

EMERGING TARGETS FOR ANALGESICS TO CONTROL INFLAMMATORY PAIN

A Thesis Submitted in Accordance with the Requirements of the Degree
of Doctors of Philosophy of Imperial College London

By

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1 Abstract

Burn injury is followed by one of the most severe inflammatory conditions and associated with one of the most excruciating persistent pain. Current therapeutics for burn injury-associated pain are limited and produce undesired side effects. The development and particularly the maintenance of persistent pain depend on plastic changes in neurons involved in the processing of noxious stimulation-evoked (nociceptive) information including nociceptive primary sensory neurons and spinal dorsal horn neurons. Those plastic changes include changes in gene expression that are regulated largely by epigenetic mechanisms including post-translational modification of histones. Here, I show inflammatory conditions including that following burn injury significantly and specifically up-regulates, both in a group of primary sensory neurons and spinal dorsal horn neurons, the expression of phosphorylated serine 10 in histone H3 (pS10H3), a transcriptionally permissive histone mark. This up-regulation is associated, in superficial spinal cord neurons, with up-regulation in the expression of cFos a known marker for nociceptive processing. In superficial spinal cord neurons, these up-regulations depend on the activation of the N-methyl-D aspartate receptor, extracellular signal-regulated kinase 1 and 2 and mitogen and stress-activated kinase 1 and 2 (MSK1/2). Importantly, deletion of MSK1/2 blocks the development of inflammatory heat hyperalgesia. Further, blocking the voltage-gated sodium channel type 1.7 significantly reduces pS10H3 up-regulation both in primary sensory neurons and superficial spinal dorsal horn neurons. I conclude that pS10H3 represents a novel marker for nociceptive processing both in primary sensory and superficial spinal dorsal horn neurons, and transcriptional changes downstream of S10H3 phosphorylation are pivotal in the

development of inflammatory heat hyperalgesia. I further conclude that Nav1.7 is a promising target for the control of heat hyperalgesia in burn injury.

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5 Declaration of originality

I hereby declare that this thesis and the work reported herein was composed by and originated entirely from me. For experimental procedure in which others participate, proper acknowledgement is given. Information derived from the published and unpublished work of others has been acknowledged in the text and references are given in the list of sources.

London, 6th of January 2017

A handwritten signature in black ink. The signature consists of the name 'Jose' followed by a stylized monogram 'V.T.' and the surname 'Torres'. A long, sweeping horizontal line is drawn across the entire signature.

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7 Acknowledgements

First and foremost, I would like to thank my first supervisor Dr. Istvan Nagy for his support and guidance throughout all these years of my Ph.D. His door has always been open for friendly talk and discussion. He truly knew how to let me do my own research and let me plan things by myself while constantly driving me through the right path. He definitely helped me to become a much better scientist.

I would also like to thank my second supervisor Dr. Carsten Bantel who, despite being in another country, was always available to offer pastoral support and to correct my writings whenever I asked. My sincere thanks also goes to the *Chelsea and Westminster Health Charity* for supporting me financially during this Ph.D; as well as to the *Guarantors of Brain*, *The Genetics Society*, *The Galton Institute*, the *EFIC Executive Board*, the *British Pharmacological Society* and the *Imperial College Trust (General Funds)* for awarding me travel grants, which allowed me to attend different congresses all over the world to learn from leading experts, increase my professional network and promote my research.

I am also thankful to my fellow labmates and friends around the C&W hospital for all the fun moments and the interesting discussions that we had through these years. In particular, I am grateful to Mr. Dominic Friston, for being always there to talk, to Dr. João Sousa-Valente, for making me feel like home at the lab, and to Dr. Sinéad Savage for keeping me sane.

Finally, I would like to thank my family and friends for supporting me and being always there either in person or virtually. If I may, I would like to address them in Catalan/Valencian: *Sobretot gràcies a Golfet, el solet de la meua vida amb qui he compartit quasi setge anys perfectes i a qui portaré sempre al cor.*

8 Abbreviations

Ili: inner lamina II of the spinal cord

Ilo: outer lamina II of the spinal cord

2mK4H3: di-methylated lysine 4 of histone H3

2mK9H3: di-methylated lysine 9 of histone H3

2mK27H3: di-methylated lysine 27 of histone H3

2mK36H3: di-methylated lysine 36 of histone H3

2mK79H3: di-methylated lysine 79 of histone H3

3mK4H3: tri-methylated lysine 4 of histone H3

3mK9H3: tri-methylated lysine 9 of histone H3

3mK27H3: tri-methylated lysine 27 of histone H3

3mK36H3: tri-methylated lysine 36 of histone H3

3mK79H3: tri-methylated lysine 79 of histone H3

5-TH: serotonin

ACC: anterior cingulate cortex

aCSF: artificial cerebrospinal fluid

A&E: accident and emergency

aK9H3: acetylated lysine 9 of histone H3

aK14H3: acetylated lysine 14 of histone H3

aK18H3: acetylated lysine 18 of histone H3

aK56H3: acetylated lysine 56 of histone H3

AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

AMPA-R: AMPA receptor

ANOVA: analysis of variance

AP1: activator protein 1

arc: activity-regulated cytoskeleton-associated protein gene

ASIC: acid-sensing ionic channels

ASK: apoptosis signal-regulating kinase

ATF: activating transcription factor

ATP: adenosine triphosphate

BDNF: brain-derived neurotrophic factor

bp: base pair

bpm: beats per minute

Brd: bromodomain

Ca²⁺: calcium ion

calca: calcitonin

CaM: calmodulin

CaMK: Ca²⁺/calmodulin-dependent protein kinase

cAMP: cyclic adenosine monophosphate

CBP/p300: CREB-binding protein with the related p300 protein

CBS: cystathionine-beta-synthetase

CD11b: complement receptor 3, also known as OX42

cdk9: cyclin-dependent kinase 9

ChIP-Seq: combined chromatin immunoprecipitation with gene sequencing

CIP: congenital insensitivity to pain

CFA: complete Freund's adjuvant

cKO: conditional KO

CGRP: calcitonin-gene-related peptide

CNS: central nervous system

COX-2: type-2 cyclooxygenase enzyme

CpG: cytosine followed by guanine residues

CRE: cAMP-response element

CREB: cAMP response element-binding protein

DAB: diaminobenzidine

DAG: diacylglycerol also known as diglyceride

DAL: disability adjusted live

DAPI: 4',6-diamidino-2-phenylindole

ddH₂O: double-distilled water

DHSC: dorsal horn of the spinal cord

DMEM: Dulbecco's modified Eagles medium

DNMT: DNA methyltransferases

DRG: dorsal root ganglion/ganglia

eIF4E: eukaryotic synthesis initiation factor 4E

Elk: ETS (E26 transformation-specific) domain-containing protein

eomes: eomesodermin

ERK: extracellular signal-regulated kinase

FSH: follicle stimulating hormone

FYN: proto-oncogene tyrosine-protein kinase fyn

GABA: gamma-aminobutyric acid

Gad65: glutamic acid decarboxylase 65

Gcn5: general control of amino acid 5 HAT

GDNF: glial-cell-derived neurotrophic factor

GDP: guanosine diphosphate

GFAP: glial fibrillary acidic protein

GPCR: G protein-coupled receptor

GR: glucocorticoid receptor

GRB2: growth factor receptor-bound protein 2

GTP: guanosine-5'-triphosphate

GzLM: generalized linear mode

HAT: histone acetyltransferase

HDAC: histone deacetylase

HDACi: HDAC inhibitor

H&E: haematoxylin and eosin

homer1: protein homolog 1

HP1: heterochromatin protein 1

HRP: horseradish peroxidase

IASP: International Association for the Study of Pain

ICC: immunocytochemistry

IEG: immediate early genes

IEM: inherited primary erythromelalgia/erythermalgia

IF: immunofluorescence

ifn β : interferon beta

Ig: immunoglobulin

igfbp5: factor-binding protein 5

IHC: immunohistochemistry

IkB: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor

Ikka: I κ B kinase- α also known as IKK1

IL: interleukin

ino1: inositol-3-phosphate synthase

i.p.: intraperitoneal

JHDM: jumonji domain-containing histone demethylase

JNK: Jun amino-terminal kinase, also known as SAPK

KO: KnockOut

LC: locus coeruleus

lgi1: leucine-rich glioma inactivated 1

LSD: histone lysine-specific demethylase

LTD: long-term depression

LTP: long-term potentiation

MAP2K: MAPK kinase

MAP3K: MAP2K kinase

MAPK: mitogen-activated protein kinase

MBD: methyl-CpG-binding domain

Mdn: median

MeCP2: methyl-CpG-binding protein 2

MEK: MAPK/ERK kinase

MEKK: MEK kinase

mK4H3: mono-methylated lysine 4 of histone H3

mK9H3: mono-methylated lysine 9 of histone H3

mK36H3: mono-methylated lysine 36 of histone H3

mK79H3: mono-methylated lysine 79 of histone H3

MKK: mitogen-activated protein kinase kinase

MKP: MAPK phosphatase

MLK: mixed lineage kinase

MLTK: MLK-like mitogen-activated protein

MNK: MAP kinase-interacting kinase

mRAR β 2: retinoic acid receptor beta 2

MSK: mitogen- and stress- activated protein kinase

Mos: proto-oncogene ser/thr-protein kinase mos

NDS: normal donkey serum

NeuN: hexaribonucleotide-binding protein 3, a marker of neuronal nuclei

NeuPSIG: IASP Special Interest Group on Neuropathic Pain

NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells

NGF: nerve growth factor

NK-1: neurokinin-1 receptor also known as SPR

NMDA: N-methyl-D-aspartic acid

NMDA-R: NMDA receptor

NRG: nucleus reticularis gigantocellularis

NRM: nucleus raphe magnus

NSAID: non-steroidal anti-inflammatory drug

NT-3: neurotrophin 3

OX42: complement receptor 3, also known as CD11b

p38: p38 MAPK

PAG: periaqueductal grey

PBS: phosphate-buffered saline	ptgs2: prostaglandin synthase 2
PBST: PBS containing 0.3% Triton-X	PTM: post-translational modification
PEPD: paroxysmal extreme pain disorders	PTSD: post-traumatic stress disorder
PFA: 4% paraformaldehyde dissolved in PBS at pH=7.4	PVDF: polyvinylidene difluoride
PGE ₂ : prostaglandin E ₂	R17: retinoblastoma-interacting PHD protein
PHD: zinc-finger	Rac1: Ras-related C3 botulinum toxin substrate 1 (small signalling GTPase)
PI3K: phosphoinositide 3-kinase	Raf: RAF proto-oncogen ser/thr-protein kinase also known as proto-oncogen c-RAF
PIL: personal licence	RasGRP: Ras guanyl-releasing protein
PIP ₂ : phosphatidylinositol 4,5-bisphosphate	RE1: neuron-restrictive silencer
PIP ₃ : phosphatidylinositol (3,4,5)-triphosphate	rest: neuron-restrictive silencer factor
PKA: protein kinase A	rgs2: regulator of G-protein signalling 2
PKC: protein kinase C	rin1: Ras and Rab interactor 1
PLC: phospholipase C	RNApol II: RNA polymerase II
PN1: peripheral nerve 1, the rat homologue of Na _v 1.7	ROI: region of interest
PN3: peripheral nerve 3, also known as SNS and Na _v 1.8	RSK: ribosomal s6 kinase
PNS: peripheral nervous system	RTK: receptor tyrosine kinase
PORN: poly-ornithine	SAHA: secreted protein acidic rich in cysteine
PP1: protein phosphatase 1	SAPK: stress-activated protein kinase, also known as JNK
pp38: phosphorylated p38	s.c.: subcutaneous
PPL: project licence	SD: standard deviation (σ)
prka: A kinase	SDS: sodium dodecyl sulphate
prdm12: PR/SET domain containing 12	SDS-PAGE: SDS-polyacrylamide gel electrophoresis
ProTxII: protoxin II	SEK: SAPK/ERK kinase
pS10H3: phosphorylated serine 10 of histone H3	SEM: standard error mean
pS28H3: phosphorylated serine 28 of histone H3	SFN: small fibre neuropathy
	sgk: serine/threonine-protein kinase SGK

Shc: SHC-transforming protein	TBSA: total burn surface area
SIE: <i>sis</i> -inducible element, Spt: suppressor of Ty protein	TEM: transmission electron microscopy
SIN3A: paired amphipathic helix protein Sin3a	TET: ten-eleven translocation enzyme
SM: supplemented media	TFIID: transcription factor II D
SMN: survival motor neuron	TGF β : transforming growth factor beta
SNL: spinal nerve ligation	tiff: tag image file format
SNS: sensory neuron specific, also known as PN3 and Na _v 1.8	TNF α : tumour necrosis factor alpha
SPR: neurokinin-1 receptor also known as NK-1	TPA: 12-o-tetradecanoylphorbol-13-acetate
SRE: serum response element	TrkA: tyrosine kinase receptor A
SRF: serum response factor	tRNA-cys: mitochondrial cysteine transfer RNA
SRG: secondary response genes	TRPV1: transient receptor potential cation channel subfamily V member 1
SWI-SNF: switch/sucrose non-fermentable	TSA: tyramide signal amplification
syngap: synaptic Ras GTPase-activating protein 1	TTX: tetrodotoxin
TAB: TAK1-binding protein	rpm: revolutions per minute
Taf: TBP associated factor	UV light: ultraviolet light
TAK1: transforming growth factor beta-activated kinase 1	VEGF: vascular endothelial growth factor
TBP: TATA-binding protein	VGSC: voltage-gated sodium channel
TBS: tris-buffered saline	VPL: ventral posterior lateral thalamus
	VPM: ventral posterior medial thalamus
	WHO: World Health Organization
	WT: wild type
	zif-268: zinc finger protein 268

9 General introduction

9.1 Burn injury associated pain and the need for new treatments

This section aims to show that pain associated to burn injuries is a top cause of concern among burn survivors and that new and better treatments are still needed. To do so, burn injury will be defined as well as its epidemiology and the associated costs. Secondly, an overview about burn quantification will be given. Finally, the problem of pain associated to burn injuries will be analysed in detail, including its prevalence, risk factors and current therapeutic strategies.

9.1.1 Definition and Epidemiology

9.1.1.1 Definition

The World Health Organization (WHO) defines burn injury as the result of an uncontrolled transfer of thermal energy to the skin or other tissue in amounts that cannot be tolerated (WHO, 2011; 2014). It can be caused by heat, radiation, radioactivity, electricity, friction or contact with chemicals. Heat burns include the ones caused by hot liquids, known as scalds, hot solids and flames. The most common cause of burns is due to flames and fires (Forjuoh, 1998; Sanghavi *et al.*, 2009).

Nearly everyone has experienced some sort of burn injury, even if just a minor scald or a small contact with a hot surface. When intense, it is felt to be “the most severe form of trauma that is survivable” (NBCRC, 2001) and is among the most

devastating of all injuries. Additionally, burn injuries are the fourth most common type of trauma worldwide (WHO, 2008a). The majority of burn injuries (around 90%) occur in low- and middle-income countries that normally do not have enough resources to reduce the risks and severity of injuries (Peck & Pressman, 2013).

Despite advances in burn care there still is a considerable mortality rate associated with burn injuries as well as intense suffering from burn-related disabilities and disfigurements (WHO, 2008b). Thus, burn injuries remain a major challenge for health care globally.

9.1.1.2 Epidemiology of burn injuries

Burn injuries have an incidence of 1.1 per 100,000 population (WHO, 2008a). However, there have been reported regional variations on the incidence according to the income group (WHO, 2008a; b; Peck & Pressman, 2013). There is also a gradual decline in mortality in the high-income countries during the last 30-40 years while the mortality rate has remained high in the low- and middle-income countries (WHO, 2008b; 2014). This inequality is mainly due to the effective enforcement of effective interventions in the high-income countries (WHO, 2008b). Nonetheless, burn risk clearly correlates with socioeconomic status within all countries among other factors (Edelman, 2007; WHO, 2014).

There are also disparities across different age groups and between genders. Children, infants and toddlers, represent nearly 1/3 of all burns (NBCRC, 2001), with an estimated hospitalization for burn treatment of 8 per 100,000 population globally, and with three times this average for the infants in Africa (WHO, 2008a). Burns are the fifth most common cause of non-fatal childhood injuries (WHO, 2014) and are considered the 11th leading reason of death in children between 1 to 9 years old (Forjuoh, 1998; Burd & Yuen, 2005; WHO, 2014). In the UK, children under 3 years

old are the most common group of age that experiences burn injuries, of which over 90% are preventable (NBCRC, 2001). In the elderly, age 60 or greater, the mortality rate from burns has remained high despite the global decrease in the high-income countries (NBCRC, 2001; Mabrouk *et al.*, 2003; Alden *et al.*, 2007). The prevalence of burns is higher in women, 0.09 per 100,000 versus 0.06 per 100,000 in men (WHO, 2008a; b). However, the American Burn Association reports a higher rate of burn admission in male, 69%, than females, 31%, in the USA (WHO, 2008b; 2011). Other factors acknowledged as risk factors are: medical conditions such as epilepsy (Minn, 2007), blindness, deafness, peripheral neuropathy and diabetes (WHO, 2014); occupation, lack of proper safety measures, alcohol abuse and smoking, different regional factors and easy access to chemicals used in assault (Forjuoh, 1998; Laloe, 2004; Edelman, 2007; WHO, 2011; ABA, 2015).

9.1.1.3 Costs of burns

Over 300,000 death are attributed to fire-related burns each year globally (WHO, 2008b). While burn injuries are responsible for an estimated loss of 10 million Disability Adjusted Live years (DAL) annually (WHO, 2008b), they also are the source of suffering and disabling that will prevent or diminish employment of burn survivors (Minister, 2011). Indirect costs of burn injury include lost wages, prolonged care for deformities, pain and psychological disturbances, and commitment of family resources (WHO, 2014).

In the UK, about 250,000 people experience burn injury each year and around 175,000 of them visit Accident and Emergency (A&E) departments. From these, about 16,100 are admitted to hospitals (NBCRC, 2001). The economic burden of fires in England and Wales was estimated at £2.5 billion in 2008 (Minister, 2011; Kalson *et al.*, 2012), including casualties, assets damaged and business disrupted.

Despite all the improvements, there are still around 300 deaths related to burns each year in UK hospitals (NBCRC, 2001; Kalson *et al.*, 2012) as many survivors suffering pain-related difficulties (see Section 9.1.3)

9.1.2 Managing and quantifying a burn injury

9.1.2.1 Managing burn injury

Once survival is guaranteed, the treatment goal is “... to recover the individuals to the pre-injury state and for them to return to their place in society with unaltered potential...” (NBCRC, 2001). Due to the complex mechanisms involved in burn injury and the associated pain, it normally is an impossible milestone in most of the occasions. Still, the goal remains with the intention to maximise recovery (NBCRC, 2001).

At the time of surgery the dead skin is removed causing a secondary trauma in the patient (a type of procedural pain), and during the healing phase the skin needs to be repaired by wound dressing or applying skin grafts, normally obtained while the individual is responding to the primary injury, from cadavers (NBCRC, 2001) or synthetics (Burke *et al.*, 1981; Yannas *et al.*, 1981). It also requires daily treatments such as changing the dressings and monitoring and control functions (especially important in face, hands and perineum) (WHO, 2007). The changing process is frequently painful with the need of general anaesthesia and/or sedation for pain control. Patients will also require great amounts of energy (up to 6,000 Kcal per day) and proteins in their diet due to the catabolism of trauma (Keyser, 1948), heat loss, infections and the process of generating new tissues (WHO, 2007).

9.1.2.2 Quantification of burn injury

Quantification of the severity of burn injury is difficult but assessment is important to determine treatment plans (NBCRC, 2001; WHO, 2007). The extension of skin injured is quantified as the total burn surface area (TBSA), expressed as the percentage of the whole individual that has been burnt (NBCRC, 2001). In adults, the TBSA is determined using the Rule of 9's, where the body is divided in anatomical portions representing 9%, or multiples, of the total body surface (Figure 9.1) (WHO, 2007). Among infants and young children this rule cannot be apply since they have different proportions than adults, making more difficult to estimate their TBSA (Figure 9.1) (WHO, 2007).

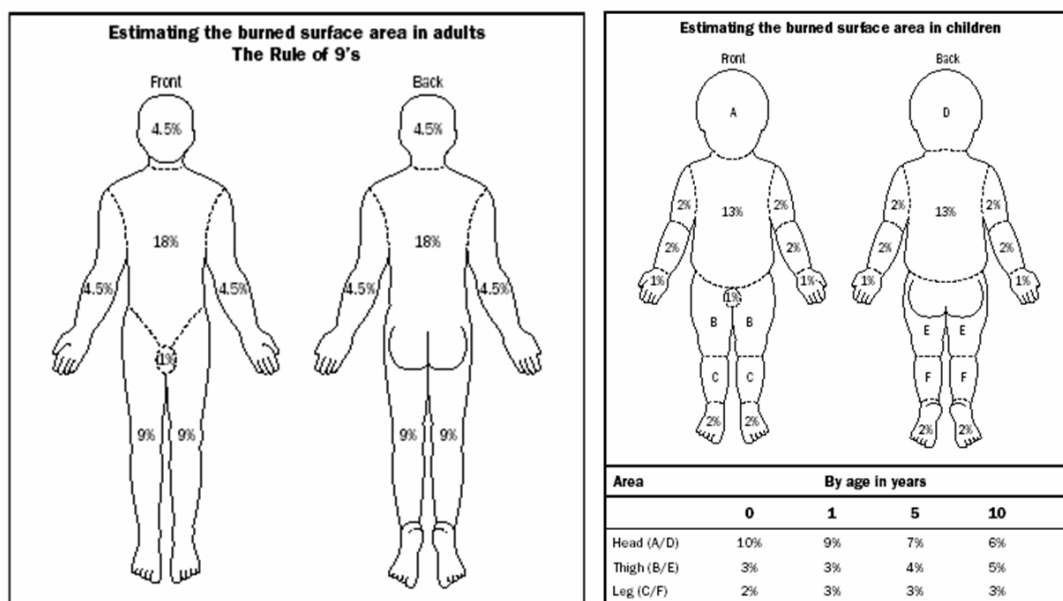


Figure 9.1. **Assessment of TBSA.** In adults, the total burn surface area can be determined applying the rule of 9's. However, it is more difficult to apply to children, due to the changes in body proportions while growing up. Adapted from (WHO, 2007).

Burns can additionally be classified according to the depth of injury (Figure 9.2) (WHO, 2007):

- First degree burns. Characterized by erythema, pain and absence of blister.
Typically, due to sunburns.

- Second degree, also known as partial thickness. They tend to look red or mottled and are normally caused by contact with hot liquids.
- Third degree or full thickness. Dry, dark and leathery. Caused by fire, electricity, lightning or prolonged exposure to hot liquids or objects. They are normally considered serious and require hospitalization.

However, burns of different degrees are commonly mixed in the same burn wound and the depth can vary through time (Figure 9.2) (WHO, 2007).

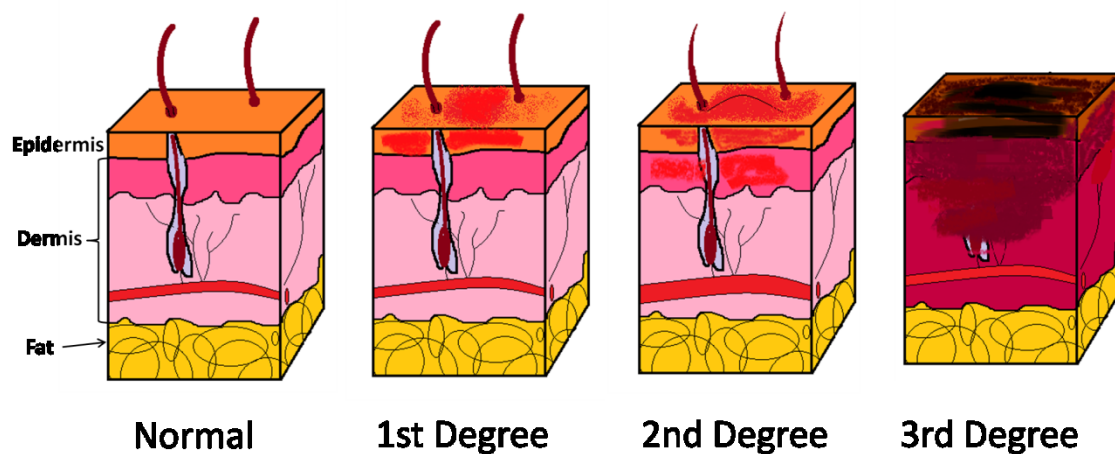


Figure 9.2. **Depth of burn injury.** Representation of normal skin and skin subjected to different degrees of burn.

9.1.3 Pain after burn injury

9.1.3.1 Understanding burn pain

The improvements in burn care during recent decades have led to an increase in the survival rate. However, little progress has been made to treat pain more effectively (Dauber *et al.*, 2002). Pain is a common feature of burn injury and recovery (Ulmer, 1997; Carrougner *et al.*, 2003; Phoenix-Society, 2011). It is one of the most important outcomes that still concerns patients (Dauber *et al.*, 2002; Sandoval *et al.*, 2014). In a survey carried between 2008 and 2013 to 753 burn survivors, pain was rated as more important than cosmetic appearance even to younger patients, female

patients and the ones suffering from head and/or neck burns (Sandoval *et al.*, 2014), and was perceived equally important as the resumption of normal function (Sandoval *et al.*, 2014).

Burn pain is complex and normally requires a multidisciplinary approach (Phoenix-Society, 2011). Pain can be felt in different ways according to its intensity, duration, timing, quality (itching, aching, shooting) or emotional impact (Phoenix-Society, 2011). Burn pain is not necessarily related to the size or seriousness of the burn (Phoenix-Society, 2011), has a changing pattern through time (Raymond *et al.*, 2004) and each patient may respond in a different manner (Giannoni-Pastor *et al.*, 2015). Additionally, since new painful sources are introduced over the treatment period, burn pain does not tend to decline with time (Raymond *et al.*, 2004).

9.1.3.2 Burn pain is a problem long after hospital discharge

Despite the improvements in the understanding and treatment of acute pain (pain related to the injury that resolves during the healing period) after burn injury, little is known about later time points, once survivors are discharged from hospital facilities (Dauber *et al.*, 2002; Browne *et al.*, 2011). However, there is not a consensus on the proportion of patients suffering or on the prognosis of burn pain. This is mainly a consequence of the heterogeneous population of burn patients (Pavoni *et al.*, 2010) together with methodological limitations and different recruitment processes (Giannoni-Pastor *et al.*, 2015), accuracy of measurements used (self-reports *versus* professional assessment), sample sizes and length of the studies (Carrougher *et al.*, 2003; Edwards *et al.*, 2007; Pavoni *et al.*, 2010; Giannoni-Pastor *et al.*, 2015).

It has been widely described in the literature that pain continues to be experienced years, even decades, after the trauma by a significant proportion of severely injured survivors (Carrougher *et al.*, 2003; Schneider *et al.*, 2006; Browne *et al.*, 2011). For

example, Dauber and colleagues observed that 52% of their study sample (n=358) experienced persistent pain (pain felt during the transition state from acute to chronic) up to 12 years post-injury (Dauber *et al.*, 2002) while Schneider and collaborators described neuropathic pain-like symptoms in 2% to 29% of their patients (n=72) starting later than four months after the burn and resolving around 8 months post-injury (Schneider *et al.*, 2006). In another population of study, moderate to severe pain was present in 79% of the patients (n=50) one year after the injury (Pavoni *et al.*, 2010). However, Shakespeare *et al.* observed 47% of the patients (n=45) with mild to severe pain (Shakespeare, 1998). Meanwhile, both Choiniere and colleagues and Malenfant and co-workers observed burn related pain up to 7-years post-injury (Choiniere *et al.*, 1991; Malenfant *et al.*, 1996).

9.1.3.3 Acute pain is the strongest risk factor to predict chronic pain

Mobility impairment, TBSA and length of hospital stay are considered predictors for persistent pain (Pavoni *et al.*, 2010; Browne *et al.*, 2011; Giannoni-Pastor *et al.*, 2015). Together with pain itself, they are related to an increased incidence of depression (Ulmer, 1997; Pavoni *et al.*, 2010), suicidal ideation (Edwards *et al.*, 2007), and anxiety (Wiechman Askay & Patterson, 2008) in burn survivors, up to 11 years post-injury (Browne *et al.*, 2011). Additionally, subjective variables related to individual personalities, the way to deal with a traumatic experience, life threat perception or coping strategies, also account for it (Ulmer, 1997; Giannoni-Pastor *et al.*, 2015).

Despite all, **acute pain severity** seems to be **the most robustly associated factor to predict burn chronic pain** (pain that extends beyond the expected period of healing) and the associated life disturbances (Ptacek *et al.*, 1995; Edwards *et al.*, 2007; Wiechman Askay & Patterson, 2008; Pavoni *et al.*, 2010; Browne *et al.*, 2011).

This was firstly described by Ptacek *et al.* in 1995 when they observed a significant correlation between procedural pain during the first 5 days of hospitalization and mental health issues one month after discharge (Ptacek *et al.*, 1995). A similar association was observed in a population of burn survivors from Western Australia: patients who recalled higher levels of acute pain during hospitalization were at significant higher risk of experiencing persistent pain (Browne *et al.*, 2011). Furthermore, pain during hospitalization has a stronger correlation with distress than any other variable investigated by Edwards and colleagues (Edwards *et al.*, 2007). Poor sleeping during the first week of hospitalization seems to be also linked to acute burn pain in a positive feedback loop: it gives higher pain intensity which leads to further sleeping complications in return and vice versa (Raymond *et al.*, 2004). It may also explain why effective pain management remains a top priority for burn injury survivors (Sandoval *et al.*, 2014).

Therefore, the aim should be to optimize burn pain control during the early phases which, apart from reducing suffering of patients during hospitalization (Ptacek *et al.*, 1995), may lower the cases of chronic pain, depression, post-traumatic stress disorder (PTSD) and suicide, among others.

9.1.4 Treating burn pain

At present, burn pain is frequently undertreated, as the strategies used are often not sufficient (Ptacek *et al.*, 1995; Ulmer, 1997; Schneider *et al.*, 2006; Demling, 2008; Wiechman Askay & Patterson, 2008; Wiechman Askay *et al.*, 2009). Effective management of burn pain is difficult and one of the most complicated aspects of burn injury (Martyn, 1986; Demling, 2008). It requires a multimodal perspective that combines both pharmacological and non-pharmacological approaches (Ulmer, 1997;

WHO, 2008b; Wiechman Askay & Patterson, 2008; Wiechman Askay *et al.*, 2009; Phoenix-Society, 2011; WHO, 2014).

Currently, opioids are the mainstay for control of burn pain (Carrougher *et al.*, 2003; Wiechman Askay *et al.*, 2009; Phoenix-Society, 2011). Potent and short-acting opioids are used when procedural pain is expected (Carrougher *et al.*, 2003) and they can be combined with non-opioid analgesics such as non-steroidal anti-inflammatory drugs (NSAID), anxiolytics and anaesthetics (Patterson *et al.*, 1997; Carrougher *et al.*, 2003; Cuignet *et al.*, 2007; Demling, 2008; Wiechman Askay *et al.*, 2009). However, there are certain limitations for the use of opioids to treat burn pain. There is a range of potential side effects, such as sedation and sleeping alterations, respiratory depression, delirium, nausea, low mood and constipation (Ptacek *et al.*, 1995; Raymond *et al.*, 2004; Wiechman Askay & Patterson, 2008; Wiechman Askay *et al.*, 2009; Phoenix-Society, 2011). Therefore, new analgesic approaches are still needed to treat burn pain more efficiently.

9.2 Pain, the warning system of an organism

In the previous section, the pain felt after burn injury was analysed. However, the question still remains: what does pain mean? Within this section, this concept will be analysed, as well as the different components of it and the way in which noxious information is transmitted to exert this sensation. Additionally, the importance of Nav1.7, as voltage-gated sodium channel, for pain processing and its involvement in burn injury-associated pain will be described.

9.2.1 Defining pain, nociception and different pain categories

The International Association for the Study of Pain (IASP) came with a definition of pain in 1996: “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. From this definition, pain is inferred as a personal and subjective ordeal, the result of multiple interactions between the signalling pathway and its modulation by higher cerebral centres (Woolf *et al.*, 2004; Marchand, 2008; Patel, 2010; Todd, 2010; Bie *et al.*, 2011; Steeds, 2013). By this statement, IASP considers that pain can only be experienced if the noxious stimulation is interpreted by the cortex to produce a multidimensional experience, which separates the concepts of pain and nociception. Nociception comes from the Latin *nocere* (to hurt) and was first appointed by Sherrington in the early 20th century. Nociception is defined as the sensory processing of a noxious stimulus, from the periphery to the brain via the pain/nociceptive pathway (Woolf *et al.*, 2004; Coutaux *et al.*, 2005; Patel, 2010; Woolf, 2011; Steeds, 2013). A nociceptive stimulus capable of activating this pathway is termed algogenic stimulus (Coutaux *et al.*, 2005).

Pain can be classified in two types: adaptive, if directed to increase the survival and/or healing of the individual; or maladaptive/dysfunctional, when presented as a pathological condition *per se*, normally persistent or recurrent and without protective or healing-promotion faculties (Woolf *et al.*, 2004; Costigan *et al.*, 2009). Furthermore, pain can also be divided in different states according to its characteristics:

- **Nociceptive pain** (also known as acute pain) can be considered as the pain resulting from a, normally, identified source that causes tissue damage and the subsequent activation of the nociceptive pathway. It is transitory and has an important role in the survival and maintenance of organism's integrity (Hunt & Mantyh, 2001; Marchand, 2008; Costigan *et al.*, 2009). It has an important adaptive nature (Woolf *et al.*, 2004; Cox *et al.*, 2006; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Costigan *et al.*, 2009; Nilsen *et al.*, 2009; Raouf *et al.*, 2010; Crow *et al.*, 2013). Nociceptive pain is normally treated with analgesics, NSAIDs, steroids and sodium channel blockers (Marchand, 2008; Wiechman Askay *et al.*, 2009). It can also be subdivided according to the origin on somatic or visceral (Marchand, 2008). Somatic nociceptive pain is characterised by a localised injury at skin, muscles, fascia and/or tendons and is caused by mechanical, thermal and/or chemical inputs (Marchand, 2008). Visceral nociceptive pain is due to distension of the guts, is poorly localised and generates autonomic responses; some examples are irritable bowel and cystitis (Marchand, 2008). In some occasion visceral pain can be felt as somatic pain due to referral to somatic areas (Marchand, 2008).
- **Inflammatory pain** is the consequence of the normal healing process that generates the organism to protect and recover from an injury, and as such is

considered adaptive (Woolf *et al.*, 2004; Marchand, 2008; Costigan *et al.*, 2009). In response to injury, the sensory system undergoes plastic changes, peripheral or central, to heighten sensitivity in the damaged site and thus aid healing and repair (Costigan *et al.*, 2009). Endothelial and immune cells in the site of injury, as well as the proper damaged cells and nociceptors, release different substances, some of which have pro-nociceptive characteristics (Coutaux *et al.*, 2005; Marchand, 2008; Costigan *et al.*, 2009). Thus, it is normally treated with NSAIDs or steroids (Marchand, 2008). Inflammatory pain normally wears off once the healing is completed. In pathological condition, the inflammatory pain could be due to hyperactivity of the neurons in the central nervous system (CNS), causing neuroinflammation (Marchand, 2008). Normally, experimental rodent models of inflammatory pain are induced by intraplantar injection of different irritants such as capsaicin, complete Freund's adjuvant (CFA), carrageenan, formalin or nerve growth factor (NGF) (Edelmayer *et al.*, 2014).

- **Persistent pain** was explained in the previous chapter on the context of burn injury (see above). This type of pain can result from sustained nociceptive input, reduced endogenous inhibitory mechanisms or hyperactivity of the endogenous excitation (Marchand, 2008; Wiechman Askay *et al.*, 2009).
- **Neurogenic/neuropathic pain.** A variety of definitions for neuropathic pain have been proposed through recent times (Woolf *et al.*, 2004; Marchand, 2008; Costigan *et al.*, 2009), however the IASP Special Interest Group on Neuropathic Pain (NeuPSIG) established the current definition in 2008 as a term that refers to different conditions due to diverse causes that are characterised by a direct lesion or disease of the somatosensory system,

either peripherally, centrally or both (Finnerup *et al.*, 2016). Some primary and secondary sensory neurons die following a peripheral nerve injury, thus connecting neuropathic pain with neurodegenerative diseases (Costigan *et al.*, 2009). Neuropathic pain is commonly treated with anticonvulsants, opioids, antidepressants and NMDA receptors antagonists (such as ketamine) (Marchand, 2008). The most used models of neuropathic pain in rodents are spinal nerve ligation (SNL) and chronic constriction injury (CCI) (Chaplan *et al.*, 1994; Edelmayer *et al.*, 2014).

Despite this classification, some aspects are shared by different pain categories. Once sensitised, an individual can perceive pain evoked by a stimulation that was previously experienced as non-painful, such as light touch, in a process known as **allodynia**. Further, an exacerbated and prolonged pain sensation can be generated in response to a noxious stimulation in a condition called **hyperalgesia** (Woolf *et al.*, 2004; Costigan *et al.*, 2009).

Furthermore, each pain category is also characterised by a unique combination of neurochemical changes at the peripheral and central levels along the nociceptive pathway (Hunt & Mantyh, 2001).

9.2.2 Nociceptive pathway: an anatomical review

The nociceptive pathway starts at the periphery, in the nociceptors, where they transmit information to the second order neurons in the spinal cord and the information is then transmitted to third order neurons. It terminates at different cortical areas in the brain, where elicits the perception of pain. It is transmitted through different areas including the spinal cord, the thalamus and brainstem. Different parallel pathways are implicated in the processing, each one contributing

differently to the final noxious experiences (Figure 9.3). The different levels of this pathway, from the periphery to the cortical areas, are explained in detail hereunder, with special focus on the nociceptors and the spinal cord.

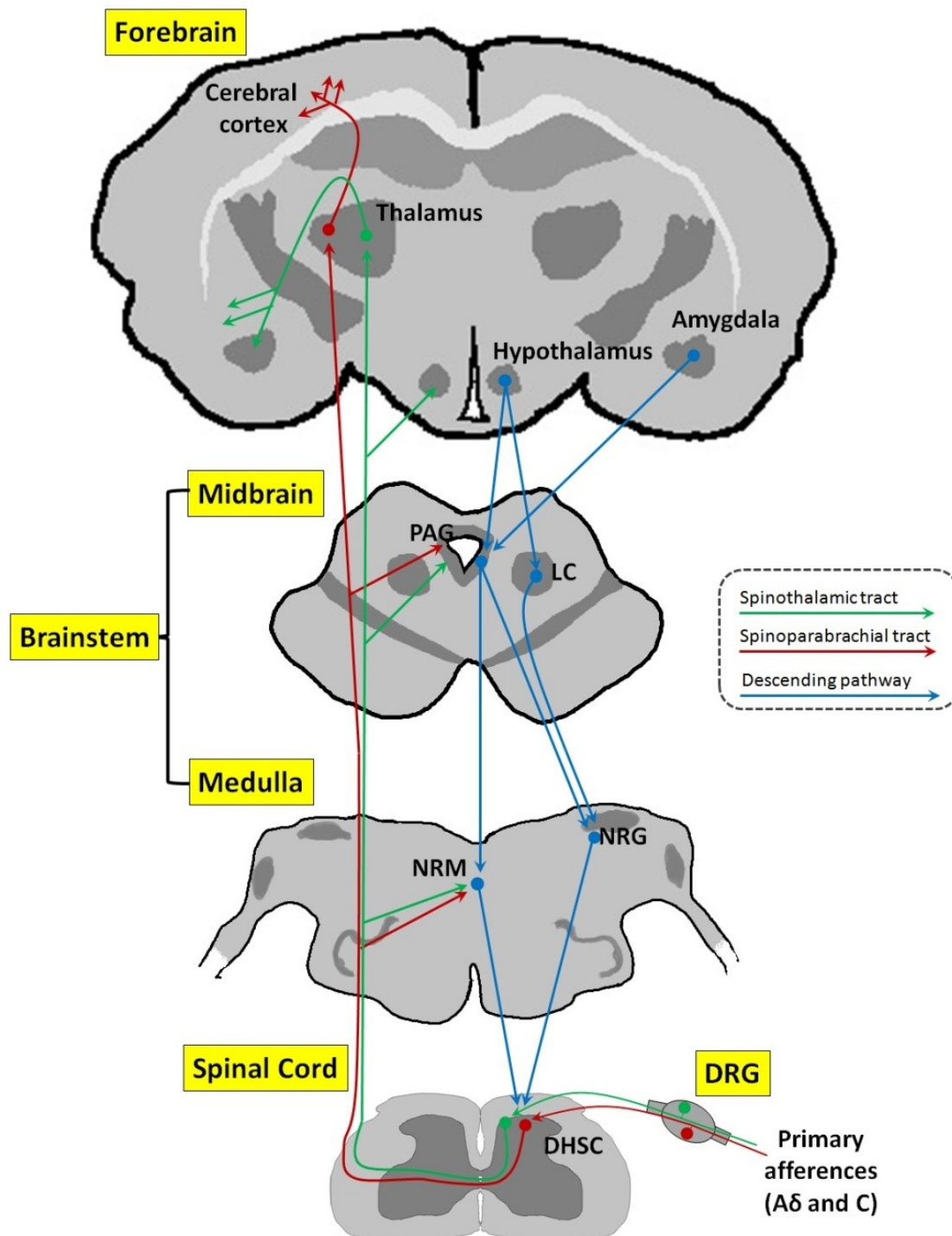


Figure 9.3. **Principal ascending and descending nociceptive pathways.** The two main ascending tracts are the spinothalamic (red) and the spinoparabrachial (green). Both originate in the dorsal horn of the spinal cord, DHSC. The descending pathway (blue) is mainly inhibitory and originates at the hypothalamus and the amygdala. DRG, dorsal root ganglion; LC, locus coeruleus; NRG, nucleus reticularis gigantocellularis; NRM, nucleus raphe magnus; PAG, periaqueductal grey. Adapted from: (Hunt & Mantyh, 2001; Todd, 2010; Steeds, 2013; Zhang *et al.*, 2014).

9.2.2.1 Nociceptors

Sensory fibres connect the skin and majority of peripheral tissues with the spinal cord. They are responsible to transmit nociceptive and non-nociceptive information. The ones that are specifically activated by noxious stimuli are termed **nociceptors** (Iggo, 1960; Woolf, 2011). Nociceptors terminate peripherally as free nerve endings, are highly specialised and responsible to carry nociceptive information (Woolf *et al.*, 2004; Patel, 2010; Steeds, 2013). The majority are polymodal, being able to respond to different stimulation modalities, such as mechanical, thermal ($>42^{\circ}\text{C}$ and $<10^{\circ}\text{C}$) or chemical (Hunt & Mantyh, 2001; Woolf *et al.*, 2004; Marchand, 2008).

Sensory fibres can be classified according with different criteria, which are not necessary exclusive, such as peripheral targets, conductivity or neurochemical properties. Nonetheless, the sensory fibres can be classified in four groups by those interrelated physical characteristics and their response:

1. $A\alpha$ fibres: have thick coat of myelin and diameters between 12 to 22 μm .
They conduct the impulse at really high velocity (70-120 m/sec) and transmit non-nociceptive stimuli (Body, 1958), therefore not relevant for nociceptive transmission.
2. $A\beta$ fibres: large myelinated fibres (diameter between 6 and 12 μm) that conduct the impulse nerve at a fast rate (35-75 m/sec). Similarly as $A\alpha$ fibres, they normally transmit innocuous stimuli like vibration, movement or light touch (Marchand, 2008). However, during chronic pain conditions they can undergo phenotypical changes and be implicated in pain modulation (see below) (Marchand, 2008; Costigan *et al.*, 2009).
3. $A\delta$ fibres: moderately large diameters (from 1 to 5 μm), poorly myelinated and conduct the signal at a relatively fast rate (5-30 m/sec). These can be divided

in two subtypes according to their response: the **mechanonociceptors** that mainly react to high-threshold mechanical forces, and the **polymodal nociceptors** that respond to mechanical, chemical and/or thermal stimulations (Julius & Basbaum, 2001; Marchand, 2008; Patel, 2010).

4. C fibres: have the smallest diameters (from 0.2 to 1.5 μm), are unmyelinated and conduct the impulse nerve quite slow (0.5-2 m/sec). They are **polymodal nociceptors** and as such respond to thermal, mechanical and chemical stimuli (Marchand, 2008; Patel, 2010; Todd, 2010). These nociceptors are mostly recruited by nociceptive information although are also implicated in non-nociceptive stimulation (Marchand, 2008). Around 10-20% of these fibres do not respond to acute nociceptive stimulations but can be gradually recruited during an inflammatory response (Hunt & Mantyh, 2001; Coutaux *et al.*, 2005).

The fast conduction through the A δ is responsible for the sharp initial pain sensation and for the rapid spinal response (the nociceptive withdrawal reflex), while the C fibres transmit a delayed, more diffuse, dull pain sensation (Julius & Basbaum, 2001; Marchand, 2008; Hunt, 2009; Patel, 2010) (Figure 9.4).

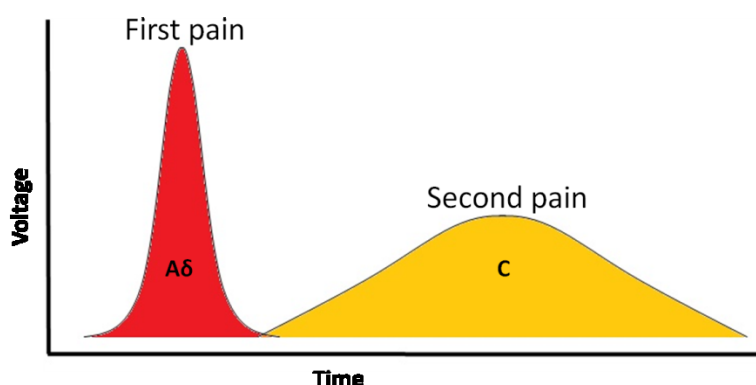


Figure 9.4. **Different conduction velocities between nociceptors.** An algogenic stimulus will initiate a relatively fast action potential through the A δ fibres (slightly myelinated and bigger diameters), responsible for the sharp pain experience. The conduction through C fibres will take more time to generate their action due to their smaller diameter and lack of myelin.

9.2.2.2 *Dorsal root ganglia*

The soma of the nociceptors, the primary sensory neurons, is collected together at either the dorsal root ganglia (DRG). These are paired ganglia situated alongside the spinal cord (Hunt & Mantyh, 2001). These neuronal somas are surrounded by satellite glial cells and their diameters reflects the type of fibres that they give rise to: bigger somas are related to myelinated rapidly-conducting fibres while small to medium diameters correspond to most of the nociceptors (Hunt & Mantyh, 2001; Julius & Basbaum, 2001). These are pseudounipolar neurons with an axon that is divided in two branches, one directed to the periphery and the other to the spinal cord. Furthermore, action potentials generated in the peripheral portion can bypass the soma and progress to the proximal branch. The fibres enter the spinal cord and send branches 1 or 2 segments up or down the spinal cord, where they make synapses with second order neurons (see below).

Histochemical studies of DRG, divide C fibres in two classes: **non-peptidergic**, which do not express peptides and express glial-cell-derived neurotrophic factor (GDNF) and the ATP-gated ion channel P2X₃, and are mainly innervating the epidermis of the skin; and **peptidergic**, characterized by expressing the peptides substance P and calcitonin-gene-related peptide (CGRP), and express tyrosine kinase receptor A (TrkA) which selectively binds nerve growth factor (NGF). The peptidergic fibres innervate different tissues as well as inner skin regions (Hunt & Mantyh, 2001; Julius & Basbaum, 2001; Hunt, 2009; Todd, 2010).

Following an algogenic stimulus such as burn injury; different events will take place. Nociceptors will initially sense the noxious input through non-selective ion channels that respond to chemical ligands, mechanical forces and/or temperature to produce inward current (into the cell) that depolarises the cell. Additionally, pro-nociceptive

inflammatory mediators, released after injury, can activate (nociceptive activators) and modulate the response of the nociceptors (nociceptive sensitizers) leading to **peripheral sensitization**: increased nociceptor sensitivity, which can lead to primary hyperalgesia and allodynia (Hunt & Mantyh, 2001; Ji *et al.*, 2003; Marchand, 2008; Costigan *et al.*, 2009; Steeds, 2013). Peripheral sensitization is a form of use-dependent fibre recruitment restricted to the injury side that involves intracellular signalling pathways and leads to altered production, transport and membrane insertion of different channels that tune-up the activation of the cells involved (Ji *et al.*, 2003; Costigan *et al.*, 2009; White *et al.*, 2011a).

