it is quite possible that the PH3S10 population described in this thesis could actually indicate that PH3S10 in these neurons is less specific to the nociceptive response, in that PH3S10 could be aiding transcription of a multitude of genes, not all of which may be specifically related to nociception. However, the set of gene programmes activated by PH3S10 could be specific to the nociceptive response. It is therefore worth considering deep sequencing techniques such as ChIP-sequencing (ChIP-seq) which could identify the main gene targets of PH3S10 regulation following injury. The other option would be to use ChIP followed by qPCR to investigate the association of PH3S10 and other candidate genes in addition to *c-Fos*. Discussions arising from the results in this thesis suggest that *Arc/Arg3.1, CREB, FKBP5, Npas4,* and *Zif268* could all be worthy targets of investigation (Géranton et al., 2007; Hossaini et al., 2010; Ji and Rupp, 1997; Soloaga et al., 2003; Spiegel et al., 2014).

7.5.2 Inhibitory/excitatory interneuron populations

The identification of a neuronal subtype for PH3S10 is another approach that could have helped to understand its function in nociception within the dorsal horn circuit. There are a number of glutamatergic excitatory markers that could be tested for colocalisation with PH3S10, including neuropeptides somatostatin (SST), neurotensin, calretinin, SP, NKB, and calretinin, provided they label cell bodies (Todd, 2010). Glutaraldehyde perfusion to optimise staining for GABAergic interneurons is another possibility. An additional technique to identify inhibitory interneurons would be to acquire genetically modified mice with a green fluorescent protein (GFP) reporter for *GAD* (GAD-GFP mice) to label GABAergic cells, and to double stain the spinal cord tissue with PH3S10 following noxious stimulation. This approach however, would require re-characterisation of formalin-induced PH3S10 expression in mice (e.g. cell specificity, colocalisation with markers of the pain pathway, time course expression).

7.5.3 Investigation of MSK1 as a regulator of homeostasis using hyperalgesic priming models

Animal models of hyperalgesic priming have been suggested to reproduce events responsible for the transition from acute to chronic pain, specifically due to the fact that an initial injury can lead to a state of sensitisation which resembles clinical situations for human increased risk of developing chronic pain (Reichling and Levine, 2009). After full behavioural recovery to a minor insult (typically peripheral inflammation with IL-6, carrageenan, or surgical incision), animals become ‘primed’ and maintain a lasting latent hyper-responsiveness to any subsequent injury (PGE_2, carrageenan, or another

surgical insult) (Aley et al., 2001; Bogen et al., 2012; Dina et al., 2008; Parada et al., 2005, 2003; Sun et al., 2013). As MSK1 has been implicated as a key homeostat in models of lasting plasticity (hours to days) (Correa et al., 2012), it would be interesting to use MSK1-/- mice in a model of hyperalgesic priming to investigate the role of MSK1 in a long-term sensitised state. The hypothesis would be that MSK-/- animals would fail to exhibit a typical response seen with primed animals; if MSK1 is to maintain balance of a sensitised state the MSK-/- mice could either exhibit more sensitivity than a normal primed response, or conversely could be hyposensitive.

7.6 General Conclusions

A growing body of evidence exists, not just within the pain field, to support the role of epigenetic mechanisms as potential targets for pain management. Human twin and animal studies have clearly indicated that epigenetic mechanisms can be differentially engaged by environmental experience, such as that experienced in early life (stress), or in adulthood (learning, addiction) (Borrelli et al., 2008; Renthal and Nestler, 2008; Weaver et al., 2004), and are even robust e250nough to encode behavioural information across generations (Dias and Ressler, 2013; Heard and Martienssen, 2014). The unique feature of epigenetic mechanisms is the ability to bridge the genome and the environment, and the ability to imprint a variety of activity-dependent experiences onto the genome (the epigenome) (Day and Sweatt, 2011; Lester et al., 2011; Sweatt, 2013). Specifically, epigenetic mechanisms refine neural networks according to experience and can allow the environment to elicit life-long biological changes in both gene expression and behaviour. The potential diversity of behavioural manifestations due to the same epigenetic processes easily highlights the fine balance that is required of these marks on the epigenome to maintain normal neural function.

Epigenetic processes have been shown, in part by experiments in this thesis and by others, to be engaged in both the induction phase as well as the maintenance phase of persistent pain states. In states of chronic pain, spinal cord nociceptive networks remain sensitised with long- lasting changes in gene expression and synaptic strength (Kuner, 2010), and could be regarded as a form of nociceptive memory (Sandkühler and Lee, 2013). It is therefore plausible that chronic pain states- in particular the events responsible for the transition from acute to chronic pain states- involve a specific epigenetic code, or possibly a contribution of epigenetic processes (Géranton and Tochiki, In press). Targeting epigenetic mechanisms could be a promising approach for the treatment of pain states; however, identifying the defining specific epigenetic events leading to, or involved in chronicity are key. Furthermore, as global regulators of gene transcription, pharmacological inhibition of epigenetic mechanisms may benefit from

refinement in the form of enzyme subtype inhibitors, or target specificity, particularly at the level of the genome. The targeting of epigenetic mechanisms should also be considered contextually, as they are sure to act in concert with a multitude of molecular events to regulate neural function. The findings in this thesis demonstrate that histone phosphorylation and histone modifying enzymes can be targeted pharmacologically to alter nociceptive behaviour and indicate a potential for epigenetic mechanisms as targets of reversibility. Furthermore, the outcomes of this thesis continue to justify the investigation into a relatively new research area which continues to increasingly gain crucial attention.

References

Abbadie, C., Taylor, B.K., Peterson, M.A., Basbaum, A.I., 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.

Abbott, F.V., Franklin, K.B., Westbrook, R.F., 1995. The formalin test: scoring properties of the first and second phases of the pain response in rats. Pain 60, 91–102.

Abrahamsen, B., Zhao, J., Asante, C.O., Cendan, C.M., Marsh, S., Martinez-Barbera, J.P., Nassar, M.A., Dickenson, A.H., Wood, J.N., 2008. The cell and molecular basis of mechanical, cold, and inflammatory pain. Science 321, 702–705. doi:10.1126/science.1156916

Abram, S.E., Yaksh, T.L., 1993. Morphine, but not inhalation anesthesia, blocks post-injury facilitation. The role of preemptive suppression of afferent transmission. Anesthesiology 78, 713–721.

Akbarian, S., 2010. The molecular pathology of schizophrenia--focus on histone and DNA modifications. Brain Res. Bull. 83, 103–107. doi:10.1016/j.brainresbull.2009.08.018

Akopian, A.N., Souslova, V., England, S., Okuse, K., Ogata, N., Ure, J., Smith, A., Kerr, B.J., McMahon, S.B., Boyce, S., Hill, R., Stanfa, L.C., Dickenson, A.H., Wood, J.N., 1999. The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways. Nat. Neurosci. 2, 541–548. doi:10.1038/9195

Aley, K.O., Martin, A., McMahon, T., Mok, J., Levine, J.D., Messing, R.O., 2001. Nociceptor sensitization by extracellular signal-regulated kinases. J. Neurosci. Off. J. Soc. Neurosci. 21, 6933–6939.

Al Ghamdi, K.S., Polgár, E., Todd, A.J., 2009. Soma size distinguishes projection neurons from neurokinin 1 receptor-expressing interneurons in lamina I of the rat lumbar spinal dorsal horn. Neuroscience 164, 1794–1804. doi:10.1016/j.neuroscience.2009.09.071

Alhaider, A.A., Lei, S.Z., Wilcox, G.L., 1991. Spinal 5-HT3 receptor-mediated antinociception: possible release of GABA. J. Neurosci. Off. J. Soc. Neurosci. 11, 1881–1888.

Al-Khater, K.M., Kerr, R., Todd, A.J., 2008. A quantitative study of spinothalamic neurons in laminae I, III, and IV in lumbar and cervical segments of the rat spinal cord. J. Comp. Neurol. 511, 1–18. doi:10.1002/cne.21811

Al-Khater, K.M., Todd, A.J., 2009. Collateral projections of neurons in laminae I, III, and IV of rat spinal cord to thalamus, periaqueductal gray matter, and lateral parabrachial area. J. Comp. Neurol. 515, 629–646. doi:10.1002/cne.22081

Alter, B.J., Zhao, C., Karim, F., Landreth, G.E., Gereau, R.W., 4th, 2010. Genetic targeting of ERK1 suggests a predominant role for ERK2 in murine pain models. J. Neurosci. Off. J. Soc. Neurosci. 30, 11537–11547. doi:10.1523/JNEUROSCI.6103-09.2010

Anderson, L.E., Seybold, V.S., 2000. Phosphorylated cAMP response element binding protein increases in neurokinin-1 receptor-immunoreactive neurons in rat spinal cord in response to formalin-induced nociception. Neurosci. Lett. 283, 29–32.

Andrew, D., 2009. Sensitization of lamina I spinoparabrachial neurons parallels heat hyperalgesia in the chronic constriction injury model of neuropathic pain. J. Physiol. 587, 2005–2017. doi:10.1113/jphysiol.2009.170290

Antognini, J.F., Wang, X.W., Carstens, E., 2000a. Isoflurane action in the spinal cord blunts electroencephalographic and thalamic-reticular formation responses to noxious stimulation in goats. Anesthesiology 92, 559–566.

Antognini, J.F., Wang, X.W., Piercy, M., Carstens, E., 2000b. Propofol directly depresses lumbar dorsal horn neuronal responses to noxious stimulation in goats. Can. J. Anaesth. J. Can. Anesth. 47, 273–279. doi:10.1007/BF03018926

Apkarian, A.V., Bushnell, M.C., Treede, R.-D., Zubieta, J.-K., 2005. Human brain mechanisms of pain perception and regulation in health and disease. Eur. J. Pain 9, 463–463. doi:10.1016/j.ejpain.2004.11.001

Arthur, J.S.C., Fong, A.L., Dwyer, J.M., Davare, M., Reese, E., Obrietan, K., Impey, S., 2004. Mitogen- and stress-activated protein kinase 1 mediates cAMP response element-binding protein phosphorylation and activation by neurotrophins. J. Neurosci. Off. J. Soc. Neurosci. 24, 4324–4332. doi:10.1523/JNEUROSCI.5227-03.2004

Atkins, C.M., Selcher, J.C., Petraitis, J.J., Trzaskos, J.M., Sweatt, J.D., 1998. The MAPK cascade is required for mammalian associative learning. Nat. Neurosci. 1, 602–609. doi:10.1038/2836

Baba, H., Ji, R.-R., Kohno, T., Moore, K.A., Ataka, T., Wakai, A., Okamoto, M., Woolf, C.J., 2003. Removal of GABAergic inhibition facilitates polysynaptic A fiber-mediated excitatory transmission to the superficial spinal dorsal horn. Mol. Cell. Neurosci. 24, 818–830.

Bai, G., Ren, K., Dubner, R., 2014. Epigenetic regulation of persistent pain. Transl. Res. J. Lab. Clin. Med. doi:10.1016/j.trsl.2014.05.012

Bai, G., Wei, D., Zou, S., Ren, K., Dubner, R., 2010. Inhibition of class II histone deacetylases in the spinal cord attenuates inflammatory hyperalgesia. Mol. Pain 6, 51. doi:10.1186/1744-8069-6-51

Bandell, M., Story, G.M., Hwang, S.W., Viswanath, V., Eid, S.R., Petrus, M.J., Earley, T.J., Patapoutian, A., 2004. Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron 41, 849–857.

Bannon, A.W., Malmberg, A.B., 2007. Models of Nociception: Hot-Plate, Tail-Flick, and Formalin Tests in Rodents, in: Crawley, J.N., Gerfen, C.R., Rogawski, M.A., Sibley, D.R., Skolnick, P., Wray, S. (Eds.), Current Protocols in Neuroscience. John Wiley & Sons, Inc., Hoboken, NJ, USA.

Bardin, L., 2011. The complex role of serotonin and 5-HT receptors in chronic pain. Behav. Pharmacol. 22, 390–404. doi:10.1097/FBP.0b013e328349aae4

Bardin, L., Jourdan, D., Alloui, A., Lavarenne, J., Eschalier, A., 1997. Differential influence of two serotonin 5-HT3 receptor antagonists on spinal serotonin-induced analgesia in rats. Brain Res. 765, 267–272.

Barrett, R.M., Wood, M.A., 2008. Beyond transcription factors: the role of chromatin modifying enzymes in regulating transcription required for memory. Learn. Mem. Cold Spring Harb. N 15, 460–467. doi:10.1101/lm.917508

Basbaum, A.I., Bautista, D.M., Scherrer, G., Julius, D., 2009. Cellular and Molecular Mechanisms of Pain. Cell 139, 267–284. doi:10.1016/j.cell.2009.09.028

Basbaum, A.I., Fields, H.L., 1979. The origin of descending pathways in the dorsolateral funiculus of the spinal cord of the cat and rat: further studies on the anatomy of pain modulation. J. Comp. Neurol. 187, 513–531. doi:10.1002/cne.901870304

Bautista, D.M., Jordt, S.-E., Nikai, T., Tsuruda, P.R., Read, A.J., Poblete, J., Yamoah, E.N., Basbaum, A.I., Julius, D., 2006. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. Cell 124, 1269–1282. doi:10.1016/j.cell.2006.02.023

Bautista, D.M., Siemens, J., Glazer, J.M., Tsuruda, P.R., Basbaum, A.I., Stucky, C.L., Jordt, S.-E., Julius, D., 2007. The menthol receptor TRPM8 is the principal detector of environmental cold. Nature 448, 204–208. doi:10.1038/nature05910

Beckmann, A.M., Wilce, P.A., 1997. Egr transcription factors in the nervous system. Neurochem. Int. 31, 477–510; discussion 517–516.

Beecher, H.K., 1946. Pain in Men Wounded in Battle. Ann. Surg. 123, 96–105.

Beggs, S., Salter, M.W., 2007. Stereological and somatotopic analysis of the spinal microglial response to peripheral nerve injury. Brain. Behav. Immun. 21, 624–633. doi:10.1016/j.bbi.2006.10.017

Bell, J.T., Loomis, A.K., Butcher, L.M., Gao, F., Zhang, B., Hyde, C.L., Sun, J., Wu, H., Ward, K., Harris, J., Scollen, S., Davies, M.N., Schalkwyk, L.C., Mill, J., Ahmadi, K.R., Ainali, C., Barrett, A., Bataille, V., Bell, J.T., Buil, A., Deloukas, P., Dermitzakis, E.T., Dimas, A.S., Durbin, R., Glass, D., Grundberg, E., Hassanali, N., Hedman, A.K., Ingle, C., Knowles, D., Krestyaninova, M., Lindgren, C.M., Lowe, C.E., McCarthy, M.I., Meduri, E., di Meglio, P., Min, J.L., Montgomery, S.B., Nestle, F.O., Nica, A.C., Nisbet, J., O'Rahilly, S., Parts, L., Potter, S., Sekowska, M., Shin, S.-Y., Small, K.S., Soranzo, N., Spector, T.D., Surdulescu, G., Travers, M.E., Tsaprouni, L., Tsoka, S., Wilk, A., Yang, T.-P., Zondervan, K.T., Williams, F.M.K., Li, N., Deloukas, P., Beck, S., McMahon, S.B., Wang, J., John, S.L., Spector, T.D., 2014. Differential methylation of the TRPA1 promoter in pain sensitivity. Nat. Commun. 5. doi:10.1038/ncomms3978

Bennett, D.L.H., Woods, C.G., 2014. Painful and painless channelopathies. Lancet Neurol. 13, 587–599. doi:10.1016/S1474-4422(14)70024-9

Bessou, P., Burgess, P.R., Perl, E.R., Taylor, C.B., 1971. Dynamic properties of mechanoreceptors with unmyelinated (C) fibers. J. Neurophysiol. 34, 116–131.

Bessou, P., Perl, E.R., 1969. Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. J. Neurophysiol. 32, 1025–1043.

Bester, H., Beggs, S., Woolf, C.J., 2000. Changes in tactile stimuli-induced behavior and c-Fos expression in the superficial dorsal horn and in parabrachial nuclei after sciatic nerve crush. J. Comp. Neurol. 428, 45–61.

Bester, H., Chapman, V., Besson, J.M., Bernard, J.F., 2000. Physiological properties of the lamina I spinoparabrachial neurons in the rat. J. Neurophysiol. 83, 2239–2259.