If the depolarising event reached a certain threshold, voltage-gated sodium channels (VGSC) will be activated and start an action potential as well as an increased calcium ion (Ca^{2+}) influx. The frequency and the duration of such will reflect the intensity and duration of the peripheral noxious stimulus. If the action potentials are generated at high-frequency, different substances are released at the central terminal (at the spinal cord) of the axons: neurotransmitters, principally glutamate and substance P; and neuromodulator such as Brain Derived Neurotrophic Factor (BDNF).

9.2.2.3 The spinal cord, a key point of control

In 1952 Bror Rexed, a Swedish neuroscientist, divided the grey matter of the spinal cord from cats in different parallel laminae, a classification that still remains widely used today and has been extended to the different species of vertebrae (Figure 9.5). There are up to 10 laminae according to the neuronal distribution, density and the type of fibres innervating them (Rexed, 1952; Hunt & Mantyh, 2001; Todd, 2010). The entry zone for sensory fibres is the most dorsal portion of the grey matter,

termed the dorsal horn of the spinal cord (DHSC) where they synapse with second order neurons (Figure 9.5).

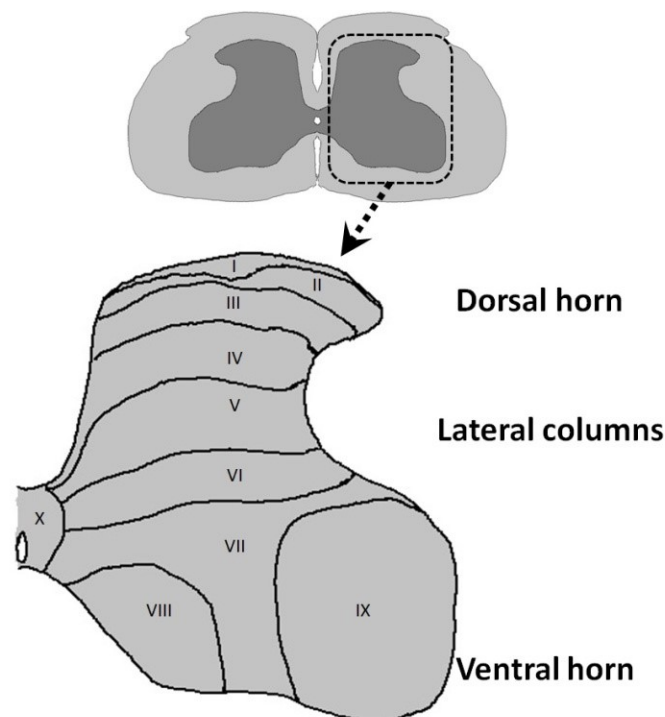


Figure 9.5. **Rexed laminae.** The grey matter of the spinal cord was originally divided by Rexed on 1952 because of his studies on cats. Following, this classification was extended to other animals. It consists of a maximum of 10 laminae (see roman numbers). Adapted from: (Rexed, 1952; Todd, 2010).

DHSC consists of a total of 6 laminae. DHSC can be subdivided in the superficial DHSC, corresponding to laminae I and II; and the lateral columns, which refer to laminae III to VI (Figure 9.5). The most marginal (outer) layer, the marginal zone, is the lamina I and contains a mix of neuronal types. There, around 10% are projecting neurons: neurons that send axons to higher central nervous centres, of which ~80% express the neurokinin-1 receptor (NK-1 receptor, also known as substance P receptor or SPR) (Hunt & Mantyh, 2001; Khasabov *et al.*, 2002; Suzuki *et al.*, 2002; Todd *et al.*, 2002; Todd, 2010). This discrete population of NK-1-positive neurons mainly respond to noxious stimulation and are the principal contributors to the ascending pathways (Khasabov *et al.*, 2002; Suzuki *et al.*, 2002; Todd *et al.*, 2002).

Following lamina I is lamina II, also known as substantia gelatinosa (although sometimes refers to both laminae I and II) due to the aspect it presents when examined in fresh tissue (Hunt & Mantyh, 2001; Todd, 2002). Lamina II is further subdivided into two parts: inner lamina II (Ili) and outer lamina II (Ilo), and is formed by a complex population of excitatory and inhibitory interneurons (Hunt & Mantyh, 2001; Todd, 2002).

The superficial DHSC is the mayor place where the primary afferents from nociceptors terminate and synapse with second-order neurons. A δ and C fibres mostly terminate in lamina I and Ilo, although some can reach deeper layers. Non-peptidergic C fibres mainly terminate within lamina Ili, while peptidergic C fibres tend to end more superficially (Hunt & Mantyh, 2001; Yoshimura & Furue, 2006; Todd, 2010).

There are different types of second order neurons in the DHSC: nociceptive specific, which respond exclusively to high-threshold noxious stimulation and are mainly situated in laminae I and Ilo, where get their input from the nociceptors; low-threshold, only activated by innocuous stimulation; and the wide dynamic range (WDR), situated in laminae V and VI that respond to both innocuous and nociceptive stimuli (Marchand, 2008; Todd, 2010; Steeds, 2013; Zhang *et al.*, 2014). As suggested by the name, the receptive field (the specific region in the space to which a neurons responds) of WDR neurons is dynamic and can change when subjected to repetitive painful stimulation (Marchand, 2008). Nonetheless, many neurons in the spinal cord are **interneurons**, non-projecting neurons. Interneurons are divided in two classes according to their effect: excitatory, which use glutamate as their neurotransmitter; and inhibitory, that release GABA (γ -aminobutyric acid) alone or in combination with glycine (Todd, 2010).

The spinal cord has long been described as a key centre in pain processing, subjected to different forms of activity-dependent plasticity (Woolf *et al.*, 2004; Yoshimura & Furue, 2006; Costigan *et al.*, 2009). The release of glutamate, the most abundant excitatory neurotransmitter within nociceptors (Julius & Basbaum, 2001), at the synaptic cleft activates α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors on the second order neurons, while neuromodulators such as BDNF will modify membrane excitability and mediate slow synaptic potentials, thus offering a chance of activity-dependent plasticity to occur at this level (Woolf *et al.*, 2004).

In 1965, Melzack and Wall first proposed the **gate-control theory** (Melzack & Wall, 1965; Torsney & MacDermott, 2006; Marchand, 2008; Woolf, 2011; Steeds, 2013). According to this theory, the first synapses between primary sensory neurons and the second order neurons in the DHSC can be modulated (Patel, 2010; Zhang *et al.*, 2014). In favour of this theory is the recruitment of new fibres during nociceptive processing: once activated by non-nociceptive stimuli, A β fibres can recruit interneurons in the substantia gelatinosa and through them inhibit the nociceptive stimulation in the same spinal segment (GABAergic or glycinergic inhibition) (Marchand, 2008; Costigan *et al.*, 2009; Steeds, 2013; Zhang *et al.*, 2014). However, during some pain conditions, this polysynaptic pathway can be recruited to transmit pain signal after a non-nociceptive stimulation in a process known as **disinhibition**, another example of use-dependent plasticity involving NMDA receptors and other molecular mediators (Hunt & Mantyh, 2001; Torsney & MacDermott, 2006; Marchand, 2008; Costigan *et al.*, 2009; Hunt, 2009; Todd, 2010; Zhang *et al.*, 2014). Further, high frequency repetitive stimulation of C fibres can lead to an increased firing response on the second order neurons in the DHSC, a

transient pain-amplifying effect known as **wind-up** (Woolf & Fitzgerald, 1983; Ji *et al.*, 2003; Woolf *et al.*, 2004; Marchand, 2008; Woolf, 2011; Steeds, 2013). NMDA is activated by glutamate and modulated via other receptors (e.g. phosphorylated), which contribute to this increased depolarization of neurons (Dubner & Ruda, 1992; Suzuki *et al.*, 2002). Wind-up is a reversible process of increased synaptic efficacy during stimulation, a homosynaptic change (Ji *et al.*, 2003; Woolf, 2011). Nonetheless, if this facilitated firing persists after the end of the noxious input, it is referred as central sensitization.

Central sensitization is a long-lasting increased excitability and spontaneous discharge (facilitation) of DHSC neurons accompanied by an augment of their receptive field, which results in secondary hyperalgesia and allodynia (Ji *et al.*, 1999; Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; Marchand, 2008; Hunt, 2009; Woolf, 2011; Steeds, 2013). It is considered a central process because, although is referred to the periphery, is due to changes at the spinal cord level (Ji *et al.*, 2003). Central sensitization is a form of long-term potentiation (LTP) similar as observed at the cortex (Ji & Woolf, 2001; Ji *et al.*, 2003; Costigan *et al.*, 2009; Woolf, 2011). This pathophysiological mechanism can appear at inflammatory and neuropathic pain states, and is considered a misadaption (Costigan *et al.*, 2009). Central sensitization principally affects WDR neurons and is dependent on activated NMDA due to a prolonged release of glutamate (Zou *et al.*, 2000; Ji & Woolf, 2001; Marchand, 2008). The projection neurons expressing NK-1 receptor, and as such responsive to substance P, have an important role on the development of this process: their loss attenuates hyperalgesia, wind-up processes and receptive field increased sizes following different noxious inputs such as inflammation and nerve injury or after experimental induction by capsaicin (Zou *et al.*, 2000; Ji & Woolf,

2001; Khasabov *et al.*, 2002; Suzuki *et al.*, 2002; Todd *et al.*, 2002; Hunt, 2009; Minett *et al.*, 2012). Neurokinins, nitric oxide and the loss of NGF seem to be triggering these changes (Ji *et al.*, 2003; Steeds, 2013). Although it was initially described in the DHSC, similar process of synaptic plasticity can happen at other central areas such as hippocampus or thalamus, therefore the term central sensitization was extended to any amplification of the neural signalling throughout the CNS (Ji *et al.*, 2003; Costigan *et al.*, 2009; Woolf, 2011).

9.2.2.4 *Ascending pathways*

The projecting second order neurons situated in the DHSC, mainly in lamina I, emit axons that cross the midline and then ascend rostrally, mainly via the spinothalamic and the spinoreticular tracts, to higher centres (Figure 9.3).

The **spinothalamic tract** is the most important ascending pain pathway (in green in Figure 9.3). It originates as second order neurons located mainly in laminae I and II. These neurons send fibres that cross the spinal midline and directly ascend to innervate the thalamus (Hunt & Mantyh, 2001; Yoshimura & Furue, 2006; Patel, 2010). The spinothalamic pathway is divided in lateral and medial tracts (Patel, 2010). The lateral spinothalamic tract sends projections to the ventral posterior lateral nucleus (VPL) of the thalamus and is involved in the discrimination aspects of the noxious input, while the medial spinothalamic tract sends its projections to the ventral posterior medial (VPM) thalamus and is involved in the autonomic and affective aspects of the pain experience. The spinothalamic tract can send collateral branches to other areas in the brainstem such as the periaqueductal grey (PAG) and nucleus raphe magnus (NRM), implicated in pain modulation (Hunt & Mantyh, 2001; Marchand, 2008; Steeds, 2013).

On the other hand, the **spinoreticular/spinoparabrachial tract** is considered the most ancient, phylogenetically speaking (in red at Figure 9.3). It starts in deeper laminae (VII and VIII) and projects to the centromedial thalamus and hypothalamus through the reticular formation in the brainstem. It also projects to structures in the brainstem implicated in pain modulation, such as PAG and NRM (Hunt & Mantyh, 2001; Marchand, 2008; Hunt, 2009; Bie *et al.*, 2011; Steeds, 2013).

Therefore, the thalamus is a key structure (Figure 9.3). From here, third order neurons project to a matrix of superior structures as follows: neurons from the VPL and VPM synapse at primary and secondary somatosensory cortices (SI and SII), while neurons from the centromedial nuclei target the limbic system (insula, anterior cingulate cortex –ACC– and prefrontal cortex)(Marchand, 2008; Hunt, 2009; Bie *et al.*, 2011; Steeds, 2013; Zhang *et al.*, 2014). The thalamus receives information from both painful and innocuous somatic stimuli (Patel, 2010). This leads to convergence of sensory information at same cortical areas (SI and SII), which allows the processing of the sensory discriminative aspects of pain (intensity, location and duration) while the projections to limbic structures are involved in the affective aspects of pain (Marchand, 2008; Patel, 2010). Furthermore, some modulation can occur at the cortical level with other types of information processed, such as person's expectation or suggestion, to modulate the final pain sensation (Marchand, 2008; Bie *et al.*, 2011).

9.2.2.5 *Descending pain modulatory pathways*

The descending tracts are important in modulation, either facilitating or inhibiting, the pain experience (in blue at Figure 9.3). Changes in the descending pathways are associated with generation and maintenance of central sensitization, which is verified

by the evaluation of secondary hyperalgesia in animal and humans (Woolf *et al.*, 2004).

The principal neurotransmitters implicated in this circuitry are noradrenaline/norepinephrine and serotonin (5-HT) (Woolf *et al.*, 2004; Yoshimura & Furue, 2006; Patel, 2010; Steeds, 2013). Endogenous opioids such as dynorphin and β -endorphine are important modulators (Yoshimura & Furue, 2006; Desroches *et al.*, 2014). The PAG is situated in the midbrain and receives projections from the thalamus, the hypothalamus and cortex, as well as collateral branches from the spinothalamic tract (Figure 9.3). Reynolds and colleagues demonstrated in 1965 that stimulation of this area results in nociceptive inhibition (Reynolds, 1969; Marchand, 2008). Neurons of this structure excite inhibitory cells from the NRM, the main source of serotonergic fibres, (Steeds, 2013) and which axons descend to the spinal cord via the dorsolateral funiculus (Yoshimura & Furue, 2006; Hunt, 2009). While the serotonergic system principally innervate neuron at the DHSC, noradrenergic-terminals are distributed throughout all spinal cord layers and produce direct inhibition of both interneurons and projecting neurons (Yoshimura & Furue, 2006). Nevertheless, both molecules cause nociceptors sensitization, although do not activate them directly under normal circumstances (Coutaux *et al.*, 2005).

The descending pathways are also implicated in facilitation of nociceptive processing (Woolf *et al.*, 2004; Marchand, 2008). There is a tonic release of serotonin, as well as GABA and glycine, at neurons of the DHSC (Yoshimura & Furue, 2006). The blockade of this tonic release can unbalance the nociceptive events transmitted to superior centres. Following a nerve injury, there is a shift of the descending pathways from inhibition to facilitation in a disinhibitory process (Costigan *et al.*, 2009). Descending systems seem subjected to plasticity as well, regulating gene

expression at the DHSC level following inflammation, and thus affecting central sensitization (Suzuki *et al.*, 2002; Ji *et al.*, 2003; Torsney & MacDermott, 2006; Yoshimura & Furue, 2006; Geranton *et al.*, 2008; Costigan *et al.*, 2009; Hunt, 2009; Geranton *et al.*, 2010; Bie *et al.*, 2011; White *et al.*, 2011b; Edelmayer *et al.*, 2014).

9.2.3 Molecular mechanisms of nociceptive processing

So far, the different types of nociceptors had been depicted as well as a brief description of the nociceptive pathway. However, to completely understand the transmission of noxious information, a molecular view must be assessed. Understanding the response at a subcellular level, identifying the transducers implicated, will help to elucidate how these cells are subjected to use-dependent plasticity, and thus how the nociceptive information gets modulated.

9.2.3.1 *Plasticity of the nervous system following an injury*

As has been described throughout the previous sections, there are different components involved in plasticity and modifiability along the nociceptive pathway. Peripheral and central sensitization, disinhibition, wind-up and processes of hyperalgesia and allodynia highlight the flexibility of the nervous system to adapt in response to tissue injury (Ji & Woolf, 2001; Ji *et al.*, 2003; Woolf *et al.*, 2004; Hunt, 2009; Steeds, 2013; Edelmayer *et al.*, 2014). These are all examples of **activity/function/use-dependent synaptic plasticity** events and in some cases, are accompanied by changes in gene expression and protein levels (Ji & Woolf, 2001; Ji *et al.*, 2003; Woolf *et al.*, 2004; Steeds, 2013; Edelmayer *et al.*, 2014). These changes are driven by cascades of intracellular transducers (e.g. intracellular increases of Ca²⁺ or cAMP) in response to the inputs received from the injury site

(Dubner & Ruda, 1992; Zou *et al.*, 2000; Ji & Woolf, 2001; Ji *et al.*, 2003; Woolf *et al.*, 2004).

- LTP: Hippocampus vs. spinal cord

Long-term potentiation (LTP) describes a type of long-lasting changes on the synaptic response in a use-dependent manner, a type of long-lasting synaptic plasticity (Hunt, 2009; Bie *et al.*, 2011). LTP was firstly described on 1973 by Bliss and Lomo at hippocampal neurons and is commonly associated with learning and memory consolidation (Bliss & Lomo, 1973; Ji *et al.*, 2003; Hunt, 2009; Woolf, 2011). This phenomenon is balanced by the opposite process, long-term depression (LTD) (Ji *et al.*, 2003). Most interestingly, LTP was also described in the spinal cord on 1993, in the context of central sensitization (Ji *et al.*, 2003; Woolf, 2011). This sensitization ensures that a protective response is exerted following and injury in an attempt to prevent further damage. Similar mechanisms as observed during hippocampal LTP seem to contribute to LTP on the projecting neurons of the superficial DHSC, especially in NK-1-expressing neurons from lamina I (Ji *et al.*, 2003; Kawasaki *et al.*, 2004; Hunt, 2009; Todd, 2010).

9.2.3.2 *Receptors implicated in nociception*

The vanilloid receptor TRPV1 normally senses noxious thermal stimulation (threshold ~44°C) and is present in around 45% of the small- to medium-size primary neurons (all C fibres and some Aδ). Thus, these fibres are also sensitive to capsaicin, the pungent ingredient on hot peppers and a selective activator of TRPV1 (Caterina *et al.*, 1997; Julius & Basbaum, 2001; Zhuang *et al.*, 2004; Coutaux *et al.*, 2005; Hunt, 2009; Nagy *et al.*, 2014). TRPV1 can be modulated by protons or capsaicin to lower its activation threshold to the point that physiological non-noxious

temperatures are able to activate nociceptors (Coutaux *et al.*, 2005; Nagy *et al.*, 2014). Despite the strong correlation between capsaicin and thermal nociception pointing at TRPV1 as the main contributor to this response, other channels may play a role on heat-stimulation such as TRPV2, insensitive to vanilloids but activated at high heat thresholds ($\sim 52^{\circ}\text{C}$) (Hunt & Mantyh, 2001; Julius & Basbaum, 2001). Interestingly, the expression of TRPV1 in the nociceptors' peripheral endings increases following peripheral inflammation, contributing to the enhanced sensitivity (Woolf *et al.*, 2004; Green *et al.*, 2013). Not surprisingly, the blockade of TRPV1 in a model of thermal injury results in a significant reduction of thermal allodynia (Green *et al.*, 2013). On the contrary, noxious cold (4°C) is detected by a different mechanism. It only activates around 15% of the fibres and seems to generate depolarizations by either blocking the maintenance of the Na^{+} and K^{+} balance or by actively promoting influx of Ca^{2+} and/or Na^{+} (Simone & Kajander, 1997; Julius & Basbaum, 2001).

Direct pressure, deformation or osmolality changes account as mechanical stressors that activate nociceptors. It is still not completely understood how mechanically gated receptors respond to these forces. However, several candidate receptors have been proposed: the channel BNC1 (also known as MDEG and ASIC2), TRPV4 and Piezo2 (Julius & Basbaum, 2001; Ranade *et al.*, 2014; Viatchenko-Karpinski & Gu, 2016). Mechanical stresses can also lead to ATP release from different cell types following an injury. ATP can then be detected by G-protein coupled receptors (e.g. P2Y) or ion channels (purinergic receptors such as P2X) present in nearby nociceptors (Julius & Basbaum, 2001; Raouf *et al.*, 2012).

Detection of protons released by an injury implicates the activation of the superfamily of Acid-Sensing Ionic Channels (ASICs). ASICs are composed of six

subtypes, which are activated at pHs below 6.9 (Coutaux *et al.*, 2005). From those, at least five subtypes are expressed in the small-diameter fibres and activated at the injury sites, where pH can reach values of 5.5 (Coutaux *et al.*, 2005). Furthermore, TRPV1 activation properties can also be modulated by protons in two ways: directly activating the channel at pH levels below 6 or by potentiating response to other stimuli such as capsaicin or heat. Interestingly, this modulation of TRPV1 by protons occurs at pHs between 6 and 8, typical levels found at injury sites (Julius & Basbaum, 2001; Nagy *et al.*, 2014). Although not its primary function, Nav1.7 and Nav1.8, two voltage gated sodium channels (see below), have also been linked to acid sensing (Minett *et al.*, 2012; Raouf *et al.*, 2012; Dib-Hajj *et al.*, 2013).

- Glutamatergic receptors

The glutamatergic receptors (Figure 9.6), ionotropic (ligand-gated non-selective cation channels) and metabotropic (mGluR, G-protein coupled receptors), are expressed on the membrane of the sensory fibres and within second order neurons. Ionotropic NMDA, AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainite receptors increase their density in response to inflammation and other noxious inputs, such as intradermal capsaicin injection or thermal injury (Zou *et al.*, 2000; Nozaki-Taguchi & Yaksh, 2002; Coutaux *et al.*, 2005; Hunt, 2009). AMPA and NMDA receptors are responsible for the fast and slow components of the excitatory synaptic currents, respectively (Bie *et al.*, 2011). Following their activation there is an influx of, Na^+ , K^+ and Ca^{2+} (Zou *et al.*, 2000).

During activity-dependent plasticity events, the NMDA and AMPA receptors are subjected to phosphorylation (kinase activity) and increased density (Dubner & Ruda, 1992; Zou *et al.*, 2000; Ji *et al.*, 2003; Woolf *et al.*, 2004; Costigan *et al.*, 2009; Todd, 2010; White *et al.*, 2011a). Phosphorylation of NMDA receptor enhances its

responsiveness by removing Mg^{2+} that normally blocks the receptor, which increases their open probability (Dubner & Ruda, 1992; Ji & Woolf, 2001; Ji *et al.*, 2003; Woolf *et al.*, 2004). Further, glutamatergic adaptation has also been observed during chronic pain on areas implicated on affective pain processing, such as ACC and amygdala (Ji *et al.*, 2003; Bie *et al.*, 2011).

- Voltage-Gated Sodium Channels (VGSC)

If a noxious input is enough to depolarize the membrane of the primary sensory neurons to a certain threshold, VGSC will open and generate the upstroke of the action potential (Coutaux *et al.*, 2005; Dib-Hajj *et al.*, 2007; Rush *et al.*, 2007; Raouf *et al.*, 2010; Black *et al.*, 2012; Eijkelkamp *et al.*, 2012). VGSC are composed by a large α -subunit, which is enough by itself to act as a functional channel (thus referred as channels by themselves within this thesis). Nonetheless, complementary β -subunits can also be associated and act as modulators of the channel activity (Baker & Wood, 2001; Pertin *et al.*, 2005; Lopez-Santiago *et al.*, 2006; Dib-Hajj *et al.*, 2010; Patino & Isom, 2010; Eijkelkamp *et al.*, 2012; Laedermann *et al.*, 2013). In mammals, there are nine different members on the family of VGSC, Nav1.1 to Nav1.9, encoded by a family of homologous genes, SCN1A-SCN5A and SCN8A-SCN11A, while there are 4 β -subunits, β 1 to β 4, encoded by SCN1B-SCN4B (England, 2008; Patino & Isom, 2010; Eijkelkamp *et al.*, 2012; Hoeijmakers *et al.*, 2012).

The nine VGSC can be classified by their sensitivity to the blocker tetrodotoxin (TTX): the sensitive ones, comprising Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.6 and Nav1.7; and the insensitive/resistant Nav1.5, Nav1.8 and Nav1.9 (Satin *et al.*, 1992; Akopian *et al.*, 1999; Baker & Wood, 2001; Ou *et al.*, 2005; Drenth & Waxman,

2007). They have a differential expression, suggesting a specialised function for each one of them (Nassar *et al.*, 2004; Eijkelkamp *et al.*, 2012; Shields *et al.*, 2012). Within nociceptors, Nav1.3, Nav1.7, Nav1.8 and Nav1.9 are preferentially expressed (Rush *et al.*, 2007; England, 2008; Dib-Hajj *et al.*, 2010; Hoeijmakers *et al.*, 2012; Shields *et al.*, 2012). The activity and expression of these sodium channels are modified in DRG neurons following injury and neuronal damage, which also suggests an important role in both inflammatory and neuropathic pain (Baker & Wood, 2001; Hunt & Mantyh, 2001; Gould *et al.*, 2004; Rush *et al.*, 2007; Costigan *et al.*, 2009; Dib-Hajj *et al.*, 2010; Hoeijmakers *et al.*, 2012). Nav1.3 for instance, is mainly present during embryonic stages although increases have been observed in adult DRG after axotomy or inflammation (Rush *et al.*, 2007; Black *et al.*, 2008; Hoeijmakers *et al.*, 2012). The other three channels, Nav1.7, Nav1.8 and Nav1.9, have a partially overlapping expression and seem to have evolved recently with a specialised role for nociception (Rush *et al.*, 2007; Dib-Hajj *et al.*, 2010; Shields *et al.*, 2012).

Nav1.9 was discovered in 1998 and is expressed in both the enteric nervous system and primary nociceptors (Eijkelkamp *et al.*, 2012). It is a TTX-resistant channel encoded by the gene SCN11A. Nav1.9 has very slow gating properties, and acts as modulator of membrane excitability rather than generator of the initial upstroke of action potentials (Raouf *et al.*, 2010; Eijkelkamp *et al.*, 2012).

Nav1.8 is also TTX-resistant and is mainly expressed in nociceptors (Akopian *et al.*, 1999; Costigan *et al.*, 2009; Raouf *et al.*, 2010; Eijkelkamp *et al.*, 2012). Nav1.8 was initially known as SNS (sensory neuron specific) or PN3 (peripheral nerve 3), and is encoded by the gene SCN10A (Eijkelkamp *et al.*, 2012). This channel is an essential regulator of the response to noxious cold and mechanical stimulations although does

not seem to contribute to neuropathic pain (Akopian *et al.*, 1999; Eijkelkamp *et al.*, 2012).

Nav1.7 is a TTX-sensitive VGSC present in peripheral sensory fibres, sympathetic neurons and olfactory epithelium that was first described in 1997 (Toledo-Aral *et al.*, 1997; Nassar *et al.*, 2004; Dib-Hajj *et al.*, 2007; Eijkelkamp *et al.*, 2012; Hoeijmakers *et al.*, 2012). It is encoded by the gene SCN9A and the homologue in rats is called peripherally nerve type 1 (PN1). There are different human inherited disorders associated with this gene that lead to aberrant pain perception (Nassar *et al.*, 2004; Fertleman *et al.*, 2006; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Rush *et al.*, 2007; Nilsen *et al.*, 2009; Raouf *et al.*, 2010; Eijkelkamp *et al.*, 2012; Hoeijmakers *et al.*, 2012). Nav1.7 is associated with both inflammatory and neuropathic pain (Cummins *et al.*, 2004; Nassar *et al.*, 2004; Yeomans *et al.*, 2005; Brochu *et al.*, 2006; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Hoyt *et al.*, 2007; McGowan *et al.*, 2009; Gingras *et al.*, 2014).

VGSC are also subjected to phosphorylation by kinases such as PKA or pERK1/2 (Baker & Wood, 2001; Drenth & Waxman, 2007; Stambouliau *et al.*, 2010). These post-translational modifications can modulate membrane excitability (Baker & Wood, 2001; Drenth & Waxman, 2007; Costigan *et al.*, 2009; Stambouliau *et al.*, 2010).

There is been a great interest during recent times on targeting VGSC for analgesic purposes. However, much work needs to be done to assess their properties in native conditions and the consequences of their inhibition (Baker & Wood, 2001; Nassar *et al.*, 2004; Costigan *et al.*, 2009; Raouf *et al.*, 2010).

9.2.3.3 Molecules released during injury and their effects

Injured tissues are responsible for the local release and production of different molecules. Damaged tissues (bradykinin, histamine, serotonin, ATP, prostaglandins

among others), immune cells (cytokines such as interleukins, interferon and tumour necrosis factors -TNF) or the neuronal cells itself (different neuropeptides: glutamate, substance P and CGRP) release their own molecules. Some of them act as nociceptive activators while the rest function as sensitizers (Julius & Basbaum, 2001; Woolf *et al.*, 2004; Coutaux *et al.*, 2005; Marchand, 2008; Costigan *et al.*, 2009) (Figure 9.6).

Different immune cells recruited to the site of injury, including lymphocytes, monocytes and macrophages, release small size proteins called **cytokines**. Some of these proteins have pro-nociceptive effects: TNF, interleukin (IL)-1 β and IL-8. While other cytokines have anti-inflammatory functions: IL-4, IL-10, IL-13 and IL-1ra. Nonetheless, some can act as both pro-and anti-inflammatory molecules (Coutaux *et al.*, 2005; Summer *et al.*, 2008). Following burn-injury pain, IL-6 is up-regulated at the damaged skin, suggesting a role of this cytokine on the induction of mechanical hyperalgesia (Summer *et al.*, 2008).

The peptides **bradykinin** and kallidin are liberated by injured tissues and exert their activating action by binding to G-protein-coupled receptors such as B1 receptors, which only appears in the membrane of nociceptors after induction by pro-inflammatory substances; and B2, present in post-synaptic neurons and with a function on central sensitization (Coutaux *et al.*, 2005; White *et al.*, 2011a). Bradykinin can modulate TRPV1 and thus exert enhanced heat-evoked currents in DRG, by either a Protein Kinase C (PKC)-dependent process or independently (Julius & Basbaum, 2001; Woolf *et al.*, 2004).

Growth factors are released at the side of injury by macrophages, fibroblast and others. They include NGF, BDNF, GDNF and neurotrophin-3 (NT-3) and are implicated on maintaining altered neuronal physiological status following an injury

(Hunt & Mantyh, 2001; Julius & Basbaum, 2001; Coutaux *et al.*, 2005). NGF acts as an adjuvant during inflammation, leading to thermal hypersensitivity (Julius & Basbaum, 2001). NGF selectively binds to TrkA, present in peptidergic C nociceptors (Zhuang *et al.*, 2004). Once NGF and TrkA are combined, they are internalised and transported to the soma, where activate different kinase pathways to alter the transcription of different peptides (such as substance P and CGRP, TRPV1), sodium channels and other growth factors (e.g. BDNF) (Ji & Woolf, 2001). BDNF, once released in the spinal cord, binds to its high-affinity receptor TrkB and indirectly phosphorylates NMDA receptors, thus implicating BDNF with central sensitization (Coutaux *et al.*, 2005). GDNF acts similarly as NGF but in non-peptidergic C fibres: preventing their loss (Hunt & Mantyh, 2001).

Further, some inflammatory agents elicit different effects. For example: IL-1 β and TNF- α are also inducing the enzyme type-2 cyclooxygenase (COX-2) that produces **prostaglandins** from the lipid messenger arachidonic acid (Julius & Basbaum, 2001; Woolf *et al.*, 2004; Coutaux *et al.*, 2005). Prostaglandins, e.g. the inflammatory mediator prostaglandin E₂ (PGE₂), can sensitize nociceptors and thus contribute to peripheral sensitization (Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; Coutaux *et al.*, 2005). Prostaglandins, and other prostanoids, act through G-protein coupled receptors, three of which are present on primary afferent fibres (Coutaux *et al.*, 2005). Nonetheless, products from COX-2 activity are found both at the periphery and centrally, at the spinal cord level (Julius & Basbaum, 2001; Ji *et al.*, 2003).

Substance P is a 11-amino acid neurotransmitter produced by around 20-30% of nociceptive primary afferents (Allen *et al.*, 1997; Ji & Woolf, 2001; Todd *et al.*, 2002). Following noxious stimulation, DRG neurons produce substance P and transport it to superficial DHSC neurons where binds to NK-1 receptor, a G-protein-coupled

receptor (Allen *et al.*, 1997; Hunt & Mantyh, 2001). Apart of activating NK-1 receptor and the subsequent cascade of intracellular mediators, substance P gets internalised by these neurons in a stimulus-intensity dependent manner (Allen *et al.*, 1997; Todd, 2010). Substance P, as well as CGRP, is up-regulated at the DHSC following inflammation although its expression decreases during neuropathic pain (Hunt & Mantyh, 2001; Khasabov *et al.*, 2002; Todd *et al.*, 2002).

9.2.3.4 Cascades of intracellular signals implicated in altered pain sensitivity

The activation of receptors by the inflammatory mediators induce the activation of kinases and other intracellular second messengers, transducing extracellular stimuli into intracellular responses (Figure 9.6). There is an intricate matrix of players situated either upstream or downstream from the effectors explained below. Ultimately, these pathways lead to post-translational modifications (PTM, covalent modifications of proteins during or after their synthesis) contributing to generation and maintenance of altered pain experiences (Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; Kawasaki *et al.*, 2004). On the one hand, PTM such as phosphorylation of different ion channels and other membrane receptors, alter the excitability of the neurons (for example increases their response). On the other hand, these pathways are important regulators of gene expression, as observed in primary and secondary sensory neurons. Gene regulation induces and maintains long-lasting functional modifications, contributing to persistent and chronic pain (Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; Kawasaki *et al.*, 2004).

Due to the influx from the exterior and release from intracellular stores, the concentration of Ca^{2+} and cAMP increase following a stimulation. Ca^{2+} activates different calcium-dependent kinases, including PKC and Ca^{2+} /calmodulin-dependent kinase (CaMK) (Ji & Woolf, 2001).

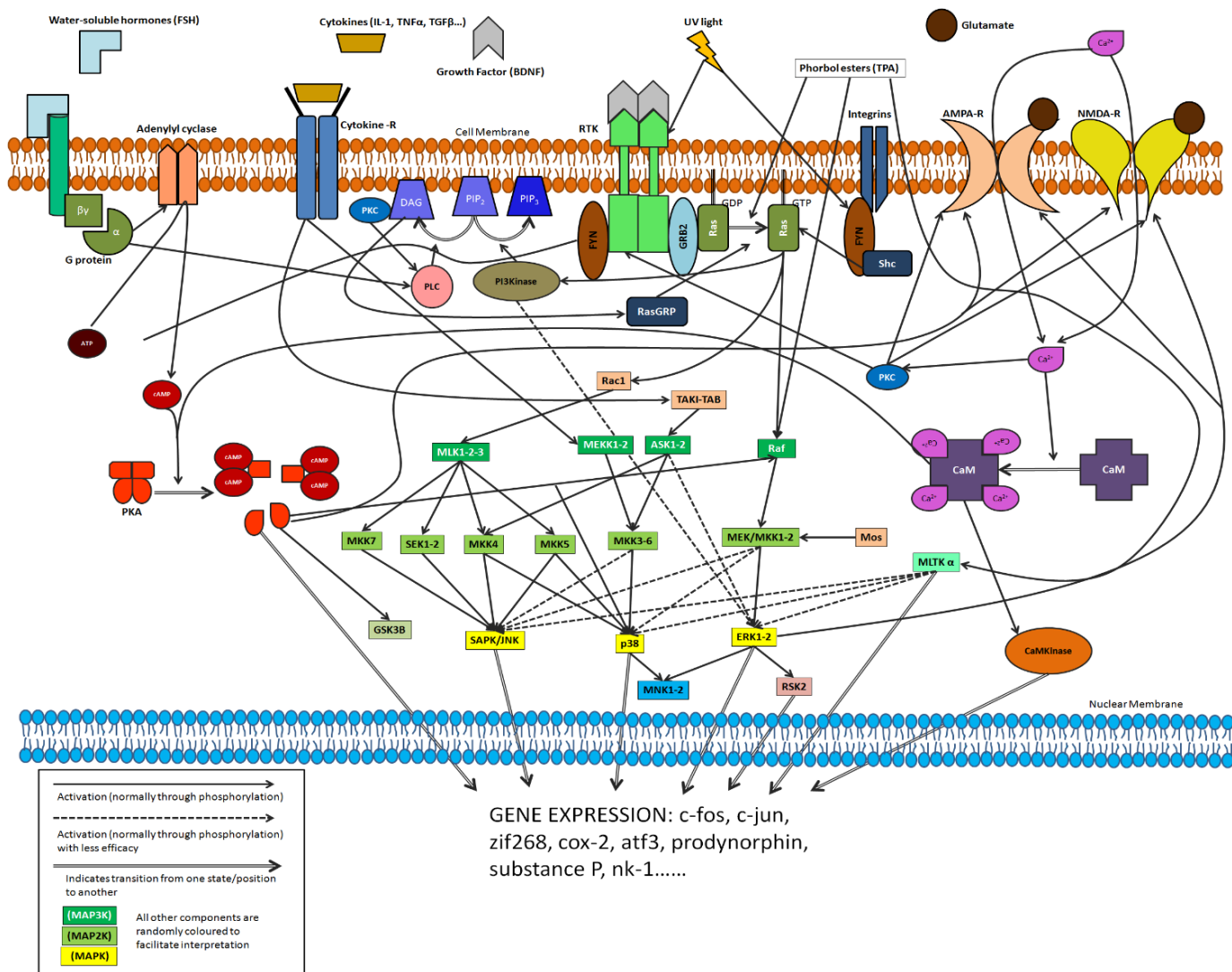


Figure 9.6. Intracellular processing of noxious stimuli. Following receptor activation, different pathways of second messenger will be activated modifying cell response and leading to changes on gene expression. AMPA-R: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, ATP: adenosine triphosphate, BDNF: brain-derived neurotrophic factor, CaM: calmodulin, CaMkinase: Ca²⁺/calmodulin-dependent protein kinase, cAMP: cyclic adenosine monophosphate, DAG: diacylglycerol *aka* diglycerade, ERK: extracellular signal-regulated kinases, FSH: follicle stimulating hormone, FYN: proto-oncogen tyrosine-protein kinase fyn, IL-1: interleukin-1, JNK: Jun amino-terminal kinase, MAP2K: MAPK kinase, MAP3K: MAP2K kinase, MAPK: mitogen-activated protein kinase, MEK: MAPK/ERK kinase, MEKK: MEK kinase, MKK: mitogen-activated protein kinase kinase, MLK: mixed lineage kinase, MLTK: MLK-like mitogen-activated protein, MNK: MAP kinase-interacting kinase, MSK: mitogen and stress activated protein kinase, NMDA-R: N-methyl-D-aspartic acid receptor, p38: p38 MAPK, Pi3kinase: phosphoinositide 3-kinase, PIP₂: phosphatidylinositol 4,5-biphosphate, PIP₃: phosphatidylinositol (3,4,5)-triphosphate, PKA: protein kinase A, PKC: protein kinase C, PLC: phospholipase C, Rac1: Ras-related C3 botulinum toxin substrate 1 (small signalling GTPase), Raf: RAF proto-oncogen ser/thr-protein kinase *aka* proto-oncogen c-RAF, RasGRP: Ras guanyl-releasing protein, RTK: receptor tyrosine kinase, SAPK: stress-activated protein kinase, SEK: SAPK/ERK kinase, Shc: SHC-transforming protein, TAK1: transforming growth factor beta-activated kinase 1, TNF α : tumor necrosis factor alpha, TPA: 12-o-tetradecanoylphorbol-13-acetate, UV light: ultraviolet light.

PKC (Figure 9.6) seems to contribute to increased thermal sensitivity (Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003). Binding of this kinase to the membrane of DHSC neurons has been observed to increase following different noxious inputs, especially the isoform γ , which is only present on neurons from the lamina III (Ji & Woolf, 2001). PKC directly phosphorylates AMPA receptors, while the phosphorylation of NMDA receptors seems to be mediated through the protein tyrosine kinase Src (Zou *et al.*, 2000; Ji & Woolf, 2001; Ji *et al.*, 2003; White *et al.*, 2011a).

CaMK proteins (Figure 9.6), especially the isoform CaMKII, are implicated in long-term synaptic plasticity by enhancing excitatory synaptic transmission in DHSC (Ji & Woolf, 2001; Ji *et al.*, 2003). Following noxious stimulation, activated CaMKII is docked to NMDA at the membrane, where it phosphorylates the AMPA receptor subunit GluR1 and thus enhances conductance of the channel (Ji *et al.*, 2003). cAMP, on the other hand, activates cAMP-dependent protein kinase A (**PKA**) (figure 2.4), a kinase implicated in both peripheral and central sensitization (Zou *et al.*, 2000; Ji & Woolf, 2001; Ji *et al.*, 2003).

Phosphatidylinositol 3-kinase, **PI3K** (Figure 9.6), is a lipid kinase that exerts its actions through downstream kinases and is activated by neurotrophic factors (Zhuang *et al.*, 2004). PI3K is also implicated on neuronal plasticity. In DRG, it can induce TRPV1 expression following NGF and capsaicin treatment but can also suppress the phosphorylation of NMDA receptors mediated by ERK (see below) (Zhuang *et al.*, 2004).

- Mitogen-activated protein kinases

Noxious stimulation also activates cascades of mitogen-activated protein kinases (**MAPK**) in neurons, as observed on DRG and DHSC, among other cell types (Figure

9.6) (Ji *et al.*, 1999; Ji & Woolf, 2001; Black *et al.*, 2008; Costigan *et al.*, 2009; Stambouliau *et al.*, 2010; White *et al.*, 2011a; White *et al.*, 2011b). These pathways of signal transduction start with MAPK kinase kinases (MAP3K) which then phosphorylate MAPK kinases (MAP2K) that consequently activate the MAPK (White *et al.*, 2011a). They, especially extracellular signal-regulated kinases (ERK, see below), have been implicated in the regulation of neuronal plasticity during processes such as acute and chronic pain (Ji & Woolf, 2001; Black *et al.*, 2008; Stambouliau *et al.*, 2010; White *et al.*, 2011a; White *et al.*, 2011b; Edelmayer *et al.*, 2014). Once activated, MAPK can act by two main mechanisms: a) modifying membrane excitability by either directly or indirectly phosphorylation of different receptor and ion channels, or b) orchestrating transcriptional changes (Ji *et al.*, 1999; Ji *et al.*, 2002b; Stambouliau *et al.*, 2010; White *et al.*, 2011a; White *et al.*, 2011b).

MAPK act on serine/threonine residues and their time course of expression/activation depends on the type of stimulation (White *et al.*, 2011a; White *et al.*, 2011b). Due to these and their dynamic characteristics and reliability, the phosphorylation of MAPK is recognised as established markers of neuronal activity (Gao & Ji, 2009; White *et al.*, 2011a; White *et al.*, 2011b; Edelmayer *et al.*, 2014; Worsley *et al.*, 2014). There are three major MAPK members implicated in mammalian neuronal plasticity: ERK, p38 MAPK, and c-Jun N-terminal kinase (JNK) (Ji & Woolf, 2001; White *et al.*, 2011a; Edelmayer *et al.*, 2014).

The **ERK** family (Figure 9.6) is composed by several enzymes including: ERK1 and ERK2, which are frequently described as ERK1/2 due to their molecular similarities, and ERK5 (White *et al.*, 2011a). The phosphorylation product of ERK (pERK) is involved in the generation of long-term memory paradigms (Ji *et al.*, 1999; Ji & Woolf, 2001; Ji *et al.*, 2003). pERK is the convergence effector of different regulators

situated up-stream (Ji & Woolf, 2001; White *et al.*, 2011a). Thus, pERK is rapidly induced on superficial spinal dorsal horn and DRG neurons following different types of noxious stimulation (heat, cold or mechanical) in an intensity-dependent manner, while non-noxious stimuli do not induce its phosphorylation (Ji *et al.*, 1999; Ji & Woolf, 2001; Ji *et al.*, 2002b; Polgar *et al.*, 2007; Black *et al.*, 2008; Gao & Ji, 2009; White *et al.*, 2011a; White *et al.*, 2011b). After different inflammatory pain models, pERK is specifically found in superficial DHSC neurons (Ji *et al.*, 1999). Following a scalding-type burn injury model in mice, there was an increase of pERK in the ipsilateral superficial DHSC as early as 5-minutes post-injury (White *et al.*, 2011b).

The up-regulation of pERK has also been observed in other areas of the CNS such as RVM and PAG following nerve injury or at the amygdala after peripheral inflammation (Geranton *et al.*, 2010; Bie *et al.*, 2011; White *et al.*, 2011a).

After cellular activation by different stressors (such as heat and ultraviolet radiation), molecules (lipopolysaccharides) and/or cytokines (IL-1 β or TNF- α), **p38** gets activated (phosphorylated, pp38). In neurons, the activation of p38 occurs in an activity-dependent manner (Ji *et al.*, 2002b). pp38 has key role responding to inflammation, NGF, cell survival and nerve injury, especially in DRG neurons of small diameter (Ji & Woolf, 2001; Edelmayer *et al.*, 2014). Similar as pERK, pp38 activates downstream processes (Hunt *et al.*, 1987; Ji & Woolf, 2001; Ji *et al.*, 2002b; Ji *et al.*, 2003).

The third type of MAPK is **JNK** (also known as stress-activated protein kinase, SAPK; Figure 9.6). JNK are activated following heat shock, DNA damage and other stressors. Increased activation of JNK has been detected in DRG neurons following peripheral axotomy (Ji & Woolf, 2001).

The role that MAPK exert in transcriptional regulation is still not completely understood. Nonetheless, it seems to involve downstream effectors. For example, phosphorylation of cAMP response element binding protein (**pCREB**) a downstream effector in most intracellular pathways (including pERK and pp38) has long been associated with the late phase of LTP. The immediate early genes (IEG) that possess the pCREB-binding sequence CRE (cAMP response element) in their promoter are subjected to its control. Interestingly, many of these genes (encoding for COX-2, NK-1, BDNF, ZIF268 and others) are induced in DRG and superficial DHSC following noxious stimulation, thus implicating pCREB with pain processing (Ji *et al.*, 2003; Kawasaki *et al.*, 2004; Bie *et al.*, 2011; Li *et al.*, 2014). Nonetheless, these downstream effectors act as transcription factors and/or chromatin remodelling molecules and their involvement will be discussed below (see Section 9.3).

9.2.3.5 Noxious stimulation results in different patterns of gene expression: immediate early genes and beyond

All these transducers will affect different parts of the cell, as well as directly or indirectly leading to changes in gene expression, which results in prolonged changes in the nervous system. In response to noxious stimulation and subsequent inflammation, **IEG** will get their expression increased, followed by upregulation of other genes such as the ones expressing neuropeptides, neuromodulators and their receptors (Dubner & Ruda, 1992; Ji & Woolf, 2001; Ji *et al.*, 2003; Drenth & Waxman, 2007).

IEG refers to genes where no synthesis *de novo* is required for their expression and thus can be accessible to the cell relatively quick after different stimuli; they include genes such as c-fos, c-jun, zif268 and cox-2 (Hunt *et al.*, 1987; Williams *et al.*, 1990;

Ji & Woolf, 2001; Ji *et al.*, 2003; Hunt, 2009). Therefore, targeting their expression can be a mechanism to regulate pain sensitivity (Ji *et al.*, 2003; Hunt, 2009).

The expression of c-Fos, the product of the gene *c-fos*, is increased in neurons from the superficial DHSC following different noxious inputs such as inflammation, and electrical or chemical stimulation (Williams *et al.*, 1990; Dubner & Ruda, 1992; Todd *et al.*, 2002; White *et al.*, 2011a). This increase was first reported by Hunt and collaborators in 1987 and seems to be related to NK-1 expression (Hunt *et al.*, 1987; Williams *et al.*, 1990; Todd *et al.*, 2002; Gao & Ji, 2009). Just after 30 minutes of heat stimulation, there is an increase of c-Fos in ipsilateral DHSC neurons, principally located in neurons innervated by C fibres (Williams *et al.*, 1990; Todd *et al.*, 2002). The levels of c-Fos correlated with an increased expression of the corresponding mRNA in these neurons, between 5 to 120 minutes of the noxious input (Williams *et al.*, 1990). Nonetheless, their expression is extended to deeper laminae at later time points (several hours), possibly due to polysynaptic pathways activating those neurons (Williams *et al.*, 1990). Importantly, c-Fos is only expressed in nuclei, does not get up-regulated following non-noxious stimulations and its basal levels are very low. Thus, c-Fos is regarded as a prototypical IEG and is commonly used as a marker of cell activation, although MAPK, such as ERK1/2, are considered better markers because of their dynamics and being selectively induced by noxious stimuli (Hunt *et al.*, 1987; Todd *et al.*, 2002; Gao & Ji, 2009; White *et al.*, 2011a). Interestingly, c-Fos is not induced in DRG (Gao & Ji, 2009).

Other IEG include *cox-2* and *c-jun*, which encode for COX-2 and c-Jun respectively. *cox-2* expression is regulated by p38 in DRG neurons (Ji & Woolf, 2001), an increase that has also been observed in DHSC neurons (Hunt *et al.*, 1987; Ji *et al.*,

2003), while c-Jun gets up-regulated in DRG following peripheral axotomy (Linda *et al.*, 2011).

Activating transcription factor 3 (ATF3) is another IEG that is induced in response to stress and influenced by growth factors, such as NGF. It is also induced in DRG following axotomy and ventral root avulsion, where it peaks after 6 hours of the stimulation. ATF3 binds to DNA sites together with c-Jun and is considered a reliable marker of neuronal regenerative capability, although is not implicated in cell survival (Linda *et al.*, 2011). One week after a burn injury, around 20% of the neurons affected express this IEG (Shields *et al.*, 2012).

Secondary-response genes (SRG), are genes activated with longer time course as IEG and include, for example, the genes that encode for prodynorphin, substance P, NK-1 and TrkB in the DHSC (Iadarola *et al.*, 1988; Williams *et al.*, 1990; Dubner & Ruda, 1992; McCarson & Krause, 1994; Ji *et al.*, 2002a; Ji *et al.*, 2003; Duric & McCarson, 2007). Although not completely understood yet, this “second event” of gene expression seems to be regulated by multiple factors including the proper expression of IEG such as c-Fos and c-Jun that together form the Activating Protein 1 (AP1) a regulator of gene transcription (Williams *et al.*, 1990).

9.2.4 Na_v1.7 is a feasible target for pain management

As explained above, VGSC play an important role on the signal transmission from the periphery to the spinal cord. From the different VGSC, Na_v1.7 seems to be critical in neuronal excitability and a major contributor to nociceptive inputs. Within this section, its distribution and function are explained, as well as assessing the importance of this channel in different animal models, with special focus on its relevance for burn injury-associated pain.

Furthermore, genetic evidences and pain disorders in humans are also described to demonstrate the importance of Nav1.7 activity and its regulation during pain sensation. Finally, current pharmacological approaches blocking the activity of this sodium channel are discussed, as a potential target for analgesic purposes.

9.2.4.1 *Distribution of Nav1.7*

Nav1.7 has a relatively restricted pattern of expression. It is predominantly found in DRG, trigeminal ganglia and sympathetic neurons (Toledo-Aral *et al.*, 1997; Nassar *et al.*, 2004; Dib-Hajj *et al.*, 2007; Minett *et al.*, 2012; Dib-Hajj *et al.*, 2013). Within DRG, this channel is highly present in small diameter neurons, principally in A δ - and C-fibres (Toledo-Aral *et al.*, 1997; Nassar *et al.*, 2004; Dib-Hajj *et al.*, 2007; Black *et al.*, 2012; Dib-Hajj *et al.*, 2013). Djouhry and colleagues described its presence in ~85% of functional nociceptors in guinea pigs (Djouhri *et al.*, 2003) while Black *et al.* observed its presence in ~65% of C-fibres and ~15% of A δ (Black *et al.*, 2012). Nonetheless, some large-diameter DRG neurons also express Nav1.7 (Gould *et al.*, 2004; Yeomans *et al.*, 2005; England, 2008; Dib-Hajj *et al.*, 2010; Dib-Hajj *et al.*, 2013). Nav1.7 has a great degree of co-expression with Nav1.8 which identifies the neurons as nociceptors (Nassar *et al.*, 2004; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Minett *et al.*, 2012; Dib-Hajj *et al.*, 2013).

Nav1.7 is not only present in the soma of these neurons, it also appears at both branches of the axon. At peripheral portion, it is found all the way throughout the nerve fibres to the free endings embedded in the skin, where is thought to function as a threshold channel. It is also present at the central projections, directed to the superficial DHSC, where seems to facilitate action potentials and/or neurotransmitter release (Toledo-Aral *et al.*, 1997; Dib-Hajj *et al.*, 2007; Black *et al.*, 2012; Minett *et al.*, 2012; Dib-Hajj *et al.*, 2013). Despite some discrepancies between data from

rodents and humans, Nav1.7 has only been detected at the PNS in humans (Toledo-Aral *et al.*, 1997; Morinville *et al.*, 2007; Dib-Hajj *et al.*, 2010; Black *et al.*, 2012; Dib-Hajj *et al.*, 2013; Minett *et al.*, 2015).

Although in much lower concentration, this VGSC is also reported in myenteric, olfactory and visceral neurons, and myocytes, as well as in non-sensory cells (Morinville *et al.*, 2007; Dib-Hajj *et al.*, 2013). It is suggested to play some roles in fluid homeostasis and cardiovascular control (Morinville *et al.*, 2007). Nonetheless, loss-of-function mutation in humans and knockout (KO) studies in mice (see below) only evidence impaired nociceptive processing (and olfaction), supports a critical role for Nav1.7 in these sensory modalities, while redundant for other autonomic functions (Dib-Hajj *et al.*, 2010; Dib-Hajj *et al.*, 2013; Minett *et al.*, 2015).

9.2.4.2 Structure and biophysical properties

VGSC have approximately 260 KDa and are composed by 1700-2000 amino acids. Each α -subunit has four domains named DI to DIV and each one of them is composed by six transmembrane segments (S1-S6) (Figure 9.7). Due to their high content on positively charged amino acids, segments 4 (S4 also named the voltage-sensor segments) undergo conformational changes following small depolarizations of the membrane ($\sim -50\text{mV}$), which leads to opening of the pore (Figure 9.7). This opening occurs within $\sim 1\text{ms}$ and allows the flux of Na^+ into the cell which then depolarizes further the membrane and initiates an action potential (Drenth & Waxman, 2007; England, 2008; Eijkelkamp *et al.*, 2012). Following, VGSCs get rapidly inactivated, which leads to decreased Na^+ current and subsequent membrane potential repolarization (Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; England, 2008; Eijkelkamp *et al.*, 2012).

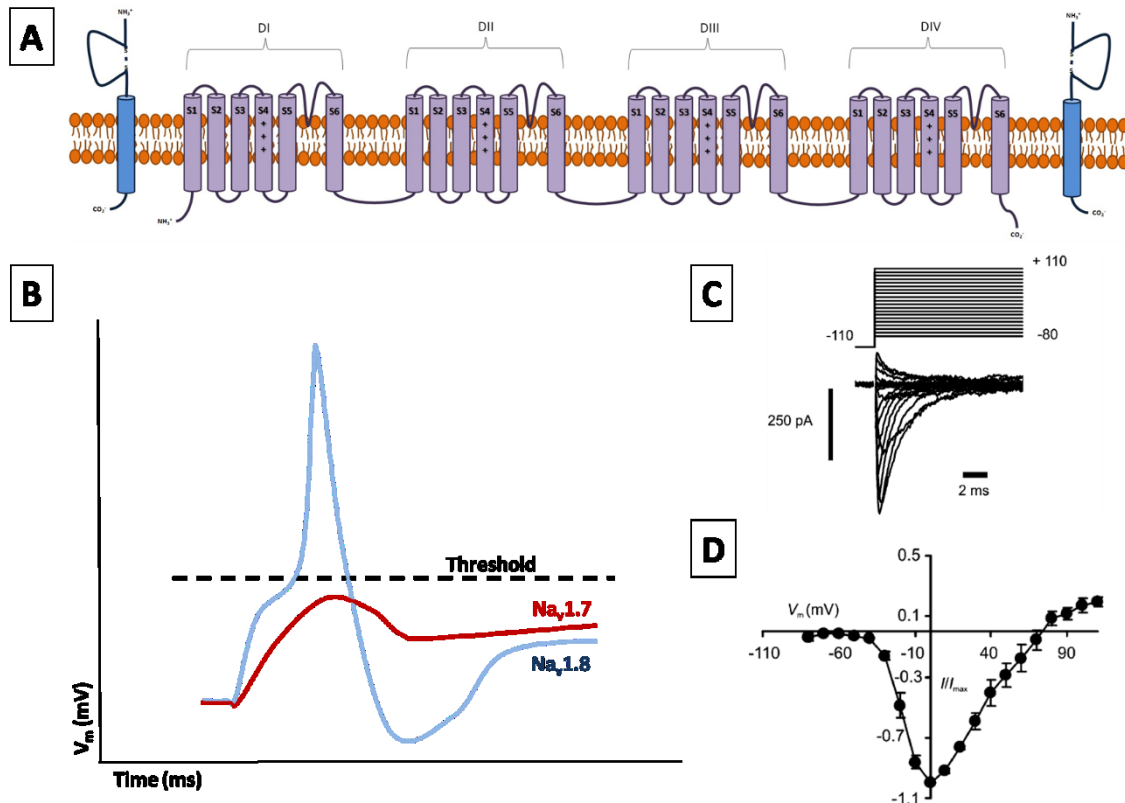


Figure 9.7. **Structure and physical properties of $\text{Na}_v1.7$.** A) Structure of VGSC: the alpha subunit (in purple) is divided in 4 domains (DI to DIV) and each domain is composed by 6 segments (S1-S6). Note the positive charges at the S4 of each domain, indicating their voltage-sensor quality. Regulatory beta subunits are shown in blue. B) Representative traces of a DRG neuron: $\text{Na}_v1.7$ responds to subthreshold stimulation while $\text{Na}_v1.8$ is triggered at suprathreshold current. C) TTX-sensitive (mainly $\text{Na}_v1.7$) currents in modified HEK293 cells in response to voltage-clamping steps. D) Current-voltage ratios for $\text{Na}_v1.7$. Note that C) and D) are adapted from: (Fertleman *et al.*, 2006).

$\text{Na}_v1.7$ is characterised by a fast activation and inactivation current with a slow-repriming from the inactivated state (Figure 9.7). Furthermore, it can respond to small or slow depolarization due to a slow closed-state inactivation. By these means, $\text{Na}_v1.7$ is capable to amplify sub-threshold potentials and generate a ramp current, a substantial current following these slow depolarizations (Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Rush *et al.*, 2007; Dib-Hajj *et al.*, 2010; Raouf *et al.*, 2010; Black *et al.*, 2012; Hoeijmakers *et al.*, 2012; Dib-Hajj *et al.*, 2013). Therefore, $\text{Na}_v1.7$ acts as a threshold channel, amplifying small depolarization and thus generating action potentials (Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Dib-Hajj *et al.*, 2010; Black *et al.*, 2012; Hoeijmakers *et al.*, 2012; Dib-Hajj *et al.*, 2013; Zhang *et al.*, 2013).

9.2.4.3 *Nav1.7 is important for nociceptive processing: animal studies*

In animal models of inflammatory pain such as carrageenan or CFA injection, the development of hyperalgesia is encompassed by an up-regulation in transcript and protein levels of Nav1.7. Stimulation with pro-inflammatory mediators also leads to this up-regulation. Similarly, the expression of Nav1.7 is increased in diabetic rats, probably contributing to the diabetic neuropathy. Although some studies do not describe a causative link, the up-regulation of Nav1.7 seems to relate to the increased sensory neurons excitability, which is further validated by studies in knockout animals (Toledo-Aral *et al.*, 1997; Cummins *et al.*, 2004; Gould *et al.*, 2004; Yeomans *et al.*, 2005; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Dib-Hajj *et al.*, 2010; Dib-Hajj *et al.*, 2013).

Until recently, Nav1.7-KO mice were not available due to their death at perinatal stages (Nassar *et al.*, 2004; Dib-Hajj *et al.*, 2007; Weiss *et al.*, 2011; Minett *et al.*, 2012; Gingras *et al.*, 2014). Nonetheless, Nassar's group created a "floxed" mice line, a type of conditional KO (cKO) in which Nav1.7 was only truncated in a subset of sensory neurons (Nassar *et al.*, 2004). Despite not showing any phenotypic deficits under normal conditions, the study of these mice following inflammatory pain models revealed a significant reduction of mechanical sensitivity and ~20% less response to noxious thermal stimulation (Nassar *et al.*, 2004). Thus, it was suggested that Nav1.7 has a relevant role for inflammatory pain (Nassar *et al.*, 2004; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Eijkelkamp *et al.*, 2012; Hoeijmakers *et al.*, 2012; Minett *et al.*, 2012). The year after this paper was published, a study applied an antisense sequence to SCN9A gene, to block Nav1.7 expression, through the hindpaw skin of mice (Yeomans *et al.*, 2005). Subsequently, they subjected these animals to the CFA model and observed that the expression of Nav1.7 protein

did not increase and that thermal hyperalgesia did not develop either, while the wild type (WT) animals (the controls) described those changes. Based on these findings, they suggested that Nav1.7 blockade can have an analgesic effect for chronic pain conditions with an inflammatory component, such as burn injury (Yeomans *et al.*, 2005).

Recently, Gingras and collaborators combined a series of genetic and husbandry techniques to overcome the death of Nav1.7-KO mice at perinatal stages (caused by their inability to feed due to anosmia, lack of the sense of smell) and by this created global Nav1.7-KO mice (Gingras *et al.*, 2014). Strikingly, these animals did not respond to any modality of nociceptive stimuli, recapitulating the phenotype of humans affected by congenital insensitivity to pain (CIP, see below), and supporting the role of Nav1.7 in acute to persistent pain conditions (Gingras *et al.*, 2014).

Furthermore, **Nav1.7 seems to have an important role in burn-injury associated pain** (Black *et al.*, 2012; Hoeijmakers *et al.*, 2012; Shields *et al.*, 2012). In 2012, Shields and colleagues studied differences between WT and Nav1.7-cKO mice following the induction of burn injury, using a hot plate at 65°C for 15 seconds (Shields *et al.*, 2012). They found that innocuous heat stimulation following the injury did not induce c-Fos in these cKO mice. Moreover, this also correlated behaviourally, where the Nav1.7-cKO mice did not display heat hypersensitivity, although showed reduced withdrawal thresholds for mechanical stimuli. Thus, Nav1.7 seems to be required to develop heat hypersensitivity and could be a feasible target to control pain in burn patients (Shields *et al.*, 2012).

9.2.4.4 Human evidence of Nav1.7 for nociceptive processing

There are different inherited pain disorders associated with mutations in the gene SCN9A, which encodes Nav1.7 (Yang *et al.*, 2004; Cox *et al.*, 2006; Fertleman *et al.*,

2006; Dib-Hajj *et al.*, 2007; Nilsen *et al.*, 2009; Dib-Hajj *et al.*, 2010; Raouf *et al.*, 2010; Hoeijmakers *et al.*, 2012; Crow *et al.*, 2013; Ramirez *et al.*, 2014). Gain-of-function mutations are found in cases of inherited primary erythromelalgia/erythermalgia (IEM), paroxysmal extreme pain disorder (PEPD), some patients with idiopathic small fibre neuropathy (SFN), and a patient with distal limb pain (Cummins *et al.*, 2004; Yang *et al.*, 2004; Fertleman *et al.*, 2006; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Dib-Hajj *et al.*, 2010; Raouf *et al.*, 2010; Eijkelkamp *et al.*, 2012; Hoeijmakers *et al.*, 2012; Dib-Hajj *et al.*, 2013; Huang *et al.*, 2014b). Interestingly, IEM, PEPD and SFN show striking similarities such as burning pain sensation and localized pain (Drenth & Waxman, 2007; Hoeijmakers *et al.*, 2012). In these cases of SCN9A gain-of-function mutations, the Nav1.7 channels show slow deactivation, increased ramp current and/or hyperpolarised activation, thus causing increased DRG neurons firing (Cummins *et al.*, 2004; Fertleman *et al.*, 2006; Dib-Hajj *et al.*, 2010; Raouf *et al.*, 2010; Eijkelkamp *et al.*, 2012; Hoeijmakers *et al.*, 2012; Dib-Hajj *et al.*, 2013; Huang *et al.*, 2014b).

On the other hand, Nav1.7 loss-of-function mutations lead to opposite phenotypes such as CIP, an autosomal recessive disorder that causes global insensitivity to any modality of pain since birth. When associated to SCN9A mutations, there are no other sensory modalities affected rather than anosmia, lack of the sense of smells (Cox *et al.*, 2006; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Nilsen *et al.*, 2009; Raouf *et al.*, 2010; Weiss *et al.*, 2011; Eijkelkamp *et al.*, 2012; Hoeijmakers *et al.*, 2012; Ramirez *et al.*, 2014). In this disorder, mutations in Nav1.7 cause loss of function, leading to a compromised nociceptive function (Cox *et al.*, 2006; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Nilsen *et al.*, 2009; Raouf *et al.*, 2010).

Apart of studying humans with inherited pain disorders related to SCN9A, alterations of Nav1.7 transcript and protein levels have also been observed following injury (Black *et al.*, 2008; Dib-Hajj *et al.*, 2010; Black *et al.*, 2012; Dib-Hajj *et al.*, 2013).

All these findings together show a causative link between Nav1.7 and nociceptive processing, which highlights the importance of this channel to regulate pain sensitivity (Black *et al.*, 2008; Dib-Hajj *et al.*, 2010; Hoeijmakers *et al.*, 2012; Dib-Hajj *et al.*, 2013).

9.2.4.5 Drugs to target Nav1.7

The evidence that Nav1.7 blockage seems to diminish the nociceptive perception has prompted the study of drugs as potential therapeutic purposes (Brochu *et al.*, 2006; Hoyt *et al.*, 2007; Williams *et al.*, 2007; England, 2008; Costigan *et al.*, 2009; McGowan *et al.*, 2009; Clare, 2010; Bregman *et al.*, 2011; Eijkelkamp *et al.*, 2012; Goldberg *et al.*, 2012; Macsari *et al.*, 2012; Yang *et al.*, 2013). Different **toxins** have been tested for specificity for Nav1.7 with different grades of success. (Middleton *et al.*, 2002; Cummins *et al.*, 2004; Schmalhofer *et al.*, 2008; Clare, 2010; Eijkelkamp *et al.*, 2012; Dib-Hajj *et al.*, 2013; Yang *et al.*, 2013; Zhang *et al.*, 2013). However, most of the toxins studied are not suitable for treatment. This is mainly due to their high molecular weights, making it difficult for them to reach the site of injury; their lack of isoform specificity, which causes unwanted side effects; and a narrow therapeutic windows (Williams *et al.*, 2007; England, 2008; McGowan *et al.*, 2009; Clare, 2010; Bregman *et al.*, 2011; Eijkelkamp *et al.*, 2012; Macsari *et al.*, 2012; Crow *et al.*, 2013; Yang *et al.*, 2013). Part of the problems could be overcome by designing state-dependent and isoform-specific drugs (referred to as **second generation sodium channel blockers**), which could help to target high-frequency firing VGSC at the site of injury while not compromising healthy tissues (Shao *et al.*, 2005; Brochu *et al.*,

2006; Hoyt *et al.*, 2007; Williams *et al.*, 2007; England, 2008; McGowan *et al.*, 2009; Clare, 2010; Bregman *et al.*, 2011; Theile & Cummins, 2011; Eijkelkamp *et al.*, 2012; Macsari *et al.*, 2012; Crow *et al.*, 2013; Dib-Hajj *et al.*, 2013).

9.3 Deciphering the epigenetic language of pain processes

Epigenetics, the changes in gene expression that do not implicate alterations in the DNA, have been linked to many biological processes. This section presents an up-to-date review of their implication in the pain field. Initially, the term epigenetics is introduced and explained carefully. Secondly, the different epigenetic modifications and the machinery implicated in their control are described. Finally, the term is unfolded in the context of the nervous system and its implications in processes such as neuronal plasticity, which is then extended into pain research. Although there are several articles summarising this knowledge (Denk & McMahon, 2012; Geranton, 2012; Crow *et al.*, 2013; Rahn *et al.*, 2013; Bai *et al.*, 2015; Geranton & Tochiki, 2015b; a), here a novel epigenetic modification, phosphorylation of serine 10 at histone H3 (pS10H3) is introduced as a potential key factor regulating nociception and a novel target for analgesic purposes, for example, for burn injury-associated pain.

9.3.1 What does epigenetics mean?

Since 1942, when the embryologist Conrad Waddington established the expression *epigenetic landscape*, many definitions have been proposed for this term (Holliday, 1990; Russo, 1996) until a consensus definition was established in 2008 at a Cold Spring Harbor meeting: “stably heritable phenotypes resulting from changes in a

chromosome without alterations in the DNA sequence” (Berger *et al.*, 2009). However, the heritability of some epigenetic mechanisms is still on debate, especially in the post-mitotic neurons (Geranton, 2012; Sweatt, 2013) (Sweatt, 2013; Geranton & Tochiki, 2015b). To solve this controversy and distinguish between alterations during development or by environmental effects, some sub-disciplines have emerged during last decade or so under names such as **behavioural epigenetics** or **neuroepigenetics** (Denk & McMahon, 2012; Geranton, 2012; Sweatt, 2013; Geranton & Tochiki, 2015b). To simplify, epigenetics is defined in this thesis as the changes in gene expression that happen in a particular cell without any change in the DNA sequence itself, independently from the heritability of those features.

Epigenetic changes, in opposition to the genomic ones, can be gathered within a single generation instead of taking millions of years (Geranton, 2012). Thus, these rapidly acquired alterations allow the organisms to adapt quicker (Geranton, 2012) by incorporating them onto the fixed DNA in an activity-dependent manner as a consequence of environmental experiences (e.g. stress, learning, addiction) (Bai *et al.*, 2010; Abramowitz & Bartolomei, 2012; Sweatt, 2013; Bai *et al.*, 2015; Geranton & Tochiki, 2015b). However, it is still too early to extrapolate most of these observations to humans (Juengst *et al.*, 2014).

9.3.2 Chromosome structure

In eukaryotes, the nuclear DNA is wrapped around proteins to form the chromatin, which enables the DNA to be compacted and physically fit in the nucleus of the cell (Baldwin *et al.*, 1975; Jenuwein & Allis, 2001; Dong & Bode, 2006; Rajapakse & Groudine, 2011; Luger *et al.*, 2012).

The smallest unit of chromatin is the **nucleosome**, a globular unit composed by ~145-147 bp of DNA wrapped around an octamer of histone proteins (Baldwin *et al.*, 1975; Cheung *et al.*, 2000a; Luger *et al.*, 2012; Ng & Cheung, 2016). The octamer of core proteins is formed by two copies of the following histones: H2A, H2B, H3 and H4; and is assembled in pairs as H2A-H2B and H3-H4 that then merge to form the histone octamer (Cheung *et al.*, 2000a; Dong & Bode, 2006) (Figure 9.8). Within each nucleosome, the DNA does one and three quarter turns around the histone octamer and the whole structure is held in place by exterior complementary proteins, like the histone H1 (Baldwin *et al.*, 1975; Jenuwein & Allis, 2001).

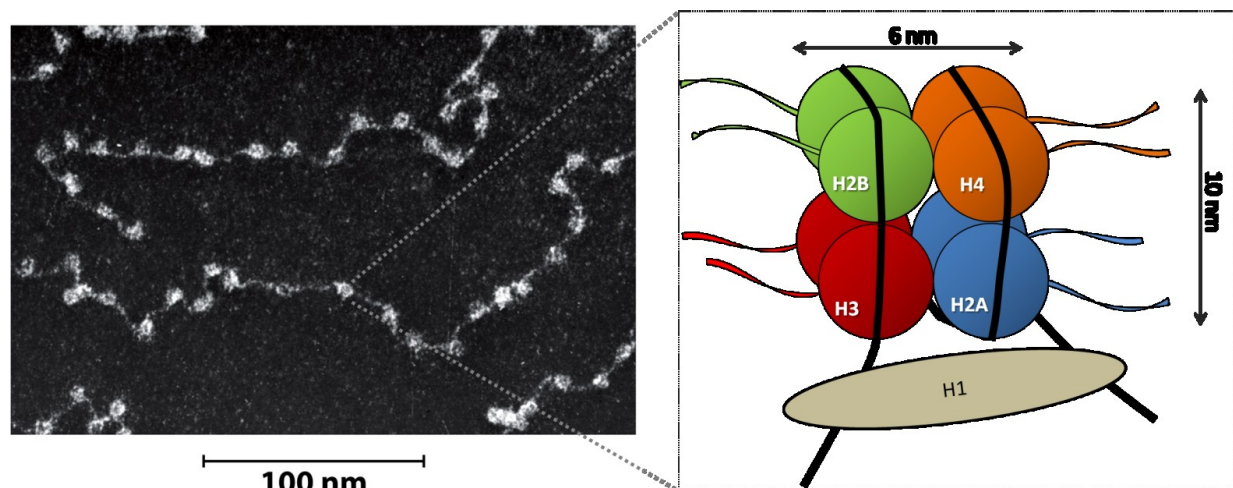


Figure 9.8. **Nucleosome structure.** Nucleosome, the structural unit of chromatin in eukaryotes, is composed by a segment of DNA of ~146bp wrapped around a histone octamer, which consist of 2 copies of each histone core proteins: H2B, H4, H2A and H3. Consecutive nucleosomes are joined together by a portion of DNA, termed *linker DNA*, with variable length (between 10-80 bp), forming the structure known as beads-on-a-string. The histone N-terminal tails are protruding outside the nucleosome structure. Additional proteins such as histone H1 help to maintain these structures. The electronic micrograph has been adapted from: (Berg, 2012).

The stability of the nucleosome is the result of interactions between histones and DNA. The C-terminal portions of the core histones have similar conformations which are critical for the assembly of the nucleosome (Cheung *et al.*, 2000a). Histones are basic proteins, positively charged, mainly due to their enrichment in lysine and arginine, that bind to the negatively charged DNA by numerous electrostatic and

hydrogen bonds (Baldwin *et al.*, 1975; Jenuwein & Allis, 2001; Dong & Bode, 2006; Luger *et al.*, 2012).

Within the nucleosome structure, the most N-terminal portions of the histone proteins, N-terminal tails also known as histone tails (accounting for ~25-30% of the protein mass), do not take part in the stability of the structure (Cheung *et al.*, 2000a; Strahl & Allis, 2000). Histone tails can make more flexible and dynamic contacts with the adjacent nuclear environment, nuclear factors, DNA or concomitant nucleosomes (Cheung *et al.*, 2000a; Luger *et al.*, 2012; Collepardo-Guevara *et al.*, 2015). These N-terminal domains protrude from the octamer and are more susceptible to be targeted to receive specific epigenetic modifications (Cheung *et al.*, 2000a; Collepardo-Guevara *et al.*, 2015) (Figure 9.8).

Each nucleosome is joint to adjacent nucleosomes by a portion of 10 to 70 bp of DNA, termed linker DNA, to form the beads-on-a-string structural model (Figure 9.8). In that conformation, the genes within can be transcriptionally active (Luger *et al.*, 2012; Ng & Cheung, 2016). Histone H1 can also interact to further package the nucleosomal chromatin into a 30nm fibre, which then can get more condensed by addition and interaction with scaffolding proteins such as histone chaperones, ATP-dependent chromatin remodelers or nucleosome-binding proteins (Agalioti *et al.*, 2002; Luger *et al.*, 2012). All this DNA packaging and compression allows the DNA to physically fit in the nucleus and controls the accessibility of the transcriptional machinery (e.g. RNA polymerase or transcription factors), and DNA replication and repair (Ng & Cheung, 2016). Thus, in the loosely packed chromatin the DNA is more accessible and transcription occurs more easily.

According to the packed stage, chromatin can be classified into **heterochromatin**, the densely compacted and consequently closed to transcription; and **euchromatin**,

the less organised, more easily accessed by the transcriptional machinery and thus also referred as open chromatin (Figure 9.9). Heterochromatin can also be divided in two types: facultative heterochromatin which differs by cell type (genes of a specific cell type), and constitutive heterochromatin which is the same in all type of cells and has a role in structural integrity (e.g. centromeres and telomers) (Jenuwein & Allis, 2001).

The DNA sequence can also affect the structure and position of the nucleosome. The nucleosome is mutable and susceptible to changes through time due to passive movements or by the activity of remodelling proteins, in a process known as nucleosome sliding (Agalioti *et al.*, 2002; Luger *et al.*, 2012). Genes that are actively transcribed tend to occupy preferred locations at the surface of chromosome territories, the peri-chromatin region, in an evolutionary conserved organization that facilitate the access of the transcriptional machinery to those genes (Rajapakse & Groudine, 2011).

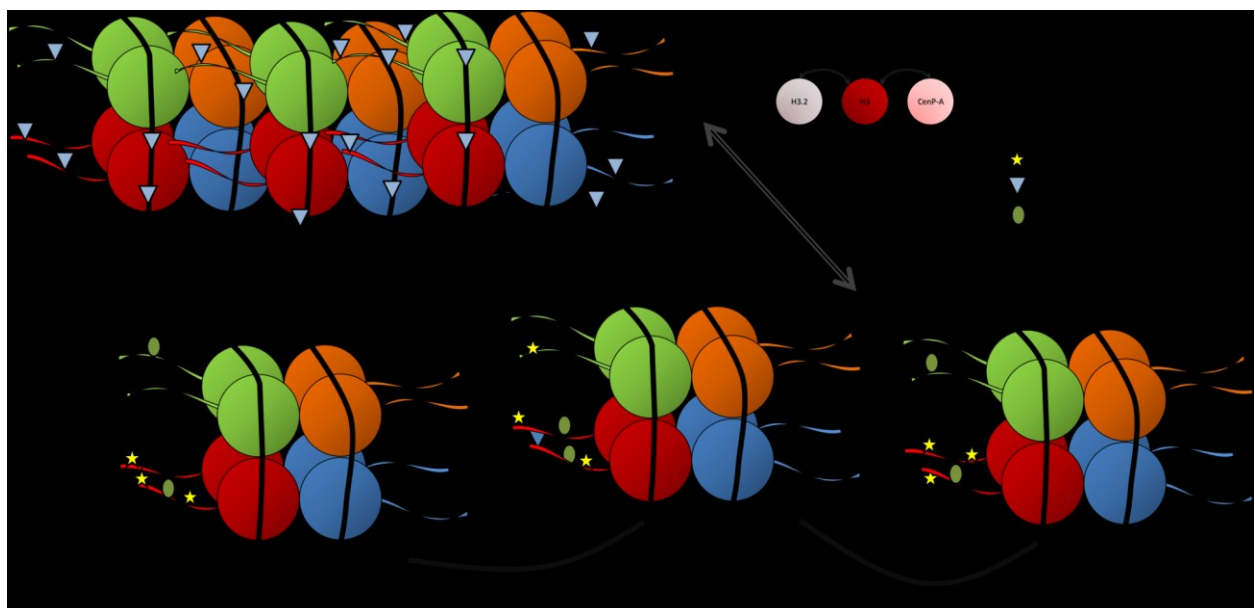


Figure 9.9. **Epigenetic mechanism can influence chromatin state.** Heterochromatin, the inactive (closed) state of chromatin, is characterised for a high level of compaction whereas euchromatin (open) is less condensed. Epigenetic mechanisms such as exchange of histone variants, DNA methylation and histone PTMs influence this process.

9.3.3 Epigenetic mechanisms.

There are different systems to achieve changes in gene expression without actually modifying the DNA sequence. The theoretical number of possible combinations of epigenetic modifications is very extensive (Monsey *et al.*, 2011; Luger *et al.*, 2012). They include DNA methylation, post-translational modifications (PTMs) of histone proteins, exchange of histone variants and the activity of non-coding RNAs, among other mechanism (Cheung *et al.*, 2000a; Strahl & Allis, 2000; Luger *et al.*, 2012; Sweatt, 2013) (Figure 9.9). DNA methylation and PTMs are due to the activity of different sets of enzymes. A common way to organise and classify them is per their ability to produce these epigenetic tags (the marks), enzymes known as *writers*, to recognise and respond to the mark, *readers*, and the enzymes responsible for the elimination of such marks, known as *erasers* (Table 9.1).

Table 9.1. **Different chromatin remodelling molecules.** The different proteins are organized for their ability to add (*writers*), to remove (*erasers*) or to bind (*readers*) epigenetic tags. Some of these molecules can also recruit additional epigenetic effectors to chromatin and can act in other proteins despite histones

		Histone PTMs			DNA methylation
		Acetylation	Methylation	Phosphorylation	
Writers	Activity	Histone acetyltransferases (HATs)	Lys Methyltransferases (KMTs)	Ser/Thr Kinases	DNA methyltransferase (DNMT)
	Examples	Gcn5 CBP/p300	KMT8/RIZ1 MLL family	MSK1-2 RSK2	DNMT1 DNMT3
Readers	Activity	Proteins with bromodomains	Proteins with chromodomains		
	Examples	Brd2 Brd4	PHD proteins Tudor-domain proteins	14-3-3 (isoforms)	MeCP2 MBD1-4
Erasers	Activity	Histone deacetylases (HDACs)	Lys demethylases (KDMs)	Phosphatases	*not completely understood*
	Examples	HDACs sirtuin	JHDM family LSD1/KDM1	MKP1 PP1	TET enzymes MBD2

Brd: Bromodomain containing, CBP/p300: CREB (cAMP response element-binding protein) binding proteins with the related p300 protein, Gcn5: general control of amino acids 5 HAT, JHDM: Jumoni domain-containing histone demethylase, LSD: histone Lys-specific demethylase, MBD: methyl-CpG-binding domain, MeCP2: methyl-CpG-binding protein 2, MKP: MAPK phosphatase, MLL: mixed-lineage leukemia, MSK: mitogen and stress activated protein kinase, PHD: zinc-finger, RIZ: retinoblastoma-interacting zinc-finger protein, PP1: protein phosphatase 1, RSK: ribosomal s6 kinase, TET: Ten-eleven translation enzyme.

9.3.3.1 *Non-coding RNAs*

This term refers to the RNAs that are not translated into protein or do not participate in protein translation *per se* (Bai *et al.*, 2015). Nonetheless, non-coding RNAs can regulate the protein translation, transcription rate or even the stability of other RNA molecules (e.g. mRNAs) (Collins, 2011; Bai *et al.*, 2015). Their expression can be influenced by environmental changes such as inflammatory mediators (Bai *et al.*, 2015). According to their size, non-coding RNAs can be classified in long, mid-sized and small (Collins, 2011; Crow *et al.*, 2013).

9.3.3.2 *DNA methylation*

In mammals, DNA methylation plays a key role in defining tissue-specificity during development (Geranton & Tochiki, 2015b). It occurs almost exclusively as a methyl addition at the carbon 5 of the pyrimidine ring of cytosine (resulting in 5-methylcytosine) followed by guanine residues (CpG dinucleotides) and is carried out by enzymes with methyltransferase activity (*writers*) (Crow *et al.*, 2013; Rahn *et al.*, 2013; Bai *et al.*, 2015; Geranton & Tochiki, 2015b). CpGs tend to cluster into CpG islands, often localised at promoters of genes (Crow *et al.*, 2013). Around 72-76% of protein-encoding genes in humans present this enrichment in their promoter (Bai *et al.*, 2015). Methylation at these CpG islands is associated to gene silencing (Borrelli *et al.*, 2008; Geranton, 2012; Rahn *et al.*, 2013; Geranton & Tochiki, 2015b). DNA methylation is mitotically heritable. The initial addition of methyl groups is done by *de novo* DNA methyltransferases (DNMT) such as DNMT3a and DNMT3b, and is mitotically inherited through other methyltransferases, such as DNMT1, that recognise the hemi-methylated DNA as their substrate (Crow *et al.*, 2013).

Methylation was considered a stable and permanent mark in the DNA, until active DNA demethylation (*eraser* activity) was observed, at first in early development and

then at later stages including adult neurons (Geranton, 2012; Yu *et al.*, 2015). Although the process is not completely understood, it seems to involve a set of different enzymes such as: TET family (Ten-eleven translocation family), Gadd45 family and the proper DNMTs (Rahn *et al.*, 2013; Geranton & Tochiki, 2015b; Yu *et al.*, 2015).

9.3.3.3 *Histone Post-Translational Modifications (PTMs)*

Although initially regarded as static components of the chromatin, histone proteins are dynamic structures susceptible to acquire different covalent modifications and involved in the regulation of the gene expression (Strahl & Allis, 2000). These PTM can occur in any part of the histones, however the N-terminal tail is where most of them happen since they protrude from the nucleosomal structure and are more easily accessed by other chromatin proteins (Strahl & Allis, 2000). Some histone tails are more susceptible to undergo epigenetic modifications than others mainly due to their positioning and length, thus happening predominantly on tails of histones H3 and H4, and less on tails of histones H2A and H2B (H4>H3>H2A/H2B) (Luger *et al.*, 2012). The evolutionary highly conserved histone H3 possesses the longest N-terminal tail (Barratt *et al.*, 1994; Davie, 2003; Bilang-Bleuel *et al.*, 2005; Collepardo-Guevara *et al.*, 2015) and is positioned at the exit point of the nucleosome, which confers this histone tail high importance mediating chromatin folding as well as recruiting other effectors (Davie, 2003; Luger *et al.*, 2012; Collepardo-Guevara *et al.*, 2015) (Figure 9.10).

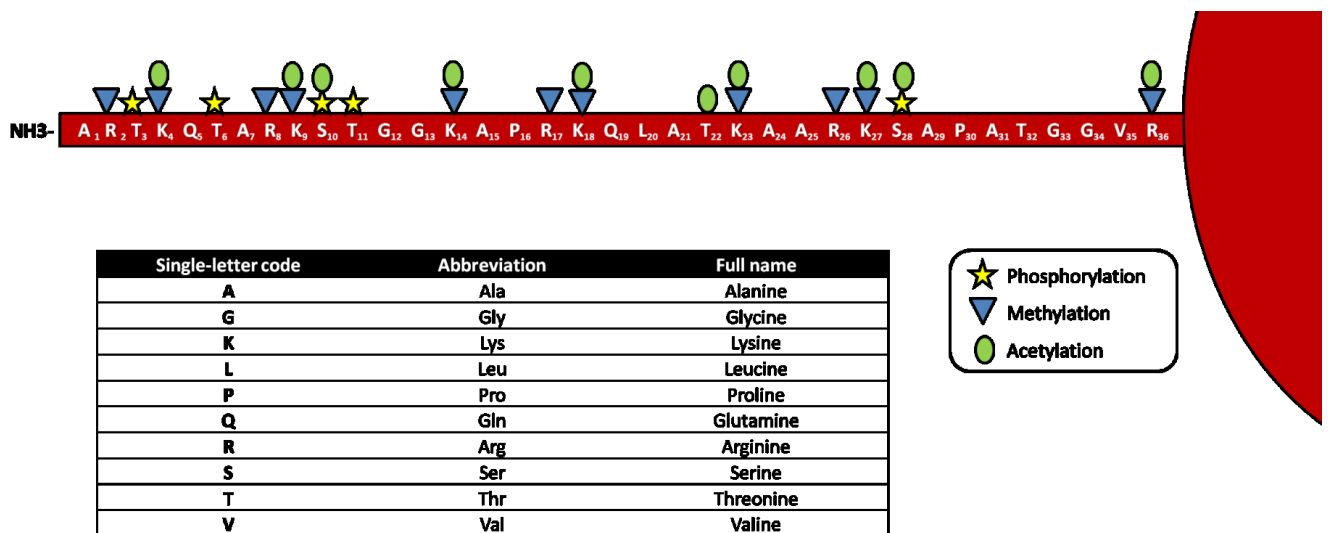


Figure 9.10. **Histone H3 tail is highly susceptible to PTMs.** The image shows the susceptibility of the different amino acids within the N-tail of histone H3 to phosphorylation, methylation or acetylation. Table describes standard abbreviations code for the amino acids presents within the tail. Adapted from (Huang *et al.*, 2014a).

Traditionally, each modification has been correlated with different functions like transcription activation, replication, condensation or repair, although histone PTMs can happen in many combinations, leading to different functional outcomes. The combinatorial effect of histone modifications was first described by Strahl and Allis in 2000, and is referred as the **Histone Code hypothesis**: a dynamic interplay between different modifications can occur on the same histone tail (Cheung *et al.*, 2000a; Cheung *et al.*, 2000b; Lo *et al.*, 2000; Strahl & Allis, 2000; Jenuwein & Allis, 2001; Agalioti *et al.*, 2002; Prigent & Dimitrov, 2003; Borrelli *et al.*, 2008; Rando, 2012; Ng & Cheung, 2016).

Histone PTMs influence chromatin structure by two mechanisms: recruiting of other proteins, and interfering with nucleosome structure (Barratt *et al.*, 1994; Lo *et al.*, 2000; Strahl & Allis, 2000; Jenuwein & Allis, 2001; Saccani *et al.*, 2002; Dong & Bode, 2006; Borrelli *et al.*, 2008; Collepardo-Guevara *et al.*, 2015).

13 different types of chemical modifications have been described in histones including: methylations (mono-, di- or tri-methyl groups), acetylations,

phosphorylations, ubiquitinations, sumoylations, ADP-ribosylations and/or citrullination (Rando, 2012; Bai *et al.*, 2015). Nonetheless acetylation, methylation and phosphorylation are the best known and characterised.

- Histone acetylation

Histone acetylation reduces the interaction between histones and DNA, by neutralising the positive charge of lysines (Allfrey *et al.*, 1964; Geranton & Tochiki, 2015b; Ng & Cheung, 2016) and is associated with activation of gene expression, whereas deacetylation is thought to suppress it (Bai *et al.*, 2015). The acetyl groups are added by histone acetyltransferases (HAT), while bromodomain proteins recognise (*reader* proteins) and bind to the acetylated amino acids (Jenuwein & Allis, 2001; Denk *et al.*, 2013; Geranton & Tochiki, 2015b; Ng & Cheung, 2016). Acetylations can then be removed by histone deacetylase (HDAC) enzymes, GCN5 and Rpd3 (Lo *et al.*, 2000; Ng & Cheung, 2016). A dynamic balance of HAT and HDAC activity is maintained in the cell to deliver a specific pattern of genes expressed (Bai *et al.*, 2015).

- Histone methylation

Some histone amino acids are susceptible to mono-, di- and tri-methylation (Figure 9.10). The addition of these methyl groups does not alter the charge of histones, although it could affect the ability of *reader* proteins to recognise and bind to them (Allfrey *et al.*, 1964; Borrelli *et al.*, 2008; Luger *et al.*, 2012). Different methylation stages have been correlated to either transcriptional activation or silencing (Jenuwein & Allis, 2001; Borrelli *et al.*, 2008; Meaney & Ferguson-Smith, 2010; Geranton & Tochiki, 2015b; Guiretti *et al.*, 2016). Histone methylation is laid down by lysine methyltransferases (KMTs, (Chen *et al.*, 1999; Ng & Cheung, 2016)), and removed by lysine demethylases (KDMs). There are different proteins that bind to

this epigenetic modification by their chromodomains: MBT, PHD and Tudor (Jenuwein & Allis, 2001).

- Histone phosphorylation

The addition of phosphate groups is an energy dependent process carried out by kinases. Phosphorylation adds negative charge to the histone tails, which may disrupt the interactions between histone H3 and the DNA sequence, also negatively charged (Mahadevan *et al.*, 1991; Cheung *et al.*, 2000a; Dong & Bode, 2006; Geranton & Tochiki, 2015b). Different serines, threonines, tyrosines and histidines are subjected to this modification and associated with chromatin remodelling (Gutierrez & Hnilica, 1967; Bai *et al.*, 2015) (Figure 9.10).

From the different amino acids susceptible to phosphorylation within histone H3 tail, the most studied is serine 10 (pS10H3) (Hendzel *et al.*, 1997; Ng & Cheung, 2016). This phosphorylation site is highly conserved among eukaryotes (Zhong *et al.*, 2000) and seems to be associated with antagonistic processes (Cheung *et al.*, 2000a; Jenuwein & Allis, 2001; He *et al.*, 2005; Dong & Bode, 2006). On the one hand, different investigations showed that pS10H3 is required for normal chromosomal segregation, condensed chromosomes during mitosis and meiosis (Gutierrez & Hnilica, 1967; Cheung *et al.*, 2000a; Cheung *et al.*, 2000b; Prigent & Dimitrov, 2003; Soloaga *et al.*, 2003; Dong & Bode, 2006; Pages, 2009; Ng & Cheung, 2016). On the other hand, pS10H3 is associated to transcriptional activation in yeast, invertebrate and mammalian cells in response to external stimulations such as stressors (UVB irradiation or heat shock), growth factors and cytokines, and pharmacological compounds (nickel, arsenite, phorbol esters, etc) (Mahadevan *et al.*, 1991; Barratt *et al.*, 1994; Cheung *et al.*, 2000a; Cheung *et al.*, 2000b; Lo *et al.*, 2000; Nowak & Corces, 2000; Zhong *et al.*, 2000; Salvador *et al.*, 2001; Sacconi *et al.*, 2002; Prigent

& Dimitrov, 2003; Dyson *et al.*, 2005; Dong & Bode, 2006). This induction is not a general process, if not only associated to a small set of IEG, like c-fos, c-jun and c-myc (Mahadevan *et al.*, 1991; Barratt *et al.*, 1994; Thomson *et al.*, 1999a; Cheung *et al.*, 2000a; Cheung *et al.*, 2000b; Clayton *et al.*, 2000; Nowak & Corces, 2000; Prigent & Dimitrov, 2003; Keum *et al.*, 2013). Interestingly, the kinases involved in this process during interphase are completely different from those observed during chromosomal condensation (kinases from Aurora family). They can be induced by different set of kinases according to each stimulation. Some of these kinases are: MAPK ERK, p38 and JNK, via downstream kinases MSK1/2 and RSK2 as the writers; I κ B Kinase and protein kinase A (PKA) (Supplementary Figure 20.5) (Gutierrez & Hnilica, 1967; Xing *et al.*, 1996; De Cesare *et al.*, 1998; Deak *et al.*, 1998; Chadee *et al.*, 1999; Jin *et al.*, 1999; Sassone-Corsi *et al.*, 1999; Thomson *et al.*, 1999a; Thomson *et al.*, 1999b; Cheung *et al.*, 2000b; Clayton *et al.*, 2000; Lo *et al.*, 2000; Nowak & Corces, 2000; Zhong *et al.*, 2000; Salvador *et al.*, 2001; Sacconi *et al.*, 2002; Davie, 2003; Ji *et al.*, 2003; Prigent & Dimitrov, 2003; Soloaga *et al.*, 2003; Yamamoto *et al.*, 2003; Kawasaki *et al.*, 2004; He *et al.*, 2005; Lo *et al.*, 2005; Dong & Bode, 2006; Pages, 2009; Bie *et al.*, 2011; Keum *et al.*, 2013; Li *et al.*, 2014; Yu *et al.*, 2015; Ng & Cheung, 2016).

The main protein identified with *reader* activity for pS10H3 is 14-3-3 ϵ (Prigent & Dimitrov, 2003) while the phosphatases in charge to remove this mark are still not fully identified. PP1 and MKP1 are the only ones so far suggested to remove this mark (*erasers*) (Supplementary Figure 20.5) (Prigent & Dimitrov, 2003; Kinney *et al.*, 2009; Pages, 2009)..

Table 9.2. **Genes regulated by epigenetic processes.** This table summarises some genes with functional links to neuronal modulation, the epigenetic modifications concomitants to their altered expression, the model/s in which they were assessed and the corresponding references. Arrows indicate increase (↑) or decrease (↓) expression.