Blackburn-Munro, G., 2004. Pain-like behaviours in animals – how human are they? Trends Pharmacol. Sci. 25, 299–305. doi:10.1016/j.tips.2004.04.008

Bliss, T.V., Collingridge, G.L., 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361, 31–39. doi:10.1038/361031a0

Bogen, O., Alessandri-Haber, N., Chu, C., Gear, R.W., Levine, J.D., 2012. Generation of a pain memory in the primary afferent nociceptor triggered by PKCε activation of CPEB. J. Neurosci. Off. J. Soc. Neurosci. 32, 2018–2026. doi:10.1523/JNEUROSCI.5138-11.2012

Bonni, A., Ginty, D.D., Dudek, H., Greenberg, M.E., 1995. Serine 133-phosphorylated CREB induces transcription via a cooperative mechanism that may confer specificity to neurotrophin signals. Mol. Cell. Neurosci. 6, 168–183. doi:10.1006/mcne.1995.1015

Borrelli, E., Nestler, E.J., Allis, C.D., Sassone-Corsi, P., 2008. Decoding the epigenetic language of neuronal plasticity. Neuron 60, 961–974. doi:10.1016/j.neuron.2008.10.012

Bosch, M., Hayashi, Y., 2012. Structural plasticity of dendritic spines. Curr. Opin. Neurobiol. 22, 383–388. doi:10.1016/j.conb.2011.09.002

Bourinet, E., Altier, C., Hildebrand, M.E., Trang, T., Salter, M.W., Zamponi, G.W., 2014. Calcium-permeable ion channels in pain signaling. Physiol. Rev. 94, 81–140. doi:10.1152/physrev.00023.2013

Bramham, C.R., Worley, P.F., Moore, M.J., Guzowski, J.F., 2008. The immediate early gene arc/arg3.1: regulation, mechanisms, and function. J. Neurosci. Off. J. Soc. Neurosci. 28, 11760–11767. doi:10.1523/JNEUROSCI.3864-08.2008

Brami-Cherrier, K., Lavaur, J., Pagès, C., Arthur, J.S.C., Caboche, J., 2007. Glutamate induces histone H3 phosphorylation but not acetylation in striatal neurons: role of mitogen- and stress-activated kinase-1. J. Neurochem. 101, 697–708. doi:10.1111/j.1471-4159.2006.04352.x

Brami-Cherrier, K., Roze, E., Girault, J.-A., Betuing, S., Caboche, J., 2009. Role of the ERK/MSK1 signalling pathway in chromatin remodelling and brain responses to drugs of abuse. J. Neurochem. 108, 1323–1335. doi:10.1111/j.1471-4159.2009.05879.x

Brami-Cherrier, K., Valjent, E., Hervé, D., Darragh, J., Corvol, J.-C., Pages, C., Arthur, S.J., Simon, A.J., Girault, J.-A., Caboche, J., 2005. Parsing molecular and behavioral effects of cocaine in mitogen- and stress-activated protein kinase-1-deficient mice. J. Neurosci. Off. J. Soc. Neurosci. 25, 11444–11454. doi:10.1523/JNEUROSCI.1711-05.2005

Bráz, J.M., Basbaum, A.I., 2010. Differential ATF3 expression in dorsal root ganglion neurons reveals the profile of primary afferents engaged by diverse noxious chemical stimuli. Pain 150, 290–301. doi:10.1016/j.pain.2010.05.005

Braz, J.M., Nassar, M.A., Wood, J.N., Basbaum, A.I., 2005. Parallel "Pain" Pathways Arise from Subpopulations of Primary Afferent Nociceptor. Neuron 47, 787–793. doi:10.1016/j.neuron.2005.08.015

Bredy, T.W., Barad, M., 2008. The histone deacetylase inhibitor valproic acid enhances acquisition, extinction, and reconsolidation of conditioned fear. Learn. Mem. 15, 39–45. doi:10.1101/lm.801108

Breese, G.R., Vogel, R.A., Mueller, R.A., 1978. Biochemical and behavioral alterations in developing rats treated with 5,7-dihydroxytryptamine. J. Pharmacol. Exp. Ther. 205, 587–595.

Breivik, H., Collett, B., Ventafridda, V., Cohen, R., Gallacher, D., 2006. Survey of chronic pain in Europe: Prevalence, impact on daily life, and treatment. Eur. J. Pain 10, 287–287. doi:10.1016/j.ejpain.2005.06.009

Brown, A.G., Fyffe, R.E., Rose, P.K., Snow, P.J., 1981. Spinal cord collaterals from axons of type II slowly adapting units in the cat. J. Physiol. 316, 469–480.

Burgess, P.R., Perl, E.R., 1967. Myelinated afferent fibres responding specifically to noxious stimulation of the skin. J. Physiol. 190, 541–562.

Burgess, S.E., Gardell, L.R., Ossipov, M.H., Malan, T.P., Vanderah, T.W., Lai, J., Porreca, F., 2002. Time-dependent descending facilitation from the rostral ventromedial medulla maintains, but does not initiate, neuropathic pain. J. Neurosci. Off. J. Soc. Neurosci. 22, 5129–5136.

Cai, Y.-Q., Wang, W., Hou, Y.-Y., Pan, Z.Z., 2014. Optogenetic activation of brainstem serotonergic neurons induces persistent pain sensitization. Mol. Pain 10, 70. doi:10.1186/1744-8069-10-70

Carr, F.B., Géranton, S.M., Hunt, S.P., 2014. Descending controls modulate inflammatory joint pain and regulate CXC chemokine and iNOS expression in the dorsal horn. Mol. Pain 10, 39. doi:10.1186/1744-8069-10-39

Carvalho, A.L., Duarte, C.B., Carvalho, A.P., 2000. Regulation of AMPA receptors by phosphorylation. Neurochem. Res. 25, 1245–1255.

Castellano, J.F., Fletcher, B.R., Kelley-Bell, B., Kim, D.H., Gallagher, M., Rapp, P.R., 2012. Age-Related Memory Impairment Is Associated with Disrupted Multivariate Epigenetic Coordination in the Hippocampus. PLoS ONE 7, e33249. doi:10.1371/journal.pone.0033249

Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., Koltzenburg, M., Basbaum, A.I., Julius, D., 2000. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288, 306–313.

Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J., Julius, D., 1999. A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 398, 436–441. doi:10.1038/18906

Cavanaugh, D.J., Lee, H., Lo, L., Shields, S.D., Zylka, M.J., Basbaum, A.I., Anderson, D.J., 2009. Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc. Natl. Acad. Sci. 106, 9075–9080. doi:10.1073/pnas.0901507106

Chandramohan, Y., Droste, S.K., Arthur, J.S.C., Reul, J.M.H.M., 2008. The forced swimming-induced behavioural immobility response involves histone H3 phospho-acetylation and c-Fos induction in dentate gyrus granule neurons via activation of the *N* -methyl-d-aspartate/extracellular signal-regulated kinase/mitogen- and stress-activated kinase signalling pathway </title>. Eur. J. Neurosci. 27, 2701–2713. doi:10.1111/j.1460-9568.2008.06230.x

Chandramohan, Y., Droste, S.K., Reul, J.M.H.M., 2007. Novelty stress induces phospho-acetylation of histone H3 in rat dentate gyrus granule neurons through coincident signalling via the N-methyl-d-aspartate receptor and the glucocorticoid receptor: relevance for c-fos induction: Stress evokes histone H3 phospho-acetylation in dentate gyrus. J. Neurochem. 101, 815–828. doi:10.1111/j.1471-4159.2006.04396.x

Chawla, S., Hardingham, G.E., Quinn, D.R., Bading, H., 1998. CBP: a signal-regulated transcriptional coactivator controlled by nuclear calcium and CaM kinase IV. Science 281, 1505–1509.

Chen, B.-S., Roche, K.W., 2007. Regulation of NMDA receptors by phosphorylation. Neuropharmacology 53, 362–368. doi:10.1016/j.neuropharm.2007.05.018

Chen, C.-L., Broom, D.C., Liu, Y., de Nooij, J.C., Li, Z., Cen, C., Samad, O.A., Jessell, T.M., Woolf, C.J., Ma, Q., 2006. Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. Neuron 49, 365–377. doi:10.1016/j.neuron.2005.10.036

Cheung, P., Allis, C.D., Sassone-Corsi, P., 2000. Signaling to chromatin through histone modifications. Cell 103, 263–271.

Chiechio, S., Zammataro, M., Morales, M.E., Busceti, C.L., Drago, F., Gereau, R.W., Copani, A., Nicoletti, F., 2009. Epigenetic Modulation of mGlu2 Receptors by Histone Deacetylase Inhibitors in the Treatment of Inflammatory Pain. Mol. Pharmacol. 75, 1014–1020. doi:10.1124/mol.108.054346

Chizh, B.A., Illes, P., 2001. P2X receptors and nociception. Pharmacol. Rev. 53, 553–568.

Chowdhury, S., Shepherd, J.D., Okuno, H., Lyford, G., Petralia, R.S., Plath, N., Kuhl, D., Huganir, R.L., Worley, P.F., 2006. Arc/Arg3.1 interacts with the endocytic machinery to regulate AMPA receptor trafficking. Neuron 52, 445–459. doi:10.1016/j.neuron.2006.08.033

Chuang, H.H., Prescott, E.D., Kong, H., Shields, S., Jordt, S.E., Basbaum, A.I., Chao, M.V., Julius, D., 2001. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. Nature 411, 957–962. doi:10.1038/35082088

Chwang, W.B., Arthur, J.S., Schumacher, A., Sweatt, J.D., 2007. The Nuclear Kinase Mitogen- and Stress-Activated Protein Kinase 1 Regulates Hippocampal Chromatin Remodeling in Memory Formation. J. Neurosci. 27, 12732–12742. doi:10.1523/JNEUROSCI.2522-07.2007

Chwang, W.B., O'Riordan, K.J., Levenson, J.M., Sweatt, J.D., 2006. ERK/MAPK regulates hippocampal histone phosphorylation following contextual fear conditioning. Learn. Mem. 13, 322–328. doi:10.1101/lm.152906

Ciccarelli, A., Calza, A., Santoru, F., Grasso, F., Concas, A., Sassoè-Pognetto, M., Giustetto, M., 2013. Morphine withdrawal produces ERK-dependent and ERK-independent epigenetic marks in neurons of the nucleus accumbens and lateral septum. Neuropharmacology 70, 168–179. doi:10.1016/j.neuropharm.2012.12.010

Ciccarelli, A., Giustetto, M., 2014. Role of ERK signaling in activity-dependent modifications of histone proteins. Neuropharmacology 80, 34–44. doi:10.1016/j.neuropharm.2014.01.039

Cingolani, L.A., Goda, Y., 2008. Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy. Nat. Rev. Neurosci. 9, 344–356. doi:10.1038/nrn2373

Coderre, T.J., Melzack, R., 1992. The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. J. Neurosci. Off. J. Soc. Neurosci. 12, 3665–3670.

Coderre, T.J., Vaccarino, A.L., Melzack, R., 1990. Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain Res. 535, 155–158.

Colburn, R.W., Lubin, M.L., Stone, D.J., Wang, Y., Lawrence, D., D'Andrea, M.R., Brandt, M.R., Liu, Y., Flores, C.M., Qin, N., 2007. Attenuated cold sensitivity in TRPM8 null mice. Neuron 54, 379–386. doi:10.1016/j.neuron.2007.04.017

Conte, D., Legg, E.D., McCourt, A.C., Silajdzic, E., Nagy, G.G., Maxwell, D.J., 2005. Transmitter content, origins and connections of axons in the spinal cord that possess the serotonin (5-hydroxytryptamine) 3 receptor. Neuroscience 134, 165–173. doi:10.1016/j.neuroscience.2005.02.013

Correa, S.A.L., Hunter, C.J., Palygin, O., Wauters, S.C., Martin, K.J., McKenzie, C., McKelvey, K., Morris, R.G.M., Pankratov, Y., Arthur, J.S.C., Frenguelli, B.G., 2012. MSK1 Regulates Homeostatic and Experience-Dependent Synaptic Plasticity. J. Neurosci. 32, 13039–13051. doi:10.1523/JNEUROSCI.0930-12.2012

Coste, B., Houge, G., Murray, M.F., Stitziel, N., Bandell, M., Giovanni, M.A., Philippakis, A., Hoischen, A., Riemer, G., Steen, U., Steen, V.M., Mathur, J., Cox, J., Lebo, M., Rehm, H., Weiss, S.T., Wood, J.N., Maas, R.L., Sunyaev, S.R., Patapoutian, A., 2013. Gain-of-function mutations in the mechanically activated ion channel PIEZO2 cause a subtype of Distal Arthrogryposis. Proc. Natl. Acad. Sci. U. S. A. 110, 4667–4672. doi:10.1073/pnas.1221400110

Coste, B., Mathur, J., Schmidt, M., Earley, T.J., Ranade, S., Petrus, M.J., Dubin, A.E., Patapoutian, A., 2010. Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. Science 330, 55–60. doi:10.1126/science.1193270

Cox, J.J., Reimann, F., Nicholas, A.K., Thornton, G., Roberts, E., Springell, K., Karbani, G., Jafri, H., Mannan, J., Raashid, Y., Al-Gazali, L., Hamamy, H., Valente, E.M., Gorman, S., Williams, R., McHale, D.P., Wood, J.N., Gribble, F.M., Woods, C.G., 2006. An SCN9A channelopathy causes congenital inability to experience pain. Nature 444, 894–898. doi:10.1038/nature05413

Crisp, T., Stafinsky, J.L., Spanos, L.J., Uram, M., Perni, V.C., Donepudi, H.B., 1991. Analgesic effects of serotonin and receptor-selective serotonin agonists in the rat spinal cord. Gen. Pharmacol. 22, 247–251.

Crook, R.J., Dickson, K., Hanlon, R.T., Walters, E.T., 2014. Nociceptive Sensitization Reduces Predation Risk. Curr. Biol. 24, 1121–1125. doi:10.1016/j.cub.2014.03.043

Crosio, C., Cermakian, N., Allis, C.D., Sassone-Corsi, P., 2000. Light induces chromatin modification in cells of the mammalian circadian clock. Nat. Neurosci. 3, 1241–1247. doi:10.1038/81767

Crosio, C., Heitz, E., Allis, C.D., Borrelli, E., Sassone-Corsi, P., 2003. Chromatin remodeling and neuronal response: multiple signaling pathways induce specific histone H3 modifications and early gene expression in hippocampal neurons. J. Cell Sci. 116, 4905–4914. doi:10.1242/jcs.00804

Cruz, C.D., Neto, F.L., Castro-Lopes, J., McMahon, S.B., Cruz, F., 2005. Inhibition of ERK phosphorylation decreases nociceptive behaviour in monoarthritic rats. Pain 116, 411–419. doi:10.1016/j.pain.2005.05.031

Culberson, J.L., Haines, D.E., Kimmel, D.L., Brown, P.B., 1979. Contralateral projection of primary afferent fibers to mammalian spinal cord. Exp. Neurol. 64, 83–97.

Dahlhaus, A., Ruscheweyh, R., Sandkühler, J., 2005. Synaptic input of rat spinal lamina I projection and unidentified neurones in vitro. J. Physiol. 566, 355–368. doi:10.1113/jphysiol.2005.088567

Davie, J.R., 2003. MSK1 and MSK2 mediate mitogen- and stress-induced phosphorylation of histone H3: a controversy resolved. Sci. STKE Signal Transduct. Knowl. Environ. 2003, PE33. doi:10.1126/stke.2003.195.pe33

Davis, J.B., Gray, J., Gunthorpe, M.J., Hatcher, J.P., Davey, P.T., Overend, P., Harries, M.H., Latcham, J., Clapham, C., Atkinson, K., Hughes, S.A., Rance, K., Grau, E., Harper, A.J., Pugh, P.L., Rogers, D.C., Bingham, S., Randall, A., Sheardown, S.A., 2000. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 405, 183–187. doi:10.1038/35012076

Davis, S., Vanhoutte, P., Pages, C., Caboche, J., Laroche, S., 2000. The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. J. Neurosci. Off. J. Soc. Neurosci. 20, 4563–4572.

Day, J.J., Sweatt, J.D., 2010. DNA methylation and memory formation. Nat. Neurosci. 13, 1319–1323. doi:10.1038/nn.2666

Day, J.J., Sweatt, J.D., 2011. Epigenetic Mechanisms in Cognition. Neuron 70, 813–829. doi:10.1016/j.neuron.2011.05.019

Day, J.J., Sweatt, J.D., 2012. Epigenetic Treatments for Cognitive Impairments. Neuropsychopharmacology 37, 247–260. doi:10.1038/npp.2011.85

Deak, M., Clifton, A.D., Lucocq, L.M., Alessi, D.R., 1998. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. EMBO J. 17, 4426–4441. doi:10.1093/emboj/17.15.4426

De Felice, M., Sanoja, R., Wang, R., Vera-Portocarrero, L., Oyarzo, J., King, T., Ossipov, M.H., Vanderah, T.W., Lai, J., Dussor, G.O., Fields, H.L., Price, T.J., Porreca, F., 2011. Engagement of descending inhibition from the rostral ventromedial medulla protects against chronic neuropathic pain. PAIN 152, 2701–2709. doi:10.1016/j.pain.2011.06.008

De Koninck, Y., Henry, J.L., 1991. Substance P-mediated slow excitatory postsynaptic potential elicited in dorsal horn neurons in vivo by noxious stimulation. Proc. Natl. Acad. Sci. U. S. A. 88, 11344–11348.

Denk, F., Huang, W., Sidders, B., Bithell, A., Crow, M., Grist, J., Sharma, S., Ziemek, D., Rice, A.S.C., Buckley, N.J., McMahon, S.B., 2013. HDAC inhibitors attenuate the development of hypersensitivity in models of neuropathic pain. PAIN 154, 1668–1679. doi:10.1016/j.pain.2013.05.021

Denk, F., McMahon, S.B., 2012. Chronic Pain: Emerging Evidence for the Involvement of Epigenetics. Neuron 73, 435–444. doi:10.1016/j.neuron.2012.01.012

Deutsch, S.I., Rosse, R.B., Mastropaolo, J., Long, K.D., Gaskins, B.L., 2008. Epigenetic therapeutic strategies for the treatment of neuropsychiatric disorders: ready for prime time? Clin. Neuropharmacol. 31, 104–119. doi:10.1097/WNF.0b013e318067e255

Deval, E., Gasull, X., Noël, J., Salinas, M., Baron, A., Diochot, S., Lingueglia, E., 2010. Acid-Sensing Ion Channels (ASICs): Pharmacology and implication in pain. Pharmacol. Ther. 128, 549–558. doi:10.1016/j.pharmthera.2010.08.006

Dhaka, A., Murray, A.N., Mathur, J., Earley, T.J., Petrus, M.J., Patapoutian, A., 2007. TRPM8 is required for cold sensation in mice. Neuron 54, 371–378. doi:10.1016/j.neuron.2007.02.024

Dhaka, A., Viswanath, V., Patapoutian, A., 2006. Trp ion channels and temperature sensation. Annu. Rev. Neurosci. 29, 135–161. doi:10.1146/annurev.neuro.29.051605.112958

Dias, B.G., Ressler, K.J., 2013. Parental olfactory experience influences behavior and neural structure in subsequent generations. Nat. Neurosci. 17, 89–96. doi:10.1038/nn.3594

Dickenson, A.H., Sullivan, A.F., 1987a. Peripheral origins and central modulation of subcutaneous formalin-induced activity of rat dorsal horn neurones. Neurosci. Lett. 83, 207–211.

Dickenson, A.H., Sullivan, A.F., 1987b. Subcutaneous formalin-induced activity of dorsal horn neurones in the rat: differential response to an intrathecal opiate administered pre or post formalin. Pain 30, 349–360.

Dina, O.A., Green, P.G., Levine, J.D., 2008. Role of interleukin-6 in chronic muscle hyperalgesic priming. Neuroscience 152, 521–525. doi:10.1016/j.neuroscience.2008.01.006

Ding, Y., Cesare, P., Drew, L., Nikitaki, D., Wood, J.N., 2000. ATP, P2X receptors and pain pathways. J. Auton. Nerv. Syst. 81, 289–294. doi:10.1016/S0165-1838(00)00131-4

Djouhri, L., Koutsikou, S., Fang, X., McMullan, S., Lawson, S.N., 2006. Spontaneous pain, both neuropathic and inflammatory, is related to frequency of spontaneous firing in intact C-fiber nociceptors. J. Neurosci. Off. J. Soc. Neurosci. 26, 1281–1292. doi:10.1523/JNEUROSCI.3388-05.2006

Djouhri, L., Lawson, S.N., 2004. Abeta-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. Brain Res. Brain Res. Rev. 46, 131–145. doi:10.1016/j.brainresrev.2004.07.015

Doehring, A., Geisslinger, G., Lötsch, J., 2011. Epigenetics in pain and analgesia: an imminent research field. Eur. J. Pain Lond. Engl. 15, 11–16. doi:10.1016/j.ejpain.2010.06.004

Dogrul, A., Ossipov, M.H., Porreca, F., 2009. Differential mediation of descending pain facilitation and inhibition by spinal 5HT-3 and 5HT-7 receptors. Brain Res. 1280, 52–59. doi:10.1016/j.brainres.2009.05.001

Dolmetsch, R.E., Pajvani, U., Fife, K., Spotts, J.M., Greenberg, M.E., 2001. Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. Science 294, 333–339. doi:10.1126/science.1063395

Dong, X., Han, S., Zylka, M.J., Simon, M.I., Anderson, D.J., 2001. A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. Cell 106, 619–632.

Doyle, C.A., Hunt, S.P., 1999. Substance P receptor (neurokinin-1)-expressing neurons in lamina I of the spinal cord encode for the intensity of noxious stimulation: a c-Fos study in rat. Neuroscience 89, 17–28.

Dubuisson, D., Dennis, S.G., 1977. The formalin test: a quantitative study of the analegesic effects of morphine, meperidine and brain stimulation in rats and cats. Pain 4, 161–174.

Eijkelkamp, N., Linley, J.E., Torres, J.M., Bee, L., Dickenson, A.H., Gringhuis, M., Minett, M.S., Hong, G.S., Lee, E., Oh, U., Ishikawa, Y., Zwartkuis, F.J., Cox, J.J., Wood, J.N., 2013. A role for Piezo2 in EPAC1-dependent mechanical allodynia. Nat. Commun. 4, 1682. doi:10.1038/ncomms2673

England, S., de Groot, M.J., 2009. Subtype-selective targeting of voltage-gated sodium channels. Br. J. Pharmacol. 158, 1413–1425. doi:10.1111/j.1476-5381.2009.00437.x

English, J.D., Sweatt, J.D., 1997. A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. J. Biol. Chem. 272, 19103–19106.

Espinosa, J.S., Stryker, M.P., 2012. Development and Plasticity of the Primary Visual Cortex. Neuron 75, 230–249. doi:10.1016/j.neuron.2012.06.009

Esteban, J.A., Shi, S.-H., Wilson, C., Nuriya, M., Huganir, R.L., Malinow, R., 2003. PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. Nat. Neurosci. 6, 136–143. doi:10.1038/nn997

Fagni, L., Chavis, P., Ango, F., Bockaert, J., 2000. Complex interactions between mGluRs, intracellular Ca2+ stores and ion channels in neurons. Trends Neurosci. 23, 80–88.

Fajardo, O., Meseguer, V., Belmonte, C., Viana, F., 2008. TRPA1 channels mediate cold temperature sensing in mammalian vagal sensory neurons:

pharmacological and genetic evidence. J. Neurosci. Off. J. Soc. Neurosci. 28, 7863–7875. doi:10.1523/JNEUROSCI.1696-08.2008

Fang, L., Wu, J., Lin, Q., Willis, W.D., 2002. Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. J. Neurosci. Off. J. Soc. Neurosci. 22, 4196–4204. doi:20026343

Fang, L., Wu, J., Lin, Q., Willis, W.D., 2003. Protein kinases regulate the phosphorylation of the GluR1 subunit of AMPA receptors of spinal cord in rats following noxious stimulation. Brain Res. Mol. Brain Res. 118, 160–165.

Fang, L., Wu, J., Zhang, X., Lin, Q., Willis, W.D., 2003. Increased phosphorylation of the GluR1 subunit of spinal cord alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor in rats following intradermal injection of capsaicin. Neuroscience 122, 237–245.

Fenselau, H., Heinke, B., Sandkühler, J., 2011. Heterosynaptic long-term potentiation at GABAergic synapses of spinal lamina I neurons. J. Neurosci. Off. J. Soc. Neurosci. 31, 17383–17391. doi:10.1523/JNEUROSCI.3076-11.2011

Ferreira, S.H., Nakamura, M., de Abreu Castro, M.S., 1978. The hyperalgesic effects of prostacyclin and prostaglandin E2. Prostaglandins 16, 31–37.

Fields, H.L., Basbaum, A.I., Clanton, C.H., Anderson, S.D., 1977. Nucleus raphe magnus inhibition of spinal cord dorsal horn neurons. Brain Res. 126, 441–453.

Finkbeiner, S., Tavazoie, S.F., Maloratsky, A., Jacobs, K.M., Harris, K.M., Greenberg, M.E., 1997. CREB: a major mediator of neuronal neurotrophin responses. Neuron 19, 1031–1047.

Fischer, A., Sananbenesi, F., Wang, X., Dobbin, M., Tsai, L.-H., 2007. Recovery of learning and memory is associated with chromatin remodelling. Nature 447, 178–182. doi:10.1038/nature05772

Fischer, M., Carli, G., Raboisson, P., Reeh, P., 2014. The interphase of the formalin test. Pain 155, 511–521. doi:10.1016/j.pain.2013.11.015

Frenguelli, B.G., Correa, S.A.L., 2013. Regulation and Role of MSK in the Mammalian Brain, in: MSKs Ed Arthur J.S.C. Landes Bioscience.

Gao, Y.-J., Ji, R.-R., 2009. c-Fos or pERK, Which is a Better Marker for Neuronal Activation and Central Sensitization After Noxious Stimulation and Tissue Injury? Open Pain J. 2, 11–17. doi:10.2174/1876386300902010011

Gao, Y.-J., Ji, R.-R., 2010. Light touch induces ERK activation in superficial dorsal horn neurons after inflammation: involvement of spinal astrocytes and JNK signaling in touch-evoked central sensitization and mechanical allodynia. J. Neurochem. 115, 505–514. doi:10.1111/j.1471-4159.2010.06946.x

Garell, P.C., McGillis, S.L., Greenspan, J.D., 1996. Mechanical response properties of nociceptors innervating feline hairy skin. J. Neurophysiol. 75, 1177–1189.

Gauriau, C., Bernard, J.-F., 2002. Pain pathways and parabrachial circuits in the rat. Exp. Physiol. 87, 251–258.

Gauriau, C., Bernard, J.-F., 2004. A comparative reappraisal of projections from the superficial laminae of the dorsal horn in the rat: The forebrain. J. Comp. Neurol. 468, 24–56. doi:10.1002/cne.10873

Gebhart, G.F., 2004. Descending modulation of pain. Neurosci. Biobehav. Rev. 27, 729–737. doi:10.1016/j.neubiorev.2003.11.008

Genoux, D., Haditsch, U., Knobloch, M., Michalon, A., Storm, D., Mansuy, I.M., 2002. Protein phosphatase 1 is a molecular constraint on learning and memory. Nature 418, 970–975. doi:10.1038/nature00928

Géranton, S.M., 2011. Targeting epigenetic mechanisms for pain relief. Curr. Opin. Pharmacol. doi:10.1016/j.coph.2011.10.012

Géranton, S.M., Fratto, V., Tochiki, K.K., Hunt, S.P., 2008. Descending serotonergic controls regulate inflammation-induced mechanical sensitivity and methyl-CpG-binding protein 2 phosphorylation in the rat superficial dorsal horn. Mol. Pain 4, 35. doi:10.1186/1744-8069-4-35

Géranton, S.M., Morenilla-Palao, C., Hunt, S.P., 2007. A role for transcriptional repressor methyl-CpG-binding protein 2 and plasticity-related gene serum- and glucocorticoid-inducible kinase 1 in the induction of inflammatory pain states. J. Neurosci. Off. J. Soc. Neurosci. 27, 6163–6173. doi:10.1523/JNEUROSCI.1306-07.2007

Géranton, S.M., Tochiki, K.K., In press. Regulation of gene expression and pain states by epigenetic mechanisms, in: Molecular Biology of Pain Eds Dussor G. and Price T.J. Elsevier.

Ghosh, A., Greenberg, M., 1995. Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science 268, 239–247. doi:10.1126/science.7716515

Gilchrist, H.D., Allard, B.L., Simone, D.A., 1996. Enhanced withdrawal responses to heat and mechanical stimuli following intraplantar injection of capsaicin in rats. Pain 67, 179–188.

Gilron, I., Quirion, R., Coderre, T.J., 1999. Pre- versus postinjury effects of intravenous GABAergic anesthetics on formalin-induced Fos immunoreactivity in the rat spinal cord. Anesth. Analg. 88, 414–420.

Ginty, D.D., Bonni, A., Greenberg, M.E., 1994. Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. Cell 77, 713–725. doi:10.1016/0092-8674(94)90055-8

Glaum, S.R., Proudfit, H.K., Anderson, E.G., 1990. 5-HT3 receptors modulate spinal nociceptive reflexes. Brain Res. 510, 12–16.

Gogas, K.R., Presley, R.W., Levine, J.D., Basbaum, A.I., 1991. The antinociceptive action of supraspinal opioids results from an increase in descending inhibitory control: correlation of nociceptive behavior and c-fos expression. Neuroscience 42, 617–628.

Goldberg, Y.P., MacFarlane, J., MacDonald, M.L., Thompson, J., Dube, M.-P., Mattice, M., Fraser, R., Young, C., Hossain, S., Pape, T., Payne, B., Radomski, C., Donaldson, G., Ives, E., Cox, J., Younghusband, H.B., Green, R., Duff, A., Boltshauser, E., Grinspan, G.A., Dimon, J.H., Sibley, B.G., Andria, G., Toscano, E., Kerdraon, J., Bowsher, D., Pimstone, S.N., Samuels, M.E., Sherrington, R., Hayden, M.R., 2007. Loss-of-function mutations in the Nav1.7 gene underlie congenital indifference to pain in multiple human populations. Clin. Genet. 71, 311–319. doi:10.1111/j.1399-0004.2007.00790.x

Gräff, J., Rei, D., Guan, J.-S., Wang, W.-Y., Seo, J., Hennig, K.M., Nieland, T.J.F., Fass, D.M., Kao, P.F., Kahn, M., Su, S.C., Samiei, A., Joseph, N., Haggarty, S.J., Delalle, I., Tsai, L.-H., 2012a. An epigenetic blockade of cognitive functions in the neurodegenerating brain. Nature. doi:10.1038/nature10849

Gräff, J., Tsai, L.-H., 2013. Histone acetylation: molecular mnemonics on the chromatin. Nat. Rev. Neurosci. 14, 97–111. doi:10.1038/nrn3427

Gräff, J., Woldemichael, B.T., Berchtold, D., Dewarrat, G., Mansuy, I.M., 2012b. Dynamic histone marks in the hippocampus and cortex facilitate memory consolidation. Nat. Commun. 3, 991. doi:10.1038/ncomms1997

Grasshoff, C., Antkowiak, B., 2006. Effects of isoflurane and enflurane on GABAA and glycine receptors contribute equally to depressant actions on spinal ventral horn neurones in rats. Br. J. Anaesth. 97, 687–694. doi:10.1093/bja/ael239

Greenberg, M.E., Ziff, E.B., Greene, L.A., 1986. Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. Science 234, 80–83.

Green, G.M., Scarth, J., Dickenson, A., 2000. An excitatory role for 5-HT in spinal inflammatory nociceptive transmission; state-dependent actions via dorsal horn 5-HT(3) receptors in the anaesthetized rat. Pain 89, 81–88.

Griffin, R.S., Costigan, M., Brenner, G.J., Ma, C.H.E., Scholz, J., Moss, A., Allchorne, A.J., Stahl, G.L., Woolf, C.J., 2007. Complement induction in spinal cord microglia results in anaphylatoxin C5a-mediated pain hypersensitivity. J.

Neurosci. Off. J. Soc. Neurosci. 27, 8699–8708. doi:10.1523/JNEUROSCI.2018-07.2007

Grudt, T.J., Perl, E.R., 2002. Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. J. Physiol. 540, 189–207.

Guan, J.-S., Haggarty, S.J., Giacometti, E., Dannenberg, J.-H., Joseph, N., Gao, J., Nieland, T.J.F., Zhou, Y., Wang, X., Mazitschek, R., Bradner, J.E., DePinho, R.A., Jaenisch, R., Tsai, L.-H., 2009. HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459, 55–60. doi:10.1038/nature07925

Guo, W., Robbins, M.T., Wei, F., Zou, S., Dubner, R., Ren, K., 2006. Supraspinal brain-derived neurotrophic factor signaling: a novel mechanism for descending pain facilitation. J. Neurosci. Off. J. Soc. Neurosci. 26, 126–137. doi:10.1523/JNEUROSCI.3686-05.2006

Gupta, S., Kim, S.Y., Artis, S., Molfese, D.L., Schumacher, A., Sweatt, J.D., Paylor, R.E., Lubin, F.D., 2010. Histone Methylation Regulates Memory Formation. J. Neurosci. 30, 3589–3599. doi:10.1523/JNEUROSCI.3732-09.2010

Gutièrrez-Mecinas, M., Trollope, A.F., Collins, A., Morfett, H., Hesketh, S.A., Kersanté, F., Reul, J.M.H.M., 2011. Long-lasting behavioral responses to stress involve a direct interaction of glucocorticoid receptors with ERK1/2-MSK1-Elk-1 signaling. Proc. Natl. Acad. Sci. U. S. A. 108, 13806–13811. doi:10.1073/pnas.1104383108

Hagenston, A.M., Bading, H., 2011. Calcium Signaling in Synapse-to-Nucleus Communication. Cold Spring Harb. Perspect. Biol. 3, a004564–a004564. doi:10.1101/cshperspect.a004564

Haley, J.E., Sullivan, A.F., Dickenson, A.H., 1990. Evidence for spinal N-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat. Brain Res. 518, 218–226.

Hamon, M., Bourgoin, S., 1999. Serotonin and its receptors in pain control., in: Sawynok, J., Cowan, A. (Eds.), Novel Aspects of Pain Management: Opioids and Beyond. Wiley, pp. 203–228.

Handwerker, H.O., Kobal, G., 1993. Psychophysiology of experimentally induced pain. Physiol. Rev. 73, 639–671.

Hannon, J., Hoyer, D., 2008. Molecular biology of 5-HT receptors. Behav. Brain Res. 195, 198–213. doi:10.1016/j.bbr.2008.03.020

Han, Z.-S., Zhang, E.-T., Craig, A.D., 1998. Nociceptive and thermoreceptive lamina I neurons are anatomically distinct. Nat. Neurosci. 1, 218–225. doi:10.1038/665

Hao, S., Mata, M., Wolfe, D., Huang, S., Glorioso, J.C., Fink, D.J., 2005. Gene transfer of glutamic acid decarboxylase reduces neuropathic pain. Ann. Neurol. 57, 914–918. doi:10.1002/ana.20483

Hardingham, G.E., Chawla, S., Cruzalegui, F.H., Bading, H., 1999. Control of recruitment and transcription-activating function of CBP determines gene regulation by NMDA receptors and L-type calcium channels. Neuron 22, 789–798.

Heard, E., Martienssen, R.A., 2014. Transgenerational Epigenetic Inheritance: Myths and Mechanisms. Cell 157, 95–109. doi:10.1016/j.cell.2014.02.045

Henry, J.L., 1976. Effects of substance P on functionally identified units in cat spinal cord. Brain Res. 114, 439–451.

Henry, J.L., Yashpal, K., Pitcher, G.M., Chabot, J., Coderre, T.J., 1999. Evidence for tonic activation of NK-1 receptors during the second phase of the formalin test in the Rat. J. Neurosci. Off. J. Soc. Neurosci. 19, 6588–6598.