Gene regulated	Epigenetic modification	Biological model	Stimulation	References
↑ bdnf IV	↓DNAmeth(Tet3)	Hippocampal neuronal culture	Electrophysiology and Tet3 presence	(Yu <i>et al.</i> , 2015)
↑ CBS	↓DNAmeth	DRG (in vivo)	CFA	(Qi <i>et al.</i> , 2013; Rahn <i>et al.</i> , 2013)
↑ c-fos	↑pS10H3, pH1, pS10aK14H3, pS28H3	Mouse fibroblasts, Ovarian granulosa cells, Hippocampal neurons (in vivo), Neurons in different brain areas (in vivo)	EGF, TPA, anisomycin, MSK1/2 presence, FSH stimulation, Novelty stimulus, Emotional stress (forced swim and immobilization)	(Chadee <i>et al.</i> , 1999; Thomson <i>et al.</i> , 1999a; Cheung <i>et al.</i> , 2000b; Salvador <i>et al.</i> , 2001; Soloaga <i>et al.</i> , 2003; Dyson <i>et al.</i> , 2005; Chandramohan <i>et al.</i> , 2008; Sarantis <i>et al.</i> , 2012; Rotllant <i>et al.</i> , 2013)
↑ c-jun	↑pS10H3, pS28H3, ↓paH	Mouse fibroblasts	EGF, anisomycin, TPA, MSK1/2 presence	(Thomson <i>et al.</i> , 1999a; Thomson <i>et al.</i> , 2001; Soloaga <i>et al.</i> , 2003; Dyson <i>et al.</i> , 2005)
↑ c-myc	↑pS10H3, pH1	Mouse fibroblasts	EGF, TPA	(Chadee <i>et al.</i> , 1999)
↑ cox-2	↑pS10H3, pS28H3, 3mK9H3; ↓pT3H3, pT11H3	Mouse epidermal cells	UVB	(Keum <i>et al.</i> , 2013)
↑ dnmt3a	↑aH3, DNAmeth	Lateral amygdala (in vivo)	Fear memory and synaptic plasticity	(Monsey <i>et al.</i> , 2011)
↑ gad65	↑aH3	Nucleus raphe (in vivo)	CFA	(Zhang <i>et al.</i> , 2011;

		vivo)		Rahn <i>et al.</i> , 2013)
↑heat shock puffs	↑pS10H3	Fruit fly	Heat shock	(Nowak & Corces, 2000)
↑ifnβ	↑aK19H3, aH4, pS10H3	HeLa cells	Viral infection	(Agalioti <i>et al.</i> , 2002)
↑IkBα	↑pS10H3, aK14H3	Mouse fibroblasts	TNFα	(Yamamoto <i>et al.</i> , 2003)
↑il-8	↑pS10H3, aK14H3	Mouse fibroblasts	TNFα	(Yamamoto <i>et al.</i> , 2003)
↑inhibin α	↑pS10H3	Ovarian granulosa cells	FSH stimulation	(Salvador <i>et al.</i> , 2001)
↑ino1	↑pS10H3, aK14H3	Yeast	Mutant cells	(Lo <i>et al.</i> , 2005)
↑mGlu2 receptors	↑aH	DRG and Spinal Cord (in vivo)	Formalin test	(Chiechio <i>et al.</i> , 2009; Bai <i>et al.</i> , 2010)
↑mkp-1	↑pS10H3, pS10aK14H3	Mouse C3H 10T1/2 cells, human endothelial cells	UV, H2O2, heat shock, arsenite, VEGF, thrombin	(Li <i>et al.</i> , 2001; Kinney <i>et al.</i> , 2009)
↑mos	↑pH1	Mouse fibroblasts	EGF, TPA	(Chadee <i>et al.</i> , 1999)
↑mRARβ2	↑pS10H3	P19 cells	Retinoid agonist	(Lefebvre <i>et al.</i> , 2002)
↑rest	↑aH3 and aH4	DRG (in vivo)	Nerve ligation	(Geranton, 2012)
↑sgk	↑pS10H3	Ovarian granulosa cells	FSH stimulation	(Salvador <i>et al.</i> , 2001)
↑synapsin1	↑pS10H3, aK14H3, aH4 ↓mK4H3	Aplysia cultures	5-HT-mediated LTF	(Guan <i>et al.</i> , 2002)
↑tRNA-cys	↑aK9H3	Spinal Cord (in vivo)	Nerve injury	(Denk <i>et al.</i> , 2013)
↑zif-268	↑pS10aK14H3	Hippocampus (in vivo)	Novelty stimulus	(Sarantis <i>et al.</i> , 2012)
↑δ-opioid receptor	↑aK9H3, ↓3mK9H3	PC12	NGF	(Agalioti <i>et al.</i> , 2002; Geranton,

2012)

↓calca(CGRP)	↓aH	Spinal Cord (in vivo)	CFA	(Crow <i>et al.</i> , 2015)
↓c-fos	↓aH	Primary hippocampal neurons, PC12 cells	Model of Huntington's disease	(Guiretti <i>et al.</i> , 2016)
↓eomes	↓aH	Primary hippocampal neurons, PC12 cells	Model of Huntington's disease	(Guiretti <i>et al.</i> , 2016)
↓homer1	↓aH	Mice brains (in vivo)	HDAC4 presence	(Sando <i>et al.</i> , 2012)
↓lgfbp5	↓aH	Primary hippocampal neurons, PC12 cells	Model of Huntington's disease	(Guiretti <i>et al.</i> , 2016)
↓lgi1	↓aH	Mice brains (in vivo)	HDAC4 presence	(Sando <i>et al.</i> , 2012)
↓mapk8	↓aH	Mice brains (in vivo)	HDAC4 presence	(Sando <i>et al.</i> , 2012)
↓miR-219	↑DNAmeth	Spinal Cord (in vivo)	CFA	(Pan <i>et al.</i> , 2014)
↓plk5	↓aH	Primary hippocampal neurons, PC12 cells	Model of Huntington's disease	(Guiretti <i>et al.</i> , 2016)
↓pp1	↑DNAmeth	Mice brains (in vivo)	Fear conditioning	(Borrelli <i>et al.</i> , 2008; Geranton & Tochiki, 2015b)
↓prdm12	↓mH	Humans	Mutation → CIP	(Chen <i>et al.</i> , 2015)
↓prka	↓aH	Mice brains (in vivo)	HDAC4 presence	(Sando <i>et al.</i> , 2012)
↓rgs2	↓aH	Mice brains (in vivo)	HDAC4 presence	(Sando <i>et al.</i> , 2012)
↓rin1	↓aH	Primary hippocampal neurons, PC12 cells	Model of Huntington's disease	(Guiretti <i>et al.</i> , 2016)
↓syngap	↓aH	Mice brains (in vivo)	HDAC4 presence	(Sando <i>et al.</i> , 2012)
↓trpv1	↓aH	Spinal Cord (in vivo)	CFA	(Crow <i>et al.</i> , 2015)

bdnf: brain-derived neurotrophic factor, calca: calcitonin, cbs: cystathionine-beta-synthetase, CGRP: calcitonin gene related peptide, cox-2: type-2 cyclooxygenase, dnmt3a: DNA methyltransferase 3A,

eomes: eomesodermin, gad65: glutamic acid decarboxylase 65, homer1: protein homolog 1, ifn β : interferon beta, igfbp5: factor-binding protein 5, ikb α : nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha, il-8: interleukin 8, ino1: inositol-3-phosphate synthase, lgi1: leucine-rich glioma inactivated 1, mapk8: mitogen-activated protein kinase 8 *aka* JNK1, mkp-1: mitogen-activated protein kinase phosphatase-1, mos: plk5: polo-like kinase 5, pp1: protein phosphatase 1, prka: A kinase, prdm12: PR/SET domain containing 12, proto-oncogene serine/threonine-protein kinase mos, mRAR β 2: retinoic acid receptor beta 2, rest: neuron-restrictive silencer factor, rgs2: regulator of G-protein signalling 2, rin1: Ras and Rab interactor 1, sgk: serine/threonine-protein kinase SGK, syngap: synaptic Ras GTPase-activating protein 1, tRNA-cys: mitochondrial cysteine transfer RNA, trpv1: transient receptor potential cation channel subfamily V member 1, zif-268: zinc finger protein 268. For the meaning of the epigenetic marks check table within Figure 9.10.

9.3.4 Epigenetic control of the nervous system and its involvement in pain processing

The number of epigenetic processes suggested to be associated with the function of the nervous systems has been increasing during recent times (Table 9.2). Epigenetic mechanisms are implicated in the reprogramming that neuronal tissues undergo during different processes, including synaptic plasticity, learning, injury, neurodegenerative diseases, stress and addiction (Zhong *et al.*, 2000; Agalioti *et al.*, 2002; Sacconi *et al.*, 2002; Chwang *et al.*, 2006; Borrelli *et al.*, 2008; Chandramohan *et al.*, 2008; Bai *et al.*, 2010; Monsey *et al.*, 2011; Zhang *et al.*, 2011; Denk & McMahon, 2012; Geranton, 2012; Kim *et al.*, 2012; Sando *et al.*, 2012; Rahn *et al.*, 2013; Sweatt, 2013; Bai *et al.*, 2015; Geranton & Tochiki, 2015b; Yu *et al.*, 2015; Guiretti *et al.*, 2016).

Epigenetic modifications are inducible marks suggested as candidate mechanism for neuronal and glial phenotypic plasticity (Borrelli *et al.*, 2008; Meaney & Ferguson-Smith, 2010; Monsey *et al.*, 2011; Rahn *et al.*, 2013; Sweatt, 2013; Geranton & Tochiki, 2015a; Yu *et al.*, 2015; Guiretti *et al.*, 2016). Some researches postulate that the nervous system could be especially sensitive to epigenetic control, being evolutionary favoured to respond and adapt to an environment always in change (Meaney & Ferguson-Smith, 2010; Sweatt, 2013). While other tissues of the body

are still in constant division and renewal and thus require this machinery to establish their different cell phenotypes, the nervous system (with low mitotic activity) would have developed this same epigenetic machinery to respond and adapt to outside cues (Meaney & Ferguson-Smith, 2010; Sweatt, 2013; Yu *et al.*, 2015). This epigenetic control could be relevant for different processes, including pain processing following a burn injury.

Many similarities at neuronal molecular level had been described between associative learning and memory (LTP formation), and nociceptive processing (Ji *et al.*, 1999; Julius & Basbaum, 2001; Ji *et al.*, 2003; Marchand, 2008; Hunt, 2009; Denk & McMahon, 2012; Geranton, 2012; Rahn *et al.*, 2013; Steeds, 2013; Geranton & Tochiki, 2015b). Both are processes involving synaptic plasticity and a similar set of molecules play relevant functions: NMDA receptors, release of BDNF, pathways of protein kinases or CREB phosphorylation (Ji *et al.*, 1999; Julius & Basbaum, 2001; Ji *et al.*, 2003; Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2008; Marchand, 2008; Monsey *et al.*, 2011; Denk & McMahon, 2012; Geranton, 2012; Rahn *et al.*, 2013; Steeds, 2013). Even more, most of these molecules are known to induce epigenetic modifications (Chandramohan *et al.*, 2008; Geranton, 2012; Sando *et al.*, 2012; Rahn *et al.*, 2013; Geranton & Tochiki, 2015b). Thus, it is feasible to hypothesize that similar epigenetic mechanisms have a key role during pain conditions and memory formation (Denk & McMahon, 2012; Geranton, 2012; Rahn *et al.*, 2013; Geranton & Tochiki, 2015b). However, there is still little research addressing their relation to pain (Zhang *et al.*, 2011; Geranton, 2012; Liu *et al.*, 2012; Chen *et al.*, 2015; Crow *et al.*, 2015; Geranton & Tochiki, 2015b; a). Few studies have revealed the influence of epigenetic factors (histone acetylation, DNA methylation and some non-coding RNAs) to regulate transcriptional alterations

during different pain states (Zhang *et al.*, 2011; Denk & McMahon, 2012; Geranton, 2012; Liu *et al.*, 2012; Crow *et al.*, 2015; Geranton & Tochiki, 2015a), and only one reported epigenetic changes in association with pain in humans (Tajerian *et al.*, 2011; Rahn *et al.*, 2013). Epigenetic regulation has even been suggested to participate in the transition of acute to chronic pain by supporting maladaptive molecular changes (Geranton *et al.*, 2007; Zhang *et al.*, 2011; Geranton, 2012; Rahn *et al.*, 2013; Geranton & Tochiki, 2015b). Thus, chronic pain could potentially be considered as a form of nociceptive memory maintenance due to a certain epigenetic landscape (Geranton & Tochiki, 2015a).

9.3.4.1 DNA methylation and pain

Many pronociceptive genes contain CpGs islands and are thus susceptible to be regulated by DNA methylation (Bai *et al.*, 2015). Nonetheless, DNA methylation was first linked to persistent pain *in vivo* by Geranton and co-workers (Geranton *et al.*, 2007; Bai *et al.*, 2015). They found that, one hour after CFA injection, there was an increase on MeCP2 phosphorylation in spinal cord neurons, mostly located in lamina I and II that was linked to up-regulation of SGK1, a serine threonine kinase expressed during consolidation of long-term memory, FKBP5, which regulates glucocorticoid receptors and Sult1a1, and to down-regulation of SIN3A, a MeCP2 co-repressor (Geranton *et al.*, 2007). Additionally, a model of neuropathic pain also resulted in downregulation of MeCP2 in DRG at 7-days post-nerve injury (Geranton *et al.*, 2007; Rahn *et al.*, 2013). In a persistent pain model of sciatic nerve's chronic constriction injury (CCI) a modest increase of global DNA methylation was detected in the spinal cord two weeks after the procedure (Wang *et al.*, 2011). A study from Pan and collaborators published in 2014 described an increase of DNA methylation at the promoter of the gene miR-219 in spinal cord following a chronic inflammatory

model (Pan *et al.*, 2014). This also correlated with a decrease of miR-219 expression and an increase in CaMKII γ , which are important for memory acquisition and retrieval (Sando *et al.*, 2012). Interestingly, the application of 5-aza-2-d, a DNMT inhibitor, was able to reduce the mechanical hypersensitivity, increased the expression of spinal miR-219 and decreased the expression of CaMKII γ in the CFA model (Geranton & Tochiki, 2015a).

Surprisingly, when studying mice with TET1 and Gadd45b genes knocked out, both important for demethylation (Geranton & Tochiki, 2015b), no alteration of thermal hypersensitivity was detected by the hot plate test following an acute noxious stimulation (although mechanical allodynia was not assessed) (Geranton & Tochiki, 2015b). However, DNMT inhibition by zebularine injected intrathecally had a dose-dependent effect in reducing thermal hyperalgesia after a CFA injection in mice. Moreover, the injection prior to the inflammatory model prevented the development of this behaviour also in a dose-dependent manner (Bai *et al.*, 2015). Additionally, a significant demethylation in the promoter of cystathionine- β -synthetase (CBS) was detected DRG neurons following CFA injection (Qi *et al.*, 2013; Rahn *et al.*, 2013), and this local inhibition of CBS reversed the mechanical hypersensitivity, thus implicating CBS on inflammatory pain. These findings suggest that only newly acquired DNA methylations have a role in the development and maintenance of persistent pain and that there is a tissue specific pattern of DNA methylation during such states (Bai *et al.*, 2015).

In both mice and humans, DNA methylation also seems to be implicated in the down-regulation of secreted protein acidic rich in cysteine (SPARC), an extracellular matrix protein linked to intervertebral disc degeneration and the subsequent low back pain (Geranton, 2012; Bai *et al.*, 2015).

9.3.4.2 *Non-coding RNAs in pain*

As well as by directly affecting the expression of pain related genes, micro-RNA can alter the epigenetic profile by directly targeting the enzymes responsible for their deposition, such as HDACs and DNMTs (Geranton, 2012; Geranton & Tochiki, 2015a). Some researchers even speculate of their role as targeting templates to direct the addition of other epigenetic marks to specific loci (Sweatt, 2013).

Micro-RNA were first directly linked to pain in 2010 by Zhao and colleagues (Zhao *et al.*, 2010; Crow *et al.*, 2013) in an experiment using cKO mice (Zhao *et al.*, 2010; Crow *et al.*, 2013). Additionally, a down-regulation of miRNAs is observed in DRG and spinal cord tissues during early to late stages of persistent pain (Bai *et al.*, 2015), a dysregulation also observed in the prefrontal cortex following a carrageenan injection (Crow *et al.*, 2013). Furthermore, abnormal profiles of micro-RNA expression were detected in the DRG of individuals that suffered from bone cancer pain (Bai *et al.*, 2015). Similar to this, long-non-coding-RNAs produced by some cancer cells seem to regulate neuronal nociceptive processing via epigenetic mechanisms (Bai *et al.*, 2015).

9.3.4.3 *Histone acetylation and pain*

Histone acetylation has been reported in different pain modalities (neuropathic, inflammatory and visceral pain) and at different levels including peripheral inflammation, pain processing and cortical areas (Zhang *et al.*, 2011; Denk & McMahon, 2012; Bai *et al.*, 2015; Geranton & Tochiki, 2015a). Similarly as observed during impairment of memory formation, an increase of CBP, which has intrinsic HAT activity, has been observed in the spinal cord and DRG during chronic pain development and linked to the expression of pro-nociceptive genes such as COX-2 or mGlu2 receptor (GRM2, figure 9.2) (Chiechio *et al.*, 2009; Rahn *et al.*, 2013; Bai

et al., 2015). In studies with PC12 cells, which are used as a model of neuronal-like cell line, the application of NGF induces the expression of δ -opioid receptor via acetylation of histone H3 at lysine 9 (aK9H3) (Agalioti *et al.*, 2002; Geranton, 2012). Histone acetylation has also been related to the development and maintenance of thermal hyperalgesia following induction of inflammation (Chiechio *et al.*, 2009; Bai *et al.*, 2010; Denk & McMahon, 2012; Bai *et al.*, 2015).

An important factor contributing to the interest of histone acetylation in pain research is the availability of inhibitors for their effectors (*writers* and *eraser*) in the pharmaceutical market with a history of efficacy in processes involving inflammation and LTP (Bai *et al.*, 2010; Crow *et al.*, 2013; Geranton & Tochiki, 2015a). Interestingly, different studies of inflammatory pain associated to arthritis, colitis and hepatitis, describe that the application of HDAC inhibitors (HDACi) has an analgesic effect (Denk & McMahon, 2012; Denk *et al.*, 2013; Bai *et al.*, 2015). Chiechio and collaborators and Bai and colleagues assessed the behaviour of rodents after a formalin test and CFA injection respectively, and the effect of administering HDACi, such as suberanilohydroxamic acid (SAHA) and MS-275, on it (Chiechio *et al.*, 2009; Bai *et al.*, 2010; Zhang *et al.*, 2011; Geranton & Tochiki, 2015a). Both groups observed impaired development of thermal hyperalgesia and linked the effect of HDACi to the up-regulation of glutamate receptors (such as mGlu2) in the DRG and spinal cord following these injury models (Chiechio *et al.*, 2009; Bai *et al.*, 2010; Zhang *et al.*, 2011; Geranton & Tochiki, 2015a). Denk and colleagues showed that the intrathecal application of HDACi was able to reduce mechanical and thermal sensitivity but only if administered before the noxious stimulation (Denk *et al.*, 2013). Even more, the administration of HDACi seems to reduce the expression of different pro-inflammatory mediators, including IL-1 β and TNF α (Denk & McMahon, 2012).

After an animal model of partial sciatic nerve ligation, hyperacetylation of pro-nociceptive genes, such as the one that encodes Nav1.8, were reported in the sciatic nerve itself (Chiechio *et al.*, 2009; Denk & McMahon, 2012; Geranton, 2012; Crow *et al.*, 2013; Geranton & Tochiki, 2015a).

Rat studies on the brain stem nucleus NRM (implicated in chronic pain) following the CFA model, showed no change of histone H3 acetylation, although a global gradual increase started to appear at 1 day post-stimulation (Zhang *et al.*, 2011). Despite the global change, a decrease of histone H3 acetylation was detected in the promoter of gad65 (glutamic acid decarboxylase 65), a GABA synthesizing enzyme (Zhang *et al.*, 2011; Rahn *et al.*, 2013), which led to impaired inhibitory function and thus contributed to sensitization. In this context, histone hyperacetylation tailored to this gene could have an analgesic effect (Zhang *et al.*, 2011).

9.3.4.4 *Histone methylation and pain*

Histone methylation has been linked to pain through mutations in PRDM12, a transcriptional regulator. Although PRDM12 does not have any *writer* activity by itself, it is required to recruit a methyltransferase that selectively demethylates histone H3 at lysine 9, 2mK9H3. Missense mutations on PRDM12 led to defects on nociceptors development and thus caused CIP. Individuals affected cannot experience acute or inflammatory pain and are unable to identify noxious heat or cold. Nonetheless, their sense of smell is not impaired as occurs in Nav1.7 mutants (Chen *et al.*, 2015).

9.3.4.5 *Histone phosphorylation is relevant to pain processing*

To date, histone phosphorylation has not been reported to participate in nociception. Nonetheless, there are different evidences suggesting that pS10H3 could be implicated in pain and inflammatory processing. In this section, some of these data

are summarized, first explaining processes in which pS10H3 is linked to important players on pain processing (Table 11.2), and secondly examples in which pS10H3 is related to neuronal memory and plasticity.

Since IEGs have been implicated in different processes including mitosis, differentiation and disease states such as inflammation, and pS10H3 (through different pathways) is required for their expression in quiescent cells, this PTM could be a potential regulator (Dong & Bode, 2006; Keum *et al.*, 2013) (Table 11.2 and Supplementary Figure 20.5). Studies using NGF-stimulated PC12 cell described the induction of ERK2 and p38 and the downstream kinase MSK1, a *writer* of pS10H3 (Deak *et al.*, 1998). Further, Fyn kinase, which is also implicated in pS10H3, can interact with many different molecules and has been linked to regulation of brain functions (He *et al.*, 2005). The expression of COX-2, an inducible enzyme implicated in inflammation-associated chronic pain, has also been linked to pS10H3 following UVB in mouse epidermal cells (Keum *et al.*, 2013). Furthermore, MKP1, a histone H3 phosphatase, seems to be modulating the expression of inflammatory genes in human endothelial cells, thus indirectly linking pS10H3 with inflammatory modulation (Kinney *et al.*, 2009).

pS10H3 has also been identified in neurons of rodents *in vivo* following exposure to stressors and contextual fear in a pattern specific for each stimulation (Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008; Sarantis *et al.*, 2012; Rotllant *et al.*, 2013; Crow *et al.*, 2015) (Table 9.2). Following different stressors (forced swimming test, exposure to predator or novel environment, and electrical shock) a transient increase of histone phosphorylation is detected in a restricted population of hippocampal neurons that, within 1 or 2 days,

return to baseline expression (Bilang-Bleuel *et al.*, 2005; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008; Crow *et al.*, 2015).

Further, pS10H3 is involved with the transcriptional activation associated to the immobility response to the forced swimming test, a behaviour regarded as the rats *learning* that there is no escape from the test (Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006). Additionally, a decrease of pS10H3 at dentate gyrus neurons was detected by long-term voluntary exercise, a behaviour that decreases anxiety and promotes stress resistance (Bilang-Bleuel *et al.*, 2005). In these paradigms, this PTM seems to be regulated by glucocorticoid, AMPA and NMDA receptors, and implicates ERK/MAPK pathways with MSK1/2 as the main effectors (Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008) (Supplementary Figure 20.5). Furthermore, this induction seems associated to expression of the IEG c-fos and zif-268, genes implicated in synaptic plasticity and memory consolidation (Bilang-Bleuel *et al.*, 2005; Chandramohan *et al.*, 2008; Sarantis *et al.*, 2012) (Table 9.2).

More recently, Rotllant and colleagues found that stress (forced immobilization) was able to increase pS10H3 in neurons of other brain areas although with regional heterogeneity in respect to c-Fos expression (Rotllant *et al.*, 2013). Additionally, Maćkowiak's group demonstrated that pS10H3 is a NMDA-dependent process in rat pyramidal neurons (glutamate-positive) from the medial prefrontal cortex (Mackowiak *et al.*, 2013).

Despite interactions with other modifications, some researchers suggest that pS10H3 itself could be serving as a rate-limiting step as well as a signal integrator for chromatin remodelling (Cheung *et al.*, 2000b; Bilang-Bleuel *et al.*, 2005; Keum *et al.*, 2013). pS10H3 could be facilitating fast and directed recruitment and activation

of the transcriptional machinery to the promoters (Saccani *et al.*, 2002). Thus, phosphorylation seems to be affecting a minor population of nucleosomes that then will help shift the transcription balance of those genes (Thomson *et al.*, 2001; Lefebvre *et al.*, 2002; Bilang-Bleuel *et al.*, 2005).

All together, these findings point at pS10H3 as a key factor in neuronal plasticity, involved in processes of LTP and possibly pain sensitization. And as such, a possible new nociceptive marker and a target for analgesic purposes.

10 Aims and objectives

Within this thesis, I worked on two main hypotheses: 1) that, due to the similarities within hippocampal LTP and processes of neuronal sensitization following nociception, pS10H3 gets also up-regulated in DRG and superficial spinal cord following a noxious stimulation and that this induction regulates the development of such nociceptive states; and 2) that blockade of Nav1.7, a VGSC described as necessary for normal burn injury nociceptive response, could be a feasible approach to treat burn-injury associated pain.

Therefore, the **aims** of this thesis are:

- To study if histone H3 gets phosphorylated at serine 10 (pS10H3) within both primary sensory neurons and superficial spinal cord neurons by burn injury and other noxious stimuli. And if so, to assess the pathway leading to such epigenetic modification, subsequent changes in gene expression and implications for persistent pain development.
- To determinate the association of Nav1.7, expressed within primary sensory neurons, with nociceptive activation by burn injury; and to test if blocking this voltage gated sodium channel can be a feasible analgesic intervention following burns.

If the first aim of this thesis is positively confirmed, pS10H3 will be used as a novel marker of neuronal activation to assess the outcome of the second aim, the analgesic effect of Nav1.7 blockade following burn injury.

To accomplish these aims, the following **objectives** were established:

- 1) Validation of the scalding type burn injury model in Sprague-Dawley rats by assessing oedema development, depth of the injury and nociceptive activation of DRG and superficial DHSC neurons; as well as quantifying the expression of Nav1.7 within primary sensory neurons following this scalding-type burn.
- 2) To quantify if pS10H3 is up-regulated in DRG and spinal cord neurons following the induction of these burn injury model.
- 3) To assess the up-regulation of pS10H3 at spinal cord neurons following other noxious models: capsaicin (*in vivo* and *in vitro*), noxious thermal and mechanical stimulation (*in vivo*) or electrical activation (*in vivo* and *in vitro*). As well as establishing the cascades of intracellular signals leading to this epigenetic modification.
- 4) Similarly, to assess if pS10H3 was also up-regulated in DRG neurons following these noxious stimuli (capsaicin injection and noxious thermal and mechanical stimulation), and elucidate the pathway leading to its lay down.
- 5) To assess the consequences of ablating MSK1/2, the postulated principal writers of pS10H3, following a peripheral injection of carrageenan (a model of persistent pain) via assessing behavioural differences in comparison to control animals and quantifying the levels of c-Fos, a prototypical IEG considered a marker of cell activation the expression of which seems regulated by pS10H3.
- 6) To quantify if blocking Nav1.7 by an intraperitoneal injection of protoxin II, a venom toxin with high selectivity for this channel, can diminish the nociceptive activation at DRG and spinal neurons following burn injury.

The first objective will confirm the validity of the model and the importance of Nav1.7 within burn injury associated pain. Objectives 2, 3, 4 and 5 will validate my first aim. If this first aim is positively confirmed, this knowledge will be used to validate the second aim as explained in the 6th objective.

11 Experimental techniques and protocols

During this chapter, all material and methods used for this thesis are described. In addition to the usual standards for *Material and Methods* sections, some methodological reviews have been included to give a complete perspective and understanding of the techniques.

Here, the different procedures include molecular and cellular techniques in both *in vivo* and *in vitro* contexts to fully assess the previously described aims of the thesis from different perspectives, and thus gain a complete knowledge on the topic.

11.1 Animal models

Although having some limitations, animal models of pain are still the most realistic and valuable to understand basic mechanisms and identify novel analgesics. However, they have to meet certain criteria such as easily reproducibility and behavioural assessment, offer a significant alteration in nociception, and share same fundamental mechanism with humans, in order to be able to transfer the learning to the clinic (Abdullahi *et al.*, 2014).

All procedures and techniques described below had been approved by the Home Office under the Animals (Scientific Procedures) Act 1986 (UK) Amendment Regulations 2012 (SI 2012/3039) in a Project Licence (PPL) and all experiments adhered to the guidelines of the Committee for Research and Ethical Issues of IASP published in Pain, 16 (1983) 109- 110. In addition, I had attended the CBS modules

1-4 and my Personal Licence (PIL) was approved by the Home Office on 22nd of April 2013. Unless states otherwise, adult male rats of the Sprague-Dawley strain (150-200g) were used in these experiments. It was decided to use males because they lack the characteristic hormonal fluctuations of the females, which can interfere with data output. Animals were housed within the animal facilities at Chelsea and Westminster Hospital and kept in a 12-hour day and night cycle at 22°C with free access to food and water.

When anaesthesia was required for experimental procedures, it was decided to use either urethane (20mg/Kg intraperitoneal, i.p.; Sigma) or sodium pentobarbital (60mg/Kg i.p.; Euthatal) as anaesthetic agents because of their minimal interference with neuronal activity. Urethane is a chemical compound also known as ethyl carbamate ($C_3H_7NO_2$) and is described as Group 2A carcinogen by IARC. Consequently, it was only used in cases when it was not possible to use sodium pentobarbital. However, sodium pentobarbital, a barbituric agent, usage in the UK is only listed for euthanasia and thus it could not be administered for techniques longer than 30 minutes, even if they were considered non-recovery procedures.

11.1.1 Scalding type burn injury model

The scalding burn injury model used was previously described in mice by my group (White *et al.*, 2011b). The aim is to induce a second degree, partial thickness, burn injury in one of the hind paws of the animal. Briefly, once the animals were anaesthetised by urethane, one of the hind paws was immersed up to the ankle into 60°C water for 2 minutes. Animals were then left for 5 minutes, 30 minutes, 1 hour or 3-hours survival times under non-recovery anaesthesia. Fluctuations in anaesthesia level were checked through monitoring the breath rate, corneal reflex and tail reflex,

and dose of anaesthetic adjusted if required. Naive animals and sham-injured animals (hind paw immersed into 37°C for 2 minutes) were used as controls (Figure 11.1).

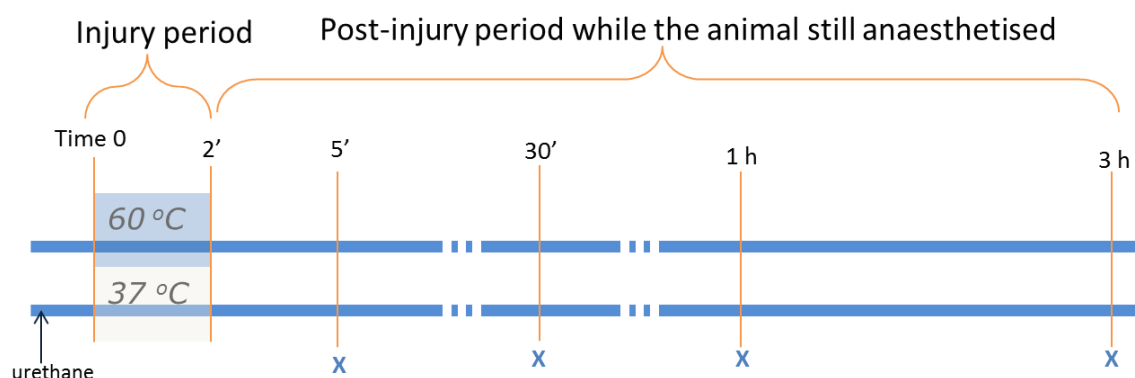


Figure 11.1. **Diagram of the scalding type burn injury model.** Xs mean end points for the animals under this injury model.

For blocking $\text{Na}_v1.7$ we used the $\text{Na}_v1.7$ blocker protoxin II (ProTxII; Tocris, 484598-36-9), which was administered by i.p. injection 15 minutes before or after the burn injury in a concentration of 0.1mg/Kg. Morphine sulphate (3 mg/Kg i.p.; Sigma, M8777) was used as positive control (Figure 11.2).

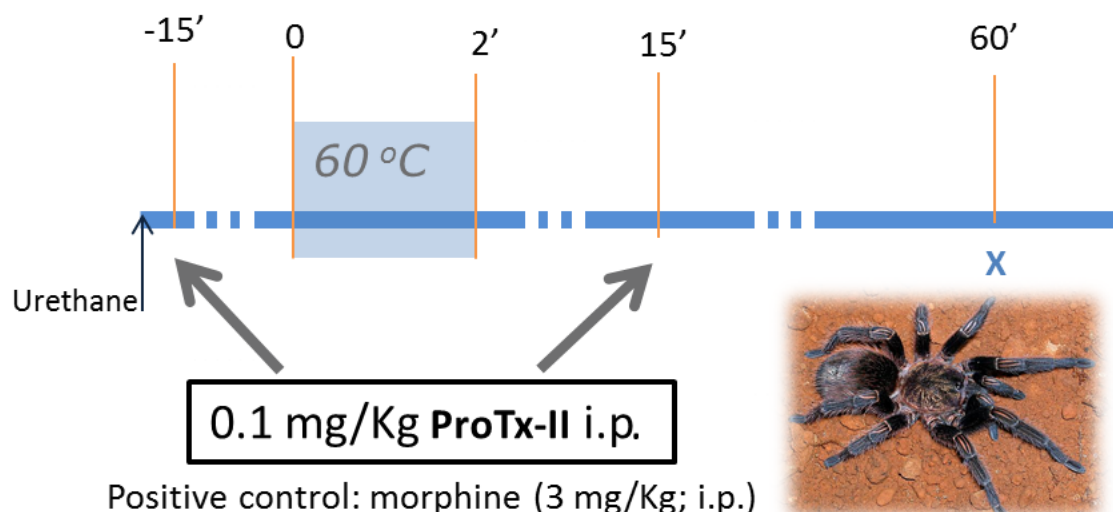


Figure 11.2. **Diagram of the scalding type burn injury model with different drug treatments.** ProTXII is found in the venom of the tarantula *Thrixopelma pruriens* (picture provided by www.birdspiders.com). Xs mean end points for the animals under the model. i.p.: intraperitoneal.

11.1.1.1 Hind paw oedema quantification

Paw volume measurement using a plethysmometer (Singh & Ghosh, 1968; Desroches *et al.*, 2014) and paw thickness evaluation with a micrometre (Petricevic *et al.*, 1978; Desroches *et al.*, 2014) are the most commonly used methods to measure paw oedema. The micrometre method (measuring paw thickness) seems to be more accurate for its evaluation. However, we lacked the equipment required for these methods in our facilities. Thus, it was decided to employ the closest approach possible to a micrometre. Adhesive tape (Fisherbrand, 15-912) was used to envelope the circumference of the ipsi- and contra-lateral hind paws both before the burn injury and at different time points after. To do so, a portion of tape was attached at the beginning of one side of the plantar surface and after the exterior tarsal pad, round through the circumference and cut at the meeting point. Then, the length of the tape portion was measured in centimetres (Figure 11.3). Interestingly, Gould and colleagues developed a similar method in which they used a string to encircle the paw and measure its circumference (Gould *et al.*, 2004).

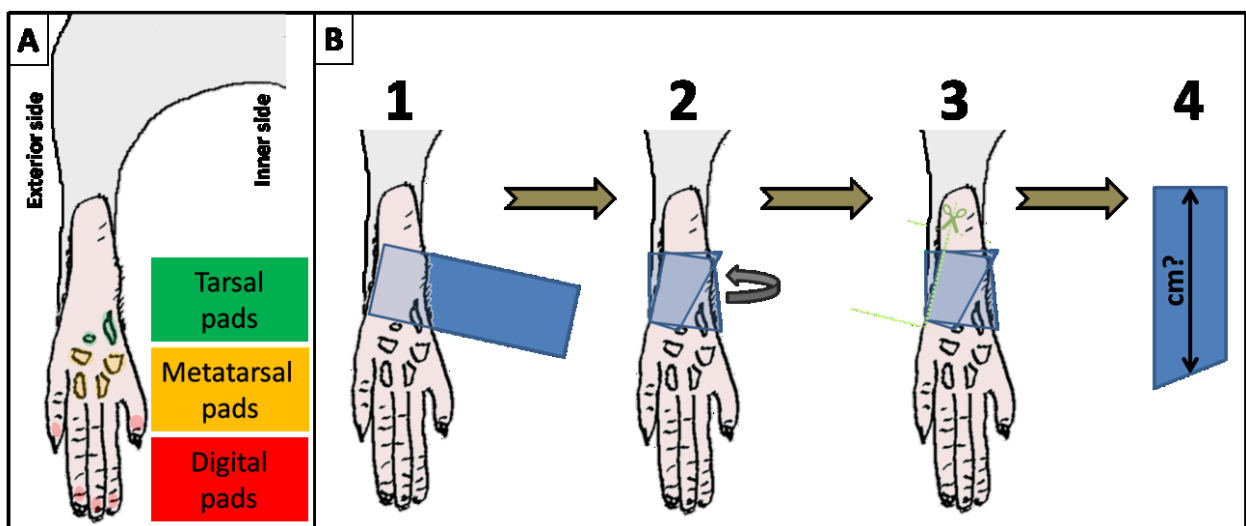


Figure 11.3. **Oedema quantification.** A) Palmar surface of left rat hind paw anatomy. B) Procedure to quantify its circumference: 1, tape attached at the beginning of the plantar surface just after the exterior tarsal pad; 2, round the tape following paw's circumference; 3, cut where the tape meets the beginning point (green line); 4, measure the length through the middle line of the resulting tape portion.

11.1.2 Intraplantar Injection of Capsaicin

When capsaicin, the pungent ingredient in hot peppers, is injected in the hind paw of a rat, it activates TRPV1 (Caterina *et al.*, 1997) and sensitizes the afferent neurons. Thus, this model mimics a noxious stimulation but caused by a specific mechanism of action.

The method used was similar as described before (Ji *et al.*, 1999). Once deeply anaesthetised with sodium pentobarbital, capsaicin (25µL of 3mg/mL dissolved in saline with 10% Tween 80; Sigma, M2028) was injected subcutaneously (s.c.) into one of the hind limbs and, at different post-injection times (5 or 30 minutes), rats were terminally anaesthetised and transcardially-perfused as described below (section 11.3.1). Naïve and saline injected animals were used as negative and sham controls respectively.

11.1.3 Cold stimulation

The aim of this model is to induce another type of noxious stimulation, in this case cold, without causing tissue damage (Ji *et al.*, 1999). To perform it, animals were anaesthetised by an i.p. injection of sodium pentobarbital. Once anaesthetised, one of the hind paw was immersed in 4°C water (icy water: water with crushed ice inside). To assure that there is not tissue damage, the immersion was done for periods of 10 seconds with 10-seconds gaps in between for a total of 2 minutes (6 immersions in 4°C water). Five minutes after the first immersion in icy water, animals were overdosed with sodium pentobarbital and then perfused as described below (section 11.3.1).

11.1.4 Heat stimulation

This animal model is intended to induce noxious heat stimulation similarly to the scalding type burn injury model but, in this occasion, without leading to any tissue harm. To do this, sodium pentobarbital-anaesthetised animals were used. One of the hind paws was submerged into 44°C water. Similar as with the cold stimulation (section 11.1.3), the intention with this method was to preserve tissues integrity by alternating 10-seconds immersions with 10-seconds rests. Thus, the heat stimulation was done by six immersions for 10 seconds in 44°C water and then 10-seconds rests. Once 5 minutes after the first stimulation were elapsed, animals were injected with an overdose of sodium pentobarbital and then processed for perfusion (section 11.3.1).

11.1.5 Carrageenan-induced oedema

Carrageenans are glycosaminoglycans from the cell walls of different marine animals (red algae). It is used to induce inflammatory nociceptive activation (Graham & Thomas, 1962) and is commonly used to test and screen anti-inflammatory treatments. Once urethane anaesthetised, animals received s.c. injection of 0.1mL of 1.5% λ -carrageenan (Sigma, 22049; diluted in saline) at the plantar side of one of the hind paws. Oedema development was assessed using tape with the micrometre-like method described above (section 11.1.1.1) at different intervals, starting from zero to one hour.

11.1.5.1 Behavioural assessments

For behavioural studies, experiments were done in mice, either WT (strain C57BL/6) or MSK1/2 KO (with C57BL/6 background; (Soloaga *et al.*, 2003; Arthur, 2008)). Mice were lightly anaesthetized with ether prior to the injection of either carrageenan

or saline (controls). The maximal survival time for those animals was 3-hours post-injection. These experiments were performed with the help of Dr João Valente, who instructed me in behavioural analysis. During the two days prior to the tests, animals were trained for 1 hour to the experimental scenario, and on the day of the experiment mice were allowed to acclimate for half an hour before experimental procedure. Mice were behaviourally assessed at the following time point: few minutes before and 30 minutes, 1 hour, 2 hours and 3 hours after the injection of carrageenan.

- von Fray Test

To detect the development of mechanical allodynia following carrageenan injection (25µL of 1.5% λ-carrageenan), animals were placed in a Perspex chamber with 0.8 cm-diameter mesh flooring. Then, series of successive calibrated von Frey hairs (from 2 to 0.008 g, Bioseb, USA) were applied to the plantar surface of the hind paw until their bending. Then, the fibres were sustained there for a total of 3 seconds or until the animal's withdrawal response. Finally, the 50% hind paw withdrawal value, the intensity of the stimulus that induces a withdrawal response 50% of the times that is applied, was calculated (Chung *et al.*, 1992; Chaplan *et al.*, 1994).

- Hot plate Test

For assessing the development of hypersensitivity to noxious thermal stimulation (heat hyperalgesia) following carrageenan injection (25µL of 1.5% λ-carrageenan), animals were placed on a hot plate at 52°C. The time until they exhibit pain-related behaviour, such as shaking or licking their limbs, was recorded. To prevent injury, the exposure to heat was stopped within 20 seconds even if mice did not show any pain-related behaviour and testing was not repeated in the same animal for a period

of 5 minutes. Paw withdrawal latency was assessed 3 times for each animal at each time point (Eddy & Leimbach, 1953).

- Hargreaves Test

This is another method to assess heat hyperalgesia (Hargreaves *et al.*, 1988). The day of the experiment, mice that received an injection of carrageenan (50 μ L of 1.5% λ -carrageenan) were placed in an elevated Perspex chamber with transparent flooring. Then, a high-intensity beam of infrared light was directed to the hind paw and paw withdrawal latencies measured. Similar as described for the hot plate test, paw withdrawal latency was assessed 3 times for each animal and each testing was not repeated in the same animal for a period of 5 minutes.

11.1.6 Electrical stimulation

The objective of the electrical stimulation is to directly induce a neurogenic inflammatory response by applying a noxious electrical charge, surpassing C-fibre activation's threshold, to the femoral nerve. Following induction of anaesthesia with sodium pentobarbital, one femoral nerve of the animal was exposed and hooked up with a platinum wire electrode for nerve stimulation (Grass Technologies Lt.). To induce C-fibres stimulation, the nerve was stimulated for 20 seconds, and 10-seconds rest in-between, with rectangular pulses to a total of 5 min (30 Volts, 10Hz, and 2ms). A continuous pulse of 15 Volts (0.5Hz and 0.1ms), to only activate A δ -fibres, was used in control animals. Due to the muscle contraction caused by electrical stimulation, a neuromuscular blocking drug was required. These drugs block the neuromuscular transmission causing paralysis of the affected skeletal muscle. Therefore, 5-10mg/mL of gallamine triethiodide (Sigma, G8134) were delivered intravenously. Since this drug can also cause paralysis of the diaphragm,

mechanical ventilation was used to ensure respiration. For legal requirements, these experiments were carried out at the Wolfson Centre for Age-Related Diseases (King's College London), under the PPL of Professor Stephen McMahon and with the supervision of Dr Anna P. Andreou.

11.1.6.1 Tracheotomy

This surgical procedure is performed to connect the animal to a respiratory pump and thus aid its breathing. A tracheotomy is essential to ensure survival when working with neuromuscular blockers.

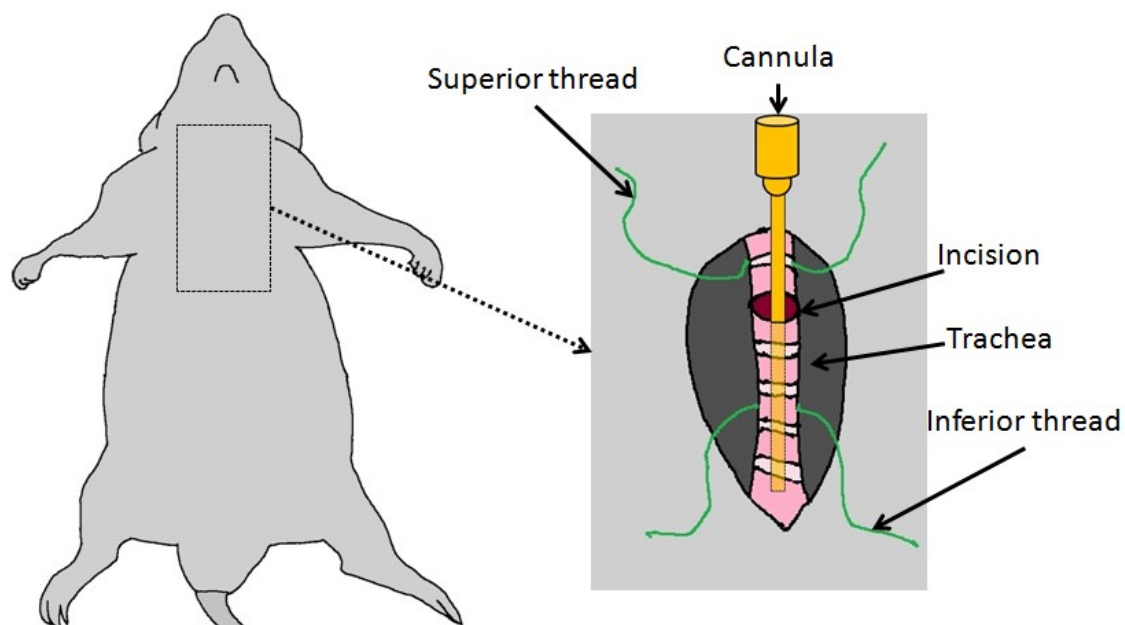


Figure 11.4. **Performing a tracheotomy.** Anatomical review of a trachea and the surgical components required to perform a tracheotomy.

Using a scalpel blade, an incision was made down the neck and until the beginning of the rib cage. Muscles were blunt-dissected until trachea was exposed and two pieces of thread were placed underneath and loosely loop. After supporting the trachea, around a third was cut transversally between two rings of cartilage to allow insertion of the cannula. Finally, approximately 1.5 cm of the cannula were inserted into the trachea and securely tied with the inferior thread, while the other was used to close the superior portion of the trachea and to further secure the cannula in place

(Figure 11.4). Once tracheotomy was completed, the cannula was connected to a pump (Harvard Apparatus) at a rate of approximately 75 beats per minute (bpm) and at a volume of 2.0L.

11.1.6.2 Femoral Vein Cannulation

A venous cannulation allows the experimenter to intravenously administer drugs such as neuromuscular blockers and, in longer experiments, is a convenient way to keep the animal hydrated by continuously pumping physiological saline (0.9% NaCl in dH₂O at pH=7.4) in.

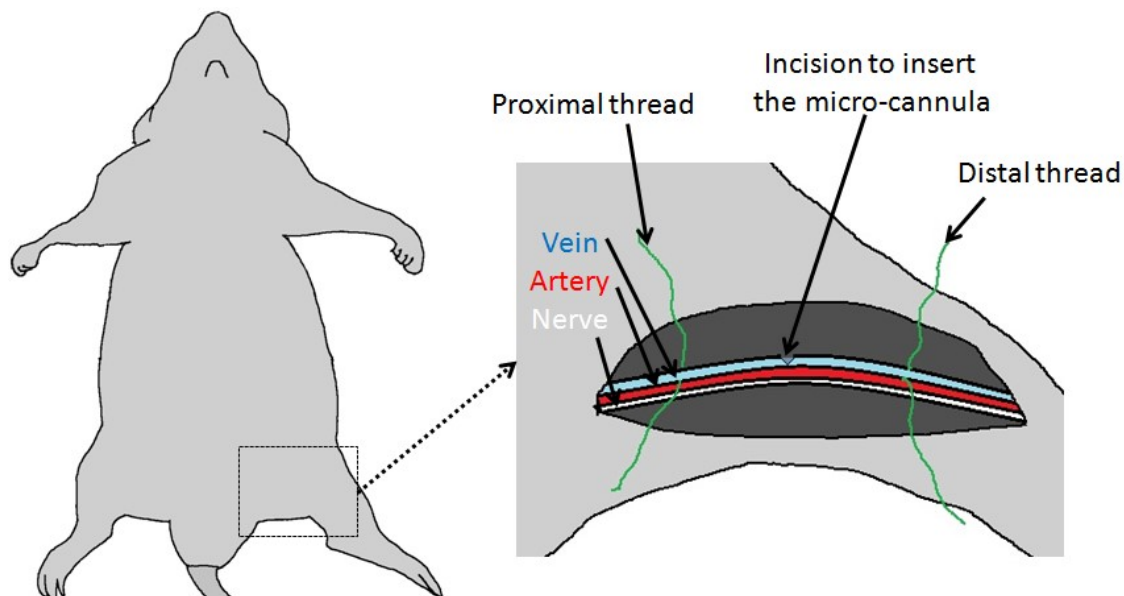


Figure 11.5. **Vein cannulation.** Anatomical review of the femoral innervations and the surgical components required to perform a femoral vein cannulation.

The contralateral femoral vein was exposed by performing an incision in the inner part of the thigh and by blunt-dissecting the muscles. Two fine threads (proximal and distal) were looped, leaving the major gap possible between them (Figure 11.5). The distal thread was then tightened with a double knot using a thread holder and the vein was gently lifted up and cut through a third/half using micro-scissors. Following, a 45° Dumont forceps was used to open the vein and insert a micro-cannula tubing filled with physiological saline. Once fully inserted, the proximal loose knot was

tightened and secured. Finally, the micro-cannula was tested to ensure functionality by drawing up a small amount of fluid, which should be blood.

11.2 Cell culture

The aim of this process is to maintain animal cells such as DRG in an *ex vivo* setup and under controlled conditions to perform different experiments with a more simplified context than in animal models. The requirements of the specific cell line, like antibiotic, supplements and growth factors, have to be met in order to maintain such cells.

11.2.1 Culture of adult rat DRG sensory neurons

Before proceeding, PORN-coated coverslips and Full Medium were prepared as follow:

- **Poly-ornathine (PORN)-coated coverslips:** batches of around 400 coverslips were previously cleaned in agitation at room temperature with 70% Ethanol (Fisher Scientific, 64-17-5) for 10 minutes, followed by two washes of 10 minutes each with ddH_2O . Then, a PORN aliquot (Sigma, P8638) was diluted 50 times from a 25mg/mL stock with ddH_2O , and used to incubate the coverslips for 3 hours in agitation at room temperature. Lastly, they were twice washed with ddH_2O and stored in Dulbecco's Modified Eagles Medium (DMEM: F12 medium; Gibco, 01475) until use. This coating process will allow the cells to attach easily to the coverslips.
- **Supplemented medium (SM)** was prepared adding together 4% Ultrosor G serums substitute (Pall Corporation, 15950), 1% L-Glutamine (Sigma, G7513)

and 1% Penicillin Streptomycin mixture (Gibco, 15140) to 500mL of DMEM:F12.

For preparation of cells for culture, all dissecting instruments and the dissecting area were sterilized with 70% alcohol. Once alcohol evaporated, an animal was terminally anaesthetised by isoflurane (Abbott, B506) and its spine presented on the dissecting area. Excess of soft tissues were removed to access the vertebrae and a bone cutter was used to cut dorsal and lateral vertebral pedicles to expose the spinal cord. With the help of micro-scissors and tweezers, the DRG were extracted; first cutting the peripheral process and then the medial of each DRG. 40 to 45 DRG were collected from each animal, from the cervical, thoracic and lumbar regions. Once removed, DRG were placed in a 35 mm Petri dish (Corning Incorporated, 3294) with 1mL DMEM and kept in ice. At this stage and unless stated differently, all steps were performed inside a sterilized fume hood. Initially, a chemical digestion was done by replacing the DMEM for new DMEM with 18% collagenase (Sigma, C2799) for 3 hours and with adding 0.1% trypsin (Gibco, 27250) for the last 15 minutes of this incubation, at 37°C in an atmosphere of 95% air and 5% CO₂. Medium was removed and dish washed by adding and removing 1mL of DMEM. Following, mechanical cell separation was performed with 1mL of SM and pipetting up and down several times until tissue was fully scattered. This cell solution was transferred to a Falcon tube, topped with more FM to a total volume of 10mL and supplemented with 50ng/mL NGF (Promega, G6257), to induce neuronal proliferation. 10µM of cytosine β-D-arabinoside hydrochloride (1:50 dilution; Sigma, C6645) were also added to restrict glial cell growth. Finally, while keeping the cells suspended in the media, 500µL were added in each well of 4-well plates (Thermo scientific, 176740) to which one PORN-coated coverslip had been placed, and kept at 37°C in an atmosphere of 95% air

and 5% CO₂. The DRGs from each animal were cultured individually in different 4-well plates. Once the cultures were established, the medium was changed to new FM every 2-3 days to maintain the cells viability.

11.2.2 Model of noxious stimulation in cultured DRG

This cell culture model studies the activation of the TRPV1 channel and the mechanisms of cell signalling afterwards. Capsaicin was used as activator of TRPV1, and capsazepine (Sigma, C191) as a blocker of TRPV1 activation. MSK1 activity was also blocked with SB747651A dihydrochloride (Tocris, 4630).

After two days of cell establishment, cultures were rinsed 3 times with DMEM to remove myelin and axonal debris and one of the following treatments was applied:

- Group one: **TRPV1 activation**. 5µM of capsaicin in extracellular buffer (150mM NaCl, 2mM MgCl, 5mM KCl, 2mM CaCl₂, 10mM HEPES, 10mM glucose in ddH₂O and pH adjusted at 7.4 with NaOH) was added to the cultures for 5 minutes. For each animal, half of the wells were used as controls and thus treated only with extracellular buffer.
- Group two: **TRPV1 blockade prior to activation**. Cultures were pre-treated with 5µM capsazepine in extracellular buffer for 2 minutes. Following, cultures were incubated with 5µM of capsaicin and 5µM capsazepine in extracellular buffer for 5 minutes. As for corresponding control, half of the wells from each animal were also pre-treated with capsazepine but then only more capsazepine was added.
- Group three: **MSK1/2 blockade prior to TRPV1 activation**. In this occasion, the cultures were pre-treated with 3µM of SB747651A dihydrochloride in extracellular buffer for two minutes and then incubated with 5µM of capsaicin

together with 3 μ M of SB747651A dihydrochloride in extracellular buffer for 5 minutes. The treatment control was done by incubating half of the wells of each animal with the MSK1/2 inhibitor and finally treated with extracellular buffer with more SB747651A dihydrochloride at the same concentration.

Incubations in all three groups were done at 37°C in 95% air with 5% CO₂. After corresponding treatment, cultures were washed three times with Phosphate-buffered saline (PBS) for 5 minutes each to guarantee treatment removal. Finally, samples were treated in the appropriate manner: for western blotting, cells were scraped and mixed with 300 μ L RIPA buffer and kept at -80°C until use (section 11.5); for immunostaining, cells were fixed and stained as detailed below (section 11.4).

11.2.3 Model of heat stress in cultured DRG

To assess the effect of heat stress on cultured primary sensory neurons, plates were incubated in an atmosphere of 100% air for 10 minutes at 45°C and then kept at 37°C in an atmosphere of 95% air and 5% CO₂ for a survival period of 30 minutes. As negative treatment control, half of the wells plated for each animal were maintained for the same time at 37°C in 95% air and 5% CO₂. Following this, cells were treated for immunostaining as stated below (section 11.4).

11.3 Staining

This term refers to a series of processes that can be done in a biological sample to enhance the contrast and facilitate its study under the microscope. It can be directed to highlight specific cell types, subcellular organelles or particular structures. Staining can be done by adding a single dye or stain or a combination of them to better differentiate the distinct compounds in a biological sample. The process can be done

in vivo, also known as vital staining, or *ex vivo*. In the second case, the sample has to previously been harvested from the initial biological context and has to undertake different treatments prior to the proper staining.

11.3.1 Fixation

The fixation process allows us to interrupt the degradation processes (autolysis, putrefaction, etc.) related to cellular death and to secure the conservation of the structure and chemical characteristics of cells and/or tissues. According to the mechanism of action, fixation can be physical, using temperature, radiation or others, or chemical. For standard procedures, a chemical agent, 4% paraformaldehyde (PFA; Acros organic, AC169650010) dissolved in PBS and at a pH=7.4 was used. Paraformaldehyde [$\text{HO}(\text{CH}_2\text{O})_n\text{H}$; $n=6-100$] is a polymerised aldehyde that acts by breaking down hydrogen bonds between adjacent proteins, destroying their helicoidally structure and exposing some active groups. It also establishes methyl bonds, leading to the formation of a reticular matrix of polypeptides. It is a great fixative on its own with optimal speed of penetration and fixation. However, it has to be prepared fresh since it progressively transforms into formic acid, which acts dissolving chromatin.

When starting from cultured DRG neurons, the method used to fix was simple immersion in the fixative solution for a period of 15 minutes. Since it is a monolayer of cells attached to a coverslip, there is no problem of uneven penetration and heterogeneous fixation.

In the cases where the experiments were performed in animals, the method used to do the fixation was transcardial perfusion, where the fixative agent is introduced through the circulatory system of the animal to guarantee efficient, homogeneous

and fast fixation. Initially, the animal was terminally anaesthetised with an overdose of sodium pentobarbital. Once the animal was in a severe state of sedation and no reflex reaction was observed, the dissection was performed. First accessing the abdominal cavity, secondly opening the diaphragm to reach the thoracic cavity and then cutting the ribs until the heart was fully exposed. At this stage, 0.1mL of heparin (Leo Pharma, 650201) was injected through the left ventricle of the animal's heart into the bloodstream to ensure that no blood clots appeared. Following, a blunt needle was injected from the left ventricle until it was visible through the walls of the *aorta ascendens* and clamped in that position. Next, the right auricle was sectioned to drain solutions injected in the circulatory system. Cold (4°C) saline solution (0.9% NaCl in ddH₂O at pH=7.4) was pumped in through the clamped blunt needle using the force of gravity. After approximately 200mL of saline solution had passed, it was switched to the PFA solution for another 200mL at 4°C. Finally, the desired tissues were collected and post-fixated by submerging them in PFA overnight.

11.3.2 Obtaining tissue sections

To process tissues for a microscopic analysis, they have to be cut in thin sections. However, to be able to perform cuts at such small thickness, tissues must have the appropriate toughness and, although the fixation confers them some, it is not enough to prevent them from cracking, maintain integrity and assure regular and even cuts. For optical microscopy, tissue was submerged in 30% sucrose (Sigma, S0389) in PBS with 0.05% sodium azide (as preservative; Sigma, S2002) just after post-fixation. This is a cryo-protective solution, preventing the formation of water crystals in the sample when frozen. The complete inclusion in the solution was guaranteed once tissue sunk. For cutting, sample was embedded in Tissue-Teck OCT

compound (VWR, 25608-930), which envelops the sample in a solid (once frozen) matrix that confers strength and allows thin cuts.

To obtain cuts at the order of micrometres (μm) desired for optical microscopy, a cryostat (Bright 5030 microtome) was used. This basically functions as a microtome in a freezer and allows collecting the frozen sections directly in slides (Superfrost Plus microscope slides from Thermo Scientific, 12727307). Sections were cut at a thickness of $10\mu\text{m}$ and serially collected in 10 slides. Thus, each $10\mu\text{m}$ section collected in a slide was separated $100\mu\text{m}$ from the next one collected in the same slide, assuring that there was a representation of the whole tissue portion in each slide without any cell overlap.

11.3.3 Haematoxylin and Eosin (H&E) staining

H&E is a type of staining used to do histological analysis that allows distinguishing topologically different components within the structure. Haematoxylin is a plant extract with positive charges due to its cationic groups, which confer affinity for acid structures like nucleic acids (DNA and RNA) and gives a blue/purple stain. Meanwhile, eosin has anionic groups, which means it has an acidophilic character, binding to acidic structures and staining with a characteristic pink colour.

After rinsing in distilled water, tissue slides were immersed in Harris haematoxylin (Sigma, HHS-32) for 6 minutes. Sections were then rinsed in running water for 10 minutes and counterstained for 15 seconds in 1% aqueous eosin (Raymond A Lamb Limited, East Sussex). Following this, slides were dehydrated with increasing concentrations of ethanol washes (3 minutes each), from 50% concentration to twice in 100% ethanol. Then cleared twice for 5 minutes with xylene (BDH Laboratory Supplies, 305756G) and mounted with DPX mounting media (BDH Laboratory

Supplies, 360294H). Once mounting media was dry, sections were examined using a Leica microscope and microphotographs were taken using the Hamamatsu Color Chilled 3CCD camera (Hamamatsu, C5810).

11.4 Immunochemical techniques

These procedures are a type of staining but normally considered as a completely different technique versus traditional ones. Here, antibodies against specific substances are used for their detection in cultured cells, **immunocytochemistry (ICC)**, and/or tissues, **immunohistochemistry (IHC)**. The two key elements are:

- *Antigen*, the substance (normally a protein) that we want to detect. Any substance that induces an immune response when injected in a host. The specific union to the antibody occurs through a sequence of 3-8 amino acids known as *epitope*.
- *Antibody*, the protein specifically designed to interact with the antigen. Antibodies are immunoglobulins (Ig), a type of glycoprotein produced by the plasmatic cells of an animal. Each Ig is made of 4 peptides chained together by disulphide bridges. The amino-terminal portions have great variability and are the responsible to the specific union to the antigen. Each Ig molecule has two binding sites. According from their origin and specificity, antibodies can be classified as polyclonal, a mix of antibodies produced by different lymphocytes against different epitopes of the same antigen, or monoclonal, from an immortalised clone that produces antibodies against a single epitope.

Once both components are in contact, a specific antigen-antibody reaction occurs. Thus, we can localize the antigen of interest by marking the antibody with a visible

tag. However, to reduce the cost and to further amplify the detectable signal, indirect methods of detection are used. Here, the primary antibody (the one that recognizes the antigen) is not labelled and a labelled secondary antibody is used to detect the primary antibody, which acts as antigen for the secondary antibody. To carry this procedure, fixation and tissue sectioning were similar as described above (Section 11.3.1). Following sectioning, there is a detailed general protocol for the different detection methods used with this technique. Table 11.1 and Table 11.2 describe the complete list of antibodies used and the specific conditions, including concentration, for each one.

11.4.1 Immunofluorescence (IF) protocol

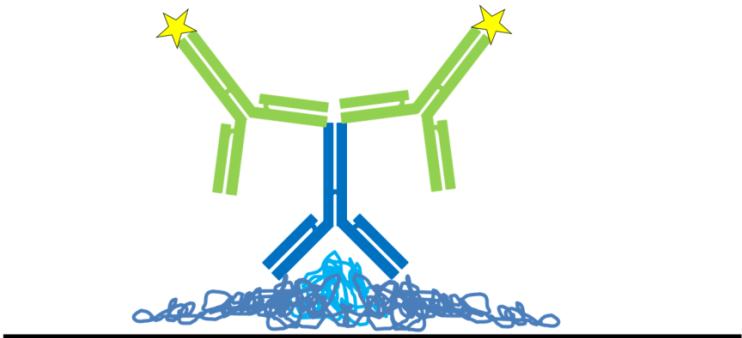
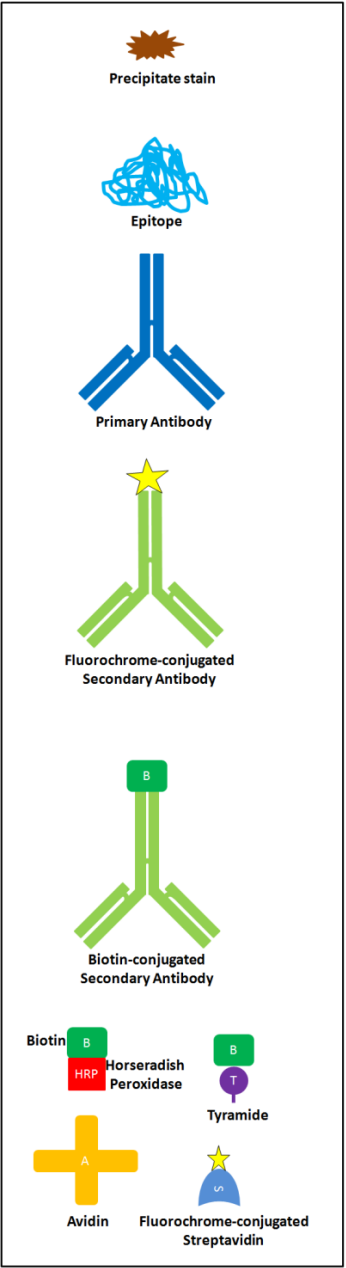
Secondary antibodies are covalently bonded with fluorochromes, chemical compounds that absorb light at a specific wavelength and re-emit light at a longer one (see 1st reaction in Figure 11.6). Slides were first washed with PBS and incubated in PBS containing 0.3% Triton-X (PBST) for 10 minutes. Sections were then covered in 10% normal donkey serum (NDS; Jackson ImmunoResearch Inc., 017-000-121) for 1 hour, to block unspecific binding. After that, they were incubated in primary antibody overnight at 4°C in a humidified chamber. The following day, slides were washed with PBST and incubated with fluorochrome-conjugated secondary antibodies raised against the species in which the primary antibody was made (Table 11.1). Some sections were also double stained following the procedure explained above in two consecutive rounds. Finally, slides were covered with the Vectashield mounting medium, with or without DAPI, (Vector Laboratories, H-1200 or H-1000) and sealed with nail varnish to prevent them from drying. All the washes and dilutions were performed at room temperature using PBST, with the addition of

2% NDS for the dilution of the antibodies. Controls were done by omitting the primary antibody (**negative control**) or using already tested antibodies (**positive control**). An antibody regarded as specific should not show any signal in the negative control, while the positive signal in the positive control assures that the technique was performed efficiently. Additionally, if the company also provides the blocking peptide (the peptide used to immunise the host animal), a **peptide neutralization control** was performed: a type of negative control in which the antibody is pre-incubated for one hour with the corresponding blocking peptide, which leads to its absorption prior to the incubation of the sample with the mix blocking peptide-antibody.

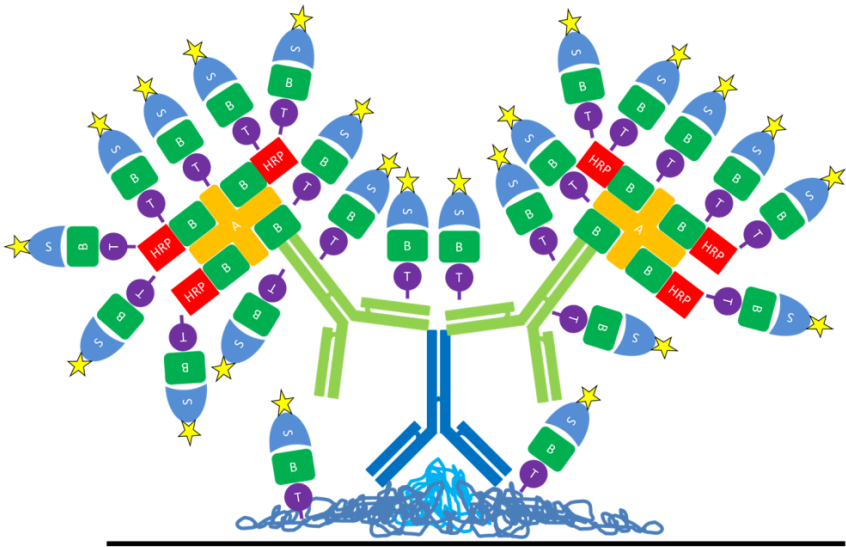
11.4.2 Tyramide Signal Amplification (TSA) protocol

This procedure is carried out whenever the normal IF technique is not efficient enough or to avoid potential cross-reaction. Further steps are added to amplify the final signal and to saturate the binding unions of the primary antibody and thus avoiding cross-reactions.

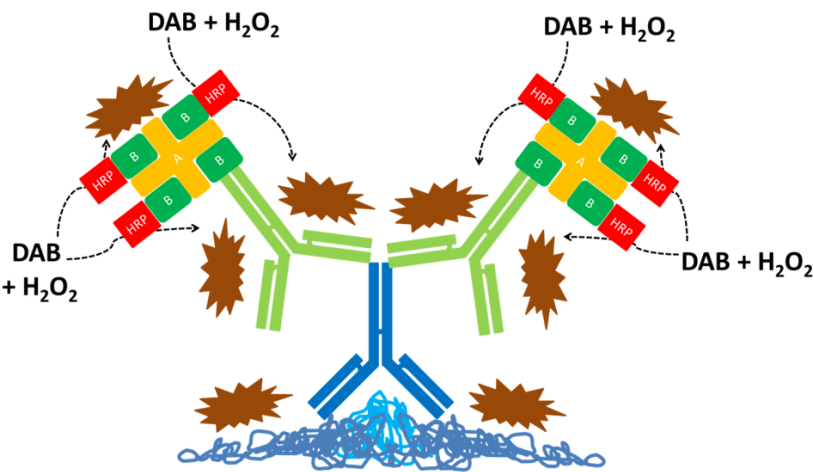
This methodology uses the strong affinity between avidin, a glycoprotein from the egg white, and biotin, a vitamin from the yolk; and the ability to combine the enzyme horseradish peroxidase (HRP) to biotin. Biotinylated tyramide is added, which uses HRP to covalently attach more biotin to the complex. It is visualized by the binding of fluorochrome-conjugated streptavidin, another biotin-binding protein (see 2nd reaction in Figure 11.6).



IF protocol



TSA protocol



DAB protocol

Figure 11.6. ←**Diagram of the different protocols to visualize immunostaining.** IF protocol is an indirect method where the secondary antibody has a fluorochrome covalently attached. For Tyramide Signal Amplification, TSA, protocol, further steps are added in order to improve the ratio between background and positive staining; here a biotinylated secondary antibody is added and signal is visualized with a fluorochrome after several signal-amplifications steps using avidin-biotin affinity, horseradish peroxidase (HRP) activity to bind biotinylated-tyramide and streptavidin marked with the fluorochrome. 3,3-diaminobenzidine, DAB, protocol uses the activity of the enzyme HRP to oxidize DAB with H₂O₂ to produce a dark-brown precipitate.

After initial washes with PBS and PBST, the endogenous peroxidase activity was quenched by incubating the sections in 70% methanol with 0.3% of H₂O₂, and endogenous biotin and avidin blocked with Avidin/Biotin blocking kit (Vector, SP-2001). Up to the step with the primary antibody incubation, the slides were treated exactly as described for the normal IF staining above (section 11.4.1). However, the antibody concentration is 25 times more diluted than the one used for that protocol. The following day, slides were washed with PBST, incubated for one hour with a biotinylated secondary antibody (Table 11.2) in 2% NDS in PBST, followed by another hour incubation with the ABC complex (1:200; Vector Labs. Inc, CA 94010) in PBST. Then, slides were incubated for 5 minutes with biotin tyramide (1:50; Perkin-Elmer Inc., NEL749A001) and subsequently incubated with fluorochrome-conjugated streptavidin (Table 11.2) in PBST for an hour. Finally, the samples were washed and covered with the Vectashield mounting medium. Unamplified slides and amplified negative control were performed to assure the specificity of signal.

11.4.3 Diaminobenzidine (DAB) protocol

The visualization of the immunostaining with this procedure is done by detecting the activity of HRP. This enzyme uses H₂O₂ to oxidize 3,3-diaminobenzidine, which resolves in a dark-brown precipitate stain in the reaction's site, and black when combined with nickel (see 3rd reaction in Figure 11.6). The slides were treated equally as for TSA protocol (section 11.4.2) up to incubation with the ABC complex.

To reveal the staining, we used peroxidase substrate kit DAB (Vector laboratories, SK-4100) as described in the kit's protocol. Nickel solution was added when required for further signal intensity. Corresponding staining controls, like IF (section 11.4.1), were carried out.

11.4.4 Isolectin IB4 staining

Even though this is not an immunochemical technique, it is included in this section because it is compatible with such procedures and was used in combination with them. Furthermore, the method of visualization and analysis was similar. Lectins are glycoproteins that bind to carbohydrates. Isolectin IB4 is 114 kDa and is obtained from the seeds of certain legumes. It was originally used to identify blood group B serotypes due to its ability to agglutinate this type of erythrocytes. It was later discovered its strong affinity for microglia and can be used to trace central and peripheral pathways along the nervous system. In the dorsal horn of the spinal cord, it is mainly present in the lamina III (Hunt & Mantyh, 2001; Julius & Basbaum, 2001; Hunt, 2009; Todd, 2010).

Slides were washed once with PBS for 10 minutes and three times with PBST, and incubated with IB4 green (Table 11.1) in PBST with 2% NDS for one hour at room temperature. Then, washed again three times with PBST and covered with Vectashield mounting medium. If used in combination with IF protocol or TSA protocol, IB4 incubation was done together with the secondary antibody or streptavidin incubation respectively.

11.4.5 Visualization and analysis

As for H&E stained sections, slides were examined using a Leica microscope and microphotographs were taken using the Hamamatsu Color Chilled 3CCD camera.

Identification of immunopositive cells was done by analysing the staining intensities for the DRG, and manually counting positive cells in spinal cord sections. All the images were taken at similar exposure time to be able to compare between them. However, and for further validation, the threshold for each image was established individually as explained below. It was decided to threshold each image individually due to a certain variability observed within different captures of the microscope despite conserving same conditions (presumably due to the alignment of the lenses). For DRG, 6 to 8 pictures were taken from each set of DRG (e.g. 8 for ipsilateral DRG and 8 for contralateral DRG for each animal) and analysed with the software ImageJ (Instrument Company Ltd., imagej.nih.gov/ij/). Initially, all neurons with their whole circumference captured in the image and with visible nucleus were outlined with the help of a design tablet (Trust, 16937) and saved as regions of interest (ROI) for later analysis. I decided to only include the cells with the complete cytoplasm, and with visible nucleus, in the analysis to avoid any under-estimation of cell size distribution. In total, between 150 and 200 cells were rounded per side in each animal, using as many pictures as required to obtain that number and always including all the valid cells within the frames used. Then, images were converted to greyscale and calibrated to corresponding scale, according to the magnification at which were taken. Following, mean pixel intensity and area of each ROI was calculated (Figure 11.7). Additionally, a ROI for the neuropil (area where cells were located, and excluding the fibres and black background surrounding the tissue) was created to obtain the mode of pixel intensity, standard deviation (SD) and the histogram of the pixel intensity distribution (Figure 11.7). To threshold the picture and objectively discriminate positive from negative cells, the value used was the mode+2SD of the neuropil for each picture (Figure 11.7). As observed (Figure 11.7 E), in the frames

where positive cells were present, a normal distribution of pixel intensity was observed with two peaks: one corresponding to non-positive cells and a second one at the right of the histogram for the positively stained. In contrast, only the first peak was observed in images where no positively stained cells were present. It was decided to use the mode and not the mean because the latter is more easily affected by extreme values and could lead to miscounting of positive cells in the images where they were relatively abundant.

When the interest was only in neurons with positive nuclear staining, each ROI value of the determined as positive cells was tracked back to the original picture and visual assessment determined if there was positive nuclear staining. For sections co-stained with several markers, same images were taken at different channels and same ROI values were used to analyse and compare them.

When studying DHSC, positive signal was determined by visual assessment. Pictures were taken to study the distribution pattern and for validation purposes. All observed ipsi- and contra-lateral side positive neurons were summed together for each side and mean and standard error mean (SEM) calculated.

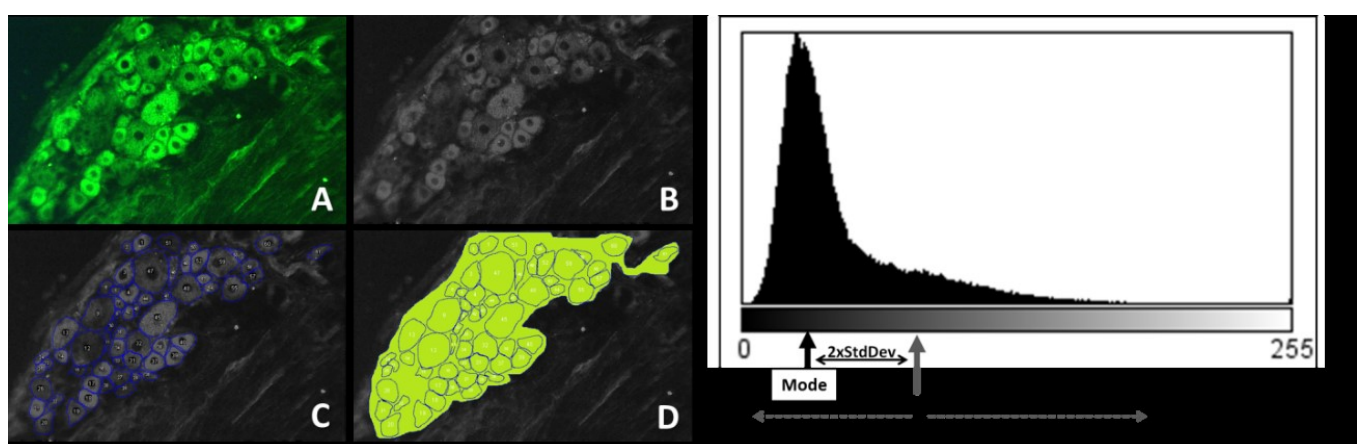


Figure 11.7. **How to find the threshold for positive staining in a DRG section.** The original image (A) is initially open with imageJ and converted to grayscale (B). ROIs corresponding to the cells are drawn (in blue; C) and measurements taken. Then the ROI for the whole neuropil area (clear green in image D) is marked and histogram and descriptive statistics obtained by pressing Ctrl+H. Finally, the threshold is established at mode+2SD (E).

On some occasions, slides were examined with a confocal microscope, Zeiss Axioplan 2 fluorescence microscope (Zeiss) and Axiovision software (Zeiss). The confocal microscope was used to ascertain co-expression and for aesthetic purposes.

11.4.6 Transmission Electron Microscopy (TEM)

TEM uses an electron-beam radiation to visualize samples. It has a better resolution power than optical microscopes and allows the study of structures at sub-cellular level. When electrons hit the sample, some are diverted by its atoms and do not reach the screen (black) while the ones that do not hit the sample are not deflected and end in the screen (white). However, the cuts need to be extremely thin in order for the electrons to penetrate and, since the main elements in a biological sample (carbon, hydrogen, oxygen, nitrogen) do not divert them much, contrast has to be enhanced with elevated atomic number elements.

For TEM, fixation was carried similarly as described above (section 11.3.1) but using Somogyi's Fixative (Somogyi & Takagi, 1982) instead: 4% paraformaldehyde, 15% saturated picric acid (Sigma, P6744) and 0.08% glutaraldehyde (Sigma, G6257) in PBS at pH=7.4. This mix combines the fixative properties of paraformaldehyde with picric acid (2,3,6-trinitrophenol), which coagulates proteins, and glutaraldehyde, with similar mechanism of action as paraformaldehyde but with rapid speed of fixation and low penetration. This mix allows an excellent structural conservation of tissues. Just after perfusion, tissue was harvested and stored in 0.1 M PB with 0.01% sodium azide.

Since we do not have the required facilities at Chelsea and Westminster Hospital, samples were processed until this stage and shipped to Dr. Akos Kulik's lab in

Freiburg, Germany, where were processed as described before (Kulik *et al.*, 2002). Briefly, sections were cut at 60µm using a vibratome, cryoprotected with 25% sucrose and 10% glycerol in 50nM PB, freeze-thawed and incubated in blocking solution (20% normal goat serum in TBS, tris-buffered saline) for one hour and then in primary antibody (with 3% normal goat serum in TBS) for an overnight at room temperature. Then, sections were washed with the same buffer and incubated with 1.4 nm gold-coupled secondary antibody (1:100) and reacted with HQ Silver kit (Nanoprobes). Sections were treated with osmium tetroxide and uranyl acetate to increase the atomic number of the lipids and nucleic acids respectively, dehydrated and embedded in Durcupan resin (Fluka, Switzerland), which provides uniform polymerization, low refraction and optimal toughness and can endure the beam of accelerated electrons. Sections were then cut (Reichert-Nissei Ultracut S; Leica, Germany) at a thickness of nanometres scale and visualized with an H-7 electron microscope (Hitachi, Japan).

11.5 Western Blotting

The aim with western blotting is to separate different proteins from a complex sample according to their size, using their electric charge, in a gel of electrophoresis and specific antibodies to identify them. Once the proteins have migrated through the gel, it is positioned next to a membrane and the proteins are transferred to the second by an electrical current. An immunoassay is performed in the resulting membrane using the primary antibodies to target the desired proteins and visualized with secondary antibodies and detection reagents.

11.5.1 Protein extraction

Just after the desired experimental procedure, samples (either tissue from animals or scraped cell cultures) were extracted. If they were not going to be used immediately, they were snap-frozen by placing them in Eppendorf tubes, immersed in liquid nitrogen and stored at -80°C until use.

When starting the protein extraction, samples were placed and kept on ice during all manipulations unless stated differently. Corresponding amount of lysate buffer and protein inhibitors was added. The lysate buffer selected was RIPA buffer (Amresco inc., N653), which is suitable for whole cell extracts, membrane-bound proteins and nuclear proteins extraction. The cocktail of protein inhibitors (Sigma, P8849) is added to preserve the proteins in the sample and avoid further degradation. 3µL/mg RIPA buffer and 5µL/mg protein inhibitors were added to each sample depending on the weight. If tissue weight was such that the volume of RIPA buffer was less than 100µL, then 100µL were added. This is due the need to have a enough liquid in the sample to be able to sonicate it effectively (see below). If working with scraped cell cultures, 17% of protease inhibitors were added to the total volume of the sample, the already existing suspension of cells in RIPA buffer.

Once all the solutions were mixed together, short intervals of sonication (Soniprep 150) of 15 microns of amplitude were used to dissociate the sample until complete visible disintegration if working from tissue, or for a 10-seconds period with the cell suspension. Specimens were then incubated at 4°C for 1 hour using a rotor (Stuart Scientific UK, Mini Orbital Shaker SO5) at 50 revolution/minute. Right after that, samples were centrifuged at 4°C for 30 minutes at 14,000 revolutions per minute (rpm) and supernatant collected for further processing.

Again, if the resulting samples were not meant to be processed immediately, they were stored at -80°C . If not, they remained in ice for the following manipulation.

11.5.2 Protein quantification

This process was done from either protein extracts obtained as mentioned above, results of immunoprecipitation (section 11.6) or histone extracts (section 11.7).

By quantifying the amount of protein, we can determinate the proper volume to be loaded in each well. It is important to load the same amount of protein in each lane to be able to do a quantitative or semi-quantitative analysis of relative protein concentrations. The assay used here is based on the spectrophotometric determination of the protein concentration using the Bradford protein assay (Bradford, 1976): the dye used shifts from 465nm to 595nm when binding to the proteins in solution. The protein concentration was estimated by comparing to a standard curve.

Starting from a stock of bovine serum albumin (BSA; Sigma, A7030) of 1.35mg/mL, the following standard curve concentrations were prepared by serial dilutions with double distilled water: 1,000; 500; 250; 12.5 and 1.25 $\mu\text{g/mL}$. To make sure that sample concentration was in the same range as the standards, samples were also diluted to 1:4. Triplicates of 10 μL per well from each protein standard or sample were added in a 96-well plate (Corning Incorporated, 3599) in the following fashion:

	1	2	3	4	5	6	7	8	...	...
A	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Blank	Sample 1	...	Sample X
B	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Blank	Sample 1	...	Sample X
C	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Blank	Sample 1	...	Sample X

The dye used, Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, 500-0006), was diluted to 1:5 in double distilled water and 200 μ L were added in each well. Lastly, plate was incubated for 5 minutes and read using an OD microplate reader (Dynex Technologies, MRX II) and the Revelation Software (Dynex Technologies).

11.5.3 Electrophoresis

This is the method used to separate the proteins from the sample. The setup used was SDS-polyacrylamide gel electrophoresis (SDS-PAGE). There, the proteins are kept denatured and consequently separated according to different molecular weights by forcing them to migrate through an acrylamide gel, from the negative electrode to the positive: the denatured protein are covered with sodium dodecyl sulphate (SDS) which has a negative charge and subsequently, the smaller proteins travel faster through the mesh of acrylamide. Protein standards, a mix of marked proteins of known molecular weights, were used in combination with the samples to allow estimation of their size.

First, the samples were prepared for the electrophoresis. With the data obtained from the protein quantification, the volumes of each sample were carefully adjusted to obtain equal amount of protein to be loaded in each well. Normally the loading is set at a certain optimal concentration. However, the amount of protein was rather low due to the nature of the samples used. Thus, it was decided to adjust them to the maximum possible. To do so, the lowest concentration detected in each experiment was used as the value to which adjust the other samples. The volume was normalized by adding either RIPA buffer or ddH_2O , depending on what was the sample previously diluted in. Following this, around 1:4 or 1:5 dilution of SDS sample

buffer (Novex, LC1676) was added (e.g. 5 μ L in 20 μ L of sample). Right after, the samples, together with the biotinylated protein ladder (Cell Signalling, 7727), were heated at 95°C for 5 minutes, immediately spun down and kept on ice.

Secondly, pre-cast electrophoresis gels were used because of their easily reproducibility. Either NuPAGE Novex 12% Bis-Tris Protein Gels (Invitrogen, NP0342BOX) or NuPAGE Novex 4-12% Bis-Tris Protein Gels (Invitrogen, NP0322BOX) were used, depending on experiment's proteins size. Gels were prepared by removing packaging, white stripe and comb. Then, they were placed in the electrophoresis tank (Invitrogen, Novex Mini-Cell) together with the other components and the ddH_2O diluted running buffer. NuPAGE MES SDS Running Buffer (20x) (Novex, NP002) was added to fill the inner chamber and around a third of the outer. Afterwards, equal volumes of each sample, plus biotinylated ladder and pre-stained ladder (Invitrogen; Novex Sharp Pre-stained Protein Standard, 57318) were loaded in each gel.

Then, the electric circuit was completed by adding the lid of the electrophoresis tank and connecting to the electricity supply (BioRad, PowerPac200). The running conditions were set at 200 Volts for approximately 30 minutes or until the blue dye band was nearly reaching the end of the gel.

11.5.4 Electrotransfer

This procedure consists in using an electric charge to transfer the proteins from the gel to a membrane in the same spatial configuration in which they are in the gel. Thus, the proteins are available for the antibodies to reach and be detected by immunostaining. The method used here was a dry transfer to a membrane of polyvinylidene difluoride (PVDF). The materials used were the iBlot Gel Transfer

Stacks (PVDF, Regular; Novex, IB4010-01) together with the iBlot Gel Transfer Device (Invitrogen, IN1001). The program used in the device was P3 for either 7 or 9 (for bigger proteins such as Nav1.7) minutes at 20 Volts, as suggested by the fabricant. The iBlot device is assembled with all the components: first the anode stack with the membrane attached to it, second the pre-run gel, thirdly a filter paper fully soaked in ddH₂O, then the cathode stack with the metal grid facing up and after that the sponge with the metal plaque contacting the metal in the lid of the device. Finally, the apparatus was closed and run for the stated time. Once the transfer was completed, the desired membrane portion was cut out of the stack and activated by a 2 minutes immersion in 100% methanol (Fisher Scientific, M4000PC17), and briefly rinsed with ddH₂O.

11.5.5 Immunoreaction

Once the transfer had taken place, the membrane is processed in order to detect the desired proteins. With the purpose to prove that there is equal loading in all the lanes of the gel and be able to quantitatively compare two sample concentrations, antibodies against a housekeeping protein such as β -actin or β -tubulin III, with different molecular weight than the protein of interest, were included (Table 11.1). In this protocol, all the incubations were carried out inside a black container and on a rocker rotor set at 50 rpm. Initially the membrane has to be blocked in order to prevent nonspecific binding of antibodies onto it. To do so, the membrane was incubated for 1 hour at room temperature with 5% non-fat milk powder (Sigma, M7409) as a blocking agent diluted in PBST. After blocking, membrane is incubated with the desired primary antibodies (Table 11.1) diluted in 3% BSA in PBST for an

overnight at 4⁰C. The following day, membrane was washed 5 times with PBST for 5 minutes each.

With the same antibody solution (3% BSA in PBST), the secondary antibodies conjugated with HRP were diluted together with [1:1,000] dilution of biotinylated detection ladder (Table 11.2) used to incubate the membrane for 1 hour at room temperature. After the stated time, the membrane was washed 5 more times with PBST for 5 minutes.

11.5.6 Visualisation and Analysis

Chemiluminescence was used to detect the immunoreaction: a substrate that reacts with the HRP in the membrane giving an emission of light that can be detected. Since this methodology is based in non-precipitating substrates, it allows further stripping (section 11.5.7) and later re-incubations (re-probings) of the membrane. The two reagents from the Luminol kit (ImmunoCruz, sc-2048) were mixed at equal amounts and used to incubate. To read it, the membrane was inserted in a G:Box (SynGene) and the GeneSnap software (Synoptics LTd, SynGene version 7.12.06) was used to capture the gel images as Tag Image File Format (tiff). The exposure times selected varied between trials but were in the range of 5 seconds to 15 minutes. Images were then converted to grey scale and analysed with the software ImageJ to obtain a plot profile and to determinate the intensity of each peak, corresponding to a protein band. Briefly, each lane was individually marked and its profile plot obtained. Then, peaks were closed off to avoid the background noise, their size measured and expressed as a percentage of total size of all the other peaks. Finally, Windows Excel (Microsoft, 2003) was used to calculate the relative densitometry of each peak by comparing with its standard

(dividing the percentage of each peak by the percentage of the standard). To obtain a densitometry ratio, the relative densitometry obtained in the ipsilateral side of each animal was divided by the value of the contralateral. This procedure was done for both bands, protein of interest and housekeeping protein (used as controls).

11.5.7 Stripping

Stripping membranes means removing the antibodies from it. This procedure is useful when wanting to analyse two different proteins in the same gel but they have similar or equal size. However, stripping can also remove some sample protein from the membrane. To prevent false data from this, when stripping was used, two gels were run in parallel with the same samples and incubated in the two possible order of antibodies. Thus, allowing a further confirmation of the observed in the samples. Nonetheless, data was considered more meaningful when coming from the gel where the most relevant detection for the study was done first (for example: when pS10H3 was done prior than total amount of histone H3).

To strip, the membrane was incubated for 15 minutes at room temperature with Restore Western Blot Stripping Buffer (Thermo Scientific, 21059). After that, the membrane was rinsed several times with PBST and processed for Visualisation and Analysis in order to confirm the absence of labelling. Finally, the membrane was washed 5 more times with PBST for 5 minutes each and processed again for immunoreaction.

11.6 Immunoprecipitation

The purpose of this technique is to isolate and concentrate a protein from a sample. This procedure can be interesting when trying to increase the amount yielded in a

western blot if the concentration is too low that cannot be detected under normal conditions. It consists on incubating the protein extract with a primary antibody for the desired protein, coupling it to a solid substrate and then precipitating the complex. Right after, heat is used to elute, to separate the different compound of the complex protein-antibody-substrate.

Initially, the proteins were extracted from the samples with the same procedure as explained for Western Blotting (section 11.5.1). Once ready, the extracted proteins were incubated overnight in constant agitation at 4°C with the primary antibody against the desired protein to immunoprecipitate. If the concentration of the antibody was stated by the supplier, the primary antibody was added at 2µg per 500µg of protein extract. If antibody's concentration was not known, a standard volume of 1µL per 500µL of sample was added. The following day, mix was incubated with a 10% of total volume of re-suspended A/G plus agarose (Santa Cruz, sc-2003) at 4°C for 2 hours. After that time, samples were centrifuged at 2,500 rpm for 5 minutes at 4°C and supernatant discharged. Pellets were washed four times by repeating cycles of reconstitution with 500µL of RIPA buffer and 5 minutes centrifugation (same conditions as before). After final wash, the pellets were re-suspended with 40µL of RIPA buffer. Finally, the samples were eluted by boiling them for 5 minutes. If this procedure was used to perform a western blotting, the corresponding SDS sample buffer amount could be added before elution. However, when running a western blot after immunoprecipitation we must consider how to validate equal loading, since possible housekeeping proteins are lost. Instead, the band corresponding to the eluted antibody (still present in the sample) can be used to standardize the ratios.

11.7 Histone extraction

The aim of this technique is to extract the histone proteins presents in a sample, from either tissue or cultured cells. This procedure can be used for multiple applications, including western blotting (from section 11.5.2 onwards) and PTM quantifications assays (section 11.8). To perform it, the EpiQuick Total Histone Extraction Kit (Epigentek, OP-0006-100) was selected due to its competitive cost and quick accomplishment of the process (Figure 11.8 A).

Briefly, it started with fresh samples or the ones previously stored at -80°C . Once the samples were weighed and acclimated into an ice container, the Pre-Lysis Buffer (10x Pre-Lysis buffer diluted in ddH_2O) was added in a 1:50 concentration, and a pestle and mortar were used to homogenize the samples for about 50 to 60 strokes. Samples were then centrifuged at 3,000 rpm for 5 minutes at 4°C , supernatant discharged and pellet re-suspended with Lysis Buffer at 1:3 ratios (according to initial amount of tissue) and kept in ice for 30 minutes. After that time, samples were centrifuged at 12,000 rpm for 5 minutes at 4°C , supernatant was collected and pellet discharged. Then, Balance-DTT Buffer (0.2% DTT Solution in Balance Buffer) was added at 1:0.3 ratios (in relation with previous amount of Lysis Buffer added) to stop reaction. Samples were quantified similarly as described in western blotting (section 11.5.2).

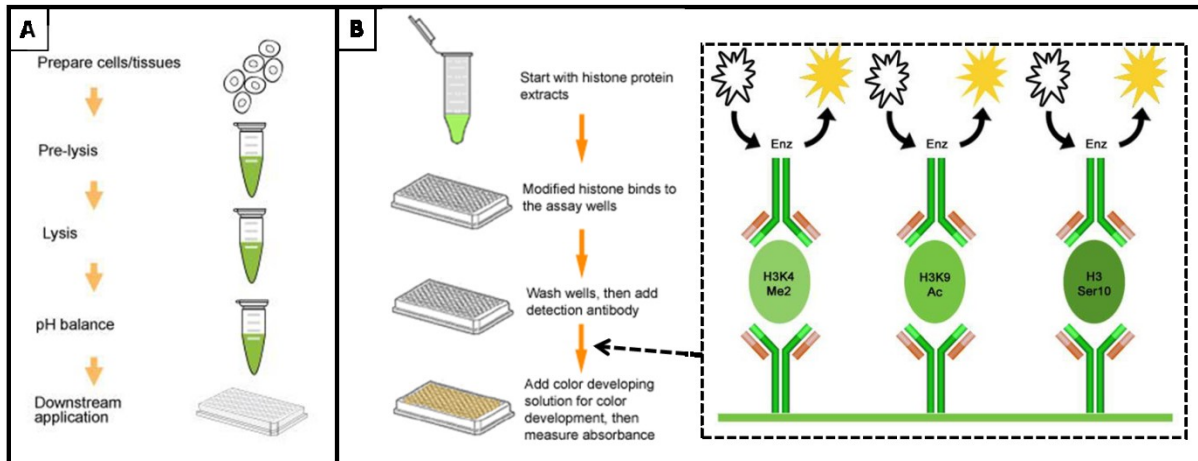


Figure 11.8. **Extracting total amount of histones in a sample, and analysing PTM.** A) Schematic procedure for using the EpiQuick Total Histone Extraction Kit (OP-0006-100). B) Schematic procedure for using EpiQuick Histone H3 Modification Multiplex Colorimetric Assay (P-3100-96) showing the working principle of the procedure. Images adapted from the corresponding user manuals.

11.8 Histone H3 PTM Assay

This methodology is used to compare amounts of different histone H3 modifications simultaneously for two samples (e.g. control and treated). The EpiQuick Histone H3 Modification Multiplex Assay Kit (Epigentek, P-3100-96) was used in order to do this procedure. This assay uses a plate with wells coated with antibodies against 21 PTM, and total histone H3, to determinate the ratio of modified histones by a colorimetric approach. It can be used from extracts of histone proteins in a concentration range between 20 to 500ng and provides its own internal controls. It is advised to run duplicates for each sample. However, the starting samples cannot contain any type of detergent, thus samples proceeding from the above immunoprecipitation method or protein extracts done as stated for western blot were not suitable.