Herz, A., Albus, K., Metys, J., Schubert, P., Teschemacher, H., 1970. On the central sites for the antinociceptive action of morphine and fentanyl. Neuropharmacology 9, 539–551.

Honoré, P., Buritova, J., Besson, J.-M., 1995. Aspirin and acetaminophen reduced both Fos expression in rat lumbar spinal cord and inflammatory signs produced by

carrageenin inflammation. Pain 63, 365–375. doi:10.1016/0304-3959(95)00065-8

Honor, P., Menning, P.M., Rogers, S.D., Nichols, M.L., Basbaum, A.I., Besson, J.M., Mantyh, P.W., 1999. Spinal substance P receptor expression and internalization in acute, short-term, and long-term inflammatory pain states. J. Neurosci. Off. J. Soc. Neurosci. 19, 7670–7678.

Hosobuchi, Y., Adams, J.E., Linchitz, R., 1977. Pain relief by electrical stimulation of the central gray matter in humans and its reversal by naloxone. Science 197, 183–186.

Hossaini, M., Duraku, L.S., Saraç, C., Jongen, J.L.M., Holstege, J.C., 2010a. Differential distribution of activated spinal neurons containing glycine and/or GABA and expressing c-fos in acute and chronic pain models. Pain 151, 356–365. doi:10.1016/j.pain.2010.07.023

Hossaini, M., Jongen, J.L., Biesheuvel, K., Kuhl, D., Holstege, J.C., 2010b. Nociceptive stimulation induces expression of Arc/Arg3.1 in the spinal cord with a preference for neurons containing enkephalin. Mol. Pain 6, 43. doi:10.1186/1744-8069-6-43

Hou, W.Y., Shyu, B.C., Chen, T.M., Lee, J.W., Shieh, J.Y., Sun, W.Z., 1997. Intrathecally administered c-fos antisense oligodeoxynucleotide decreases formalin-induced nociceptive behavior in adult rats. Eur. J. Pharmacol. 329, 17–26.

Hsu, J.Y., Sun, Z.W., Li, X., Reuben, M., Tatchell, K., Bishop, D.K., Grushcow, J.M., Brame, C.J., Caldwell, J.A., Hunt, D.F., Lin, R., Smith, M.M., Allis, C.D., 2000. Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. Cell 102, 279–291.

Huang, J., Spier, A.D., Pickel, V.M., 2004. 5-HT3A receptor subunits in the rat medial nucleus of the solitary tract: subcellular distribution and relation to the serotonin transporter. Brain Res. 1028, 156–169. doi:10.1016/j.brainres.2004.09.009

Hucho, T., Levine, J.D., 2007. Signaling pathways in sensitization: toward a nociceptor cell biology. Neuron 55, 365–376. doi:10.1016/j.neuron.2007.07.008

Hughes, P., Dragunow, M., 1995. Induction of immediate-early genes and the control of neurotransmitter-regulated gene expression within the nervous system. Pharmacol. Rev. 47, 133–178.

Hu, H.-J., Alter, B.J., Carrasquillo, Y., Qiu, C.-S., Gereau, R.W., 2007. Metabotropic glutamate receptor 5 modulates nociceptive plasticity via extracellular signal-regulated kinase-Kv4.2 signaling in spinal cord dorsal horn neurons. J. Neurosci. Off. J. Soc. Neurosci. 27, 13181–13191. doi:10.1523/JNEUROSCI.0269-07.2007

Hu, H.-J., Glauner, K.S., Gereau, R.W., 2003. ERK integrates PKA and PKC signaling in superficial dorsal horn neurons. I. Modulation of A-type K+ currents. J. Neurophysiol. 90, 1671–1679. doi:10.1152/jn.00340.2003

Hunter, J.C., Woodburn, V.L., Durieux, C., Pettersson, E.K., Poat, J.A., Hughes, J., 1995. c-fos antisense oligodeoxynucleotide increases formalin-induced nociception and regulates preprodynorphin expression. Neuroscience 65, 485–492.

Hunt, S.P., Mantyh, P.W., 2001. The molecular dynamics of pain control. Nat. Rev. Neurosci. 2, 83–91. doi:10.1038/35053509

Hunt, S.P., Pini, A., Evan, G., 1987. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature 328, 632–634. doi:10.1038/328632a0

Hunt, S.P., Rossi, J., 1985. Peptide- and non-peptide-containing unmyelinated primary afferents: the parallel processing of nociceptive information. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 308, 283–289.

Iannetti, G.D., Mouraux, A., 2010. From the neuromatrix to the pain matrix (and back). Exp. Brain Res. 205, 1–12. doi:10.1007/s00221-010-2340-1

Ibrahim, M.M., Mata, H.P., Chawla, M., Lai, J., Porreca, F., Malan, T.P., 2001. Allodynia and hyperalgesia produced by specific inhibition of spinal c-fos expression: lack of correlation with dynorphin content. J. Pain Off. J. Am. Pain Soc. 2, 241–249. doi:10.1054/jpai.2001.23135

Ikeda, H., Heinke, B., Ruscheweyh, R., Sandkühler, J., 2003. Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. Science 299, 1237–1240. doi:10.1126/science.1080659

Ikeda, H., Stark, J., Fischer, H., Wagner, M., Drdla, R., Jäger, T., Sandkühler, J., 2006. Synaptic amplifier of inflammatory pain in the spinal dorsal horn. Science 312, 1659–1662. doi:10.1126/science.1127233

Imbe, H., Okamoto, K., Okamura, T., Kumabe, S., Nakatsuka, M., Aikawa, F., Iwai-Liao, Y., Senba, E., 2005. Effects of peripheral inflammation on activation of ERK in the rostral ventromedial medulla. Brain Res. 1063, 151–158. doi:10.1016/j.brainres.2005.09.057

Impey, S., Fong, A.L., Wang, Y., Cardinaux, J.R., Fass, D.M., Obrietan, K., Wayman, G.A., Storm, D.R., Soderling, T.R., Goodman, R.H., 2002. Phosphorylation of CBP mediates transcriptional activation by neural activity and CaM kinase IV. Neuron 34, 235–244.

Impey, S., Mark, M., Villacres, E.C., Poser, S., Chavkin, C., Storm, D.R., 1996. Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. Neuron 16, 973–982.

Impey, S., Obrietan, K., Storm, D.R., 1999. Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. Neuron 23, 11–14.

International Association for the Study of Pain, n.d. International Association for the Study of Pain | IASP Taxonomy [WWW Document]. Int. Assoc. Study Pain.

Jasmin, L., Wang, H., Tarczy-Hornoch, K., Levine, J.D., Basbaum, A.I., 1994. Differential effects of morphine on noxious stimulus-evoked fos-like immunoreactivity in subpopulations of spinoparabrachial neurons. J. Neurosci. Off. J. Soc. Neurosci. 14, 7252–7260.

Jenuwein, T., Allis, C.D., 2001. Translating the histone code. Science 293, 1074–1080. doi:10.1126/science.1063127

Jinks, S.L., Antognini, J.F., Martin, J.T., Jung, S.-W., Carstens, E., Atherley, R., 2002. Isoflurane, but not halothane, depresses c-fos expression in rat spinal cord at concentrations that suppress reflex movement after supramaximal noxious stimulation. Anesth. Analg. 95, 1622–1628, table of contents.

Ji, R.-R., Baba, H., Brenner, G.J., Woolf, C.J., 1999. Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nat. Neurosci. 2, 1114–1119. doi:10.1038/16040

Ji, R.-R., Befort, K., Brenner, G.J., Woolf, C.J., 2002a. ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity. J. Neurosci. Off. J. Soc. Neurosci. 22, 478–485.

Ji, R.-R., Gereau, R.W., Malcangio, M., Strichartz, G.R., 2009. MAP kinase and pain. Brain Res. Rev. 60, 135–148. doi:10.1016/j.brainresrev.2008.12.011

Ji, R.-R., Kohno, T., Moore, K.A., Woolf, C.J., 2003. Central sensitization and LTP: do pain and memory share similar mechanisms? Trends Neurosci. 26, 696–705.

Ji, R.R., Rupp, F., 1997. Phosphorylation of transcription factor CREB in rat spinal cord after formalin-induced hyperalgesia: relationship to c-fos induction. J. Neurosci. Off. J. Soc. Neurosci. 17, 1776–1785.

Ji, R.-R., Samad, T.A., Jin, S.-X., Schmoll, R., Woolf, C.J., 2002b. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. Neuron 36, 57–68.

Julius, D., Basbaum, A.I., 2001. Molecular mechanisms of nociception. Nature 413, 203–210. doi:10.1038/35093019

Kandel, E.R., Dudai, Y., Mayford, M.R., 2014. The Molecular and Systems Biology of Memory. Cell 157, 163–186. doi:10.1016/j.cell.2014.03.001

Karashima, Y., Talavera, K., Everaerts, W., Janssens, A., Kwan, K.Y., Vennekens, R., Nilius, B., Voets, T., 2009. TRPA1 acts as a cold sensor in vitro and in vivo. Proc. Natl. Acad. Sci. U. S. A. 106, 1273–1278. doi:10.1073/pnas.0808487106

Karelina, K., Hansen, K.F., Choi, Y.-S., Devries, A.C., Arthur, J.S.C., Obrietan, K., 2012. MSK1 regulates environmental enrichment-induced hippocampal plasticity and cognitive enhancement. Learn. Mem. Cold Spring Harb. N 19, 550–560. doi:10.1101/lm.025775.112

Karim, F., Wang, C.C., Gereau, R.W., 4th, 2001. Metabotropic glutamate receptor subtypes 1 and 5 are activators of extracellular signal-regulated kinase signaling required for inflammatory pain in mice. J. Neurosci. Off. J. Soc. Neurosci. 21, 3771–3779.

Kawasaki, Y., Kohno, T., Zhuang, Z.-Y., Brenner, G.J., Wang, H., Van Der Meer, C., Befort, K., Woolf, C.J., Ji, R.-R., 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. J. Neurosci. Off. J. Soc. Neurosci. 24, 8310–8321. doi:10.1523/JNEUROSCI.2396-04.2004

Kayser, V., Elfassi, I.E., Aubel, B., Melfort, M., Julius, D., Gingrich, J.A., Hamon, M., Bourgoin, S., 2007. Mechanical, thermal and formalin-induced nociception is differentially altered in 5-HT1A-/-, 5-HT1B-/-, 5-HT2A-/-, 5-HT3A-/- and 5-HTT-/- knock-out male mice. Pain 130, 235–248. doi:10.1016/j.pain.2006.11.015

Kelleher, R.J., 3rd, Govindarajan, A., Jung, H.-Y., Kang, H., Tonegawa, S., 2004. Translational control by MAPK signaling in long-term synaptic plasticity and memory. Cell 116, 467–479.

Kelleher, R.J., Govindarajan, A., Tonegawa, S., 2004. Translational Regulatory Mechanisms in Persistent Forms of Synaptic Plasticity. Neuron 44, 59–73. doi:10.1016/j.neuron.2004.09.013

Khasabov, S.G., Rogers, S.D., Ghilardi, J.R., Peters, C.M., Mantyh, P.W., Simone, D.A., 2002. Spinal neurons that possess the substance P receptor are required for the development of central sensitization. J. Neurosci. Off. J. Soc. Neurosci. 22, 9086–9098.

Kiguchi, N., Kobayashi, Y., Maeda, T., Fukazawa, Y., Tohya, K., Kimura, M., Kishioka, S., 2012. Epigenetic augmentation of the macrophage inflammatory protein 2/C-X-C chemokine receptor type 2 axis through histone H3 acetylation in injured peripheral nerves elicits neuropathic pain. J. Pharmacol. Exp. Ther. 340, 577–587. doi:10.1124/jpet.111.187724

Kiguchi, N., Kobayashi, Y., Saika, F., Kishioka, S., 2013. Epigenetic upregulation of CCL2 and CCL3 via histone modifications in infiltrating macrophages after peripheral nerve injury. Cytokine. doi:10.1016/j.cyto.2013.09.019

Kim, S.E., Coste, B., Chadha, A., Cook, B., Patapoutian, A., 2012. The role of Drosophila Piezo in mechanical nociception. Nature 483, 209–212. doi:10.1038/nature10801

Kobayashi, K., Fukuoka, T., Obata, K., Yamanaka, H., Dai, Y., Tokunaga, A., Noguchi, K., 2005. Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with adelta/c-fibers and colocalization with trk receptors. J. Comp. Neurol. 493, 596–606. doi:10.1002/cne.20794

Kosai, K., Tateyama, S., Ikeda, T., Uno, T., Nishimori, T., Takasaki, M., 2001. MK-801 reduces non-noxious stimulus-evoked Fos-like immunoreactivity in the spinal cord of rats with chronic constriction nerve injury. Brain Res. 910, 12–18.

Koshibu, K., Graff, J., Beullens, M., Heitz, F.D., Berchtold, D., Russig, H., Farinelli, M., Bollen, M., Mansuy, I.M., 2009. Protein Phosphatase 1 Regulates the Histone Code for Long-Term Memory. J. Neurosci. 29, 13079–13089. doi:10.1523/JNEUROSCI.3610-09.2009

Koshibu, K., Gräff, J., Mansuy, I.M., 2011. Nuclear protein phosphatase-1: an epigenetic regulator of fear memory and amygdala long-term potentiation. Neuroscience 173, 30–36. doi:10.1016/j.neuroscience.2010.11.023

Kouzarides, T., 2007. Chromatin modifications and their function. Cell 128, 693–705. doi:10.1016/j.cell.2007.02.005

Kumar, A., Choi, K.-H., Renthal, W., Tsankova, N.M., Theobald, D.E.H., Truong, H.-T., Russo, S.J., LaPlant, Q., Sasaki, T.S., Whistler, K.N., 2005. Chromatin Remodeling Is a Key Mechanism Underlying Cocaine-Induced Plasticity in Striatum. Neuron 48, 303–314. doi:10.1016/j.neuron.2005.09.023

Kuner, R., 2010. Central mechanisms of pathological pain. Nat. Med. 16, 1258–1266. doi:10.1038/nm.2231

Kwan, K.Y., Allchorne, A.J., Vollrath, M.A., Christensen, A.P., Zhang, D.-S., Woolf, C.J., Corey, D.P., 2006. TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. Neuron 50, 277–289. doi:10.1016/j.neuron.2006.03.042

Kwan, K.Y., Glazer, J.M., Corey, D.P., Rice, F.L., Stucky, C.L., 2009. TRPA1 modulates mechanotransduction in cutaneous sensory neurons. J. Neurosci. Off. J. Soc. Neurosci. 29, 4808–4819. doi:10.1523/JNEUROSCI.5380-08.2009

Kwiat, G.C., Basbaum, A.I., 1992. The origin of brainstem noradrenergic and serotonergic projections to the spinal cord dorsal horn in the rat. Somatosens. Mot. Res. 9, 157–173.

Lacroix-Fralish, M.L., Tawfik, V.L., Tanga, F.Y., Spratt, K.F., DeLeo, J.A., 2006. Differential spinal cord gene expression in rodent models of radicular and neuropathic pain. Anesthesiology 104, 1283–1292.

Lagraize, S.C., Guo, W., Yang, K., Wei, F., Ren, K., Dubner, R., 2010. Spinal cord mechanisms mediating behavioral hyperalgesia induced by neurokinin-1 tachykinin receptor activation in the rostral ventromedial medulla. Neuroscience 171, 1341–1356. doi:10.1016/j.neuroscience.2010.09.040

Lai, H.H., Qiu, C.-S., Crock, L.W., Morales, M.E.P., Ness, T.J., Gereau, R.W., 4th, 2011. Activation of spinal extracellular signal-regulated kinases (ERK) 1/2 is associated with the development of visceral hyperalgesia of the bladder. Pain 152, 2117–2124. doi:10.1016/j.pain.2011.05.017

Laing, I., Todd, A.J., Heizmann, C.W., Schmidt, H.H., 1994. Subpopulations of GABAergic neurons in laminae I-III of rat spinal dorsal horn defined by coexistence with classical transmitters, peptides, nitric oxide synthase or parvalbumin. Neuroscience 61, 123–132.

Lanahan, A., Worley, P., 1998. Immediate-early genes and synaptic function. Neurobiol. Learn. Mem. 70, 37–43. doi:10.1006/nlme.1998.3836

Landry, C.D., Kandel, E.R., Rajasethupathy, P., 2013. New mechanisms in memory storage: piRNAs and epigenetics. Trends Neurosci. 36, 535–542. doi:10.1016/j.tins.2013.05.004

Langley, C.K., Aziz, Q., Bountra, C., Gordon, N., Hawkins, P., Jones, A., Langley, G., Nurmikko, T., Tracey, I., 2008. Volunteer studies in pain research--opportunities and challenges to replace animal experiments: the report and recommendations of a Focus on Alternatives workshop. NeuroImage 42, 467–473. doi:10.1016/j.neuroimage.2008.05.030

Larsson, M., Broman, J., 2008. Translocation of GluR1-containing AMPA receptors to a spinal nociceptive synapse during acute noxious stimulation. J. Neurosci. Off. J. Soc. Neurosci. 28, 7084–7090. doi:10.1523/JNEUROSCI.5749-07.2008

Latremoliere, A., Woolf, C.J., 2009. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J. Pain Off. J. Am. Pain Soc. 10, 895–926. doi:10.1016/j.jpain.2009.06.012

Lau, C.G., Zukin, R.S., 2007. NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. Nat. Rev. Neurosci. 8, 413–426. doi:10.1038/nrn2153

Lawson, S.N., Crepps, B.A., Perl, E.R., 1997. Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. J. Physiol. 505 (Pt 1), 177–191.

Lawson, S.N., Waddell, P.J., 1991. Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. J. Physiol. 435, 41–63.

Le Bars, D., Gozariu, M., Cadden, S.W., 2001. Animal models of nociception. Pharmacol. Rev. 53, 597–652.

Lee, J.-H., Park, C.-K., Chen, G., Han, Q., Xie, R.-G., Liu, T., Ji, R.-R., Lee, S.-Y., 2014. A monoclonal antibody that targets a NaV1.7 channel voltage sensor for pain and itch relief. Cell 157, 1393–1404. doi:10.1016/j.cell.2014.03.064

Lee, K.K., Workman, J.L., 2007. Histone acetyltransferase complexes: one size doesn't fit all. Nat. Rev. Mol. Cell Biol. 8, 284–295. doi:10.1038/nrm2145

Lee, S.-J.R., Escobedo-Lozoya, Y., Szatmari, E.M., Yasuda, R., 2009. Activation of CaMKII in single dendritic spines during long-term potentiation. Nature 458, 299–304. doi:10.1038/nature07842

Legrain, V., Iannetti, G.D., Plaghki, L., Mouraux, A., 2011. The pain matrix reloaded: a salience detection system for the body. Prog. Neurobiol. 93, 111–124. doi:10.1016/j.pneurobio.2010.10.005

Lesburgueres, E., Gobbo, O.L., Alaux-Cantin, S., Hambucken, A., Trifilieff, P., Bontempi, B., 2011. Early Tagging of Cortical Networks Is Required for the Formation of Enduring Associative Memory. Science 331, 924–928. doi:10.1126/science.1196164

Lester, B.M., Tronick, E., Nestler, E., Abel, T., Kosofsky, B., Kuzawa, C.W., Marsit, C.J., Maze, I., Meaney, M.J., Monteggia, L.M., Reul, J.M.H.M., Skuse, D.H., Sweatt, J.D., Wood, M.A., 2011. Behavioral epigenetics: Behavioral epigenetics. Ann. N. Y. Acad. Sci. 1226, 14–33. doi:10.1111/j.1749-6632.2011.06037.x

Levenson, J.M., O'Riordan, K.J., Brown, K.D., Trinh, M.A., Molfese, D.L., Sweatt, J.D., 2004. Regulation of histone acetylation during memory formation in the hippocampus. J. Biol. Chem. 279, 40545–40559. doi:10.1074/jbc.M402229200

Lever, I.J., Pezet, S., McMahon, S.B., Malcangio, M., 2003. The signaling components of sensory fiber transmission involved in the activation of ERK MAP kinase in the mouse dorsal horn. Mol. Cell. Neurosci. 24, 259–270.

Liang, D.-Y., Li, X., Clark, J.D., 2013. Epigenetic Regulation of Opioid-Induced Hyperalgesia, Dependence, and Tolerance in Mice. J. Pain 14, 36–47. doi:10.1016/j.jpain.2012.10.005

Light, A.R., Perl, E.R., 1979. Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. J. Comp. Neurol. 186, 133–150. doi:10.1002/cne.901860203

Li, J.L., Kaneko, T., Nomura, S., Li, Y.Q., Mizuno, N., 1997. Association of serotonin-like immunoreactive axons with nociceptive projection neurons in the caudal spinal trigeminal nucleus of the rat. J. Comp. Neurol. 384, 127–141.

Li, K., Lin, T., Cao, Y., Light, A.R., Fu, K.-Y., 2010. Peripheral formalin injury induces 2 stages of microglial activation in the spinal cord. J. Pain Off. J. Am. Pain Soc. 11, 1056–1065. doi:10.1016/j.jpain.2010.01.268

Lima, D., Coimbra, A., 1986. A Golgi study of the neuronal population of the marginal zone (lamina I) of the rat spinal cord. J. Comp. Neurol. 244, 53–71. doi:10.1002/cne.902440105

Lin, Y., Bloodgood, B.L., Hauser, J.L., Lapan, A.D., Koon, A.C., Kim, T.-K., Hu, L.S., Malik, A.N., Greenberg, M.E., 2008. Activity-dependent regulation of inhibitory synapse development by Npas4. Nature 455, 1198–1204. doi:10.1038/nature07319

Littlewood, N.K., Todd, A.J., Spike, R.C., Watt, C., Shehab, S.A., 1995. The types of neuron in spinal dorsal horn which possess neurokinin-1 receptors. Neuroscience 66, 597–608.

Liu, Q., Vrontou, S., Rice, F.L., Zylka, M.J., Dong, X., Anderson, D.J., 2007. Molecular genetic visualization of a rare subset of unmyelinated sensory neurons that may detect gentle touch. Nat. Neurosci. 10, 946–948. doi:10.1038/nn1937

Lonze, B.E., Ginty, D.D., 2002. Function and regulation of CREB family transcription factors in the nervous system. Neuron 35, 605–623.

Lorenzo, L.-E., Ramien, M., St Louis, M., De Koninck, Y., Ribeiro-da-Silva, A., 2008. Postnatal changes in the Rexed lamination and markers of nociceptive afferents in the superficial dorsal horn of the rat. J. Comp. Neurol. 508, 592–604. doi:10.1002/cne.21691

Lubin, F.D., Roth, T.L., Sweatt, J.D., 2008. Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. J. Neurosci. Off. J. Soc. Neurosci. 28, 10576–10586. doi:10.1523/JNEUROSCI.1786-08.2008

Luo, C., Gangadharan, V., Bali, K.K., Xie, R.-G., Agarwal, N., Kurejova, M., Tappe-Theodor, A., Tegeder, I., Feil, S., Lewin, G., Polgar, E., Todd, A.J., Schlossmann, J., Hofmann, F., Liu, D.-L., Hu, S.-J., Feil, R., Kuner, T., Kuner, R., 2012. Presynaptically Localized Cyclic GMP-Dependent Protein Kinase 1 Is a Key Determinant of Spinal Synaptic Potentiation and Pain Hypersensitivity. PLoS Biol. 10, e1001283. doi:10.1371/journal.pbio.1001283

Lu, Y., Dong, H., Gao, Y., Gong, Y., Ren, Y., Gu, N., Zhou, S., Xia, N., Sun, Y.-Y., Ji, R.-R., Xiong, L., 2013. A feed-forward spinal cord glycinergic neural circuit gates mechanical allodynia. J. Clin. Invest. 123, 4050–4062. doi:10.1172/JCI70026

Lynn, B., Carpenter, S.E., 1982. Primary afferent units from the hairy skin of the rat hind limb. Brain Res. 238, 29–43. doi:10.1016/0006-8993(82)90768-5

Macpherson, L.J., Xiao, B., Kwan, K.Y., Petrus, M.J., Dubin, A.E., Hwang, S., Cravatt, B., Corey, D.P., Patapoutian, A., 2007. An ion channel essential for sensing chemical damage. J. Neurosci. Off. J. Soc. Neurosci. 27, 11412–11415. doi:10.1523/JNEUROSCI.3600-07.2007

Magerl, W., Fuchs, P.N., Meyer, R.A., Treede, R.D., 2001. Roles of capsaicin-insensitive nociceptors in cutaneous pain and secondary hyperalgesia. Brain J. Neurol. 124, 1754–1764.

Mahadevan, L.C., Willis, A.C., Barratt, M.J., 1991. Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors. Cell 65, 775–783.

Mahan, A.L., Mou, L., Shah, N., Hu, J.-H., Worley, P.F., Ressler, K.J., 2012. Epigenetic Modulation of Homer1a Transcription Regulation in Amygdala and Hippocampus with Pavlovian Fear Conditioning. J. Neurosci. 32, 4651–4659. doi:10.1523/JNEUROSCI.3308-11.2012

Malmberg, A.B., Chen, C., Tonegawa, S., Basbaum, A.I., 1997. Preserved acute pain and reduced neuropathic pain in mice lacking PKCgamma. Science 278, 279–283.

Mannion, R.J., Costigan, M., Decosterd, I., Amaya, F., Ma, Q.P., Holstege, J.C., Ji, R.R., Acheson, A., Lindsay, R.M., Wilkinson, G.A., Woolf, C.J., 1999. Neurotrophins: peripherally and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity. Proc. Natl. Acad. Sci. U. S. A. 96, 9385–9390.

Mantyh, P., DeMaster, E., Malhotra, A., Ghilardi, Rogers, S., Mantyh, C., Liu, H., Basbaum, A., Vigna, Maggio, J., et al., 1995. Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. Science 268, 1629–1632. doi:10.1126/science.7539937

Mantyh, P.W., Rogers, S.D., Honore, P., Allen, B.J., Ghilardi, J.R., Li, J., Daughters, R.S., Lappi, D.A., Wiley, R.G., Simone, D.A., 1997. Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. Science 278, 275–279.

Ma, Q.P., Woolf, C.J., 1995a. Noxious stimuli induce an N-methyl-D-aspartate receptor-dependent hypersensitivity of the flexion withdrawal reflex to touch: implications for the treatment of mechanical allodynia. Pain 61, 383–390.

Ma, Q.P., Woolf, C.J., 1995b. Involvement of neurokinin receptors in the induction but not the maintenance of mechanical allodynia in rat flexor motoneurones. J. Physiol. 486 (Pt 3), 769–777.

Ma, Q.P., Woolf, C.J., 1996. Basal and touch-evoked fos-like immunoreactivity during experimental inflammation in the rat. Pain 67, 307–316.

Marshall, G.E., Shehab, S.A.S., Spike, R.C., Todd, A.J., 1996. Neurokinin-1 receptors on lumbar spinothalamic neurons in the rat. Neuroscience 72, 255–263. doi:10.1016/0306-4522(95)00558-7

Matsushita, Y., Araki, K., Omotuyi, O. idowu, Mukae, T., Ueda, H., 2013. HDAC inhibitors restore C-fibre sensitivity in experimental neuropathic pain model. Br. J. Pharmacol. 170, 991–998. doi:10.1111/bph.12366

Maxwell, D.J., Kerr, R., Rashid, S., Anderson, E., 2003. Characterisation of axon terminals in the rat dorsal horn that are immunoreactive for serotonin 5-HT3A receptor subunits. Exp. Brain Res. 149, 114–124. doi:10.1007/s00221-002-1339-7

Mayer, M.L., Westbrook, G.L., Guthrie, P.B., 1984. Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. Nature 309, 261–263.

McCormack, K., Santos, S., Chapman, M.L., Krafte, D.S., Marron, B.E., West, C.W., Krambis, M.J., Antonio, B.M., Zellmer, S.G., Printzenhoff, D., Padilla, K.M., Lin, Z., Wagoner, P.K., Swain, N.A., Stupple, P.A., de Groot, M., Butt, R.P., Castle, N.A., 2013. Voltage sensor interaction site for selective small molecule inhibitors of voltage-gated sodium channels. Proc. Natl. Acad. Sci. U. S. A. 110, E2724–2732. doi:10.1073/pnas.1220844110

McCoy, C.E., Campbell, D.G., Deak, M., Bloomberg, G.B., Arthur, J.S.C., 2005. MSK1 activity is controlled by multiple phosphorylation sites. Biochem. J. 387, 507–517. doi:10.1042/BJ20041501

McNamara, C.R., Mandel-Brehm, J., Bautista, D.M., Siemens, J., Deranian, K.L., Zhao, M., Hayward, N.J., Chong, J.A., Julius, D., Moran, M.M., Fanger, C.M., 2007. TRPA1 mediates formalin-induced pain. Proc. Natl. Acad. Sci. U. S. A. 104, 13525–13530. doi:10.1073/pnas.0705924104

McQuown, S.C., Barrett, R.M., Matheos, D.P., Post, R.J., Rogge, G.A., Alenghat, T., Mullican, S.E., Jones, S., Rusche, J.R., Lazar, M.A., Wood, M.A., 2011. HDAC3 Is a Critical Negative Regulator of Long-Term Memory Formation. J. Neurosci. 31, 764–774. doi:10.1523/JNEUROSCI.5052-10.2011

Meaney, M.J., Ferguson-Smith, A.C., 2010. Epigenetic regulation of the neural transcriptome: the meaning of the marks. Nat. Neurosci. 13, 1313–1318. doi:10.1038/nn1110-1313

Melzack, R., Wall, P.D., Ty, T.C., 1982. Acute pain in an emergency clinic: latency of onset and descriptor patterns related to different injuries. Pain 14, 33–43.

Mendell, L.M., 1966. Physiological properties of unmyelinated fiber projection to the spinal cord. Exp. Neurol. 16, 316–332. doi:10.1016/0014-4886(66)90068-9

Menétrey, D., Giesler, G.J., Besson, J.M., 1977. An analysis of response properties of spinal cord dorsal horn neurones to nonnoxious and noxious stimuli in the spinal rat. Exp. Brain Res. 27, 15–33.

Millan, M.J., 2002. Descending control of pain. Prog. Neurobiol. 66, 355–474. doi:10.1016/S0301-0082(02)00009-6

Millan, M.J., Girardon, S., Bervoets, K., 1997. 8-OH-DPAT-induced spontaneous tail-flicks in the rat are facilitated by the selective serotonin (5-HT)2C agonist, RO 60-0175: blockade of its actions by the novel 5-HT2C receptor antagonist SB 206,553. Neuropharmacology 36, 743–745.

Minett, M.S., Nassar, M.A., Clark, A.K., Passmore, G., Dickenson, A.H., Wang, F., Malcangio, M., Wood, J.N., 2012. Distinct Nav1.7-dependent pain sensations

require different sets of sensory and sympathetic neurons. Nat. Commun. 3, 791. doi:10.1038/ncomms1795

Miraucourt, L.S., Dallel, R., Voisin, D.L., 2007. Glycine inhibitory dysfunction turns touch into pain through PKCgamma interneurons. PloS One 2, e1116. doi:10.1371/journal.pone.0001116

Mogil, J.S., 2009. Animal models of pain: progress and challenges. Nat. Rev. Neurosci. 10, 283–294. doi:10.1038/nrn2606

Molander, C., Grant, G., 1986. Laminar distribution and somatotopic organization of primary afferent fibers from hindlimb nerves in the dorsal horn. A study by transganglionic transport of horseradish peroxidase in the rat. Neuroscience 19, 297–312.

Molander, C., Hongpaisan, J., Persson, J.K., 1994. Distribution of c-fos expressing dorsal horn neurons after electrical stimulation of low threshold sensory fibers in the chronically injured sciatic nerve. Brain Res. 644, 74–82.

Molander, C., Xu, Q., Grant, G., 1984. The cytoarchitectonic organization of the spinal cord in the rat. I. The lower thoracic and lumbosacral cord. J. Comp. Neurol. 230, 133–141. doi:10.1002/cne.902300112

Molander, C., Xu, Q., Rivero-Melian, C., Grant, G., 1989. Cytoarchitectonic organization of the spinal cord in the rat: II. The cervical and upper thoracic cord. J. Comp. Neurol. 289, 375–385. doi:10.1002/cne.902890303

Molina‐Serrano, D., Schiza, V., Kirmizis, A., 2013. Cross-talk among epigenetic modifications: lessons from histone arginine methylation. Biochem. Soc. Trans. 41, 751–759. doi:10.1042/BST20130003

Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., Doster, K., Wen, D., Yan, Q., Snider, W.D., 1997. IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron 19, 849–861.

Mouraux, A., Diukova, A., Lee, M.C., Wise, R.G., Iannetti, G.D., 2011. A multisensory investigation of the functional significance of the “pain matrix.” NeuroImage 54, 2237–2249. doi:10.1016/j.neuroimage.2010.09.084

Munton, R.P., Vizi, S., Mansuy, I.M., 2004. The role of protein phosphatase-1 in the modulation of synaptic and structural plasticity. FEBS Lett. 567, 121–128. doi:10.1016/j.febslet.2004.03.121

Nagy, G.G., Watanabe, M., Fukaya, M., Todd, A.J., 2004. Synaptic distribution of the NR1, NR2A and NR2B subunits of the N-methyl-d-aspartate receptor in the rat lumbar spinal cord revealed with an antigen-unmasking technique. Eur. J. Neurosci. 20, 3301–3312. doi:10.1111/j.1460-9568.2004.03798.x

Nagy, J.I., Hunt, S.P., 1982. Fluoride-resistant acid phosphatase-containing neurones in dorsal root ganglia are separate from those containing substance P or somatostatin. Neuroscience 7, 89–97.

Naim, M.M., Shehab, S.A., Todd, A.J., 1998. Cells in laminae III and IV of the rat spinal cord which possess the neurokinin-1 receptor receive monosynaptic input from myelinated primary afferents. Eur. J. Neurosci. 10, 3012–3019.

Naim, M., Spike, R.C., Watt, C., Shehab, S.A., Todd, A.J., 1997. Cells in laminae III and IV of the rat spinal cord that possess the neurokinin-1 receptor and have dorsally directed dendrites receive a major synaptic input from tachykinin-containing primary afferents. J. Neurosci. Off. J. Soc. Neurosci. 17, 5536–5548.