All the steps for the histone H3 PTM assay were done as suggested by the company's manual (Figure 11.8 B). Thus, a short description will be provided. Initially, the wells of the plate designated for control of the assay were filled with 1µL of corresponding control protein previously prepared (5ng/µL and 25ng/µL, from a

stock of 100 µg/mL and ddH₂O). Then, the histone extracts of interest were diluted to the range specified above and 1 µL was added in all the corresponding wells. 49 µL of Antibody Buffer were included in all the wells of the plate, including the ones designated for blank controls. Then, the plate was covered with adhesive covering film and incubated for 90 minutes at 37°C. Once the time was elapsed, the solution was eliminated and the plate was washed three times using the washing buffer (10X diluted in ddH₂O). Following, 50 µL of detection antibody (a final concentration of 1:1000 from 1000x detection antibody diluted in washing buffer) was added in each well of the plate, covered with parafilm (Parafilm, PM-996) and incubated for one hour at room temperature. Right after, the plate was washed twice with washing buffer and 100 µL per well of developer solution were added and incubated for up to 10 minutes at room temperature. The development process was monitored and when it turned to blue colour in the solutions with enough products, such as the assay control and samples, the enzymatic reaction was stopped by adding 100 µL per well of stop solution. This gave a change of colour from blue to yellow. Absorbance was read using the OD microplate reader and Revelation Software. A dual wavelength analysis was done: subtracting the reference wavelength of 620nm from the test wavelength of 450nm. Once the data was collected, different calculations were performed in order to quantify histone H3 PTM changes:

1. Average of duplicate readings
2. Simple quantification amount
3. Percentage of each histone H3 PTM respect total H3
4. Percentage of relative change of each histone H3 PTM between samples
(control and treatment)

11.9 Statistics

Before any experiment was carried out, **power calculations** were used to estimate sufficient sample size, the number of replicates needed in each group to achieve meaningful statistics. For this purpose, statistical significance was assumed at 0.5 and magnitude of effect when comparing treatments versus controls was assessed with indirect measurements of effect size, with a common standard deviation (σ) of 0.2 for all data. Online based software (<http://homepage.stat.uiowa.edu/~rlenth/Power/>) was used to estimate power calculation prior to each experiment (Lenth, 2006-9).

All following statistical analyses were carried out using SPSS program (IBM SPSS statistics 22.0 for Windows) and data, including significant differences, was graphically represented using GraphPad Prism v.6 software (GraphPad Software Inc.). Prior to any study, outliers were assessed based on mean+2SD values, and excluded.

To avoid any possible bias, experimenter was blinded from the different experimental groups. However, there were some exceptions when this was not possible due to the nature of the procedure. For example: oedema developed after burn injury or carrageenan injection was so clearly evident that blinding was not possible.

Once data from staining experiments was gathered, it was analysed with the **generalized linear model** (GzLM), which is more flexible than the general linear model and can be used even if the variables do not meet the homogeneity criteria. GzLM is a more supple generalization of standard linear regression that allows using different types of data distribution and different data covariance (McCulloch *et al.*, 2008). The significance using this method was assessed with the Wald chi square test. Whenever a statistically significant interaction was found, the pertinent pairwise

comparisons was carried out. Differences were regarded significant at $p < 0.05$. This same analytical method was applied for the paw circumferences measurements, to assess oedema development.

To analyse densitometry ratios from western blotting, either t -test or Mann-Whitney U test were used. In the scenarios where normalization of densitometry ratios was possible, **independent t -test** was used to analyse whether the mean of the obtained densitometry ratios for each group, control and treated, were different. Here normal distribution and homoscedasticity were assumed. However, when it was not possible to normalise the densitometry ratios (e.g. when using samples obtained by immunoprecipitation where no housekeeping protein was present), **Mann-Whitney U test** was used. Mann-Whitney U test is a non-parametric test equivalent of the independent t -test, it is used when to compare two conditions but data violates any assumption of the independent t -test. Mann-Whitney U test ranks the sum of each group and then looks at differences between the different scores. Differences were also regarded significant at $p < 0.05$.

Behavioural responses were compared between treatment groups by two-way repeated measures analysis of variance (**ANOVA**) followed by **Bonferroni's post-test**, with differences regarded as significant at $p < 0.05$.

Table 11.1. **List of primary antibodies used during thesis and specific characteristics of each one.** It includes the company, catalogue number, host species, protocol used for and concentration, immunogen and controls. Isolectine IB4 is included in this list despite not being an antibody and Na_v1.7-KO tissue was provided by John Wood's lab (Nassar *et al.*, 2004).

Primary antibodies										
Target	Company	Catalogue Number	Host Species	Staining Protocol	Concentration used	Western Blot	Clonality	Immunogen	Company's control	Our controls
ATF3	Santa Cruz	sc-188	rabbit	IF	0.5625	X	polyclonal	Around 15-25 aa, located between aa 138 and aa 188 of the C-terminus of ATF-3 of human origin*	Blocking peptide provided	Blocking peptide
c-Fos	Santa Cruz	sc-52	rabbit	IF	1:1,000	X	polyclonal	Around 15-25 aa, located between the first 50 aa of the N-terminus of c-Fos of human origin*	-	-
CGRP	Abcam	ab10987	mouse	IF	1:2,000	X	monoclonal	FAESGVNTPV; C-terminal ten amino acids of rat alpha CGRP	Rat alpha-CGRP peptide	-

GFAP	Abcam	ab53554	goat	IF	1:2,000	X	monoclonal	DGEVIKESKQEHKD; C terminal aa of Human GFAP	Human and mouse brain lysate	-
Histone H3, CT, pan	EMB millipore	07-690	rabbit	-	-	✓ 1:2,000	polyclonal	KLH-conjugated, synthetic peptide corresponding to the C-terminus of human Histone H3	HeLa cell extract	Embrionic tissues with high mitotic factor
IB4 Alexa Fluor 488	Molecular Probes	121411	G. <i>simplicifolia</i>	-	1:1,000	X	-	-	-	-
MAP2	Abcam	ab5392	chicken	IF	1:5,000	X	polyclonal	Full length cow brain MAP2 purified from the material that pellets with GTP polymerized tubulin	cultured rat cortical neurons	-

MSK1/2	Bioss	bs-2995R	rabbit	IF	0.3888889	X	polyclonal	KLH-conjugated, synthetic peptide derived from human MSK1	-	KO tissue
Na _v 1.7	Alomone	ASC-008	rabbit	IF	0.3888889	X	polyclonal	Peptide (C)EFTSIGRSR IMGLSE, corresponding to amino acid residues 446-460 of rat Na _v 1.7 (Accession O08562). Intracellular loop between domains I and II	Confirmed by amino acid analysis	Na _v 1.7 conditional KO
Na _v 1.7	Millipore	ab5390	rabbit	IF	0.5625	✓ 1:2,000	polyclonal	EFTSIGRSRIMGLSE; purified peptide from rat Na _v 1.7 (amino acids 446-460)	Control antigen included	-

NeuN	Millipore	mab377	mouse	IF	1:2,000	X	monoclonal	Purified cell nuclei from mouse brain	Positive control -Brain Tissue.	-
OX42 (CD11b)	ABDserotec	MCA275R	mouse	IF	1:1,000	X	monoclonal	whole resident rat peritoneal macrophages	Clone validated on Rat Peritoneal Macrophages by flow Cytometry	-
pan-histone H3 CT	Millipore	07-690	rabbit	X	-	✓ 1:4,000	polyclonal	KLH-conjugated, synthetic peptide corresponding to the C-terminus of human Histone H3	Immunoblot:acid extracted proteins from untreated, sodium butyrate or colcemid treated HeLa cells.	Embryonic tissues with high mitotic factor and blocking peptide provided by the company

pCREB	Cell Signaling	9198	rabbit	IF	1:5,000	✗	monoclonal	CSRRPsYRKI; synthetic phosphopeptide corresponding to residues surrounding Ser133 of human CREB	Western: C6 +/- forskolin and IBMX +/- lambda phosphatase; IHC: SKNMC +/- forskolin & IBMX (paraffin-embedded cell pellets) Human tumor array mouse lung +/- antigen peptide block +/- lambda phosphatase treatment	-

pERK1/2	Neuromics	RA15002	rabbit	IF	1:1,500	X	polyclonal	Synthetic phosphopeptide corresponding to residues surrounding T202/Y204 of human, rat and mouse ERK*	Validated by Western Blot (lysates of HeLa human cervical epithelial carcinoma cell line untreated or treated with 200 nM PMA) and IHC (rat brain; cortex)	-
pp38	Cell Signaling	9216	mouse	IF	0.3888889	X	monoclonal	CLARHTDDEMtGyVA; synthetic phosphopeptide corresponding to residues around Thr180/Tyr182 of human p38 MAPK	C6 -/+ anisomycin; 3T3 -/+ UV; HeLa -/+ UV	-

pp38	Cell Signaling	9211	rabbit	IF	1:1,000	✗	polyclonal	LARHTDDEMtGyVAC; synthetic phosphopeptide corresponding to residues around Thr180/Tyr182 of human p38 MAPK	C6 -/+ anisomycin; 3T3 -/+ UV; HeLa-/+ UV	-
pS10H3	Santa Cruz	sc-8656-R	rabbit	TSA/DAB	0.5625	✓ 1:500	polyclonal	Around 15-25 aa, located between the first 50 aa of the N-terminus of pS10H3 of human origin*	Blocking peptide provided and HeLa + Calyculin A whole cell lysates	Embryonic tissues with high mitotic factor and blocking peptide provided by the company
β Tubulin III	Sigma	T8578	mouse	IF	1:7,000	✓ 1:1,000	monoclonal	MYEDDEEESEAQGPK; Synthetic peptide corresponding to amino acids 436-450 of neuronal specific bIII tubulin	-	-

TRPV1	Neuromics	GP141000	guinea pig	IF	1:1,500	X	monoclonal	YTGSLKPEDAEVFKDSMVPGEK; C terminal aa of rat VR1	Immunohistochemistry on rat dorsal root ganglion (PFA perfusion fizzed frozen sections at a dilution of 1/1000)	-
β actin	Millipore	mab1522	mouse	X	-	✓ 1:2,000	monoclonal	Chicken gizzard actin; smooth muscle gamma & alpha actin	Actin from Chicken, Rabbit, Rat, Monkey, or Human	-

* company does not share the specific sequence

Table 11.2. **List of secondary antibodies used during this thesis and specific characteristics for each one.** It includes the company, catalogue number, host specie, protocol used for and concentration. Note: streptavidin molecules have been included in this list.

Secondary antibodies						
Target	Company	Catalogue number	Host	Staining Protocol	Concentration for staining	Western Blott
Chicken Alexa Fluor 546	Molecular Probes	A11040	goat	IF	1:1,000	✗
Chicken Alexa Fluor 594	Molecular Probes	A11042	goat	IF	1:1,000	✗
Goat Alexa Fluor 468	Molecular Probes	A11057	donkey	IF	1:1,000	✗
Goat Alexa Fluor 488	Molecular Probes	A-11055	donkey	IF	1:1,000	✗
Guinea pig Alexa Fluor 488	Molecular Probes	A11075	donkey	IF	1:1,000	✗
Biotin HRP-linked	Cell Signalling	7075	goat	✗	✗	✓ 1:1,000
Mouse Alexa Fluor 488	Molecular Probes	A21202	donkey	IF	1:1,000	✗
Mouse Alexa Fluor 568	Molecular Probes	A10037	donkey	IF	1:1,000	✗
Mouse biotin-SP-conjugated	Jackson Immuno Research	715-066-150	donkey	TSA/DAB	1:1,000	✗
Mouse HRP-conjugated	Cell Signaling	7076S	horse	✗	✗	✓ 1:1,000
Rabbit Alexa Fluor 350	Molecular Probes	A10039	donkey	IF	1:1,000	✗
Rabbit Alexa Fluor 488	Molecular Probes	A21206	donkey	IF	1:1,000	✗
Rabbit Alexa Fluor 568	Molecular Probes	A10042	donkey	IF	1:1,000	✗
Rabbit biotin-SP-conjugated	Jackson Immuno Research	711-066-152	donkey	TSA/DAB	0.3888889	✗
Rabbit HRP-conjugated	Cell Signaling	7074S	goat	✗	✗	✓ 1:1,000
Streptavidin Alexa Fluor 350	Invitrogen	S-11249	-	TSA	1:1,000	✗
Streptavidin Alexa Fluor 488	Invitrogen	S-11223	-	TSA	1:1,000	✗
Streptavidin Alexa Fluor 546	Invitrogen	S-11225	-	TSA	1:1,000	✗

**12 Establishing a scalding type burn
injury model in Sprague-Dawley rats
and assessing Na_v1.7 expression
throughout the nociceptive pathway.**

12.1 Background

Burn injury is one of the main sources of accidental tissue damage and a major health problem globally (NBCRC, 2001; WHO, 2008b; Laycock *et al.*, 2013; Abdullahi *et al.*, 2014). Advances in burn prevention, management and other aspects of burn care have increased the survival rate after a major trauma (WHO, 2008b; ABA, 2015). However, the pain associated with burns is still an unmet medical need: it is not treated efficiently in most cases, and is one of the major concerns for burn survivors (Ptacek *et al.*, 1995; Ulmer, 1997; Dauber *et al.*, 2002; Demling, 2008; Wiechman Askay & Patterson, 2008; Laycock *et al.*, 2013; Sandoval *et al.*, 2014). Effective management of burn pain is difficult and one of the most complicated aspects of burn injury (Martyn, 1986; Demling, 2008). Thus, further research is needed to assess this issue.

In vitro models are very limited and cannot reliably replicate clinical and pathophysiological features of human burn injury. Therefore, animal models of burn injury are still needed to reproduce the conditions of a burn and to test novel treatment options (Abdullahi *et al.*, 2014). Different animal models have been described in the literature, each one centred on a different aspect of burn injury as the emphasis of its research. While in the 60s and 70s the focus was to study the skin physiology and metabolic changes, nowadays most experimental models are directed to investigate the molecular mediators involved, the so called “burn injury soup”, and the nociceptive processing associated to this type of trauma (Urban *et al.*, 1993; White *et al.*, 2011b; Shields *et al.*, 2012; Green *et al.*, 2013; Laycock *et al.*, 2013; Abdullahi *et al.*, 2014).

Most of the animal models of burn injury are caused by thermal burns. This is mainly due to their reliability and easy reproducibility (Walker & Mason, 1968; Abdullahi *et*

al., 2014), in conjunction with the uncontrollable diffusion of chemical burns (Kim *et al.*, 1994) and the need of higher order animals to replicate electrical burns (Zelt *et al.*, 1988; Chilbert *et al.*, 1990). A heat-induced injury procedure can be achieved by different means such as contact with hot surfaces (Suzuki *et al.*, 1991; Shields *et al.*, 2012) or scalds (Walker & Mason, 1968; White *et al.*, 2011b; Green *et al.*, 2013). In 2010, White *et al.*, at the laboratory where I was conducting experiments at the Chelsea and Westminster Hospital, developed a scalding-type burn injury model using C57BL/6 mice: one of the hind paw of the rodents was immersed, up to the ankle, in 60°C water for a total of 2 minutes (White *et al.*, 2011b). This is a non-recovery model of burn injury, with a maximum post-injury period of six hours that reliably produces a second degree burn in the hind paw (White *et al.*, 2011b).

Following a burn, nociceptors get activated. The soma of these nociceptors, the primary sensory neurons, are collected together at DRG. Centrally directed afferent fibres from those DRG cells mostly terminate and synapse with second order neurons situated at superficial laminae of the dorsal horn of the spinal cord (DHSC) (Hunt & Mantyh, 2001; Yoshimura & Furue, 2006; Todd, 2010). Both DRG and DHSC are key centres to control pain perception that can be subjected to function-dependent plasticity and thus are implicated in peripheral and central sensitization respectively (Ji *et al.*, 1999; Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; Marchand, 2008; Hunt, 2009; Woolf, 2011; Steeds, 2013).

If a noxious stimulation resulting from a burn injury is enough to depolarize the membrane of the primary sensory neurons to a certain threshold, voltage gated sodium channels (VGSC) will open and generate the upstroke of the action potentials (Coutaux *et al.*, 2005; Dib-Hajj *et al.*, 2007; Rush *et al.*, 2007; Raouf *et al.*, 2010; Black *et al.*, 2012; Eijkelkamp *et al.*, 2012). In mammals, there are nine

members on the VGSC family (Nav1.1 to Nav1.9), from which Nav1.7, Nav1.8 and Nav1.9 are preferentially expressed within nociceptors (Rush *et al.*, 2007; England, 2008; Dib-Hajj *et al.*, 2010; Hoeijmakers *et al.*, 2012; Shields *et al.*, 2012). The activity and expression of these sodium channels is modified in DRG neurons following injury and neuronal damage (Baker & Wood, 2001; Hunt & Mantyh, 2001; Gould *et al.*, 2004; Rush *et al.*, 2007; Costigan *et al.*, 2009; Dib-Hajj *et al.*, 2010; Hoeijmakers *et al.*, 2012)

Nav1.7 is associated with both inflammatory and neuropathic pain (Cummins *et al.*, 2004; Nassar *et al.*, 2004; Yeomans *et al.*, 2005; Brochu *et al.*, 2006; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Hoyt *et al.*, 2007; McGowan *et al.*, 2009; Gingras *et al.*, 2014) and seems to have an important role in burn-injury associated pain (Black *et al.*, 2012; Hoeijmakers *et al.*, 2012; Shields *et al.*, 2012). Thus, Nav1.7 seems to be a feasible target to control pain in burn patients.

Mitogen-activated protein kinases (MAPK) are kinase enzymes dynamically activated by phosphorylation in response to different extracellular stimuli (Stambouliau *et al.*, 2010; Edelmayer *et al.*, 2014). MAPK are implicated in neuronal plasticity during processes such as inflammation and neuropathic pain (Ji & Woolf, 2001; Black *et al.*, 2008; White *et al.*, 2011a; White *et al.*, 2011b; Edelmayer *et al.*, 2014). ERKs are a group of MAPK with important roles in nociceptive response following different types of injuries (White *et al.*, 2011a). pERK1/2 are the convergence effectors of different regulators situated upstream (Ji & Woolf, 2001; White *et al.*, 2011a). They are rapidly induced on superficial spinal dorsal horn neurons following different types of noxious stimulation (heat, cold or mechanical) in an intensity-dependent manner (Ji *et al.*, 1999; Ji & Woolf, 2001; Polgar *et al.*, 2007; Black *et al.*, 2008; Gao & Ji, 2009; White *et al.*, 2011a; White *et al.*, 2011b).

Following the scalding type burn model, the activation of pERK1/2 can be detected as early as 5 minutes after the injury (White *et al.*, 2011b).

p38 is also a MAPK that, similarly to pERK1/2, is phosphorylated by noxious stimuli in DRG neurons of small diameter and has a role on inflammation (Ji & Woolf, 2001; Edelmayer *et al.*, 2014). However, pp38 is not detected in neurons from the spinal cord (Ji *et al.*, 2002b; Geranton *et al.*, 2010). Following inflammation, pp38 seems to regulate neuronal responses such as the expression of TRPV1, cytokines, sodium channels and COX-2 enzyme (Hunt *et al.*, 1987; Ji & Woolf, 2001; Ji *et al.*, 2003).

These intracellular cascades lead to different changes in the neurons, including phosphorylation of cAMP response element binding protein (pCREB). pCREB is a downstream effector that controls the expression of different IEG and late-response genes in neurons (Chiechio *et al.*, 2009; Rahn *et al.*, 2013). Activating transcription factor 3 (ATF3) is one of those IEG closely related to pCREB that gets induced in DRG and seems implicated with neuronal regeneration following an injury (Linda *et al.*, 2011).

12.2 Aims

The **aims** of this chapter are:

- To validate the use of the scalding type burn injury model previously developed in mice (White *et al.*, 2011b) for its use in Sprague-Dawley rats.
- To study the expression of Nav1.7 within the primary and secondary order neurons together with different nociceptive markers at different time point following the trauma.

The goal of this chapter is to develop the means to use Sprague-Dawley rats for burn research and to establish the implication of Nav1.7 on the nociception input following this type of injury.

12.3 Material and methods

Rats from the Sprague-Dawley strain of 150-200 grams were subjected to the **scalding type burn injury model** (White *et al.*, 2011b). Briefly, once anaesthetised with urethane, one of the hind paw of the animal was submerged up until the ankle in a beaker containing 60°C water for two minutes. Once that time was elapsed, rats were maintained with controlled levels of anaesthesia for different post-injury periods: 5 minutes (n=8), 30 minutes (n=4), 1 hour (n=4), and 3 hours (n=8). Naïve (n=4) and sham (n=4, 37°C water instead of 60°C) animals were used as controls. During this procedure, oedema development was quantified by measuring the increase of paw circumference.

After the corresponding stimulation, rodents were terminally-anaesthetised with an overdose of sodium pentobarbital and ended by either neck dislocation, if tissues were intended for western blotting, or fixed by transcardial perfusion with PFA to obtain tissues for immunostaining techniques.

For **western blotting**, L4 and L5 DRG were collected from the ipsi- and contra-lateral side of rats subjected to the burn with a post-damage period of 5 minutes (n=4) and 3 hours (n=4). Proteins were extracted from DRG tissues, ran in a NuPAGE Novex 4-12% Bis-Tris Protein Gel (Invitrogen, NP0322BOX), transferred to a PVDF membrane and stained against both Nav1.7 (Millipore, AB5390, [1:2000]), and β Tubulin III (Sigma, T8578, [1:1000]) as a housekeeping protein control. Nav1.7 normalised densitometry ratios between ipsi- and contra-lateral sides of each animal were plotted and independent *t*-test used to analyse whether group means, 5 minutes or 3-hour post-injury periods, were different.

Following transcardial perfusion, different tissues were extracted for **immunostaining** purposes: skin from the dorsal surface of hind paw, sciatic nerve,

L4-L5 DRG and corresponding spinal cord portions from all experimental groups (n=4 in each group). These tissues were post-fixed for an overnight, cryoprotected with 30% sucrose in PBS and cut at sections of 10µm each using a cryostat. Skin preparations were stained with H&E to study the anatomy of the skin at different time point: prior to the injury (naïve) and at 5 minutes and 3 hours after. Additional skin sections, together with sciatic nerve, DRG and spinal cord preparations, from naïve animals were stained with immunofluorescence against Nav1.7 (Millipore, AB5390, [1:750]), β Tubulin III ([1:7000]) and NeuN (Millipore, MAB377, [1:2000]) to assess the distribution of this voltage gated sodium channel. Additionally, Nav1.7 staining was also IHC-developed using DAB-Niquel.

To evaluate nociceptive activation, spinal cord sections from naïve (n=4), sham (n=4), and animals subjected to the burn model with post-injury times of 5 minutes (n=4), 30 minutes (n=4), 1 hour (n=4) and 3 hours (n=4), were used for immunofluorescence against pERK1/2 (Neuromics, RA15002, [1:1500]). Similarly, DRG sections from each experimental group (n=4 for each) were immunofluorescently marked against Nav1.7 (Millipore, AB5390, [1:750]), pCREB (Cell Signaling, 9198, [1:5000]), pp38 (Cell Signaling, 9216, [1:500]; Cell Signaling, 9211, [1:1000]), ATF3 (Santa Cruz, SC188, [1:750]). Following, microphotographs were processed and analysed using the GzLM statistical analysis to detect any significant difference.

For each procedure, corresponding negative and positive controls were run in parallel to guarantee signal specificity. WT mice as well as Nav1.7-cKO mice from Professor John N Woods' lab (Nassar *et al.*, 2004) were used to assess the specificity of the Nav1.7 antibodies (Millipore, AB5390, [1:750]; Alomone, ASC008, [1:500]).

12.4 Results

An antibody against Nav1.7 (Millipore, AB5390) was tested for its specificity and validation in DRG and other relevant tissues for our scalding-type burn injury model (White *et al.*, 2011b). Additionally, the burn-injury model, previously assessed in mice (White *et al.*, 2011b), was studied and validated for its use in Sprague-Dawley rats. To do so, H&E staining was performed in skin sections to evaluate the development of a second-degree burn, and the oedema developing at the paw monitored by measuring leg's circumference. Further, the expression of different markers of nociceptive activation in DRG and spinal cord were assessed by western blotting and immunostaining.

Once the antibody and the burn injury model in rats were validated, Nav1.7, which was previously implicated in the development of hyperalgesia following heat stimulation (Shields *et al.*, 2012), was studied in the context of this burn injury model (White *et al.*, 2011b).

12.4.1 Nav1.7 antibody specificity in mice DRG

L4 and L5 DRG sections from Nav1.7-cKO (Nassar *et al.*, 2004), and WT (C57BL/6) mice killed under baseline conditions were immunostained for Nav1.7 (Millipore, AB5390) to test its specificity against the target in which it was raised.

In the WT mice (Figure 12.1, second column), there was a great proportion (see below) of positive cells. The staining was located outside the nucleus, within the cytoplasmic compartment. Additionally, some dotted pattern was observed in the positive cells, indicating that the stain was also located superficially at the cells, at the membrane. However, when applying the same thresholding criteria to the cKO tissue (Figure 12.1, first column) no positive staining was detected. Similarly, the

negative control for the staining process, which was carried out in DRG sections of WT mice that lacked the primary antibody incubation, did not show any positive signal (Figure 12.1, third column).

These findings were consistently observed when revealing the staining with both immunofluorescence (Figure 12.1, first row) and DAB (Figure 12.1, second row).

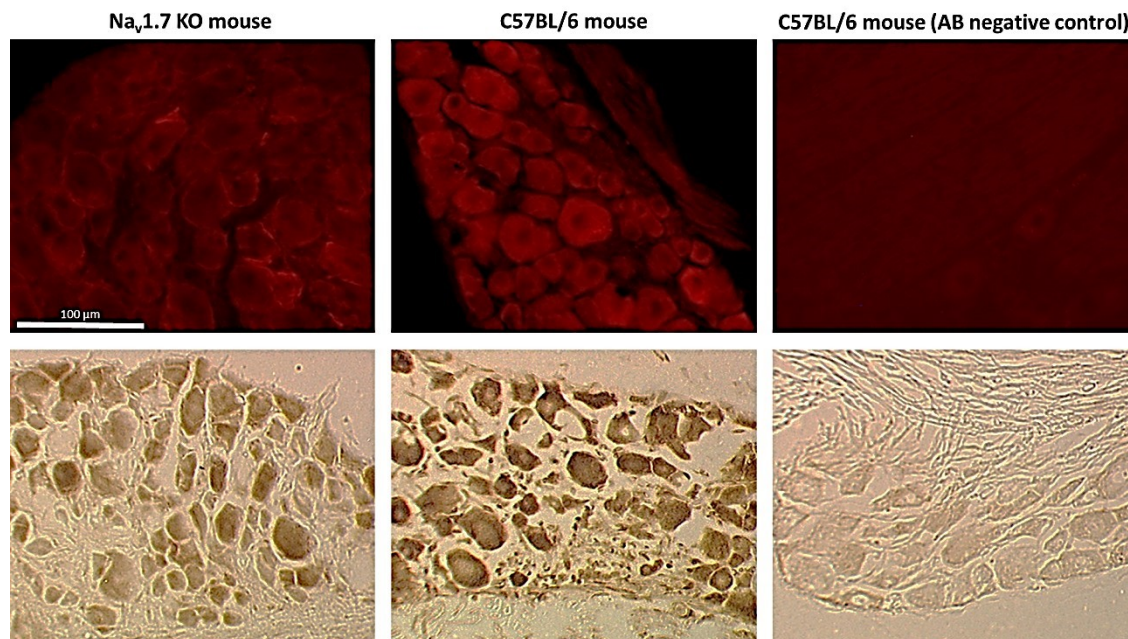


Figure 12.1. **$\text{Na}_v1.7$ antibody: control of specificity.** $\text{Na}_v1.7$ staining (Millipore, AB5390) was performed in DRG sections from $\text{Na}_v1.7$ -cKO (first column) and naïve mice (C57BL/6, second column), together with corresponding negative (third column) and positive (not shown) controls and revealed by both fluorescence (first row) and DAB (second row). The scale bar equals 100 μm . KO animals were provided by Professor John Wood's lab (Nassar et al., 2004).

12.4.2 Validation of the $\text{Na}_v1.7$ antibody in the DRG from Sprague-Dawley rats

In order to validate the use of this antibody for our model of study, L4 and L5 DRG sections from Sprague-Dawley rats killed under baseline conditions were used for immunostaining against the $\text{Na}_v1.7$ antibody (Millipore, AB5390).

While normal IF (Figure 12.2) showed a similar pattern as described before for WT mice (Figure 12.1), in the negative control (Figure 12.2, primary antibody not included in the immunostaining process) the signal was completely abolished.

Furthermore, it was also reduced within the blocking peptide control (Figure 12.2). Here the antibody was previously incubated with the peptide used to its generation in the rabbit host and DRG sections incubated with this resulting mix.

For positive control (Figure 12.2), the already-validated TRPV1 antibody (Neuromics, GP14100) was used, which produced the expected positive signal (Caterina *et al.*, 1997). Thus, indicating that the procedure was carried out effectively.

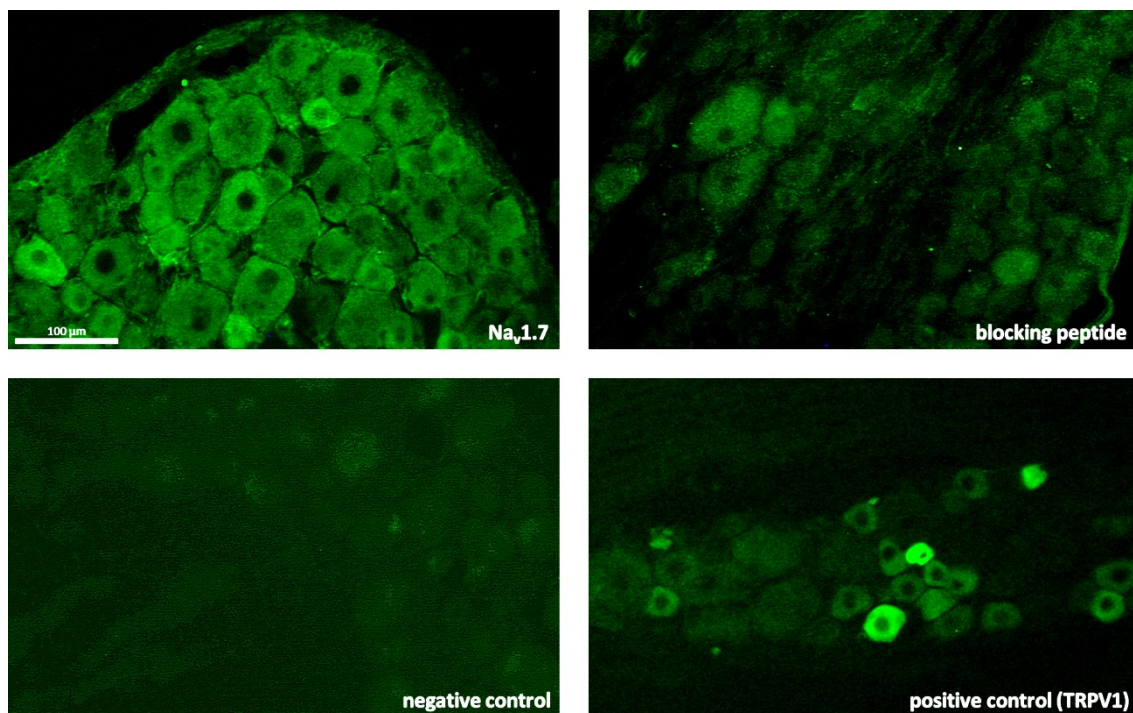


Figure 12.2. **Na_v1.7 antibody: quality control.** Na_v1.7 staining (Millipore, AB5390) was performed in DRG sections from Sprague-Dawley rats together with corresponding controls to test the quality and specificity of the signal. In the blocking control, the blocking peptide (provided together with the antibody) was used to block/complete with antibody binding. In the negative control, no primary antibody was added. Staining with TRPV1 antibody (Neuromics, GP141000) was used as positive control. The scale bar equals 100 μm.

12.4.3 Na_v1.7 is present at different parts of the primary nociceptive neurons

Tissues were collected from Sprague-Dawley rats killed under naïve conditions and used to assess the expression patter of Na_v1.7.

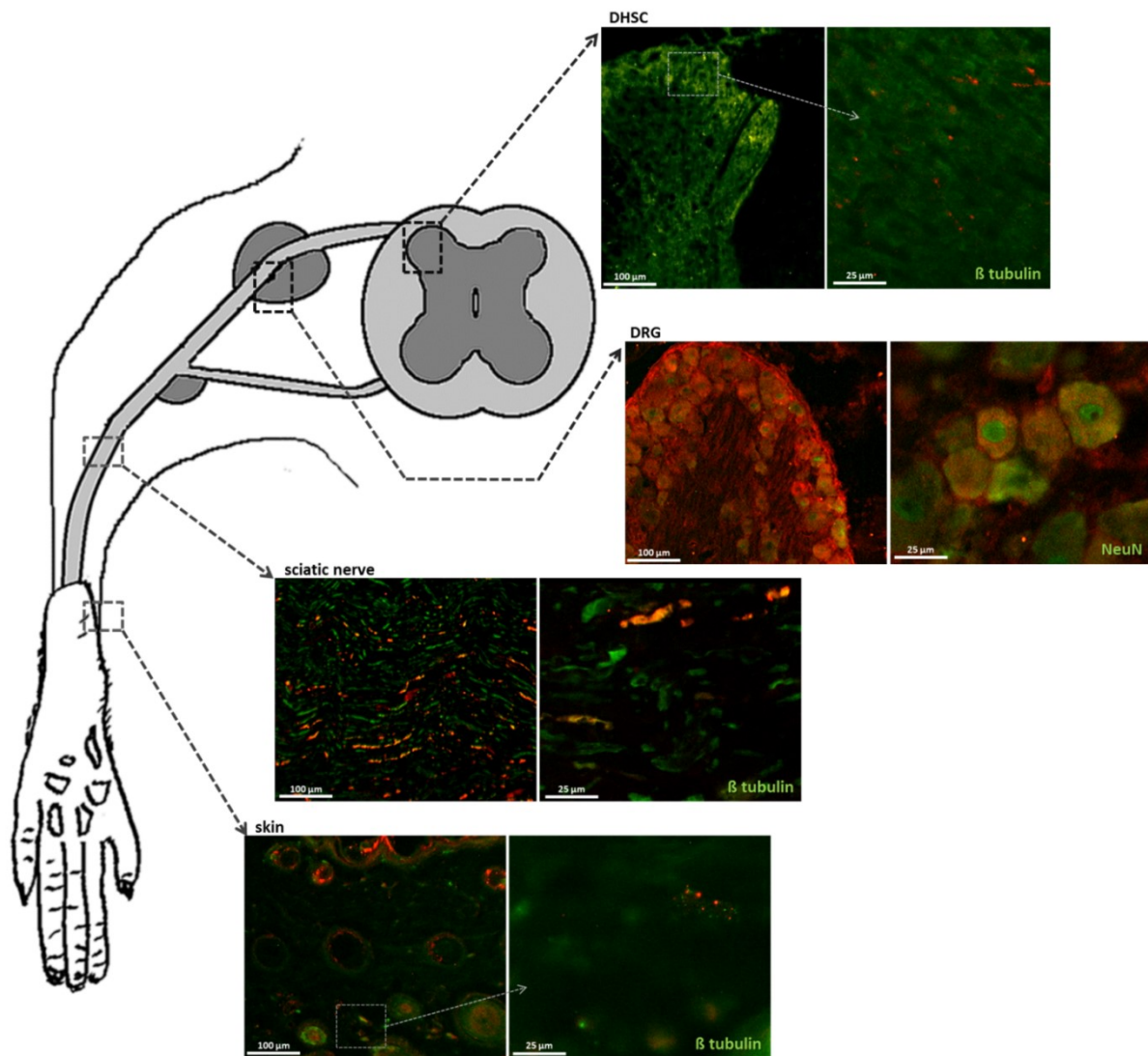


Figure 12.3. **Nav1.7: distribution pattern.** Nav1.7 is expressed in DRG and is also localised in both peripheral- and centrally-directed fibres. In all pictures, red channel is for Nav1.7 and green is either β Tubulin (row 1, 3 and 4) or NeuN (row 2). White bars show the corresponding scale. Note that Nav1.7 is only present in neurons, NeuN+ cells.

Nav1.7 was detected in all levels studied, from skin to spinal cord (Figure 12.3). In the skin sections, it was co-expressed with β tubulin III, an element of microtubules exclusive of neurons (Katsetos *et al.*, 1993; Draberova *et al.*, 1998). Next, there was a high co-expression of this marker with Nav1.7 at the sciatic nerve preparations studied. The pattern of staining in the L4-L5 spinal cord sections revealed that Nav1.7 was only expressed in small terminal neuronal fibres (co-expressing with β tubulin III) and was restricted to the most superficial laminae of the dorsal horn.

In L4 and L5 DRG sections, Nav1.7 co-expressed exclusively with NeuN, a marker of neuronal nucleus (Mullen *et al.*, 1992), indicating that the cells positives for Nav1.7 are indeed neurons. The study of these slides showed that around 40% ($37 \pm 11.6\%$, $n=4$) of the neurons within the DRG expressed Nav1.7, mainly small and middle size neurons ($200\text{-}600\ \mu\text{m}^2$, Figure 12.4).

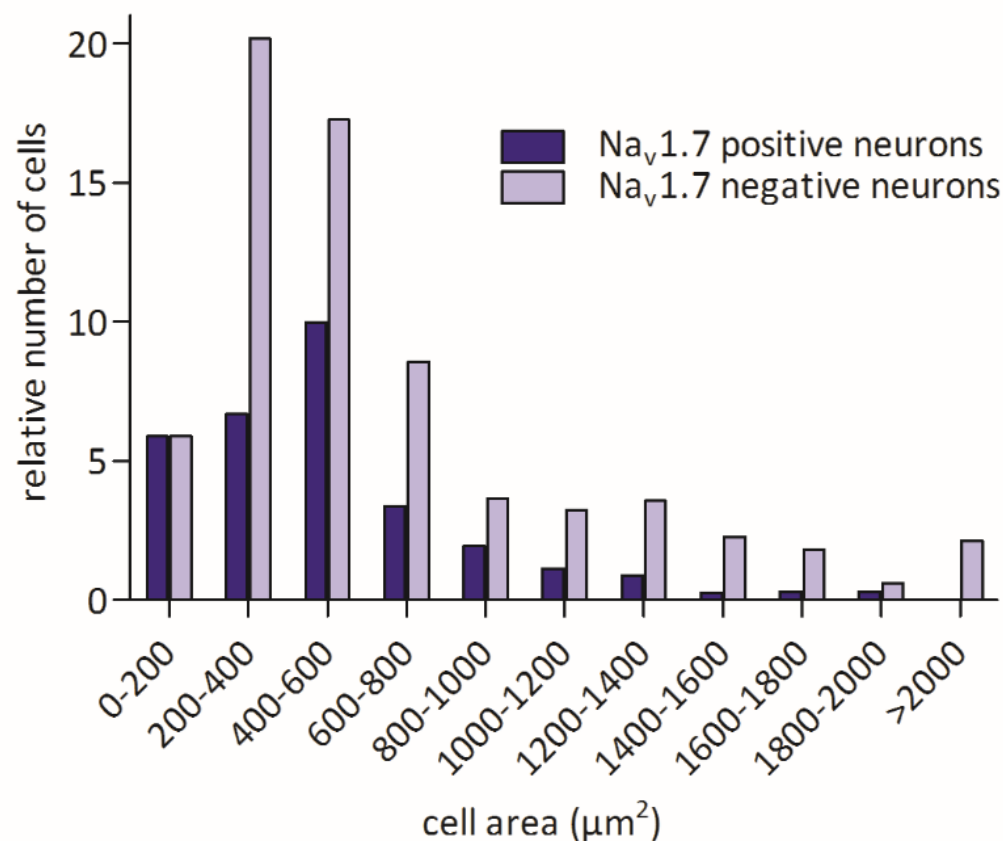


Figure 12.4. **Nav1.7 is highly expressed in neurons within DRG.** This graph represents the cell size distribution of Nav1.7+ neurons in the DRG of control Sprague-Dawley rats. Note that most of the Nav1.7+ neurons are small and middle sized.

12.4.4 Assessment of the burn injury model in Sprague-Dawley rats

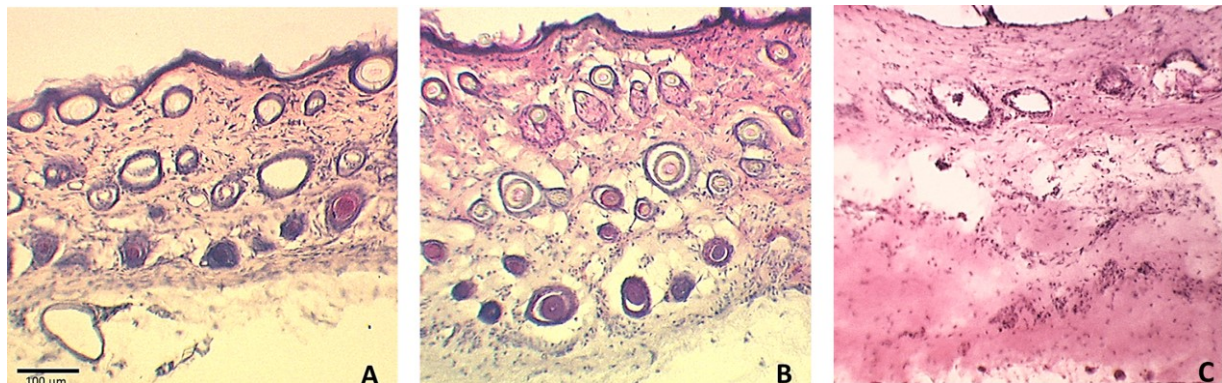


Figure 12.5. **Histopathology of the skin after burn injury.** Microphotographs of H&E stained skin from (A) ipsilateral hind paw of a naive animal, (B) an animal from 5-minutes group (60°C water for 2 minutes, 5 minutes post-injury period); and (C) an animal from 180 minutes group (60°C water for 2 minutes, 3 hours post-injury period). Scale bar equals 100μm. Note that in B and C the structure of the skin is damaged by the thermal injury.

The scalding type burn injury model used was previously established in mice (White *et al.*, 2011b) and here it was validated for Sprague-Dawley rats. To do so, hind paw skin sections under baseline conditions and after 5 minutes and 3 hours following the induction of the injury were stained with H&E for their histopathological analysis (Figure 12.5).

The skin under naïve conditions (Figure 12.5 A) showed the normal histological features of undamaged skin. From upper layers to most inner structures: the epidermis is highly compacted and strongly stained in violet due to its negative charges; below, the dermis and right after is the subcutis with vessels embedded in it. 5 minutes after the immersion in 60°C water (Figure 12.5 B), the epidermis is still present although some fragmentation is observed. There also is an infiltration of liquid in the dermis, consistent with the development of an oedema in the skin (Moritz, 1947). Once 3 hours of the injury are elapsed (Figure 12.5 C), a major destruction is observed in the skin: the superior layers are totally or partially missing,

the integrity of the cells in the dermis is visibly compromised and there is a bigger infiltration of fluid that threatens the macrostructure of the skin.

To further validate the development of the burn injury, oedema formation was assessed throughout different time points, from 0 minutes up to 3-hours post-injury (Figure 12.6). Initially, at time zero, no significant variation in hind paw circumference was observed between the ipsi- and contra-lateral limbs (ipsilateral: 3.11 ± 0.03 , $n=5$; contralateral: 3.07 ± 0.05 , $n=5$; $p=0.518$, one-way ANOVA). This fashion was constantly observed in the contralateral side, where no significant difference in hind paw circumference was detected at any time studied (5 min: 3.06 ± 0.06 , $n=5$, $p=0.898$; 30 min: 2.94 ± 0.04 , $n=5$, $p=0.067$; 1 hour: 2.98 ± 0.06 , $n=5$, $p=0.271$; 2 hours: 3.09 ± 0.08 , $n=4$, $p=0.851$; 3 hours: 2.96 ± 0.11 , $n=4$, $p=0.380$; one-way ANOVA). However, 5 minutes after the injury there was a significant increase in the circumference of the ipsilateral hind paw (5 min: 3.39 ± 0.04 , $n=5$, $p=0.001$, one-way ANOVA), which was further exacerbated at later time-points (30 min: 3.56 ± 0.06 , $n=5$, $p<0.001$; 1 hour: 3.55 ± 0.08 , $n=5$, $p=0.001$; 2 hours: 3.79 ± 0.08 , $n=4$, $p<0.001$; 3 hours: 3.62 ± 0.06 , $n=4$, $p<0.001$; one-way ANOVA), reaching a significant difference in comparison to the one observed at 5 minutes post-injury period (30 min: $p=0.04$, 1 hour: $p=0.101$, 2 hours: $p=0.002$, 3 hours: $p=0.016$, one-way ANOVA).

It also needs to be mentioned that during the assessment of hind paw circumference some fluid leakage out of the limb was detected in two animals (one at 60 minutes and another at 180-minutes post-injury), which could be hiding higher oedema development.

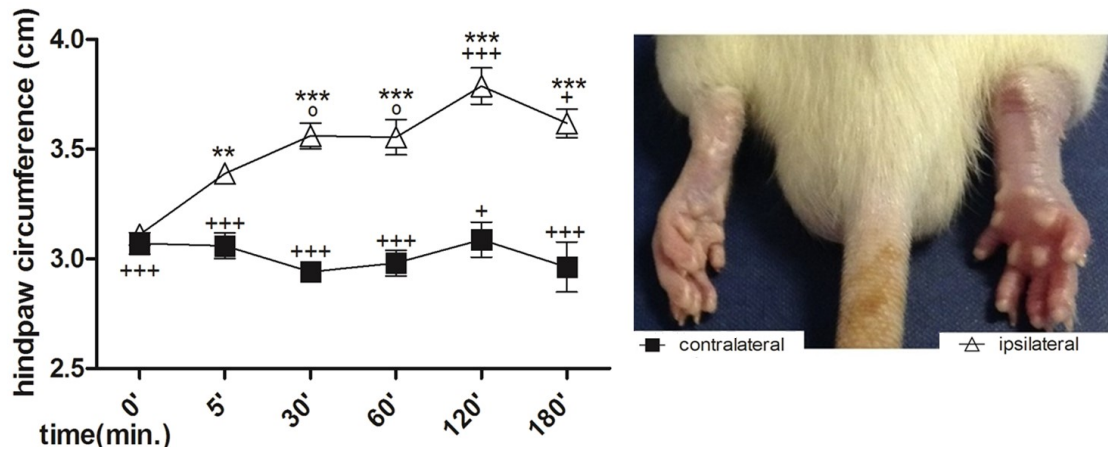


Figure 12.6. **Oedema quantification.** Time course of the effect of thermal injury in the circumference of both contra- and ipsi-lateral hind paws of 5 animals: 4 from 60°C water for 2-minutes and 180-minutes post-injury period; and 1 animal from 60°C water for 2 minutes and 60-minutes post-injury period. The data is presented as mean±SEM. ** p<0.01 vs. ipsilateral 0'; *** p<0.001 vs. ipsilateral 0'; + p<0.05 vs. ipsilateral 5'; +++ p<0.001 vs. ipsilateral 5'; o p<0.05 vs. ipsilateral 120'.

12.4.5 Expression of Nav1.7 in the L4-L5 DRG after the burn injury model

Since previous studies have shown a direct relationship between Nav1.7 and nociception after burn (Shields *et al.*, 2012), it was then decided to assess its expression in the L4-L5 DRG during all time points of the burn injury model.

Western blotting analysis of the protein extracts from ipsi- and contra-lateral DRG of animals after 5 minutes and 3 hours following burn injury showed two bands corresponding to Nav1.7 at ~130 and ~210KDa molecular masses (Figure 12.7 A and Supplementary Figure 20.1) (Toledo-Aral *et al.*, 1997; Laedermann *et al.*, 2013). The analysis of the normalised densitometry ratios showed a significant increase of Nav1.7 protein in the ipsilateral DRG after 3 hours of the injury that was not observed at 5 minutes post-noxious stimulation (3 hours: 1.23±0.09, n=4; 5 min: 0.94±0.02, n=4, p=0.041, student's t test; Figure 12.7 B and Supplementary Figure 20.1).