Naqvi, S., Macdonald, A., McCoy, C.E., Darragh, J., Reith, A.D., Arthur, J.S.C., 2012. Characterization of the cellular action of the MSK inhibitor SB-747651A. Biochem. J. 441, 347–357. doi:10.1042/BJ20110970

Nardi, A., Damann, N., Hertrampf, T., Kless, A., 2012. Advances in targeting voltage-gated sodium channels with small molecules. ChemMedChem 7, 1712–1740. doi:10.1002/cmdc.201200298

Nassar, M.A., Stirling, L.C., Forlani, G., Baker, M.D., Matthews, E.A., Dickenson, A.H., Wood, J.N., 2004. Nociceptor-specific gene deletion reveals a major role for

Nav1.7 (PN1) in acute and inflammatory pain. Proc. Natl. Acad. Sci. U. S. A. 101, 12706–12711. doi:10.1073/pnas.0404915101

Neher, E., Sakaba, T., 2008. Multiple Roles of Calcium Ions in the Regulation of Neurotransmitter Release. Neuron 59, 861–872. doi:10.1016/j.neuron.2008.08.019

Neumann, S., Braz, J.M., Skinner, K., Llewellyn-Smith, I.J., Basbaum, A.I., 2008. Innocuous, not noxious, input activates PKCgamma interneurons of the spinal dorsal horn via myelinated afferent fibers. J. Neurosci. Off. J. Soc. Neurosci. 28, 7936–7944. doi:10.1523/JNEUROSCI.1259-08.2008

Neumann, S., Doubell, T.P., Leslie, T., Woolf, C.J., 1996. Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature 384, 360–364. doi:10.1038/384360a0

Nichols, M.L., Allen, B.J., Rogers, S.D., Ghilardi, J.R., Honore, P., Luger, N.M., Finke, M.P., Li, J., Lappi, D.A., Simone, D.A., Mantyh, P.W., 1999. Transmission of Chronic Nociception by Spinal Neurons Expressing the Substance P Receptor. Science 286, 1558 –1561. doi:10.1126/science.286.5444.1558

Nielsen, C.S., Knudsen, G.P., Steingrímsdóttir, Ó.A., 2012. Twin studies of pain. Clin. Genet. 82, 331–340. doi:10.1111/j.1399-0004.2012.01938.x

Nitanda, A., Yasunami, N., Tokumo, K., Fujii, H., Hirai, T., Nishio, H., 2005. Contribution of the peripheral 5-HT 2A receptor to mechanical hyperalgesia in a rat model of neuropathic pain. Neurochem. Int. 47, 394–400. doi:10.1016/j.neuint.2005.06.002

Nowak, S.J., Corces, V.G., 2004. Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation. Trends Genet. TIG 20, 214–220. doi:10.1016/j.tig.2004.02.007

Oatway, M.A., Chen, Y., Weaver, L.C., 2004. The 5-HT3 receptor facilitates at-level mechanical allodynia following spinal cord injury. Pain 110, 259–268. doi:10.1016/j.pain.2004.03.040

Obata, H., Saito, S., Sakurazawa, S., Sasaki, M., Usui, T., Goto, F., 2004. Antiallodynic effects of intrathecally administered 5-HT(2C) receptor agonists in rats with nerve injury. Pain 108, 163–169. doi:10.1016/j.pain.2003.12.019

O'Connor, T.C., Abram, S.E., 1994. Halothane enhances suppression of spinal sensitization by intrathecal morphine in the rat formalin test. Anesthesiology 81, 1277–1283.

Okamoto, K., Imbe, H., Tashiro, A., Kumabe, S., Senba, E., 2004. Blockade of peripheral 5HT3 receptor attenuates the formalin-induced nocifensive behavior in persistent temporomandibular joint inflammation of rat. Neurosci. Lett. 367, 259–263. doi:10.1016/j.neulet.2004.06.017

Ossipov, M.H., Dussor, G.O., Porreca, F., 2010. Central modulation of pain. J. Clin. Invest. 120, 3779–3787. doi:10.1172/JCI43766

Oyama, T., Ueda, M., Kuraishi, Y., Akaike, A., Satoh, M., 1996. Dual effect of serotonin on formalin-induced nociception in the rat spinal cord. Neurosci. Res. 25, 129–135.

Papadatos, G.A., Wallerstein, P.M.R., Head, C.E.G., Ratcliff, R., Brady, P.A., Benndorf, K., Saumarez, R.C., Trezise, A.E.O., Huang, C.L.-H., Vandenberg, J.I., Colledge, W.H., Grace, A.A., 2002. Slowed conduction and ventricular tachycardia after targeted disruption of the cardiac sodium channel gene Scn5a. Proc. Natl. Acad. Sci. U. S. A. 99, 6210–6215. doi:10.1073/pnas.082121299

Parada, C.A., Reichling, D.B., Levine, J.D., 2005. Chronic hyperalgesic priming in the rat involves a novel interaction between cAMP and PKCepsilon second messenger pathways. Pain 113, 185–190. doi:10.1016/j.pain.2004.10.021

Parada, C.A., Yeh, J.J., Reichling, D.B., Levine, J.D., 2003. Transient attenuation of protein kinase Cepsilon can terminate a chronic hyperalgesic state in the rat. Neuroscience 120, 219–226.

Patel, A.J., Honoré, E., Lesage, F., Fink, M., Romey, G., Lazdunski, M., 1999. Inhalational anesthetics activate two-pore-domain background K+ channels. Nat. Neurosci. 2, 422–426. doi:10.1038/8084

Paul, D., Yao, D., Zhu, P., Minor, L.D., Garcia, M.M., 2001. 5-hydroxytryptamine3 (5-HT3) receptors mediate spinal 5-HT antinociception: an antisense approach. J. Pharmacol. Exp. Ther. 298, 674–678.

Peixoto, L., Abel, T., 2013. The role of histone acetylation in memory formation and cognitive impairments. Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol. 38, 62–76. doi:10.1038/npp.2012.86

Peleg, S., Sananbenesi, F., Zovoilis, A., Burkhardt, S., Bahari-Javan, S., Agis-Balboa, R.C., Cota, P., Wittnam, J.L., Gogol-Doering, A., Opitz, L., Salinas-Riester, G., Dettenhofer, M., Kang, H., Farinelli, L., Chen, W., Fischer, A., 2010. Altered Histone Acetylation Is Associated with Age-Dependent Memory Impairment in Mice. Science 328, 753–756. doi:10.1126/science.1186088

Pertovaara, A., Keski-Vakkuri, U., Kalmari, J., Wei, H., Panula, P., 2001. Response properties of neurons in the rostroventromedial medulla of neuropathic rats: attempted modulation of responses by [1DMe]NPYF, a neuropeptide FF analogue. Neuroscience 105, 457–468.

Pertovaara, A., Wei, H., Hämäläinen, M.M., 1996. Lidocaine in the rostroventromedial medulla and the periaqueductal gray attenuates allodynia in neuropathic rats. Neurosci. Lett. 218, 127–130.

Petrovic, P., Kalso, E., Petersson, K.M., Ingvar, M., 2002. Placebo and opioid analgesia-- imaging a shared neuronal network. Science 295, 1737–1740. doi:10.1126/science.1067176

Pezet, S., Malcangio, M., Lever, I.J., Perkinton, M.S., Thompson, S.W.N., Williams, R.J., McMahon, S.B., 2002. Noxious stimulation induces Trk receptor and downstream ERK phosphorylation in spinal dorsal horn. Mol. Cell. Neurosci. 21, 684–695.

Pezet, S., Marchand, F., D'Mello, R., Grist, J., Clark, A.K., Malcangio, M., Dickenson, A.H., Williams, R.J., McMahon, S.B., 2008. Phosphatidylinositol 3-kinase is a key mediator of central sensitization in painful inflammatory conditions. J. Neurosci. Off. J. Soc. Neurosci. 28, 4261–4270. doi:10.1523/JNEUROSCI.5392-07.2008

Plath, N., Ohana, O., Dammermann, B., Errington, M.L., Schmitz, D., Gross, C., Mao, X., Engelsberg, A., Mahlke, C., Welzl, H., Kobalz, U., Stawrakakis, A., Fernandez, E., Waltereit, R., Bick-Sander, A., Therstappen, E., Cooke, S.F., Blanquet, V., Wurst, W., Salmen, B., Bösl, M.R., Lipp, H.-P., Grant, S.G.N., Bliss, T.V.P., Wolfer, D.P., Kuhl, D., 2006. Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. Neuron 52, 437–444. doi:10.1016/j.neuron.2006.08.024

Plenderleith, M.B., Snow, P.J., 1993. The plant lectin Bandeiraea simplicifolia I-B4 identifies a subpopulation of small diameter primary sensory neurones which innervate the skin in the rat. Neurosci. Lett. 159, 17–20.

Polgár, E., Al Ghamdi, K.S., Todd, A.J., 2010. Two populations of neurokinin 1 receptor-expressing projection neurons in lamina I of the rat spinal cord that differ in AMPA receptor subunit composition and density of excitatory synaptic input. Neuroscience 167, 1192–1204. doi:10.1016/j.neuroscience.2010.03.028

Polgár, E., Al-Khater, K.M., Shehab, S., Watanabe, M., Todd, A.J., 2008. Large projection neurons in lamina I of the rat spinal cord that lack the neurokinin 1 receptor are densely innervated by VGLUT2-containing axons and possess GluR4-containing AMPA receptors. J. Neurosci. Off. J. Soc. Neurosci. 28, 13150–13160. doi:10.1523/JNEUROSCI.4053-08.2008

Polgár, E., Campbell, A.D., MacIntyre, L.M., Watanabe, M., Todd, A.J., 2007. Phosphorylation of ERK in neurokinin 1 receptor-expressing neurons in laminae

III and IV of the rat spinal dorsal horn following noxious stimulation. Mol. Pain 3, 4. doi:10.1186/1744-8069-3-4

Polgár, E., Fowler, J.H., McGill, M.M., Todd, A.J., 1999. The types of neuron which contain protein kinase C gamma in rat spinal cord. Brain Res. 833, 71–80.

Polgár, E., Hughes, D.I., Riddell, J.S., Maxwell, D.J., Puskár, Z., Todd, A.J., 2003. Selective loss of spinal GABAergic or glycinergic neurons is not necessary for development of thermal hyperalgesia in the chronic constriction injury model of neuropathic pain. Pain 104, 229–239.

Polgár, E., Sardella, T.C.P., Tiong, S.Y.X., Locke, S., Watanabe, M., Todd, A.J., 2013. Functional differences between neurochemically-defined populations of inhibitory interneurons in the rat spinal dorsal horn. PAIN. doi:10.1016/j.pain.2013.05.001

Polgár, E., Sardella, T.C.P., Watanabe, M., Todd, A.J., 2011. Quantitative study of NPY-expressing GABAergic neurons and axons in rat spinal dorsal horn. J. Comp. Neurol. 519, 1007–1023. doi:10.1002/cne.22570

Polgár, E., Shehab, S.A., Watt, C., Todd, A.J., 1999. GABAergic neurons that contain neuropeptide Y selectively target cells with the neurokinin 1 receptor in laminae III and IV of the rat spinal cord. J. Neurosci. Off. J. Soc. Neurosci. 19, 2637–2646.

Presley, R.W., Menétrey, D., Levine, J.D., Basbaum, A.I., 1990. Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. J. Neurosci. Off. J. Soc. Neurosci. 10, 323–335.

Prigent, C., Dimitrov, S., 2003. Phosphorylation of serine 10 in histone H3, what for? J. Cell Sci. 116, 3677–3685. doi:10.1242/jcs.00735

Puig, S., Sorkin, L.S., 1996. Formalin-evoked activity in identified primary afferent fibers: systemic lidocaine suppresses phase-2 activity. Pain 64, 345–355.

Puskár, Z., Polgár, E., Todd, A.J., 2001. A population of large lamina I projection neurons with selective inhibitory input in rat spinal cord. Neuroscience 102, 167–176.

Putignano, E., Lonetti, G., Cancedda, L., Ratto, G., Costa, M., Maffei, L., Pizzorusso, T., 2007. Developmental downregulation of histone posttranslational modifications regulates visual cortical plasticity. Neuron 53, 747–759. doi:10.1016/j.neuron.2007.02.007

Qi, F., Zhou, Y., Xiao, Y., Tao, J., Gu, J., Jiang, X., Xu, G.-Y., 2013. Promoter demethylation of cystathionine-β-synthetase gene contributes to inflammatory pain in rats. Pain 154, 34–45. doi:10.1016/j.pain.2012.07.031

Qureshi, I.A., Mehler, M.F., 2010. Impact of nuclear organization and dynamics on epigenetic regulation in the central nervous system: implications for neurological disease states. Ann. N. Y. Acad. Sci. 1204 Suppl, E20–37. doi:10.1111/j.1749-6632.2010.05718.x

Rahman, W., Suzuki, R., Webber, M., Hunt, S.P., Dickenson, A.H., 2006. Depletion of endogenous spinal 5-HT attenuates the behavioural hypersensitivity to mechanical and cooling stimuli induced by spinal nerve ligation. Pain 123, 264–274. doi:10.1016/j.pain.2006.02.033

Rahn, E.J., Guzman-Karlsson, M.C., David Sweatt, J., 2013. Cellular, molecular, and epigenetic mechanisms in non-associative conditioning: implications for pain and memory. Neurobiol. Learn. Mem. 105, 133–150. doi:10.1016/j.nlm.2013.06.008

Rajasethupathy, P., Antonov, I., Sheridan, R., Frey, S., Sander, C., Tuschl, T., Kandel, E.R., 2012. A role for neuronal piRNAs in the epigenetic control of memory-related synaptic plasticity. Cell 149, 693–707. doi:10.1016/j.cell.2012.02.057

Rakhit, S., Clark, C.J., O'shaughnessy, C.T., Morris, B.J., 2005. N-methyl-D-aspartate and brain-derived neurotrophic factor induce distinct profiles of extracellular signal-regulated kinase, mitogen- and stress-activated kinase, and ribosomal s6

kinase phosphorylation in cortical neurons. Mol. Pharmacol. 67, 1158–1165. doi:10.1124/mol.104.005447

Ramsey, I.S., Delling, M., Clapham, D.E., 2006. An introduction to TRP channels. Annu. Rev. Physiol. 68, 619–647. doi:10.1146/annurev.physiol.68.040204.100431

Raouf, R., Quick, K., Wood, J.N., 2010. Pain as a channelopathy. J. Clin. Invest. 120, 3745–3752. doi:10.1172/JCI43158

Reichling, D.B., Green, P.G., Levine, J.D., 2013. The fundamental unit of pain is the cell. Pain 154 Suppl 1, S2–9. doi:10.1016/j.pain.2013.05.037

Reichling, D.B., Levine, J.D., 2009. Critical role of nociceptor plasticity in chronic pain. Trends Neurosci. 32, 611–618. doi:10.1016/j.tins.2009.07.007

Reid, K.J., Harker, J., Bala, M.M., Truyers, C., Kellen, E., Bekkering, G.E., Kleijnen, J., 2011. Epidemiology of chronic non-cancer pain in Europe: narrative review of prevalence, pain treatments and pain impact. Curr. Med. Res. Opin. 27, 449–462. doi:10.1185/03007995.2010.545813

Ren, K., Dubner, R., 1999. Inflammatory Models of Pain and Hyperalgesia. ILAR J. 40, 111–118. doi:10.1093/ilar.40.3.111

Renthal, W., Nestler, E.J., 2008. Epigenetic mechanisms in drug addiction. Trends Mol. Med. 14, 341–350. doi:10.1016/j.molmed.2008.06.004

Rexed, B., 1952. The cytoarchitectonic organization of the spinal cord in the cat. J. Comp. Neurol. 96, 414–495.

Rexed, B., 1954. A cytoarchitectonic atlas of the spinal cord in the cat. J. Comp. Neurol. 100, 297–379.

Reynolds, D.V., 1969. Surgery in the rat during electrical analgesia induced by focal brain stimulation. Science 164, 444–445.

Riccio, A., 2010. Dynamic epigenetic regulation in neurons: enzymes, stimuli and signaling pathways. Nat. Neurosci. 13, 1330–1337. doi:10.1038/nn.2671

Roozendaal, B., Hernandez, A., Cabrera, S.M., Hagewoud, R., Malvaez, M., Stefanko, D.P., Haettig, J., Wood, M.A., 2010. Membrane-associated glucocorticoid activity is necessary for modulation of long-term memory via chromatin modification. J. Neurosci. Off. J. Soc. Neurosci. 30, 5037–5046. doi:10.1523/JNEUROSCI.5717-09.2010

Roth, T.L., Sweatt, J.D., 2009. Regulation of chromatin structure in memory formation. Curr. Opin. Neurobiol. 19, 336–342. doi:10.1016/j.conb.2009.05.011

Rotllant, D., Armario, A., 2011. Brain pattern of histone H3 phosphorylation after acute amphetamine administration: its relationship to brain c-fos induction is strongly dependent on the particular brain area. Neuropharmacology. doi:10.1016/j.neuropharm.2011.10.019

Roze, E., Betuing, S., Deyts, C., Marcon, E., Brami-Cherrier, K., Pages, C., Humbert, S., Merienne, K., Caboche, J., 2007. Mitogen- and stress-activated protein kinase-1 deficiency is involved in expanded-huntingtin-induced transcriptional dysregulation and striatal death. FASEB J. 22, 1083–1093. doi:10.1096/fj.07-9814

Ruda, M.A., 1988. Spinal dorsal horn circuitry involved in the brain stem control of nociception. Prog. Brain Res. 77, 129–140.

Rygh, L.J., Suzuki, R., Rahman, W., Wong, Y., Vonsy, J.L., Sandhu, H., Webber, M., Hunt, S., Dickenson, A.H., 2006. Local and descending circuits regulate long-term potentiation and zif268 expression in spinal neurons. Eur. J. Neurosci. 24, 761–772. doi:10.1111/j.1460-9568.2006.04968.x

Salter, M.W., Henry, J.L., 1991. Responses of functionally identified neurones in the dorsal horn of the cat spinal cord to substance P, neurokinin A and physalaemin. Neuroscience 43, 601–610.