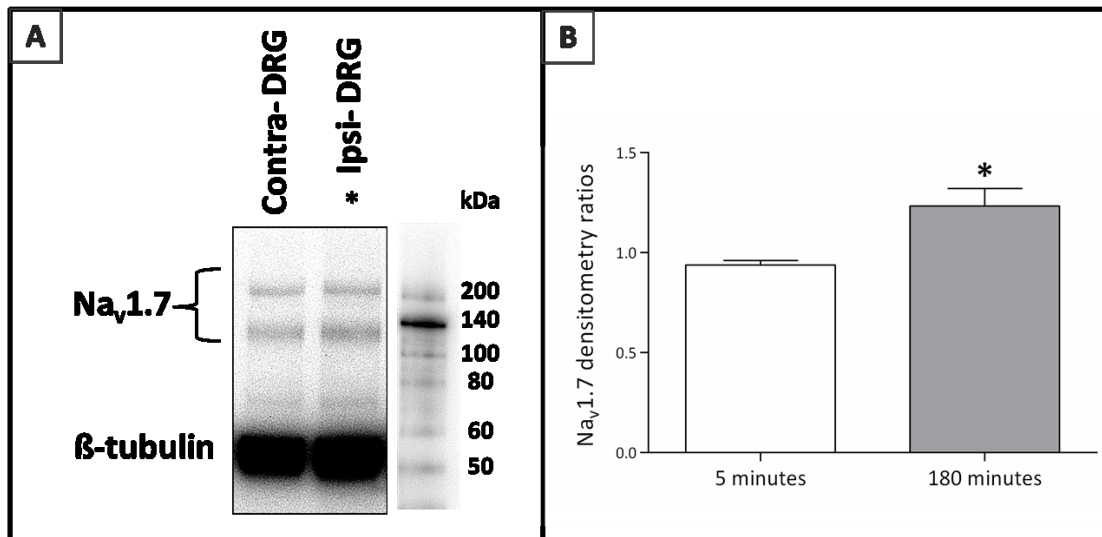


Figure 12.7. **There is an increase of Nav_v1.7 in DRG 3 hours after burn injury.** **A)** Representative western blot showing an increase of Nav_v1.7 within L4 and L5 DRG after 3 hours of burn stimulation (for 5-minutes western blot see Supplementary Figure 20.1). **B)** This graph shows the normalized densitometry ratios between contra- and ipsi-lateral DRG of 5' and 180' burn injury animals (n=4). Data is presented as mean±SEM. * p<0.05. Note that both Nav_v1.7 bands were considered for analysis.

Immunofluorescent staining against Nav_v1.7 of ipsi- and contra-lateral DRG section from the different time points studied also showed a significant increase in the number of neurons expressing Nav_v1.7 in the ipsilateral DRG at 3 hours after the induction of burn injury (3 hours: 55.81±14.11, Mdn=68.26, n=4; sham: 25.72±9.81, Mdn=24.51, n=4; p=0.005, GzLM; Figure 12.8). This increase was not observed at earlier time points in the ipsilateral DRG (5 min: 35.27±9.1, Mdn=37.84, n=4, p=0.377; 30 min: 29.96±7.27, Mdn=26.70, n=4, p=0.695; 1 hour: 35.45±7.83, Mdn=34.48, p=0.368; GzLM) or at any point in the contralateral DRG (sham: 27.67±5.97, Mdn=24.05, n=4, p=0.857; 5 min: 22.24±2.57, Mdn=22.69, n=4, p=0.747; 30 min: 40.05±9.55, Mdn=41.59, n=4, p=0.185; 1 hour: 22.32±8.28, Mdn=17.56, n=4, p=0.753; 3 hours: 18.96±9.18, Mdn=12.16, n=4, p=0.532; GzLM; Figure 12.8). Interestingly, the proportion of Nav_v1.7 positive cells according to their size remained similar as observed in basal conditions (not shown).

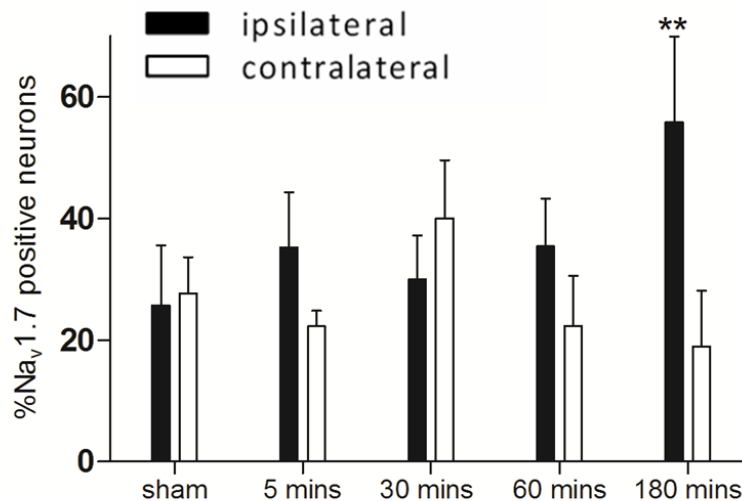


Figure 12.8. **The number of Na_v1.7+ neurons slowly increases 3 hours after burn injury.** Immunofluorescence study of the percentage of Na_v1.7 positive neurons within L4 and L5 DRG at different time points following burn-injury. Data is presented as mean±SEM. Note that, despite great variations, the number of Na_v1.7+ cells is only significantly different (** p<0.01 vs. 5 minutes ipsilateral) in the ispsi-lateral DRG at 3-hours post burn-injury.

12.4.6 pERK1/2 as a marker of nociceptive activation in DHSC after burn injury

Previous studies have shown the importance of phosphorylated ERK1 and ERK2 (pERK1/2) as markers of nociceptive activation in the spinal cord (Ji *et al.*, 1999; White *et al.*, 2011a). Hence, we evaluated its presence in the rat scalding type burn injury model.

The study of L4-L5 spinal cord sections revealed that a discrete population of pERK1/2 positive cells appeared in superficial laminae of the ipsilateral DHSC at 5 minutes following the noxious induction, that were not present in the contralateral side (Figure 12.9) and neither appeared under naïve or sham conditions (images not shown). Consequently, it was decided to perform statistical analysis on the number of positive cells obtained by visual assessment of the L4-L5 spinal cord sections at the different time points (Figure 12.10).

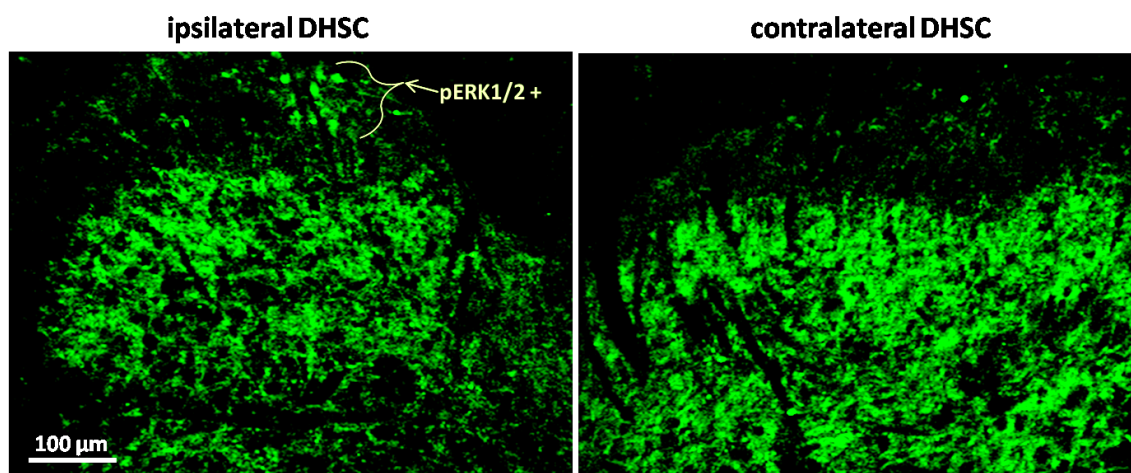


Figure 12.9. **Number of pERK1/2+ cells increases in the ipsi-lateral DHSC at 5 minutes after burn-injury.** Animals used were from the 5-minutes group (60°C water for 2 minutes and 5 minutes post-injury period). There is significant increase of pERK1/2+ cells in the superficial laminae of the ipsi-lateral DHSC (left) after burn-injury. This does not appear in the contra-lateral side (right). Scale bar equals 100μm. Positive cells have been further signalled in order to facilitate their differentiation from the reactive neuropil.

The number of positive cells in the ipsilateral DHSC was significantly increased after 5 minutes of the noxious activation (5 min: 90.75 ± 27.61 , Mdn=82.00, n=4; naïve: 5.00 ± 1.35 , Mdn=4.00, n=4; $p < 0.001$, GzLM; Figure 12.10). This was not observed under sham conditions, meaning immersion in 37°C water instead of 60°C and a survival period of 5 minutes (sham: 2.75 ± 1.25 , Mdn=2.50, n=4, $p = 0.784$, GzLM). The increase on the number of pERK1/2 positive cells in the ipsilateral DHSC was significantly maintained at 30 minutes (30 min: 38.25 ± 6.29 , Mdn=33.00, n=4, $p < 0.001$, GzLM) and 60 minutes (1 hour: 40.00 ± 5.05 , Mdn=42.00, n=4, $p < 0.001$, GzLM), although returned to naïve values after 3 hours of the noxious stimulation (3 hours: 8.20 ± 3.56 , Mdn=3.00, n=4, $p = 0.682$, GzLM). Nonetheless, the peak of pERK1/2 in the ipsilateral DHSC after 5 minutes was significantly higher than the number observed at 30 minutes ($p < 0.001$, GzLM) or 60 minutes ($p < 0.001$, GzLM) following the thermal injury. No significant increase on the number of pERK1/2 positive cells was detected in the contralateral DHSC at any time of the study (naïve: 3.50 ± 1.32 , Mdn=3.00, n=4, $p = 0.855$; sham: 0.75 ± 0.48 , Mdn=0.50, n=4, $p = 0.605$; 5

min: 9.50 ± 3.75 , Mdn=7.00, n=4, $p=0.584$; 30 min: 3.50 ± 0.96 , Mdn=4.00, n=4, $p=0.855$; 1 hour: 10.75 ± 5.39 , Mdn=11.00, n=4, $p=0.485$; 3 hours: 0.80 ± 0.37 , Mdn=1.00, n=4, $p=0.590$; GzLM; Figure 12.10).

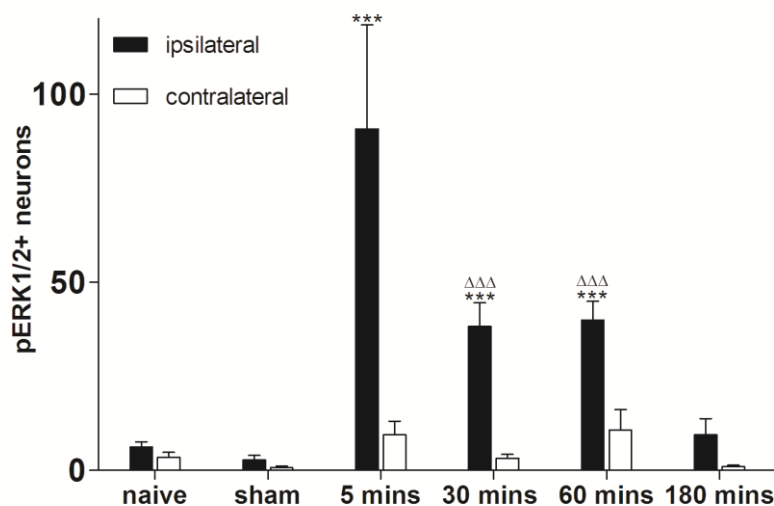


Figure 12.10. **Immunolabelled pERK1/2 neurons in DHSC of the burn-injury model.** Data is presented as mean±SEM. The experimental groups (n=4) were naive animals (naive), 37°C water for 2 minutes and 5-minutes post-injury period (sham), 60°C water for 2 minutes and 5 minutes post-injury period (5 mins), 60°C water for 2 minutes and 30 minutes post-injury period (30 mins), 60°C water for 2 minutes and 60 minutes post-injury period (60 mins) and 60°C water for 2 minutes and 180 minutes post-injury period (180 mins). *** $p < 0.001$ vs. naive ipsilateral; ΔΔΔ $p < 0.001$ vs. 5' ipsilateral.

12.4.7 pCREB as a marker of nociceptive activation at L4-L5

DRG after burn injury

Since phosphorylation of p38 (pp38) has been previously associated with thermal hyperalgesia and nerve injury in small sized DRG neurons (Ji *et al.*, 2002b; Jin *et al.*, 2003), it was decided to study its presence in the rat scalding-type burn injury model. L4-L5 DRG sections of both ipsi- and contra-lateral sides after 5-minutes post-injury were stained against pp38 and the number of positive cells was statistically analysed. No significant difference on the number of pp38 was detected between the ipsilateral and the contralateral DRG (ipsilateral: 16.88%, n=2; contralateral: 16.82%, n=2) when using a pp38 antibody rose in rabbit (Cell Signalling, 9211). Then, a similar analysis was repeated using another antibody against pp38, this time rose in

mouse (Cell Signalling, 9216) and no statistical difference was assessed either when using this second antibody (ipsilateral: 12.38%, n=2; contralateral: 12.80%, n=2). Thus, we decided to study the expression of the activating transcription factor 3 (ATF3), a transcription factor up-regulated during regeneration following a peripheral injury (Schwaiger *et al.*, 2000; Nascimento *et al.*, 2011). Unfortunately, no positive cells were detected after immunostaining against this marker in our model of scalding type burn injury (numbers not shown).

Therefore, burn injury does not up-regulate pp38 and ATF3 expression in primary sensory neurons.

Next, we analysed the expression of phosphorylated cAMP response element-binding protein (pCREB), a downstream inducible factor of the MAPKinases pathway following stress-activation, which has also been related to the induction of IEG such as *c-fos* in areas of the nervous system (Haghighparast *et al.*, 2014; Pahlevani *et al.*, 2014) and in DRG following injury (Li *et al.*, 2009). For this purpose, ipsi- and contralateral L4-L5 DRG sections of all time-points studied were immunostained against pCREB (Figure 12.11). Although the marker was observed in both neurons and satellite cells (Figure 12.11), analysis was only done on neurons. Statistical analysis revealed that there is a significant increase on the number of pCREB positive neurons in the ipsilateral DRG as promptly as 5 minutes after the burn injury (5 min: 10.98 ± 3.16 , Mdn=10.66, n=4; naïve: 0.41 ± 0.41 , Mdn=0.00, n=4; $p < 0.001$, GzLM; Figure 12.12) that was not sustained at either 30 minutes (30 min: 2.95 ± 2.68 , Mdn=0.43, n=4, $p=0.393$, GzLM) or 60 minutes (1 hour: 3.85 ± 2.38 , Mdn=1.53, n=4, $p=0.287$, GzLM) post-stimulation, but peaked again after 3 hours (3 hours: 6.10 ± 2.41 , Mdn=5.99, n=4, $p=0.041$, GzLM) to a similar number as observed at 5 minutes ($p=0.097$, GzLM). These differences were not observed at the ipsilateral

DRG of sham controls (sham: 5.37 ± 3.30 , Mdn=3.40, n=4, $p=0.087$, GzLM) or at the contralateral DRG of any point of the study (naïve: 0.22 ± 0.22 , Mdn=0.00, n=4, $p=0.948$; sham: 4.41 ± 2.10 , Mdn=3.80, n=4, $p=0.167$; 30 min: 0.37 ± 0.37 , Mdn=0.00, n=4, $p=0.989$; 1 hour: 1.95 ± 1.95 , Mdn=0.00, n=4, $p=0.623$; 3 hours: 2.89 ± 1.43 , Mdn=1.53, n=4, $p=0.087$; GzLM; Figure 12.12). Interestingly, most neurons describing positive staining for pCREB were small- and medium-sized ($200\text{--}1000\mu\text{m}^2$, Figure 12.13).

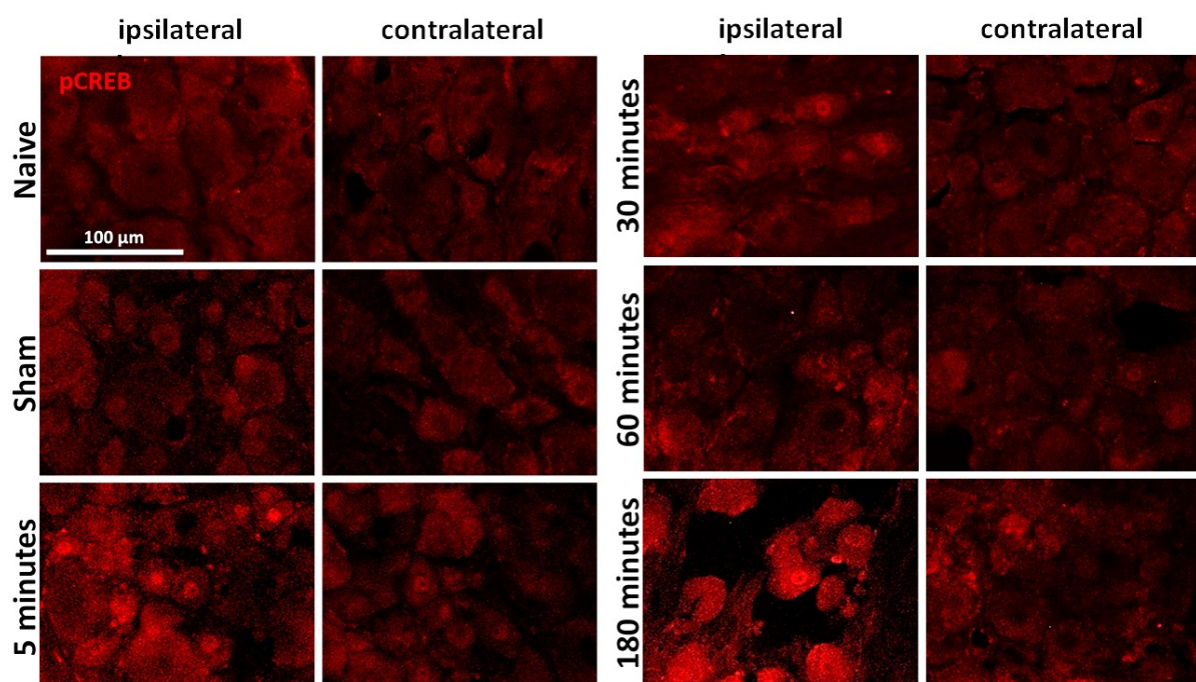


Figure 12.11. **Time course of pCREB induction in DRG after burn injury model.** In all pictures red is for pCREB. The scale bar equals $100\mu\text{m}$. The experimental groups (n=4) were naive animals (naive), 37°C water for 2 minutes and 5-minutes post-injury period (sham), 60°C water for 2 minutes and 5 minutes post-injury period (5 minutes), 60°C water for 2 minutes and 30 minutes post-injury period (30 minutes), 60°C water for 2 minutes and 60 minutes post-injury period (60 minutes) and 60°C water for 2 minutes and 180 minutes post-injury period (180 minutes).

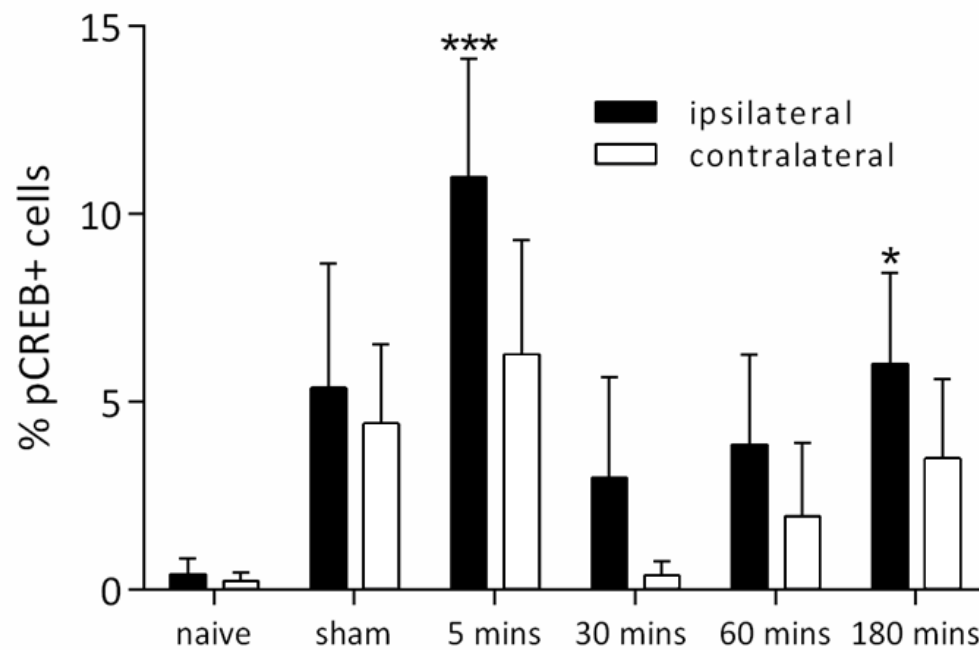


Figure 12.12. **Immunolabelled pCREB neurons in DRG of the burn-injury model.** Data is presented as mean $\pm$ SEM. The experimental groups (n=4) were naive animals (naive), 37°C water for 2 minutes and 5-minutes post-injury period (sham), 60°C water for 2 minutes and 5-minutes post-injury period (5 mins), 60°C water for 2 minutes and 30 minutes post-injury period (30 mins), 60°C water for 2 minutes and 60 minutes post-injury period (60 mins) and 60°C water for 2 minutes and 180 minutes post-injury period (180 mins). *** p<0.001 vs. naive ipsilateral; ** p<0.05 vs. naive ipsilateral.

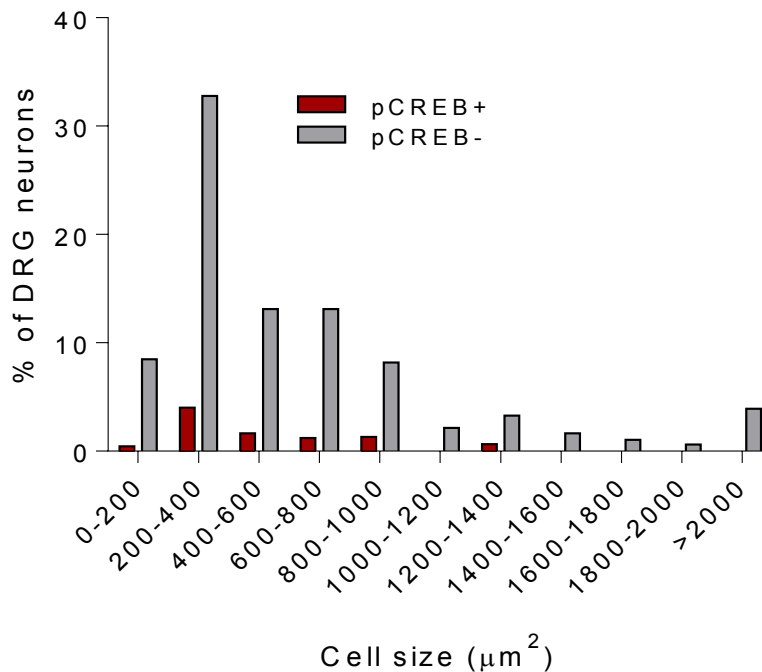


Figure 12.13. **Cell size distribution of pCREB positive neurons in DRG following the burn-injury model.** Data obtained from ipsilateral DRG from 5 minutes after burn injury. Most DRG neurons positive for pCREB are small and medium sized.

12.4.8 pCREB and Nav1.7 co-expression in DRG after the induction of burn injury

Ipsi- and contra-lateral L4 and L5 DRG sections corresponding to the different ending points studied in the scalding type burn injury model were immunostained against both pCREB and Nav1.7 with the objective to study their pattern of distribution and co-expression under the different conditions.

In the baseline group (Figure 12.14), a low number of neurons positive for pCREB were also Nav1.7 positive (25.00 ± 25.00 , $n=4$). Nonetheless, 5 minutes after burn injury there is a significant increase ($p < 0.001$, GzLM) in the number of pCREB positive neurons that were also Nav1.7 positive to ~80% ($87.5 \pm 7.98\%$, $n=4$; Figure 12.14). This proportion was significantly similar to the one observed in the burnt animals that survived a total of 3 hours ($p=0.983$, GzLM; Figure 12.14 A).

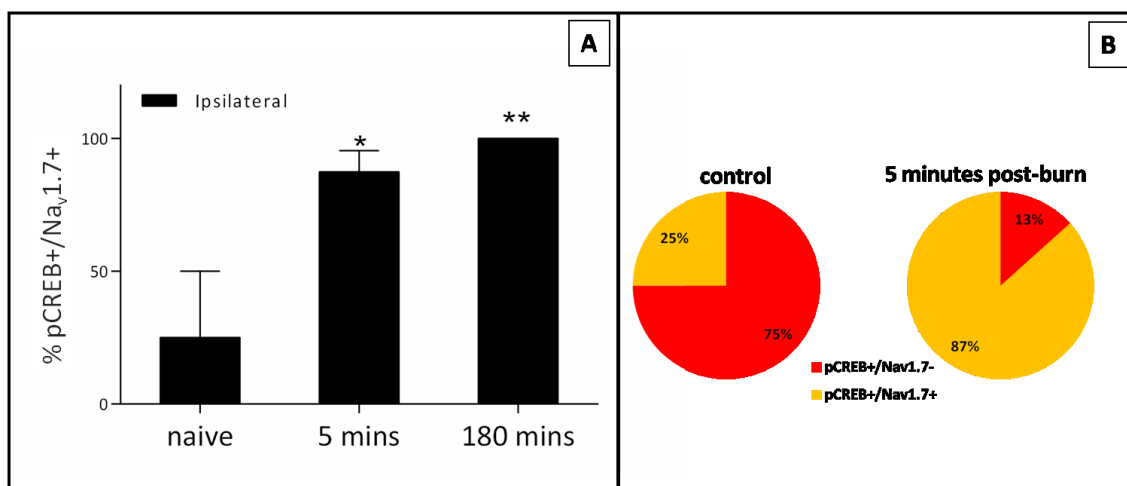


Figure 12.14. Co-expression between Nav1.7 and pCREB in L4 and L5 ipsilateral DRG after burn injury model. The experimental groups ($n=4$) were naive animals (naive), 60°C water for 2 minutes and 5-minutes post-injury period (5 mins) and 60°C water for 2 minutes and 180 minutes post-injury period (180 mins). **A)** Graph showing pCREB+ neurons that are also Nav1.7+. Data is presented as mean $\pm$ SEM. * $p < 0.05$ vs. naive ipsilateral; ** $p < 0.01$ vs. naive ipsilateral. **B)** Pie chart shows the higher proportion of pCREB+ neurons co-expressing with Nav1.7 after a burn injury.

Despite the elevated co-expression between pCREB and Nav1.7, a closer evaluation of the staining pattern at a subcellular level (Figure 12.15) revealed a different

compartmentalization: while pCREB is localised in the neuronal nuclei (Figure 12.15, red staining), Nav1.7 was mainly localised within the cytoplasm and the lipid membrane (see above and the green staining in Figure 12.15).

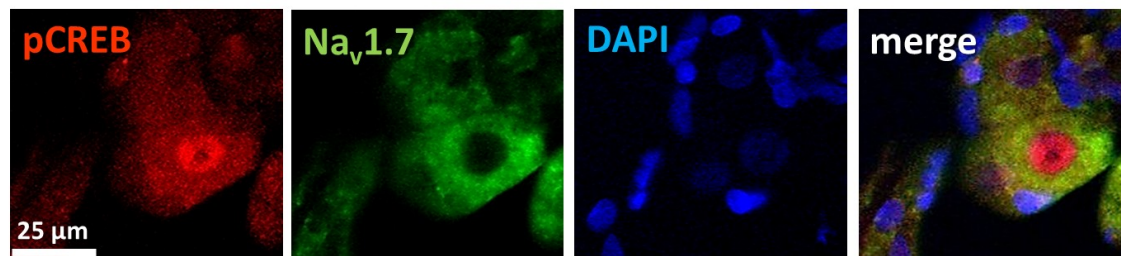


Figure 12.15. **Sub-cellular distribution of pCREB in the ipsilateral DRG after burn injury and its relationship with Nav1.7.** DRG of animals from the 5-minutes group (60°C water for 2-minutes and 5-minutes post-injury period) were stained for pCREB (red), Nav1.7 (green) and nuclei (DAPI; in blue). The scale bar equals 25µm. pCREB is present intra-nuclear while Nav1.7 is observed at the membrane surface and cytoplasm.

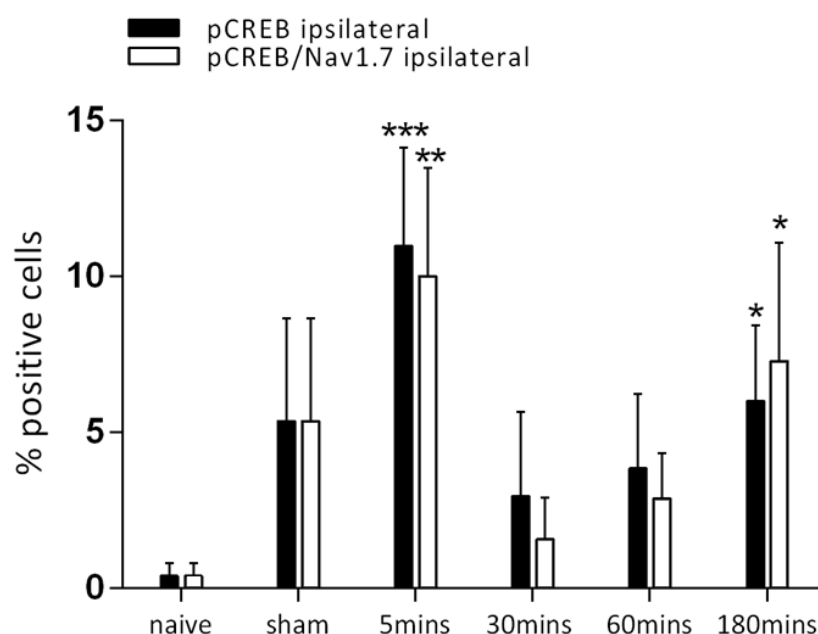


Figure 12.16. **Percentage of pCREB+ cells and co-expression with Nav1.7 in the ipsilateral DRG of the burn injury model.** Data is presented as mean±SEM. The experimental groups (n=4) were naive animals (naive), 37°C water for 2 minutes and 5-minutes post-injury period (sham), 60°C water for 2 minutes and 5 minutes post-injury period (5 mins), 60°C water for 2 minutes and 30 minutes post-injury period (30 mins), 60°C water for 2 minutes and 60 minutes post-injury period (60 mins) and 60°C water for 2 minutes and 180 minutes post-injury period (180 mins). * p<0.05 vs. naive ipsilateral pCREB; ** p<0.01 vs. naive ipsilateral pCREB; *** p<0.001 vs. naive ipsilateral pCREB.

Finally, the number of cells positive to both, pCREB and Nav1.7, from the total number of cells counted was statistically analysed and compared against the pattern

of pCREB positive neurons from the total number of cells counted. The purpose of this was to check for any possible discrepancy between both staining schemes (Figure 12.16). The significant increase on pCREB positive neurons in the ipsilateral DRG at time points 5 minutes and 3 hours (Figure 12.11 and Figure 12.16) was equally observed when accounting for the number of positive neurons for both markers from the total sample of cells studied (5 min: 10.02 ± 3.46 , Mdn=9.05, n=4, $p=0.001$; 3 hours: 6.10 ± 2.42 , Mdn=5.99, n=4, $p=0.041$; GzLM; Figure 12.16). These changes in the expression were not significantly different between both measurements of cells analysed at either 5 minute ($p=0.748$, GzLM) or 3 hours post-injury ($p=1$, GzLM). Thus, indicating that there was no dysfunctional expression between pCREB and Nav1.7.

12.5 Discussion

Here, the scalding type burn injury model, that was previously assessed in mice (White *et al.*, 2011b), has shown to also induce a second degree burn in Sprague-Dawley rats. Additionally, the distribution of the sodium channel Nav1.7, a channel implicated in the processing of noxious heat (Shields *et al.*, 2012), has been studied throughout DRG, together with its possible link and implications to nociceptive processing following this model. To do so, first an antibody against this voltage channel has been validated. Secondly, this antibody has been used to study Nav1.7 pattern of expression.

Furthermore, nociception following this burn model has also been assessed at two key processing levels, superficial spinal cord and primary sensory neurons, using the MAPK pERK1/2 and the transcription factor pCREB respectively.

12.5.1 The antibody against Nav1.7 from Millipore selectively reacts to Nav1.7

Initially, the selectivity of the antibody was tested on Nav1.7-cKO and WT mice. The lack of staining in the cKO mice indicates that the antibody reacts selectively to the Nav1.7 protein. Similarly, there was no staining in the negative controls, which demonstrates that the antibody can be efficiently used for this technique without causing a false positive staining.

Secondly, the antibody was tested in Sprague-Dawley rats. As observed in mice, the staining with this antibody produced a specific and reliable labelling in DRG sections from Sprague-Dawley rats.

These data together clearly support the use of this Nav1.7 antibody (Millipore, AB5390) in our assessment.

Throughout the literature, different antibodies have been used to study the distribution of Nav1.7 (Morinville *et al.*, 2007; Black *et al.*, 2008; Stambouliau *et al.*, 2010; Black *et al.*, 2012). However, the most reliable data using immunoglobulins to detect Nav1.7 have all been achieved with a non-commercially available antibody produced by Toledo-Aral's group in 1997 (Toledo-Aral *et al.*, 1997). Nonetheless, the Millipore's antibody used here has been validated as a promising tool to study Nav1.7 distribution pattern in rodents.

12.5.2 Nav1.7 is highly present in small/medium-sized DRG neurons

In agreement with previous findings (Toledo-Aral *et al.*, 1997; Nassar *et al.*, 2004; Dib-Hajj *et al.*, 2007; Black *et al.*, 2012; Dib-Hajj *et al.*, 2013), Nav1.7 was found in the soma and axons of DRG neurons, mainly present in small- and middle-sized

neurons (400-600 μm^2), while very few large cells express it. In total, Nav1.7 was observed in nearly 40% of the identified neurons. This pattern correlates with the high numbers described by other studies, mainly identified in nociceptors (Djouhri *et al.*, 2003; Black *et al.*, 2012). Nonetheless, some variability on the absolute value can be justified by the use of different antibodies (with different sensitivity), animal models or the intrinsic variability of Nav1.7 expression (Yeomans *et al.*, 2005) and analysis techniques.

At a sub-cellular level, Nav1.7 seems to be at the cytoplasmic compartment and at the plasmatic membrane (the dotted pattern observed), although this should be confirmed by confocal microscopy. This sub-cellular distribution could be showing different states of protein maturation, as well as, Nav1.7 stored in the cytoplasm and ready to be incorporated to the membrane following appropriate cues (Toledo-Aral *et al.*, 1997; Gould *et al.*, 2004; Black *et al.*, 2008; Dib-Hajj *et al.*, 2010; Stambouliau *et al.*, 2010).

Within fibres, Nav1.7 is present in both centrally directed branches and peripheral portions. This has also previously been observed by others (Toledo-Aral *et al.*, 1997; Dib-Hajj *et al.*, 2007; Black *et al.*, 2012; Minett *et al.*, 2012; Dib-Hajj *et al.*, 2013). Interestingly, the Nav1.7 staining seems to centrally terminate at pre-synaptic terminals within the superficial DHSC, where synapses are established with second order neurons and Nav1.7 is suggested to regulate neurotransmitter release and firing (Toledo-Aral *et al.*, 1997; Dib-Hajj *et al.*, 2007; Black *et al.*, 2012; Minett *et al.*, 2012; Dib-Hajj *et al.*, 2013).

Peripherally, this VGSC is found all the way through the nervous fibres to their endings at the epidermis. There, Nav1.7 seems to play a key role in the generation of action potential following depolarisation of the membrane at the nerve terminal. Its

presence throughout the whole length of the nerves also points at its role on action potential propagation (Toledo-Aral *et al.*, 1997; Dib-Hajj *et al.*, 2007; Black *et al.*, 2012; Minett *et al.*, 2012; Dib-Hajj *et al.*, 2013). The speckled presence of Nav1.7 throughout the nerves could be due to a heterogeneous distribution: more clustered at nodes of Ranvier as suggested by Black's investigation (Black *et al.*, 2012).

12.5.3 Scalding-type burn injury model causes a partial thickness, second degree, burn in Sprague-Dawley rats.

The histopathological analyses of skin biopsies, together with oedema quantification, clearly show that the scalding model does induce a significant burn in Sprague-Dawley rats.

From the skin preparations, an infiltration of liquid in the injured side can be observed as early as 5 minutes after the burn, which also correlates with the significant increase in paw circumference detected. Further, a partial loss of skin histoarchitecture can also be observed. Together, these data describe a second-degree burn (also known as partial thickness), characterised by the blistering and the deepness of the injury (Moritz, 1947; WHO, 2007; Abdullahi *et al.*, 2014). Hence, it confirms that the model, previously validated in mice (White *et al.*, 2011b), does induce a similar extent of damage in rats.

Two positive characteristic of this model are the extent of the injury and its localisation. It affects ~10% of TBSA (according to Meeh's equation) (Walker & Mason, 1968; Gouma *et al.*, 2012), while other scalding burn injury models that also provide a second degree burn extended to smaller percentages (Shields *et al.*, 2012; Green *et al.*, 2013) or are localised to other areas of the skin that were previously shaved (Walker & Mason, 1968). The skin at rodents' limbs does not have the dense

fur coat characteristic of the rest of the animal, hence the burn injury response in these areas is more like that in humans (Moritz, 1947; Seok *et al.*, 2013; Abdullahi *et al.*, 2014). Thus, the model used here affects a considerable area in a structure with less fur, which makes the data more reliable and translational to burn survivor's scenarios (Abdullahi *et al.*, 2014). Furthermore, the injury is localised to one hind paw; it is circumscribed to the same area where the majority of inflammatory pain models are tested (Edelmayer *et al.*, 2014), which facilitates their comparison.

At later time points, the oedema developed further. Moreover, assessment of the skin structure at 3 hours post-injury showed a major trauma. These could be explained by the subsequent death after the injury, the increased extravasations of fluids and other injury mediators released into the area of trauma.

Taking these together, the scalding type burn injury assessed here is reliably producing a second degree burn injury in Sprague-Dawley rats. Thus, it is a feasible model to study burn injury trauma in rodents. However, this model also has some limitations. Due to the extent and severity of the injury and in order to meet legal standards of animal research in the UK, the animals subjected to it have to remain under anaesthesia during the post-injury period, which was limited to 3 hours due to ethical considerations. Hence, this model cannot be used to do behavioural studies or to assess changes at later time points, while other models that produce more modest burns can be used for these purposes (Shields *et al.*, 2012; Green *et al.*, 2013; Desroches *et al.*, 2014).

12.5.4 Nav1.7 expression increases following a burn injury

The immunostaining and western blot studies revealed an increase in the number of DRG neurons, associated to the injury site, that express Nav1.7 at 3-hours post-

injury and suggests that Nav1.7 has an important role in burn-injury associated nociception.

The fact that the increment is proportionally greater from immunostaining data than from the western blot could be explained by an increase of the number of cells expressing this VGSC rather than its up-regulation in cells that already express it.

In any case, the up-regulation of Nav1.7 in DRG innervating an injury site had been observed previously (Gould *et al.*, 1998; Black *et al.*, 2004; Gould *et al.*, 2004; Yeomans *et al.*, 2005; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Dib-Hajj *et al.*, 2010; Hoeijmakers *et al.*, 2012; Dib-Hajj *et al.*, 2013). Nonetheless, majority of investigations detected this increase at much later time points. For example: Black's group found an up-regulation of Nav1.7 at 4 days following injection of carrageenan (Black *et al.*, 2004), while Gould and colleagues described an up-regulation at 24 hours following CFA injection (Gould *et al.*, 1998; Gould *et al.*, 2004).

The increase on Nav1.7 detected here as early as 3-hours post-injury could be due to the greater extent of our injury model. Furthermore, certain intrinsic variability in the expression of Nav1.7 was observed in this experiment, which matches observations from other investigations (Yeomans *et al.*, 2005) and could be masking greater up-regulations.

Interestingly, the increase in Nav1.7 was not directed to a certain population of DRG neurons, according to their cell size. It was spread across all types. This contrasts with other's data, where the increased expression of Nav1.7 was mainly found in small- and medium-diameter neurons (Gould *et al.*, 1998; Gould *et al.*, 2004). Possibly, both large diameter neurons and additional small-diameter nociceptors got recruited following this model of burn injury.

Overall, these results show plasticity of Nav1.7 expression and point at this voltage channel as a key regulator of nociception following the scalding-type burn injury model. This is in agreement with previous data; Shields and colleagues linked Nav1.7 to the development of heat hyperalgesia following another burn injury model (Shields *et al.*, 2012) or Yeoman's research, where they showed overexpression of Nav1.7 as critical to inflammatory pain hyperalgesia (Yeomans *et al.*, 2005). Additionally, Toledo-Aral and colleagues suggested that NGF, which is liberated during inflammatory processes, can induce the expression of this sodium channel; a process that correlates with increased membrane excitability (Toledo-Aral *et al.*, 1997).

12.5.5 pERK1/2 is a dynamic nociceptive-specific marker at DHSC following the burn injury model

Phosphorylation of the MAPK ERK1/2, pERK1/2, is observed in a population of cells from the superficial DHSC after the induction of thermal injury, but not in naïve or sham animals. Hence, this activation appears in a nociceptive-specific manner. It peaked as early as 5 minutes after the burn injury and gradually reduced its expression.

These findings support previous research (Ji *et al.*, 1999; Ji & Woolf, 2001; Polgar *et al.*, 2007; Black *et al.*, 2008; Gao & Ji, 2009; White *et al.*, 2011a; White *et al.*, 2011b). Ji and collaborators established similar data in their injury model, a peak at ~5 minutes following an inflammatory model in an intensity-dependent manner (Ji *et al.*, 1999). Similarly, previous studies of this same scalding type burn injury model in mice also showed a peak at 5-minutes post-injury (White *et al.*, 2011b). Nonetheless, a second event of pERK1/2 appeared after 3 hours in both ipsi- and contra-lateral

sides, which was not restricted to neurons (White *et al.*, 2011b). This second wave was not observed here in rats, a difference that could be due to a differential processing and/or with different molecular kinetics between rats and mice.

pERK1/2 has previously been implicated in processes of neuronal plasticity, including LTP and central sensitization (Ji & Woolf, 2001; Ji *et al.*, 2003; Woolf *et al.*, 2004; Steeds, 2013; Edelmayer *et al.*, 2014). It is valued as a nociceptive-specific marker due to its highly dynamic changes and intensity-specific induction (Gao & Ji, 2009). Hence, the data presented here together with previous researches validate the use of pERK1/2 as a marker of nociception and shows that our model of scalding-type burn injury is capable to induce a significant nociceptive activation at DHSC.

12.5.6 pCREB is induced in DRG following the burn injury model in a nociceptive-specific fashion

Due to the inability to obtain a reliable signal using antibodies against MAPK or ATF3 in DRG, it was decided to study a common downstream effector of those pathways: pCREB (Ji *et al.*, 2003; Kawasaki *et al.*, 2004; Bie *et al.*, 2011; Li *et al.*, 2014). The phosphorylation of this transduction factor appeared in a nociceptive-specific manner as early as 5 minutes post-injury in the nuclear compartment and mainly localised to small- and medium-sized neurons. Moreover, it rapidly returned to basal number, until 3-hours post-injury where peaked again.

Knowing that pCREB induces the expression of different genes (Thomson *et al.*, 1999b; Strahl & Allis, 2000), these two peaks could be explained as two events of gene expression: the first one immediately after the injury due to the injury itself and the inflammatory mediators liberated by the cells dying, and the second one due to

the initial expression of other IEG (e.g. ATF3) or late-response genes (e.g. the ones regulated by AP1, a dimer of c-Fos and c-Jun) by the recruited immune cells (Iadarola *et al.*, 1988; Williams *et al.*, 1990; Dubner & Ruda, 1992; McCarson & Krause, 1994; Ji *et al.*, 2002a; Ji *et al.*, 2003; Duric & McCarson, 2007). Nonetheless, this should be confirmed by looking at later time points and with other techniques such as RNA extraction and posterior PCR. In any case, the data here shows that this burn model significantly induces nociceptive activation in DRG, and that pCREB is induced following this injury.

Furthermore, pCREB was not only present at neuronal nuclei but in the nuclei of satellite cells as well. This is in favour with the view that satellite cells play an active role during nociceptive processing (Ohara *et al.*, 2009), hence getting activated following an injury. Despite the interest in this fact, satellite cells were not the focus of this thesis and no further analysis were carried on them, although it could be an interesting topic for further studies.

The lack of pp38 induction within this model does not necessarily mean that it is not induced by the stimuli. It could also be due to limitations of the techniques used to detect it, as well as sensitivity of antibodies and other conditions of the experimental setup. Nonetheless, it is possible that neurons favour the induction of such stimuli throughout other pathways, involving different MAPK or others such as PKC, CaMK or PKA (Zou *et al.*, 2000; Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; White *et al.*, 2011a) (see Supplementary Figure 20.5). Notwithstanding, it was possible to detect the activation through pCREB because it is a common downstream effector in most of these pathways (Thomson *et al.*, 1999b; Ji *et al.*, 2003; Kawasaki *et al.*, 2004; Bie *et al.*, 2011; Li *et al.*, 2014).

The absence of the ATF3 could be explained because it requires longer times to get expressed. ATF3 is an IEG associated with neuronal regeneration (Linda *et al.*, 2011; Shields *et al.*, 2012). In models of axotomy and ventral root avulsion, its induction was not observed until 6 hours of the stimulation (Linda *et al.*, 2011). Shields *et al.* described ATF3 present in around 20% of DRG neurons one week after burn injury (Shields *et al.*, 2012). Thus, the last time point in this study was simply not late enough to detect an induction of ATF3.

12.5.7 pCREB induction following a burn injury co-expresses with Nav1.7 in DRG neurons

Following the induction of the scalding-type burn injury, ~80% of the neurons that expressed pCREB were also Nav1.7 positive. On the one hand, this finding further validates pCREB as a nociceptive marker. The majority of neurons where this marker was induced were Nav1.7 and thus nociceptors. On the other hand, this data shows the importance of this VGSC in the nociceptive processing following this scalding-type burn injury model.

The co-expression did not change through time (statistically similar at 5 minutes and 3-hours post-stimuli), which is in favour with a functional link between Nav1.7 and pCREB in these cells.

Hypothetically, different nociceptors depolarise following the induction of burn injury that, upon reaching a certain threshold, generate action potentials involving the activity of Nav1.7. Once action potentials reach the soma, different molecular transducers lead to up-regulation in pCREB expression. Then, the activation of CREB, together with other molecular events, regulates the cellular response as well as modulating its answer to future inputs.

12.5.8 Conclusion and future work

The scalding type burn injury model effectively induces a partial thickness, second degree, burn in the hind paw of Sprague-Dawley rats. It produces a substantial oedema, compromises the structure of the skin paw and affects ~10% TBSA, which validates its use on rats to assess burn injury as a tool to potentially translate the findings to humans.

This damage consequently induces the activation of pERK1/2 at superficial neurons of the DHSC and pCREB in DRG neurons. Both are dynamic markers of nociceptive processing that were only present following the noxious inputs. Hence, this burn injury model effectively activates the nociceptive pathway in a substantial way. pERK1/2 is activated as early as 5-minutes post injury and gradually returns to basal numbers after 3 hours of the noxious stimulation. pCREB is also rapidly induced at 5 minutes post-injury, although it drops at later time points and peaks again at 3 hours of the injury.

Nav1.7, a VGSC, is highly expressed at the soma of small- and middle- sized DRG neurons and at both branches of the unipolar axon. Interestingly, its expression gets up-regulated at 3-hours post-injury and the channel is highly present in the DRG neurons that show activation by this algogenic stimulus, as assessed by pCREB. Thus, Nav1.7 seems implicated in pain processing following burn injury. Furthermore, the antibody studied to assess such expression is a reliable source to perform future studies of this channel. The data presented here validated the use of this scalding burn injury model, and the implication of Nav1.7 in the afterwards nociceptive processing. This investigation sets the bases to study burn injury in rats with a novel method, and suggests that blocking Nav1.7 could be a feasible target for analgesic purposes, issues that are assessed in the following chapters.

13 Phosphorylated histone H3 at serine 10 identifies activated DRG and spinal neurons following a burn injury

13.1 Background

Burn related injuries are one of the main sources of accidental tissue damage and constitute a major health and economic burden globally (NBCRC, 2001; WHO, 2008b; Laycock *et al.*, 2013; Abdullahi *et al.*, 2014). Despite the advances in burn prevention and management, the pain associated with it is still not efficiently managed and remains an unmet medical need (Martyn, 1986; Ptacek *et al.*, 1995; Ulmer, 1997; Dauber *et al.*, 2002; Demling, 2008; WHO, 2008b; Wiechman Askay & Patterson, 2008; Laycock *et al.*, 2013; Sandoval *et al.*, 2014; ABA, 2015). Thus, a greater understanding of burn pain aetiology is required with the goal to ameliorate its treatment. Accordingly, animal models of burn injury are needed to reproduce this severe trauma and to test novel analgesics (Abdullahi *et al.*, 2014). In the previous chapter (Chapter 12), a scalding type burn injury model was validated for its use on Sprague-Dawley rats. This is a non-recovery model initially developed for C57BL/6 mice by White and collaborators (White *et al.*, 2011b). It consists on immersing one of the hind paws of the animal in 60°C water for a total of 2 minutes with different post-injury periods (White *et al.*, 2011b).