Sandkühler, J., 2009. Models and mechanisms of hyperalgesia and allodynia. Physiol. Rev. 89, 707–758. doi:10.1152/physrev.00025.2008

Sandkühler, J., 2010. Central sensitization versus synaptic long-term potentiation (LTP): a critical comment. J. Pain Off. J. Am. Pain Soc. 11, 798–800. doi:10.1016/j.jpain.2010.05.002

Sandkühler, J., Gruber-Schoffnegger, D., 2012. Hyperalgesia by synaptic long-term potentiation (LTP): an update. Curr. Opin. Pharmacol. 12, 18–27. doi:10.1016/j.coph.2011.10.018

Sandkühler, J., Lee, J., 2013. How to erase memory traces of pain and fear. Trends Neurosci. 36, 343–352. doi:10.1016/j.tins.2013.03.004

Sardella, T.C.P., Polgár, E., Watanabe, M., Todd, A.J., 2011. A quantitative study of neuronal nitric oxide synthase expression in laminae I-III of the rat spinal dorsal horn. Neuroscience 192, 708–720. doi:10.1016/j.neuroscience.2011.07.011

Scholz, J., Woolf, C.J., 2002. Can we conquer pain? Nat. Neurosci. 5, 1062–1067. doi:10.1038/nn942

Schuck, S., Soloaga, A., Schratt, G., Arthur, J.S.C., Nordheim, A., 2003. The kinase MSK1 is required for induction of c-fos by lysophosphatidic acid in mouse embryonic stem cells. BMC Mol. Biol. 4, 6. doi:10.1186/1471-2199-4-6

Seagrove, L.C., Suzuki, R., Dickenson, A.H., 2004. Electrophysiological characterisations of rat lamina I dorsal horn neurones and the involvement of excitatory amino acid receptors. Pain 108, 76–87. doi:10.1016/j.pain.2003.12.004

Seal, R.P., Wang, X., Guan, Y., Raja, S.N., Woodbury, C.J., Basbaum, A.I., Edwards, R.H., 2009. Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. Nature 462, 651–655. doi:10.1038/nature08505

Sgambato, V., Pagès, C., Rogard, M., Besson, M.J., Caboche, J., 1998. Extracellular signal-regulated kinase (ERK) controls immediate early gene induction on corticostriatal stimulation. J. Neurosci. Off. J. Soc. Neurosci. 18, 8814–8825.

Shepherd, J.D., Bear, M.F., 2011. New views of Arc, a master regulator of synaptic plasticity. Nat. Neurosci. 14, 279–284. doi:10.1038/nn.2708

Shepherd, J.D., Rumbaugh, G., Wu, J., Chowdhury, S., Plath, N., Kuhl, D., Huganir, R.L., Worley, P.F., 2006. Arc/Arg3.1 mediates homeostatic synaptic scaling of AMPA receptors. Neuron 52, 475–484. doi:10.1016/j.neuron.2006.08.034

Sherrington, C.S., 1903. Qualitative difference of spinal reflex corresponding with qualitative difference of cutaneous stimulus. J. Physiol. 30, 39–46.

Sherrington, C.S., 1906. Observations on the scratch-reflex in the spinal dog. J. Physiol. 34, 1–50.

Shields, S.D., Cavanaugh, D.J., Lee, H., Anderson, D.J., Basbaum, A.I., 2010. Pain behavior in the formalin test persists after ablation of the great majority of C-fiber nociceptors. PAIN 151, 422–429. doi:10.1016/j.pain.2010.08.001

Shimada, M., Nakadai, T., Fukuda, A., Hisatake, K., 2010. cAMP-response element-binding protein (CREB) controls MSK1-mediated phosphorylation of histone H3 at the c-fos promoter in vitro. J. Biol. Chem. 285, 9390–9401. doi:10.1074/jbc.M109.057745

Shortland, P., Woolf, C.J., Fitzgerald, M., 1989. Morphology and somatotopic organization of the central terminals of hindlimb hair follicle afferents in the rat lumbar spinal cord. J. Comp. Neurol. 289, 416–433. doi:10.1002/cne.902890307

Siegfried, B., Frischknecht, H.R., Nunes de Souza, R.L., 1990. An ethological model for the study of activation and interaction of pain, memory and defensive systems in the attacked mouse. Role of endogenous opioids. Neurosci. Biobehav. Rev. 14, 481–490.

Silveira, J.W.S., Dias, Q.M., Del Bel, E.A., Prado, W.A., 2010. Serotonin receptors are involved in the spinal mediation of descending facilitation of surgical incision-induced increase of Fos-like immunoreactivity in rats. Mol. Pain 6, 17. doi:10.1186/1744-8069-6-17

Simonetti, M., Hagenston, A.M., Vardeh, D., Freitag, H.E., Mauceri, D., Lu, J., Satagopam, V.P., Schneider, R., Costigan, M., Bading, H., Kuner, R., 2013. Nuclear calcium signaling in spinal neurons drives a genomic program required for persistent inflammatory pain. Neuron 77, 43–57. doi:10.1016/j.neuron.2012.10.037

Slack, S.E., Pezet, S., McMahon, S.B., Thompson, S.W.N., Malcangio, M., 2004. Brain-derived neurotrophic factor induces NMDA receptor subunit one phosphorylation via ERK and PKC in the rat spinal cord. Eur. J. Neurosci. 20, 1769–1778. doi:10.1111/j.1460-9568.2004.03656.x

Snider, W.D., McMahon, S.B., 1998. Tackling Pain at the Source: New Ideas about Nociceptors. Neuron 20, 629–632. doi:10.1016/S0896-6273(00)81003-X

Soloaga, A., Thomson, S., Wiggin, G.R., Rampersaud, N., Dyson, M.H., Hazzalin, C.A., Mahadevan, L.C., Arthur, J.S.C., 2003. MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. EMBO J. 22, 2788–2797. doi:10.1093/emboj/cdg273

Sommers, M.G., Nguyen, N.-K., Veening, J.G., Vissers, K.C., Ritskes-Hoitinga, M., van Egmond, J., 2008. Suppression of noxious-induced c-fos expression in the rat lumbar spinal cord by isoflurane alone or combined with fentanyl. Anesth. Analg. 106, 1303–1308, table of contents. doi:10.1213/ane.0b013e3181678831

South, S.M., Kohno, T., Kaspar, B.K., Hegarty, D., Vissel, B., Drake, C.T., Ohata, M., Jenab, S., Sailer, A.W., Malkmus, S., Masuyama, T., Horner, P., Bogulavsky, J., Gage, F.H., Yaksh, T.L., Woolf, C.J., Heinemann, S.F., Inturrisi, C.E., 2003. A conditional deletion of the NR1 subunit of the NMDA receptor in adult spinal cord dorsal horn reduces NMDA currents and injury-induced pain. J. Neurosci. Off. J. Soc. Neurosci. 23, 5031–5040.

Spiegel, I., Mardinly, A.R., Gabel, H.W., Bazinet, J.E., Couch, C.H., Tzeng, C.P., Harmin, D.A., Greenberg, M.E., 2014. Npas4 Regulates Excitatory-Inhibitory Balance within Neural Circuits through Cell-Type-Specific Gene Programs. Cell 157, 1216–1229. doi:10.1016/j.cell.2014.03.058

Spike, R.C., Puskár, Z., Andrew, D., Todd, A.J., 2003. A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord. Eur. J. Neurosci. 18, 2433–2448.

Stefanko, D.P., Barrett, R.M., Ly, A.R., Reolon, G.K., Wood, M.A., 2009. Modulation of long-term memory for object recognition via HDAC inhibition. Proc. Natl. Acad. Sci. 106, 9447–9452. doi:10.1073/pnas.0903964106

Stein, C., Millan, M.J., Herz, A., 1988. Unilateral inflammation of the hindpaw in rats as a model of prolonged noxious stimulation: alterations in behavior and nociceptive thresholds. Pharmacol. Biochem. Behav. 31, 445–451.

Stone, L.S., Szyf, M., 2013. The emerging field of pain epigenetics. Pain 154, 1–2. doi:10.1016/j.pain.2012.10.016

Story, G.M., Peier, A.M., Reeve, A.J., Eid, S.R., Mosbacher, J., Hricik, T.R., Earley, T.J., Hergarden, A.C., Andersson, D.A., Hwang, S.W., McIntyre, P., Jegla, T., Bevan, S., Patapoutian, A., 2003. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell 112, 819–829.

Strahl, B.D., Allis, C.D., 2000. The language of covalent histone modifications. Nature 403, 41–45. doi:10.1038/47412

Stucky, C.L., Lewin, G.R., 1999. Isolectin B(4)-positive and -negative nociceptors are functionally distinct. J. Neurosci. Off. J. Soc. Neurosci. 19, 6497–6505.

Suganuma, T., Workman, J.L., 2012. MAP kinases and histone modification. J. Mol. Cell Biol. 4, 348–350. doi:10.1093/jmcb/mjs043

Sullivan, A.F., Dickenson, A.H., Roques, B.P., 1989. Delta-opioid mediated inhibitions of acute and prolonged noxious-evoked responses in rat dorsal horn neurones. Br. J. Pharmacol. 98, 1039–1049.

Sun, Y., Sahbaie, P., Liang, D.-Y., Li, W.-W., Li, X.-Q., Shi, X.-Y., Clark, J.D., 2013. Epigenetic Regulation of Spinal CXCR2 Signaling in Incisional Hypersensitivity in Mice. Anesthesiology. doi:10.1097/ALN.0b013e31829ce340

Suzuki, R., Morcuende, S., Webber, M., Hunt, S.P., Dickenson, A.H., 2002. Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways. Nat. Neurosci. 5, 1319–1326. doi:10.1038/nn966

Svensson, C.I., Tran, T.K., Fitzsimmons, B., Yaksh, T.L., Hua, X.-Y., 2006. Descending serotonergic facilitation of spinal ERK activation and pain behavior. FEBS Lett. 580, 6629–6634. doi:10.1016/j.febslet.2006.11.012

Sweatt, J.D., 2009. Experience-dependent epigenetic modifications in the central nervous system. Biol. Psychiatry 65, 191–197. doi:10.1016/j.biopsych.2008.09.002

Sweatt, J.D., 2013. The Emerging Field of Neuroepigenetics. Neuron 80, 624–632. doi:10.1016/j.neuron.2013.10.023

Swett, J.E., Woolf, C.J., 1985. The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord. J. Comp. Neurol. 231, 66–77. doi:10.1002/cne.902310106

Tabuchi, Y., Takasaki, I., Doi, T., Ishii, Y., Sakai, H., Kondo, T., 2006. Genetic networks responsive to sodium butyrate in colonic epithelial cells. FEBS Lett. 580, 3035–3041. doi:10.1016/j.febslet.2006.04.048

Takamura, H., Ichisaka, S., Watanabe, K., Toigawa, M., Hata, Y., 2008. Effects of anesthesia on immunohistochemical detection of phosphorylated extracellular signal-regulated kinase in cerebral cortex. J. Neurosci. Methods 170, 300–304. doi:10.1016/j.jneumeth.2008.01.005

Takasusuki, T., Yamaguchi, S., Hamaguchi, S., Yaksh, T.L., 2013. Effects of General Anesthetics on Substance P Release and c-Fos Expression in the Spinal Dorsal Horn: Anesthesiology 119, 433–442. doi:10.1097/ALN.0b013e31829996b6

Tao, X., Finkbeiner, S., Arnold, D.B., Shaywitz, A.J., Greenberg, M.E., 1998. Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20, 709–726.

Tappe-Theodor, A., Kuner, R., 2014. Studying ongoing and spontaneous pain in rodents--challenges and opportunities. Eur. J. Neurosci. 39, 1881–1890. doi:10.1111/ejn.12643

Taylor, B.K., Peterson, M.A., Basbaum, A.I., 1995. Persistent cardiovascular and behavioral nociceptive responses to subcutaneous formalin require peripheral nerve input. J. Neurosci. Off. J. Soc. Neurosci. 15, 7575–7584.

Taylor, B.K., Peterson, M.A., Basbaum, A.I., 1997. Early nociceptive events influence the temporal profile, but not the magnitude, of the tonic response to subcutaneous formalin: effects with remifentanil. J. Pharmacol. Exp. Ther. 280, 876–883.

Terman, G.W., Shavit, Y., Lewis, J.W., Cannon, J.T., Liebeskind, J.C., 1984. Intrinsic mechanisms of pain inhibition: activation by stress. Science 226, 1270–1277.

Thomson, S., Mahadevan, L.C., Clayton, A.L., 1999. MAP kinase-mediated signalling to nucleosomes and immediate-early gene induction. Semin. Cell Dev. Biol. 10, 205–214. doi:10.1006/scdb.1999.0302

Tiong, S.Y., Polgár, E., van Kralingen, J.C., Watanabe, M., Todd, A.J., 2011. Galanin-immunoreactivity identifies a distinct population of inhibitory interneurons in laminae I-III of the rat spinal cord. Mol. Pain 7, 36. doi:10.1186/1744-8069-7-36

Tjølsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H., Hole, K., 1992. The formalin test: an evaluation of the method. Pain 51, 5–17.

Tochiki, K.K., Cunningham, J., Hunt, S.P., Géranton, S.M., 2012. The expression of spinal methyl-CpG-binding protein 2, DNA methyltransferases and histone deacetylases is modulated in persistent pain states. Mol. Pain 8, 14. doi:10.1186/1744-8069-8-14

Todd, A.J., 2010. Neuronal circuitry for pain processing in the dorsal horn. Nat. Rev. Neurosci. 11, 823–836. doi:10.1038/nrn2947

Todd, A.J., McGill, M.M., Shehab, S.A., 2000. Neurokinin 1 receptor expression by neurons in laminae I, III and IV of the rat spinal dorsal horn that project to the brainstem. Eur. J. Neurosci. 12, 689–700.

Todd, A.J., Puskar, Z., Spike, R.C., Hughes, C., Watt, C., Forrest, L., 2002. Projection neurons in lamina I of rat spinal cord with the neurokinin 1 receptor are selectively innervated by substance p-containing afferents and respond to noxious stimulation. J. Neurosci. Off. J. Soc. Neurosci. 22, 4103–4113. doi:20026261

Todd, A.J., Spike, R.C., Polgár, E., 1998. A quantitative study of neurons which express neurokinin-1 or somatostatin sst2a receptor in rat spinal dorsal horn. Neuroscience 85, 459–473.

Todd, A.J., Sullivan, A.C., 1990. Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. J. Comp. Neurol. 296, 496–505. doi:10.1002/cne.902960312

Torsney, C., 2011. Inflammatory pain unmasks heterosynaptic facilitation in lamina I neurokinin 1 receptor-expressing neurons in rat spinal cord. J. Neurosci. Off. J. Soc. Neurosci. 31, 5158–5168. doi:10.1523/JNEUROSCI.6241-10.2011

Torsney, C., MacDermott, A.B., 2006. Disinhibition opens the gate to pathological pain signaling in superficial neurokinin 1 receptor-expressing neurons in rat spinal cord. J. Neurosci. Off. J. Soc. Neurosci. 26, 1833–1843. doi:10.1523/JNEUROSCI.4584-05.2006

Tracey, I., Mantyh, P.W., 2007. The cerebral signature for pain perception and its modulation. Neuron 55, 377–391. doi:10.1016/j.neuron.2007.07.012

Treede, R.D., Kenshalo, D.R., Gracely, R.H., Jones, A.K., 1999. The cortical representation of pain. Pain 79, 105–111.

Treede, R.D., Meyer, R.A., Campbell, J.N., 1998. Myelinated mechanically insensitive afferents from monkey hairy skin: heat-response properties. J. Neurophysiol. 80, 1082–1093.

Trevino, D.L., Carstens, E., 1975. Confirmation of the location of spinothalamic neurons in the cat and monkey by the retrograde transport of horseradish peroxidase. Brain Res. 98, 177–182.

Tsankova, N.M., Kumar, A., Nestler, E.J., 2004. Histone modifications at gene promoter regions in rat hippocampus after acute and chronic electroconvulsive seizures. J. Neurosci. Off. J. Soc. Neurosci. 24, 5603–5610. doi:10.1523/JNEUROSCI.0589-04.2004

Tsankova, N., Renthal, W., Kumar, A., Nestler, E.J., 2007. Epigenetic regulation in psychiatric disorders. Nat. Rev. Neurosci. 8, 355–367. doi:10.1038/nrn2132

Tsou, K., Jang, C.S., 1964. STUDIES ON THE SITE OF ANALGESIC ACTION OF MORPHINE BY INTRACEREBRAL MICRO-INJECTION. Sci. Sin. 13, 1099–1109.

Tsuda, M., Beggs, S., Salter, M.W., Inoue, K., 2013. Microglia and intractable chronic pain. Glia 61, 55–61. doi:10.1002/glia.22379

Turrigiano, G., 2011. Too Many Cooks? Intrinsic and Synaptic Homeostatic Mechanisms in Cortical Circuit Refinement. Annu. Rev. Neurosci. 34, 89–103. doi:10.1146/annurev-neuro-060909-153238

Uchida, H., Ma, L., Ueda, H., 2010. Epigenetic gene silencing underlies C-fiber dysfunctions in neuropathic pain. J. Neurosci. Off. J. Soc. Neurosci. 30, 4806–4814. doi:10.1523/JNEUROSCI.5541-09.2010

Uchida, H., Matsushita, Y., Ueda, H., 2013. Epigenetic regulation of BDNF expression in the primary sensory neurons after peripheral nerve injury: Implications in the development of neuropathic pain. Neuroscience 240, 147–154. doi:10.1016/j.neuroscience.2013.02.053

Uchida, H., Sasaki, K., Ma, L., Ueda, H., 2010. Neuron-restrictive silencer factor causes epigenetic silencing of Kv4.3 gene after peripheral nerve injury. Neuroscience 166, 1–4. doi:10.1016/j.neuroscience.2009.12.021

Urban, M.O., Gebhart, G.F., 1997. Characterization of Biphasic Modulation of Spinal Nociceptive Transmission by Neurotensin in the Rat Rostral Ventromedial Medulla. J. Neurophysiol. 78, 1550–1562.

Urban, M.O., Gebhart, G.F., 1999. Supraspinal contributions to hyperalgesia. Proc. Natl. Acad. Sci. U. S. A. 96, 7687–7692.

Urban, M.O., Smith, D.J., 1993. Role of neurotensin in the nucleus raphe magnus in opioid-induced antinociception from the periaqueductal gray. J. Pharmacol. Exp. Ther. 265, 580–586.

Valder, C.R., Liu, J.-J., Song, Y.-H., Luo, Z.D., 2003. Coupling gene chip analyses and rat genetic variances in identifying potential target genes that may contribute to neuropathic allodynia development. J. Neurochem. 87, 560–573.

Vecsey, C.G., Hawk, J.D., Lattal, K.M., Stein, J.M., Fabian, S.A., Attner, M.A., Cabrera, S.M., McDonough, C.B., Brindle, P.K., Abel, T., Wood, M.A., 2007. Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent transcriptional activation. J. Neurosci. Off. J. Soc. Neurosci. 27, 6128–6140. doi:10.1523/JNEUROSCI.0296-07.2007

Vega-Avelaira, D., Géranton, S.M., Fitzgerald, M., 2009. Differential regulation of immune responses and macrophage/neuron interactions in the dorsal root ganglion in young and adult rats following nerve injury. Mol. Pain 5, 70. doi:10.1186/1744-8069-5-70

Vera-Portocarrero, L.P., Zhang, E.-T., Ossipov, M.H., Xie, J.Y., King, T., Lai, J., Porreca, F., 2006. Descending facilitation from the rostral ventromedial medulla maintains nerve injury-induced central sensitization. Neuroscience 140, 1311–1320. doi:10.1016/j.neuroscience.2006.03.016

Vierck, C.J., Hansson, P.T., Yezierski, R.P., 2008. Clinical and pre-clinical pain assessment: Are we measuring the same thing? PAIN 135, 7–10. doi:10.1016/j.pain.2007.12.008

Vikman, K.S., Rycroft, B.K., Christie, M.J., 2008. Switch to Ca2+-permeable AMPA and reduced NR2B NMDA receptor-mediated neurotransmission at dorsal horn nociceptive synapses during inflammatory pain in the rat. J. Physiol. 586, 515–527. doi:10.1113/jphysiol.2007.145581

Waddington, C.H., 1942. Canalization of Development and the Inheritance of Acquired Characters. Nature 150, 563–565. doi:10.1038/150563a0

Wang, R., King, T., De Felice, M., Guo, W., Ossipov, M.H., Porreca, F., 2013. Descending facilitation maintains long-term spontaneous neuropathic pain. J. Pain Off. J. Am. Pain Soc. 14, 845–853. doi:10.1016/j.jpain.2013.02.011

Wang, Y., Liu, C., Guo, Q.-L., Yan, J.-Q., Zhu, X.-Y., Huang, C.-S., Zou, W.-Y., 2011. Intrathecal 5-azacytidine inhibits global DNA methylation and methyl- CpG-binding protein 2 expression and alleviates neuropathic pain in rats following chronic constriction injury. Brain Res. 1418, 64–69. doi:10.1016/j.brainres.2011.08.040

Wang, Y.-Y., Wei, Y.-Y., Huang, J., Wang, W., Tamamaki, N., Li, Y.-Q., Wu, S.-X., 2009. Expression patterns of 5-HT receptor subtypes 1A and 2A on GABAergic neurons within the spinal dorsal horn of GAD67-GFP knock-in mice. J. Chem. Neuroanat. 38, 75–81. doi:10.1016/j.jchemneu.2009.04.003

Waxman, S.G., Zamponi, G.W., 2014. Regulating excitability of peripheral afferents: emerging ion channel targets. Nat. Neurosci. 17, 153–163. doi:10.1038/nn.3602

Weaver, I.C.G., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., Meaney, M.J., 2004. Epigenetic programming by maternal behavior. Nat. Neurosci. 7, 847–854. doi:10.1038/nn1276

Wee, S., Dhanak, D., Li, H., Armstrong, S.A., Copeland, R.A., Sims, R., Baylin, S.B., Liu, X.S., Schweizer, L., 2014. Targeting epigenetic regulators for cancer

therapy: Targeting epigenetic regulators for cancer therapy. Ann. N. Y. Acad. Sci. 1309, 30–36. doi:10.1111/nyas.12356

Wei, F., Dubner, R., Ren, K., 1999a. Dorsolateral funiculus-lesions unmask inhibitory or disfacilitatory mechanisms which modulate the effects of innocuous mechanical stimulation on spinal Fos expression after inflammation. Brain Res. 820, 112–116.

Wei, F., Dubner, R., Ren, K., 1999b. Nucleus reticularis gigantocellularis and nucleus raphe magnus in the brain stem exert opposite effects on behavioral hyperalgesia and spinal Fos protein expression after peripheral inflammation. Pain 80, 127–141.

Wei, F., Dubner, R., Zou, S., Ren, K., Bai, G., Wei, D., Guo, W., 2010. Molecular depletion of descending serotonin unmasks its novel facilitatory role in the development of persistent pain. J. Neurosci. Off. J. Soc. Neurosci. 30, 8624–8636. doi:10.1523/JNEUROSCI.5389-09.2010

Wei, F., Vadakkan, K.I., Toyoda, H., Wu, L.-J., Zhao, M.-G., Xu, H., Shum, F.W.F., Jia, Y.H., Zhuo, M., 2006. Calcium calmodulin-stimulated adenylyl cyclases contribute to activation of extracellular signal-regulated kinase in spinal dorsal horn neurons in adult rats and mice. J. Neurosci. Off. J. Soc. Neurosci. 26, 851–861. doi:10.1523/JNEUROSCI.3292-05.2006

Widmann, C., Gibson, S., Jarpe, M.B., Johnson, G.L., 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. 79, 143–180.

Wiggin, G.R., Soloaga, A., Foster, J.M., Murray-Tait, V., Cohen, P., Arthur, J.S.C., 2002. MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. Mol. Cell. Biol. 22, 2871–2881.

Willis, W.D., Trevino, D.L., Coulter, J.D., Maunz, R.A., 1974. Responses of primate spinothalamic tract neurons to natural stimulation of hindlimb. J. Neurophysiol. 37, 358–372.

Winegar, B.D., MacIver, M.B., 2006. Isoflurane depresses hippocampal CA1 glutamate nerve terminals without inhibiting fiber volleys. BMC Neurosci. 7, 5. doi:10.1186/1471-2202-7-5

Wisden, W., Errington, M.L., Williams, S., Dunnett, S.B., Waters, C., Hitchcock, D., Evan, G., Bliss, T.V., Hunt, S.P., 1990. Differential expression of immediate early genes in the hippocampus and spinal cord. Neuron 4, 603–614.

Wittmann, M., Queisser, G., Eder, A., Wiegert, J.S., Bengtson, C.P., Hellwig, A., Wittum, G., Bading, H., 2009. Synaptic Activity Induces Dramatic Changes in the Geometry of the Cell Nucleus: Interplay between Nuclear Structure, Histone H3 Phosphorylation, and Nuclear Calcium Signaling. J. Neurosci. 29, 14687–14700. doi:10.1523/JNEUROSCI.1160-09.2009

Woolf, C.J., 1983. Evidence for a central component of post-injury pain hypersensitivity. Nature 306, 686–688.

Woolf, C.J., 2010. What is this thing called pain? J. Clin. Invest. 120, 3742–3744. doi:10.1172/JCI45178

Woolf, C.J., Fitzgerald, M., 1983. The properties of neurones recorded in the superficial dorsal horn of the rat spinal cord. J. Comp. Neurol. 221, 313–328. doi:10.1002/cne.902210307

Woolf, C.J., Ma, Q., 2007. Nociceptors--noxious stimulus detectors. Neuron 55, 353–364. doi:10.1016/j.neuron.2007.07.016

Woolf, C.J., Salter, M.W., 2000. Neuronal plasticity: increasing the gain in pain. Science 288, 1765–1769.

Woolf, C.J., Thompson, S.W., 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.

Woo, S.-H., Ranade, S., Weyer, A.D., Dubin, A.E., Baba, Y., Qiu, Z., Petrus, M., Miyamoto, T., Reddy, K., Lumpkin, E.A., Stucky, C.L., Patapoutian, A., 2014. Piezo2 is required for Merkel-cell mechanotransduction. Nature 509, 622–626. doi:10.1038/nature13251

Wu, W., Wessendorf, M.W., 1992. Organization of the serotonergic innervation of spinal neurons in rats--I. Neuropeptide coexistence in varicosities innervating some spinothalamic tract neurons but not in those innervating postsynaptic dorsal column neurons. Neuroscience 50, 885–898.

Xiao, H.-S., Huang, Q.-H., Zhang, F.-X., Bao, L., Lu, Y.-J., Guo, C., Yang, L., Huang, W.-J., Fu, G., Xu, S.-H., Cheng, X.-P., Yan, Q., Zhu, Z.-D., Zhang, X., Chen, Z., Han, Z.-G., Zhang, X., 2002. Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. Proc. Natl. Acad. Sci. U. S. A. 99, 8360–8365. doi:10.1073/pnas.122231899

Xu, W., Qiu, X.C., Han, J.S., 1994. Serotonin receptor subtypes in spinal antinociception in the rat. J. Pharmacol. Exp. Ther. 269, 1182–1189.

Yaksh, T.L., Wilson, P.R., 1979. Spinal serotonin terminal system mediates antinociception. J. Pharmacol. Exp. Ther. 208, 446–453.

Yaksh, T.L., Yeung, J.C., Rudy, T.A., 1976. Systematic examination in the rat of brain sites sensitive to the direct application of morphine: observation of differential effects within the periaqueductal gray. Brain Res. 114, 83–103.

Yang, S., Xiao, Y., Kang, D., Liu, J., Li, Y., Undheim, E.A.B., Klint, J.K., Rong, M., Lai, R., King, G.F., 2013. Discovery of a selective NaV1.7 inhibitor from centipede venom with analgesic efficacy exceeding morphine in rodent pain models. Proc. Natl. Acad. Sci. U. S. A. 110, 17534–17539. doi:10.1073/pnas.1306285110

Yasaka, T., Tiong, S.Y.X., Hughes, D.I., Riddell, J.S., Todd, A.J., 2010. Populations of inhibitory and excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined electrophysiological and anatomical approach. Pain 151, 475–488. doi:10.1016/j.pain.2010.08.008

Yashpal, K., Mason, P., McKenna, J.E., Sharma, S.K., Henry, J.L., Coderre, T.J., 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.

Ying, S.-W., Futter, M., Rosenblum, K., Webber, M.J., Hunt, S.P., Bliss, T.V.P., Bramham, C.R., 2002. Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. J. Neurosci. Off. J. Soc. Neurosci. 22, 1532–1540.

Zaugg, M., Lucchinetti, E., Uecker, M., Pasch, T., Schaub, M.C., 2003. Anaesthetics and cardiac preconditioning. Part I. Signalling and cytoprotective mechanisms. Br. J. Anaesth. 91, 551–565.

Zeitz, K.P., Guy, N., Malmberg, A.B., Dirajlal, S., Martin, W.J., Sun, L., Bonhaus, D.W., Stucky, C.L., Julius, D., Basbaum, A.I., 2002. The 5-HT3 subtype of serotonin receptor contributes to nociceptive processing via a novel subset of myelinated and unmyelinated nociceptors. J. Neurosci. Off. J. Soc. Neurosci. 22, 1010–1019.

Zhang, H.-H., Hu, J., Zhou, Y.-L., Hu, S., Wang, Y.-M., Chen, W., Xiao, Y., Huang, L.-Y.M., Jiang, X., Xu, G.-Y., 2013. Promoted Interaction of Nuclear Factor- B with Demethylated Cystathionine- -Synthetase Gene Contributes to Gastric Hypersensitivity in Diabetic Rats. J. Neurosci. 33, 9028–9038. doi:10.1523/JNEUROSCI.1068-13.2013

Zhang, J., Cavanaugh, D.J., Nemenov, M.I., Basbaum, A.I., 2013. The modality-specific contribution of peptidergic and non-peptidergic nociceptors is manifest at the level of dorsal horn nociresponsive neurons. J. Physiol. 591, 1097–1110. doi:10.1113/jphysiol.2012.242115

Zhang, X., Giesler, G.J., 2005. Response characterstics of spinothalamic tract neurons that project to the posterior thalamus in rats. J. Neurophysiol. 93, 2552–2564. doi:10.1152/jn.01237.2004

Zhang, X., Huang, J., McNaughton, P.A., 2005. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. EMBO J. 24, 4211–4223. doi:10.1038/sj.emboj.7600893

Zhang, Z., Cai, Y.-Q., Zou, F., Bie, B., Pan, Z.Z., 2011. Epigenetic suppression of GAD65 expression mediates persistent pain. Nat. Med. doi:10.1038/nm.2442

Zhao, Z.-Q., Chiechio, S., Sun, Y.-G., Zhang, K.-H., Zhao, C.-S., Scott, M., Johnson, R.L., Deneris, E.S., Renner, K.J., Gereau, R.W., 4th, Chen, Z.-F., 2007. Mice lacking central serotonergic neurons show enhanced inflammatory pain and an impaired analgesic response to antidepressant drugs. J. Neurosci. Off. J. Soc. Neurosci. 27, 6045–6053. doi:10.1523/JNEUROSCI.1623-07.2007

Zhou, R., Gong, A.-Y., Chen, D., Miller, R.E., Eischeid, A.N., Chen, X.-M., 2013. Histone Deacetylases and NF-kB Signaling Coordinate Expression of CX3CL1 in Epithelial Cells in Response to Microbial Challenge by Suppressing miR-424 and miR-503. PLoS ONE 8, e65153. doi:10.1371/journal.pone.0065153

Zhou, V.W., Goren, A., Bernstein, B.E., 2011. Charting histone modifications and the functional organization of mammalian genomes. Nat. Rev. Genet. 12, 7–18. doi:10.1038/nrg2905

Zhuo, M., Gebhart, G.F., 1991. Spinal serotonin receptors mediate descending facilitation of a nociceptive reflex from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. Brain Res. 550, 35–48.

Zhuo, M., Gebhart, G.F., 1992. Characterization of descending facilitation and inhibition of spinal nociceptive transmission from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. J. Neurophysiol. 67, 1599–1614.

Zhuo, M., Gebhart, G.F., 1997. Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat. J. Neurophysiol. 78, 746–758.

Zhu, X., Li, Q., Chang, R., Yang, D., Song, Z., Guo, Q., Huang, C., 2014. Curcumin Alleviates Neuropathic Pain by Inhibiting p300/CBP Histone Acetyltransferase Activity-Regulated Expression of BDNF and Cox-2 in a Rat Model. PLoS ONE 9, e91303. doi:10.1371/journal.pone.0091303

Zhu, X.-Y., Huang, C.-S., Li, Q., Chang, R., Song, Z., Zou, W., Guo, Q.-L., 2012. p300 exerts an epigenetic role in chronic neuropathic pain through its acetyltransferase activity in rats following chronic constriction injury (CCI). Mol. Pain 8, 84. doi:10.1186/1744-8069-8-84

Zocchi, L., Sassone-Corsi, P., 2010. Joining the dots: from chromatin remodeling to neuronal plasticity. Curr. Opin. Neurobiol. 20, 432–440. doi:10.1016/j.conb.2010.04.005

Zou, X., Lin, Q., Willis, W.D., 2000. Enhanced phosphorylation of NMDA receptor 1 subunits in spinal cord dorsal horn and spinothalamic tract neurons after intradermal injection of capsaicin in rats. J. Neurosci. Off. J. Soc. Neurosci. 20, 6989–6997.

Zylka, M.J., Rice, F.L., Anderson, D.J., 2005. Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. Neuron 45, 17–25. doi:10.1016/j.neuron.2004.12.015

Appendix

Solutions

Immunohistochemistry

TTBS (1L) (All Sigma, UK)
0.05M (v/v) Tris HCl
0.3% (v/v) Triton X-100
0.9% (w/v) NaCl

Western Blot

TX Homogenisation Buffer (All Sigma, UK)
50 mM Tris HCl
150 mM NaCl
2 mM EDTA
1% (v/v) Triton X100

TX Resuspension/Lysis Buffer
TX Buffer + 0.2M HCl

CHAPS Buffer (All Sigma, UK)
10mM Tris pH 8.0
5mM Magnesium Acetate
8M urea
4% CHAPS

Loading Buffer
0.267M DTT
66.6% (v/v) NuPage LDS 4X loading buffer (Life Technologies, UK)

MOPS Running Buffer (All Sigma, UK)
0.05M 3-(N-Morpholino) propane sulphonic acid (MOPS)
0.05M Tris Base
3.5mM SDS
1mM EDTA

Transfer Buffer (All Sigma except methanol)
48mM Tris Base
39mM Glycine
0.037% (v/v) SDS
10% (v/v) Methanol (VWR, UK)

iBlock
0.24% (w/v) iblock (Tropix/Applied Biosystems, UK)
1% Tris HCl (v/v)
0.15M NaCl
0.05% (v/v) Tween 20

PBS-Tween
0.1% (v/v) Tween 20
0.1M PBS