Burn injury induces the activation of nociceptors, the fibres specialised to sense noxious stimulation. If the activation reaches certain threshold, action potentials are generating and traveling through the fibres up to the soma of these nociceptors, which are collected together forming the DRG (Hunt & Mantyh, 2001; Julius & Basbaum, 2001). Here, cascades of second messenger are initiated leading to altered activation of the cells involved (Ji *et al.*, 2003; Costigan *et al.*, 2009; White *et al.*, 2011a). This modulation tunes up their response, a use-dependent process termed peripheral sensitization. Moreover, the centrally directed fibres also transport action potentials that end up synapsing with second order neurons situated at the

superficial DHSC (Hunt & Mantyh, 2001; Yoshimura & Furue, 2006; Todd, 2010). DHSC neurons are also subjected to use-dependent plasticity (Woolf *et al.*, 2004; Yoshimura & Furue, 2006; Costigan *et al.*, 2009), a process particularly important in these first synapses, because it forms the “gateway” of nociceptive information towards superior neuronal centres where the pain is interpreted (Torsney & MacDermott, 2006; Marchand, 2008; Todd, 2010; Woolf, 2011; Steeds, 2013). Thus, both DRG and DHSC neurons are regarded as important centres for nociceptive processing (Ji *et al.*, 1999; Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; Marchand, 2008; Hunt, 2009; Woolf, 2011; Steeds, 2013).

VGSC are responsible for the upstroke of the action potentials at nociceptors following an injury (Coutaux *et al.*, 2005; Dib-Hajj *et al.*, 2007; Rush *et al.*, 2007; Raouf *et al.*, 2010; Black *et al.*, 2012; Eijkelkamp *et al.*, 2012). From the 9 different VGSC expressed in mammals, Nav1.7 (together with Nav1.8 and Nav1.9) is mainly expressed within nociceptors. Due to its biophysical properties, Nav1.7 acts as a threshold channel for firing action potentials (Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Rush *et al.*, 2007; Dib-Hajj *et al.*, 2010; Raouf *et al.*, 2010; Black *et al.*, 2012; Hoeijmakers *et al.*, 2012; Dib-Hajj *et al.*, 2013; Zhang *et al.*, 2013). Interestingly, this channel is associated with both inflammatory and neuropathic pain (Cummins *et al.*, 2004; Nassar *et al.*, 2004; Yeomans *et al.*, 2005; Brochu *et al.*, 2006; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Hoyt *et al.*, 2007; McGowan *et al.*, 2009; Gingras *et al.*, 2014). Furthermore, studies in Nav1.7-cKO mice show that it is needed to develop heat hyperalgesia following burn injury (Black *et al.*, 2012; Hoeijmakers *et al.*, 2012; Shields *et al.*, 2012). These, together with the findings from the previous chapter (Chapter 12), suggest that Nav1.7 could be a feasible analgesic target for burn patients.

The phosphorylation of MAPK, such as pERK1/2 or pp38, is implicated with neuronal plasticity during processes such as inflammation and neuropathic pain (Ji & Woolf, 2001; Black *et al.*, 2008; White *et al.*, 2011a; White *et al.*, 2011b; Edelmayer *et al.*, 2014). They are dynamically activated in neurons (including DRG and DHSC) following different extracellular stimuli (Stambouliau *et al.*, 2010; Edelmayer *et al.*, 2014). Once activated, MAPK can alter the neuronal excitability by modifying the distribution and physical properties of different cellular components such as the VGSC and other receptors and by regulating gene expression, thus inducing/maintaining long-lasting functional modifications that contribute to persistent and chronic pain (Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; Kawasaki *et al.*, 2004).

The activations of those MAPK can also lead to phosphorylation of histone H3 at serine 10 (pS10H3), an epigenetic post-translational modification (PTM). Following external stimuli of quiescent cells, induction of pS10H3 is associated to transcriptional activation of a small set of genes, including IEG c-fos, c-jun and c-myc (Mahadevan *et al.*, 1991; Barratt *et al.*, 1994; Chadee *et al.*, 1999; Cheung *et al.*, 2000a; Cheung *et al.*, 2000b; Lo *et al.*, 2000; Nowak & Corces, 2000; Zhong *et al.*, 2000; Salvador *et al.*, 2001; Sacconi *et al.*, 2002; Davie, 2003; Prigent & Dimitrov, 2003; Dyson *et al.*, 2005; Dong & Bode, 2006). Interestingly, the same pathways also direct CREB phosphorylation, a mediator of gene expression (Xing *et al.*, 1996; Clayton *et al.*, 2000; Ji *et al.*, 2003; Kawasaki *et al.*, 2004; Bie *et al.*, 2011; Li *et al.*, 2014).

pS10H3 has also been identified in neurons of rodents following different paradigms such as contextual fear, exposure to stressor or drugs of abuse (Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008;

Sarantis *et al.*, 2012; Rotllant *et al.*, 2013; Crow *et al.*, 2015). In neurons, this induction seems to be an NMDA-dependent process (Mackowiak *et al.*, 2013) also associated to the induction of IEG c-fos and zif-268 (Bilang-Bleuel *et al.*, 2005; Chandramohan *et al.*, 2008; Sarantis *et al.*, 2012). Hence, pS10H3 is implicated in synaptic plasticity and memory consolidation. Due to the striking similarities between memory formation (LTP) and nociception (Ji *et al.*, 1999; Julius & Basbaum, 2001; Ji *et al.*, 2003; Marchand, 2008; Hunt, 2009; Denk & McMahon, 2012; Geranton, 2012; Rahn *et al.*, 2013; Steeds, 2013; Geranton & Tochiki, 2015b), it is possible to speculate that this PTM is also implicated on nociceptive processing.

13.2 Aims

The **aims** of this chapter are:

- To assess if pS10H3 is induced in spinal cord neurons following the induction of the scalding type burn injury model (White *et al.*, 2011b).
- To assess if pS10H3 is induced in DRG neurons following the induction of the scalding type burn injury model (White *et al.*, 2011b).

13.3 Material and Methods

Burn injury was also induced using the scalding type model (White *et al.*, 2011b) as explained above. Following this burn injury model, rats were maintained under anaesthesia for different post-injury periods ranging from 5 minutes (n=8), 30 minutes (n=4), one hour (n=4) to 3 hours (n=4). Additionally, two control groups were done in parallel: naïve (n=9), rats not subjected to any treatment and maintained in their cages until the end of the experiment; and sham controls (n=4), rodents that received similar treatment to the 5-minutes post-injury group but instead of immersing the hind paw into 60°C water it was at 37°C, a non-noxious stimulation.

After corresponding treatment, an overdose of sodium pentobarbital was injected intraperitoneally into the animal and then subjected to either neck dislocation, if samples intended for western blotting (n=8, 4 from 5-minutes post-injury and 4 naïve), or transcardial perfusion with PFA to fix tissues for posterior immunostaining (n=24).

To perform **western blot**, spinal dorsal horn from ipsi- and contra-lateral sides of corresponding L4-L5 spinal cords were harvested from controls (n=4) and animals from the 5-minutes post-injury group (n=4). Following, tissues were homogenized and total histone protein content isolated using the EpiQuick Total Histone Extraction Kit (Epigentek, OP-0006-100) as advised by the manufacturer. The resulting extracts were separated by electrophoresis using NuPAGE Novex 12% Bis-Tris Protein Gels (Invitrogen, NP0342BOX), electrotransferred to a PVDF membranes and immunolabelled. The immunoreactions were done as follow: initially reacted against pS10H3 (Santa Cruz, SC8656R, [1:500]) and β actin (Millipore, MAB1522, [1:2000]) and visualised with chemiluminescence, then the membrane was stripped and reacted again against pan-histone H3 (Millipore, 07-690, [1:4000]) and visualised

also with chemiluminescence. Finally, densitometry values were obtained, normalised, and used to assess the ratios for each animal (intensity in the ipsilateral side divided by the contralateral). Differences were regarded as significant using the independent *t*-test at $p < 0.05$. As further control, a duplicate gel was run in parallel, in which the order of immunoreactions was inverted: first reacted against pan-histone H3, secondly stripped and thirdly reacted for pS10H3 and β actin.

For **immunostaining** purposes, animals were overdosed with pentobarbital and, when no corneal reflex was observed, transcardially perfused with saline buffer and PFA. Following, L4-L5 DRG and corresponding spinal cord portions from both ipsi- and contra-lateral sides of each rat were harvested, post-fixed for an overnight and cryoprotected with 30% sucrose in PBS. Then, tissues were cut at a thickness of 10 μ m using a cryostat and mounted in slides for their immunological study.

Spinal cord sections from naive (n=4), sham (n=4), and 5 minutes (n=4), 30 minutes (n=4), one hour (n=4), and three hours post-burn (n=4) were immunoreacted against pS10H3 ([1:750]) and pERK1/2 (Neuromics, RA15002, [1:1500]) to evaluate their expression following this peripheral injury. Further, slides from animals subjected to the burn with a post-injury period of 5 minutes were stained for pS10H3 together with CGRP (Abcam, AB10987, [1:2000]), NeuN (Millipore, MAB377, [1:2000]), GFAP (Abcam, AB53554, [1:2000]), OX42 (ABDserotec, MCA275R, [1:1000]), IB4 (Molecular Probes, 121411, [1:1,000]) and Nav1.7 (Millipore, AB5390, [1:750]) in order to characterise the cells expressing this epigenetic mark. Also, animals from the 30 minutes post-injury period were co-stained for pS10H3 and c-Fos (Santa Cruz, SC52, [1:1000]) to assess co-expression. In all cases, immunoreaction was detected by normal immunofluorescence (using corresponding secondary antibodies

with a fluorescent tag), except pS10H3 that was further amplified by the TSA method or detected by DAB-Niquel.

Similar as for spinal cord, DRG sections corresponding to the different groups (n=4 for each) were immunoreacted against pS10H3 (as above) and Na_v1.7. Moreover, DRG sections from animals subjected to a post-burn period of 5 minutes were used to assess the co-expression between pS10H3 and pCREB (Cell Signaling, 9198, [1:5000]).

Following, slides were examined using a Leica microscope and microphotographs taken with a Hamamatsu Color Chilled 3CCD camera. Immunopositive cells were assessed by manually counting them at spinal cord sections and by assessing staining intensity with the software ImageJ (Instrument Company Ltd.) at DRG. Then, statistical differences were regarded as significant at $p < 0.05$, calculated using the GzLM. Additionally, corresponding negative and positive controls were done in parallel to validate the specificity of the procedures.

In order to analyse the sub-cellular distribution of pS10H3 in DRG neurons, two additional experiments were carried out. First, an in vitro heat stress model: DRG from a control rat were harvested, dissociated and plated. Once primary sensory neurons were established in the culture, a heat stress model was induced by incubating them in an atmosphere of 100% air for 10 minutes at 45°C and then transferred back to the incubator (37°C in an atmosphere of 95% air and 5% CO₂) for a post-stress period of 30 minutes. The corresponding control group consisted on maintaining half of the wells plated for this animal in the incubator. Following, cells were fixed by a 15 minutes immersion in PFA and immunostained against pS10H3 and Na_v1.7 as stated above. In the second experiment, DRG sections were processed for Transmission Electronic Microscopy (TEM) and stained against

pS10H3: an animal subjected to the burn and a post-injury period of 5 minutes was transcardially perfused with Somogyi's Fixative solution. Then, DRG were harvested, stored in 0.1 M PB with 0.01% sodium azide and shipped to Dr. Akos Kulik's lab in Freiburg, Germany, where were processed for TEM as described before (Kulik *et al.*, 2002).

13.4 Results

Within this chapter, studies on the expression of pS10H3 by western blotting and immunostaining before and after the induction of the scalding-type burn injury model are described (White *et al.*, 2011b). First, studying the changes of pS10H3 observed in the spinal dorsal horn and characterising these positive population of cells, and second, looking at its expression pattern and distribution in DRG sections.

13.4.1 Immunoblotting anti-pS10H3 of histone extracts from DHSC after induction of the burn injury model

Histone extracts from ipsi- and contra-lateral DHSC of control and after 5 minutes survival were run for western blotting, immunoreacted against pS10H3 and the obtained densitometry ratios statistically assessed in order to check for a differential expression of this histone H3 PTM.

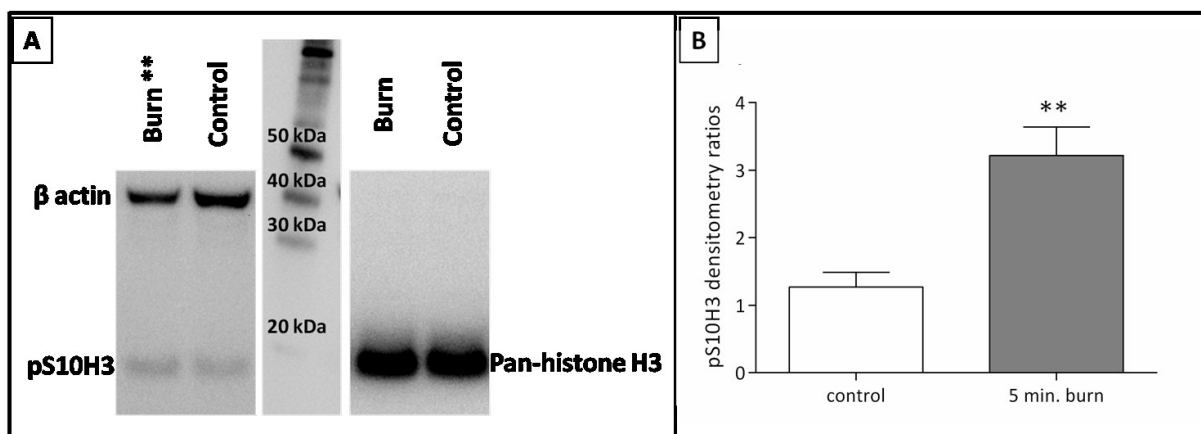


Figure 13.1. **Increase of pS10H3 in DHSC after burn injury shown by western blotting.** Four animals from 5 minutes group (60°C water for 2 minutes and 5 minutes post-injury period) and four control rats were used **A)** Representative western blot of histone H3 extracts from ipsi- and contra-lateral DHSC after burn injury. First, membrane was stained for pS10H3 (left), stripped and then reacted for pan-histone H3 (right). **B)** Western blot quantification of pS10H3 in control and burn groups. Data is presented as mean±SEM. T test was performed with normalized densitometry ratios. ** p<0.01

A band at ~15 KDa mass was detected corresponding to pS10H3 (Figure 13.1). The statistical analysis revealed a significant up-regulation of pS10H3 in the ipsilateral

DHSC of the animals injured that was not present in naïve controls (burn: 3.22 ± 0.42 , $n=4$, control: 1.27 ± 0.22 , $n=4$; $p=0.006$, Student's t-test; Figure 13.1 B).

13.4.2 Characterising pS10H3 staining in the spinal cord after burn injury

Next, spinal cord sections were immunostained against pS10H3 to confirm these findings. A population of pS10H3 positive cells was detected in the ipsilateral DHSC after induction of the burn injury (Figure 13.2). Studies of co-expression with calcitonin gene-related peptide (CGRP), marker of peptidergic afferent fibres (Ju *et al.*, 1987), and isolectin B4 (IB4), for non-peptidergic c-fibres (Wang *et al.*, 1994), showed that these positive cells are mainly localised in the superficial laminae of the ipsilateral DHSC, together with CGRP (laminae I and II) and not with IB4 (lamina III) (Todd, 2010), Figure 13.3).

The pS10H3 positive signal was only found co-expressing with hexaribonucleotide-binding protein-3 (NeuN), a marker of neuronal nuclei (Mullen *et al.*, 1992). No pS10H3 expressing structures were found surrounded with structures expressing either glial fibrillary acidic protein (GFAP) or complement receptor 3 (CD11b, also known as OX42), markers specific for astrocytes or microglia respectively (Robinson *et al.*, 1986; Reeves *et al.*, 1989; Shaw *et al.*, 1990; Eng *et al.*, 2000) (Figure 13.4). Confocal microscopy revealed that pS10H3 staining was also positive for the dye 4',6-diamidino-2-phenylindole (DAPI), a marker that attaches to DNA regions (Kapuscinski, 1995) (Figure 13.5). And these positive cells were in close proximity to Nav1.7 fibres (see Chapter 12 and Figure 13.5). For all above, pS10H3 staining was regarded as positive for neuronal nuclei of, mostly, second order neurons.

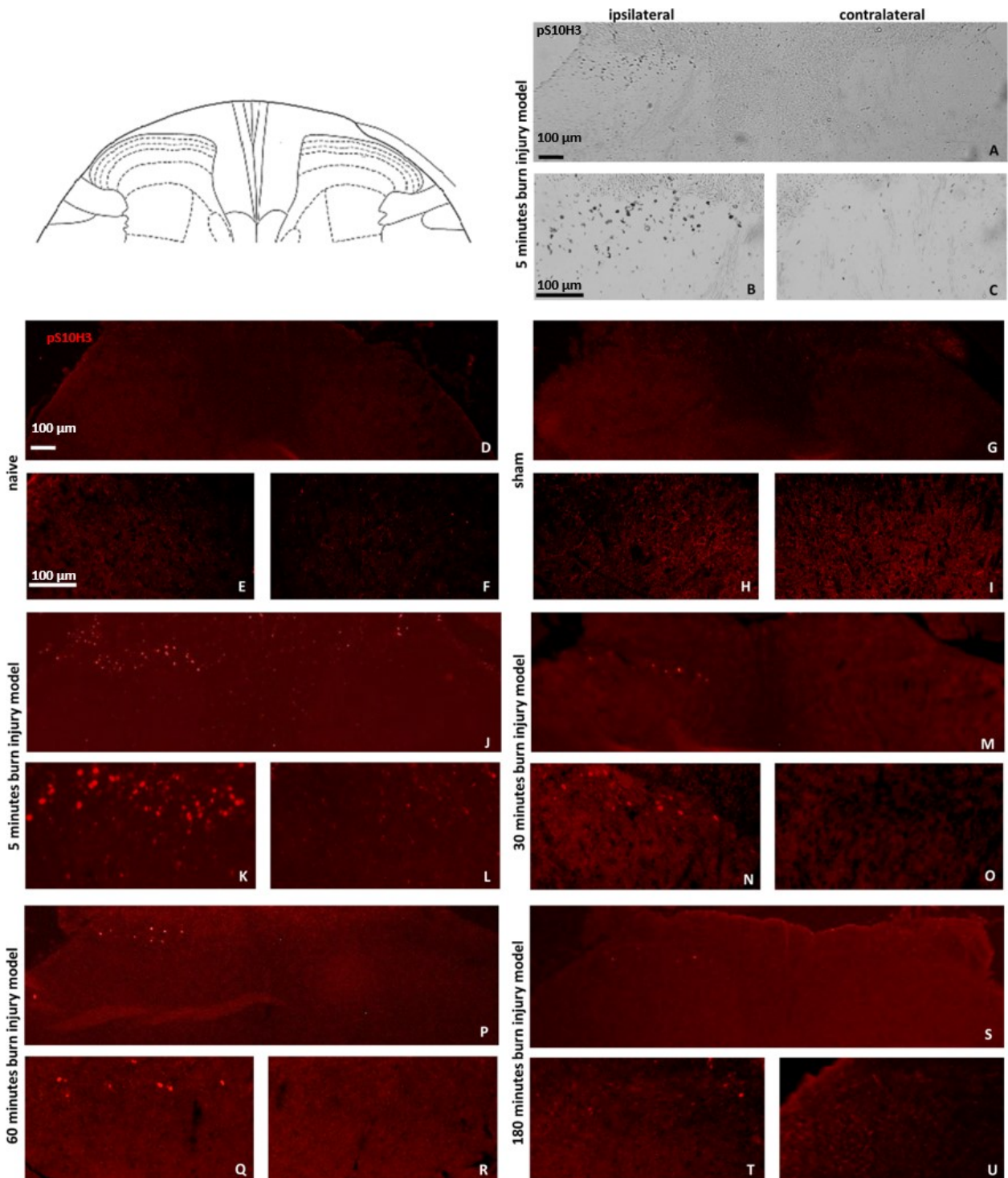


Figure 13.2. **Time course of pS10H3 induction in DHSC after burn injury model.** In all pictures red is for pS10H3. The experimental groups (n=4) were naive animals (naive), 37°C water for 2 minutes and 5 minutes post-injury period (sham), 60°C water for 2 minutes and 5 minutes post-injury period (5 mins), 60°C water for 2 minutes and 30 minutes post-injury period (30 mins), 60°C water for 2 minutes and 60 minutes post-injury period (60 mins) and 60°C water for 2 minutes and 180 minutes post-injury period (180 mins). The scale bar equals 100μm. Pictures A, D, G, J, M, P and S were taken at the same magnification (5x) and pictures B, C, E, F, H, I, K, L, N, O, G, R, T and U were taken at a higher magnification (20x). Pictures A, B and C were processed with DAB-niquel. The drawing of the DHSC is adapted from www.christopherreeve.org.

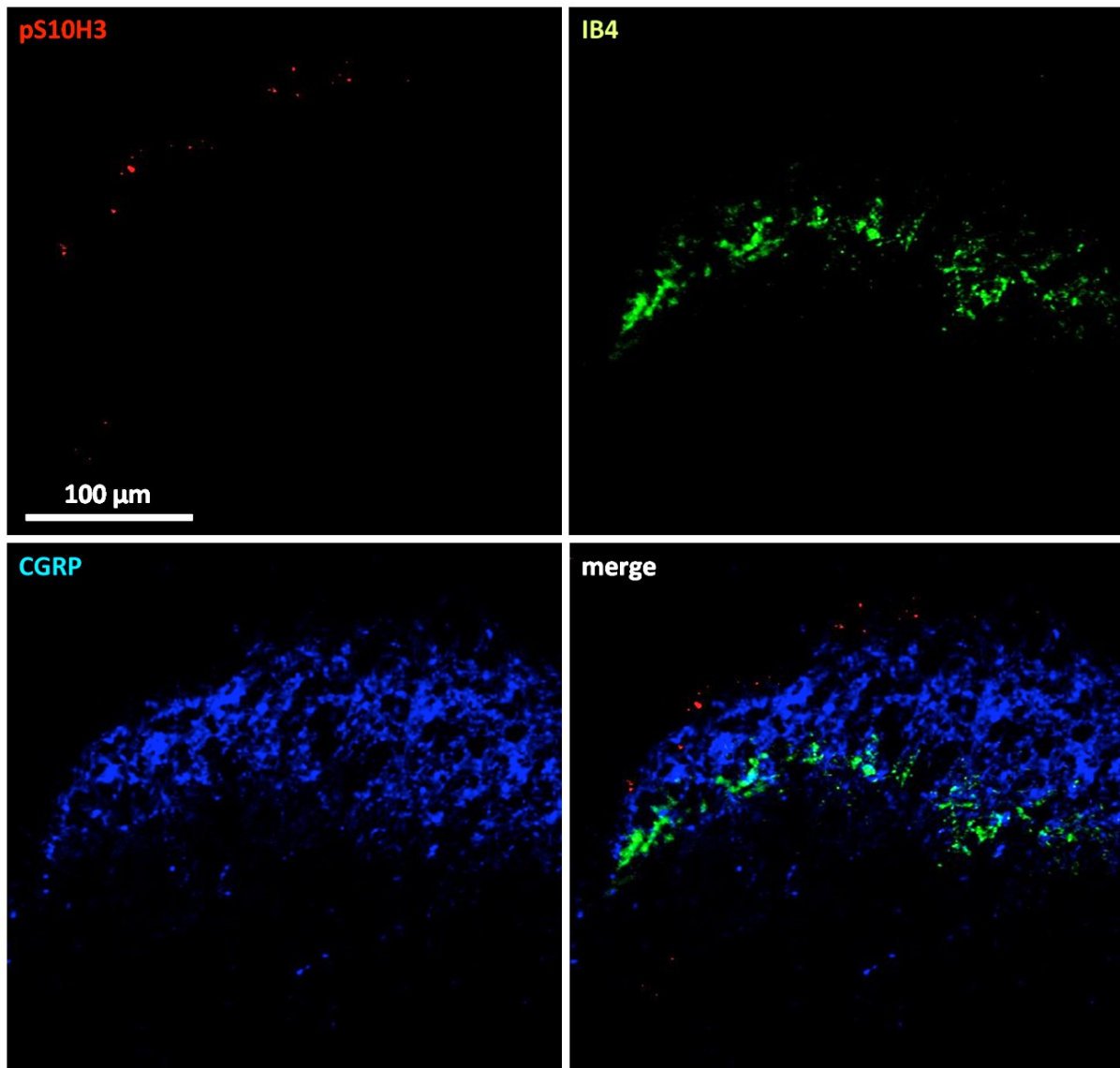


Figure 13.3. **pS10H3 distribution in DHSC after burn injury model.** Sections of spinal cord were labelled with pS10H3 (red), IB4 (green) and CGRP (blue). CGRP labelling is present in lamina I and Lamina II while IB4 is mainly restricted to lamina III. pS10H3+ neurons are mainly present at superficial laminae, co-localizing with CGRP. Scale bar represents 100μm.

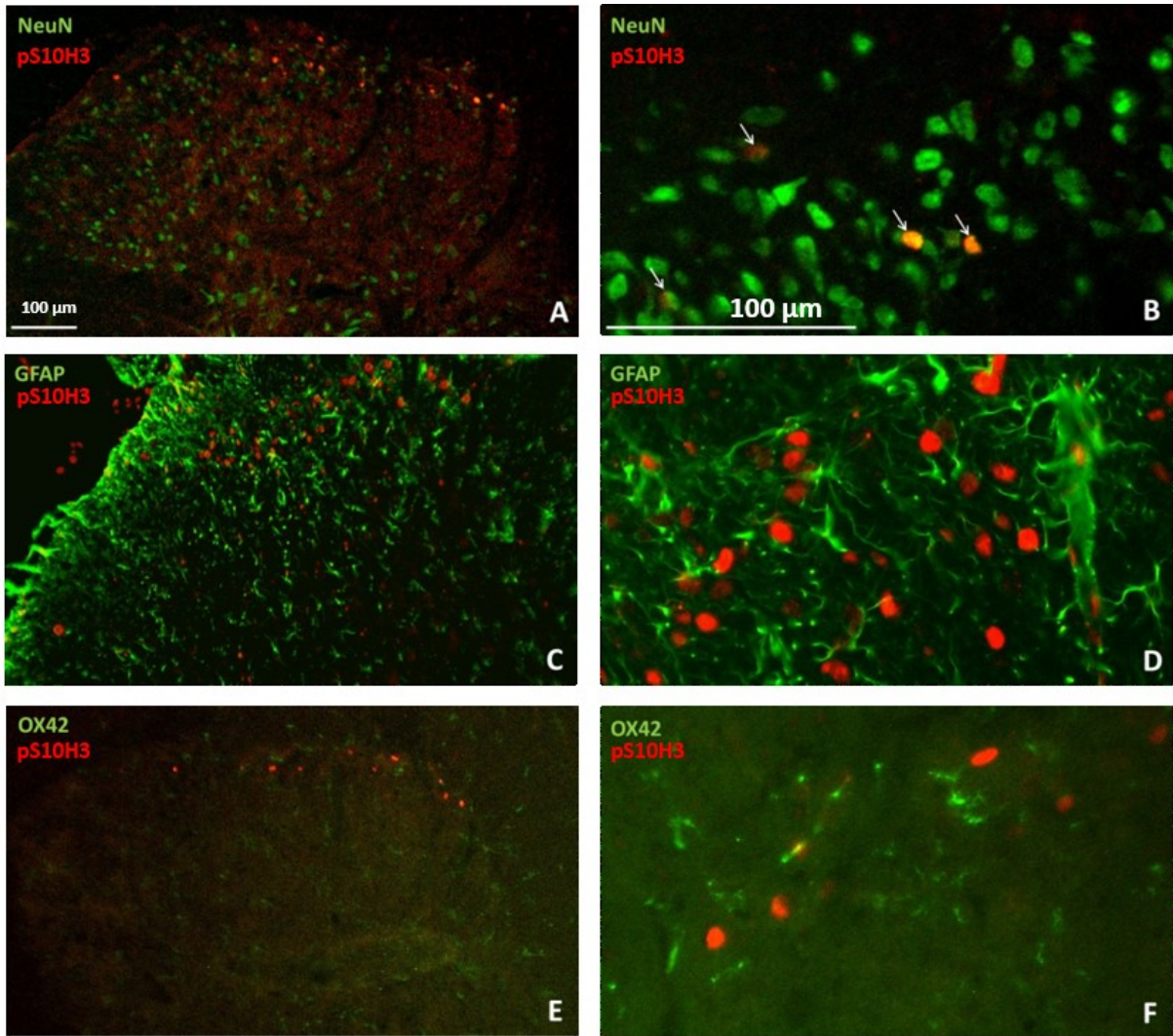


Figure 13.4. **Characterization of immunolabelled pS10H3 cells in the ipsilateral DHSC after burn injury model.** Animals from the 5' group (60°C water for 2 minutes and 5 minutes post-injury period) were used to study the characterization. In all pictures, the red is for pS10H3. The scale bars equal 100μm. Pictures A, C and E were taken at the same magnification (10x) and pictures B, D and F were taken at a higher magnification (40x). Arrows indicate cells where both markers co-localize. Note that pS10H3 only co-localize with NeuN (100%).

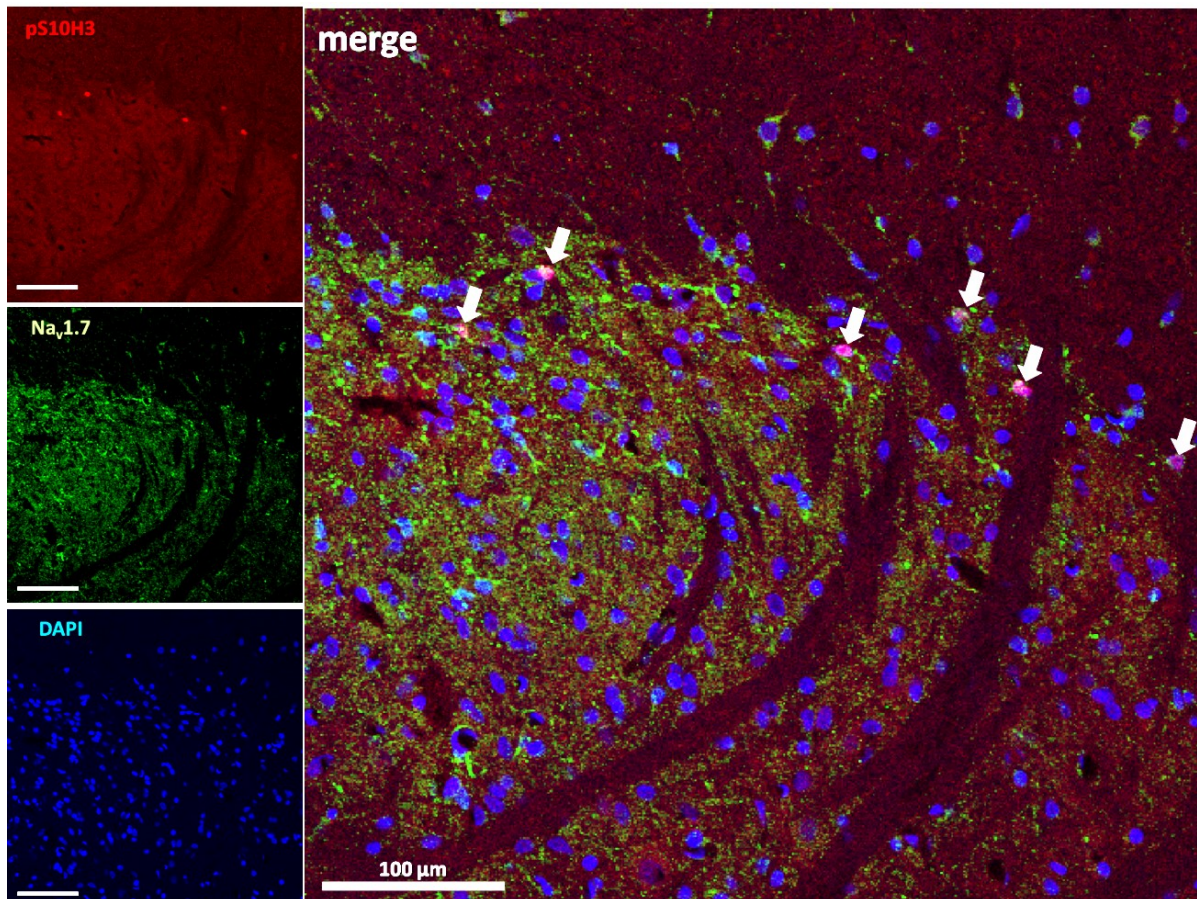


Figure 13.5. **Sub-cellular distribution of pS10H3 in the ipsilateral DHSC after burn injury and its relationship with Na_v1.7 fibres.** DHSC of animals from the 5' group (60°C water for 2 minutes and 5 minutes post-injury period) were stained for pS10H3 (red), Na_v1.7 (green) and nuclei (DAPI; in blue). The scale bars equal 100μm. Pictures were taken with confocal microscopy. The diagonal arrows indicate pS10H3+ neurons with Na_v1.7+ fibres from peripheral origin close to them. Note that in all occasions, pS10H3 is only present together with DAPI.

Statistical analysis of the total number of neurons counted in each set of sections (Figure 13.6) showed an increase of pS10H3 positive neurons in the ipsilateral DHSC after 5 minutes post-injury (5 min: 44.25 ± 13.33 , Mdn= 48.50, n=4), that significantly different ($p < 0.001$, GzLM) from the numbers observed in the ipsilateral DHSC of naïve animals (naïve: 2.50 ± 1.04 , Mdn=29.50, n=4). This increase in pS10H3 positive cells was also found at 30-minutes post-injury (30 min: 42.00 ± 3.08 , Mdn=42.50, n=4, $p < 0.001$, GzLM) and up to 60 minutes (1 hour: 27.25 ± 3.52 , Mdn=29.50, n=4, $p = 0.003$, GzLM), although a significant reduction of ~30% was observed in the latest ($p = 0.001$, GzLM) respect the number at 30 minutes. The up-

regulation of pS10H3 positive neurons returned to baseline numbers at 3-hours post-injury (3 hours: 11.40 ± 3.19 , Mdn=14.00, $n=4$, $p=0.254$, GzLM). Similarly, the increase was not observed in the ipsilateral side of sham-injured controls (sham: 1.75 ± 0.63 , Mdn= 2.00, $n=4$, $p=0.927$, GzLM) or in any of the contralateral DHSC studied (naïve: 1.50 ± 1.50 , Mdn=0.00, $n=4$, $p=0.103$; sham: 0.25 ± 0.25 , Mdn=0.00, $n=4$, $p=0.784$; 5 min: 3.00 ± 3.00 , Mdn=0.00, $n=4$, $p=0.952$; 30 min: 0.00 ± 0.00 , Mdn=0.00, $n=4$, $p=0.761$; 1 hour: 2.50 ± 0.87 , Mdn=2.5, $n=4$, $p=1$; 3 hours: 0.60 ± 0.60 , Mdn=0.00, $n=4$, $p=0.808$; GzLM).

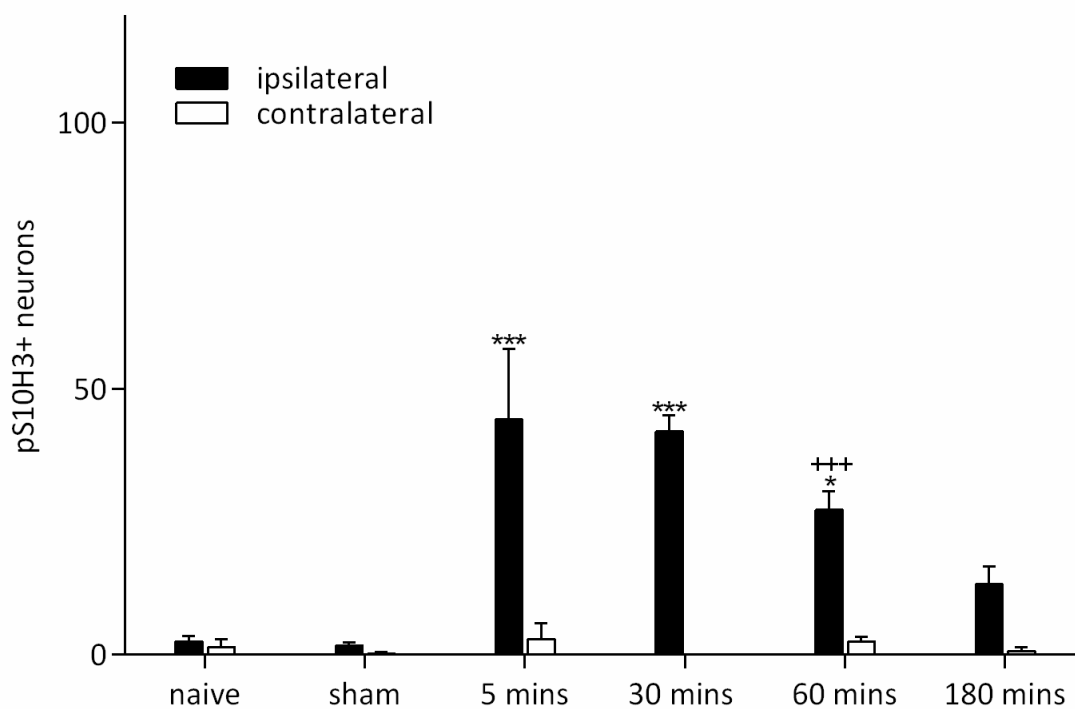


Figure 13.6. **immunolabelled pS10H3 neurons in DHSC of the burn-injury model.** The data is presented as mean±SEM. The experimental groups ($n=4$) are naive animals (naive), 37°C water for 2 minutes and 5 minutes post-injury period (sham), 60°C water for 2 minutes and 5 minutes post-injury period (5 mins), 60°C water for 2 minutes and 30 minutes post-injury period (30 mins), 60°C water for 2 minutes and 60 minutes post-injury period (60 mins) and 60°C water for 2 minutes and 180 minutes post-injury period (180 mins). * $p<0.05$ vs. naive ipsilateral; *** $p<0.001$ vs. naive ipsilateral, +++ $p<0.001$ vs. 30 mins ipsilateral.

13.4.3 pS10H3 co-expression with pERK1/2 and c-Fos in the spinal cord after induction by the peripheral burn injury model

As described previously, burn injury also increases the expression of pERK1/2 in neurons from DHSC at 5 minutes after a thermal injury (see above and (Ji *et al.*, 1999; Ji *et al.*, 2003)) and is an upstream event leading to pS10H3 (Zhong *et al.*, 2000). Furthermore, c-Fos expression (the protein product of the IEG *fos*) is also up-regulated after a nociceptive activation and pS10H3 has been associated with the activation of *fos* in neurons (Gutierrez-Mecinas *et al.*, 2011). Hence, the co-expression of pS10H3 with pERK1/2 and c-Fos after the induction of burn injury was studied (Figure 13.7).

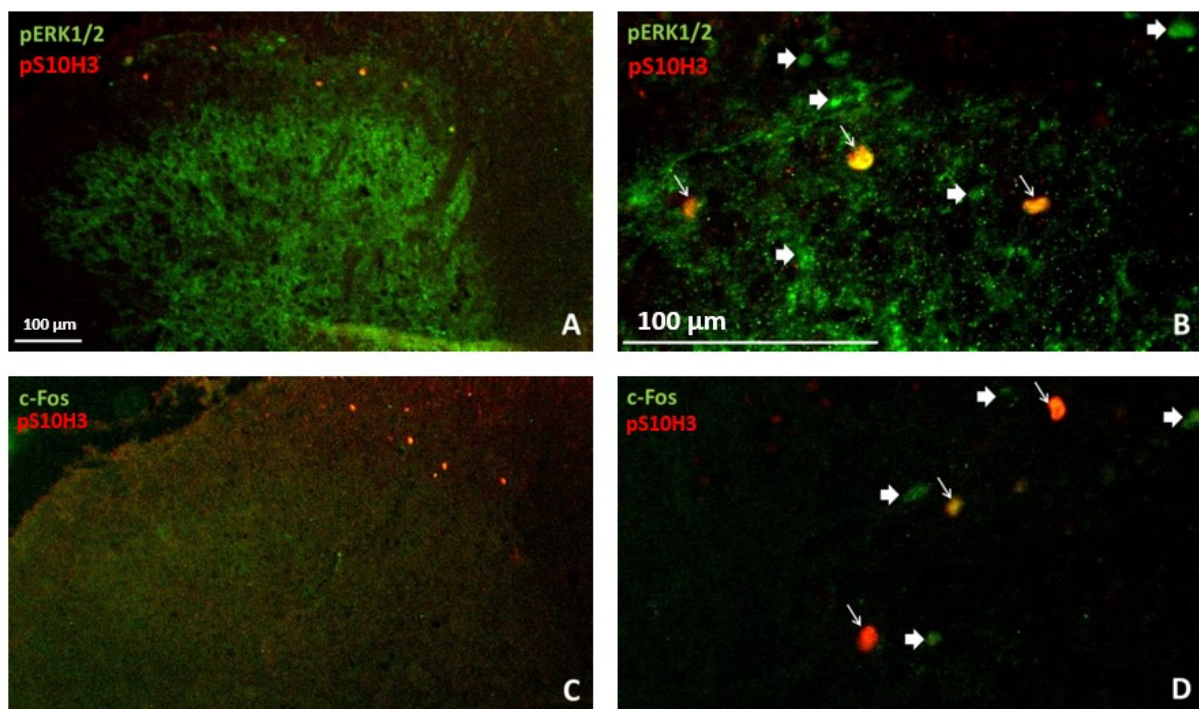


Figure 13.7. **Co-localization of pS10H3 with pERK1/2 and c-Fos in the ipsilateral DHSC after burn injury model.** Animals from the 5' group (60°C water for 2 minutes and 5 minutes post-injury period) were used to study the co-localization with pERK1/2 and from the 30' group (60°C water for 2 minutes and 30 minutes post-injury period) for the co-localization with c-Fos. In all images, red is for pS10H3 and scale bars equal 100µm. Pictures A and C were taken at the same magnification (10x) and pictures B and D were taken at a higher magnification (40x). Diagonal fine arrows indicate neurons where both markers co-localize and the right arrows indicate the cells positive for the other marker but not for pS10H3.

Five minutes following burn injury, $84.74 \pm 1.85\%$ ($n=4$) of the neurons positive for pERK1/2 were also immunopositive for pS10H3 and $57 \pm 8.12\%$ ($n=4$) of pS10H3 immunopositive neurons were also positive for pERK1/2. The study of spinal cord sections after 30 minutes post-injury showed that $66.98 \pm 14.54\%$ ($n=4$) of cells immunopositive for c-Fos had labelled nuclei for pS10H3 whereas $72.2 \pm 7.73\%$ ($n=4$) of neurons pS10H3 positive exhibited c-Fos immunopositive staining.

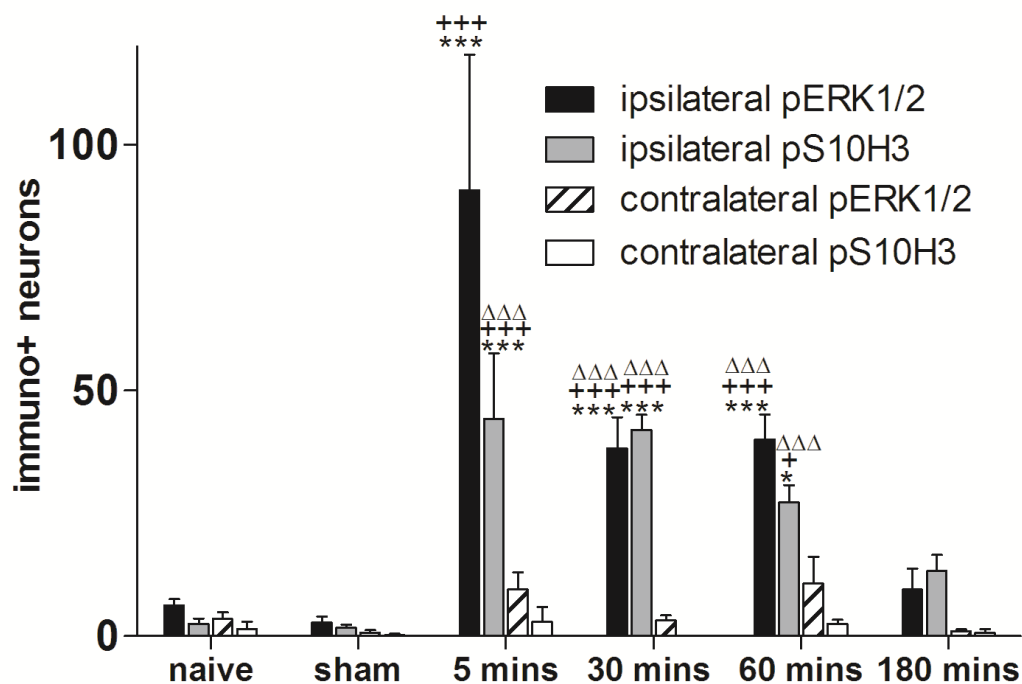


Figure 13.8. **immunolabelled pERK1/2 and pS10H3 neurons in DHSC of the burn-injury model.** Data is presented as mean $\pm$ SEM. The experimental groups ($n=4$ each) were naive animals (naive), 37°C water for 2 minutes and 5 minutes post-injury period (sham), 60°C water for 2 minutes and 5 minutes post-injury period (5 mins), 60°C water for 2 minutes and 30 minutes post-injury period (30 mins), 60°C water for 2 minutes and 60 minutes post-injury period (60 mins) and 60°C water for 2 minutes and 180 minutes post-injury period (180 mins). * $p < 0.05$ vs. naive ipsilateral pERK1/2; *** $p < 0.001$ vs. naive ipsilateral pERK1/2; + $p < 0.05$ vs. naive ipsilateral pS10H3; +++ $p < 0.001$ vs. naive ipsilateral pS10H3; ΔΔΔ $p < 0.001$ vs. 5' ipsilateral pERK1/2.

Due to the high co-expression between pERK1/2 and pS10H3, it was decided to study the up-regulation pattern of pERK1/2 in comparison with the increase in pS10H3 (Figure 13.6) throughout the burn injury model. The up-regulation observed using both pERK1/2 and pS10H3 in the ipsilateral DHSC was significant within 5, 30

and 60-minutes post-injury periods (see Figure 13.8 and further above). The increase in pERK1/2 at 5 minutes post-injury was significantly higher than the up-regulation observed with pS10H3 ($p < 0.001$, GzLM). No other significant differences were observed within both patterns (p -values not shown).

13.4.4 Expression pattern of pS10H3 in L4-5 DRG after induction of the burn injury model

After assessing the expression of pS10H3 in spinal cord following the burn-injury model, it was decided to check its presence and/or expression in the DRG.

L4 and L5 DRG section of all end points studied in the burn injury model were immunolabelled against pS10H3 (Figure 13.9). The statistical analysis of the percentage of pS10H3 positive nuclei revealed that there was a significant up-regulation in the ipsilateral DRG as early as 5 minutes post-injury (5 min: 9.70 ± 0.95 , Mdn=10.47, $n=4$; naïve: 0.00 ± 0.00 , Mdn=0.00, $n=4$; $p < 0.001$, GZLM) that was maintained until after 3 hours, the last time point studied (30 min: 9.03 ± 2.13 , Mdn=9.06, $n=4$, $p < 0.001$; 1 hour: 9.12 ± 2.62 , Mdn=11.07, $n=4$, $p < 0.001$; 3 hours: 7.53 ± 2.24 , Mdn=8.39, $n=4$, $p < 0.001$; GzLM; Figure 13.10). Further, no significant difference was observed in the ipsilateral DRG of the sham controls (sham: 2.07 ± 0.29 , Mdn=1.94, $n=4$, $p=0.243$, GzLM) and in the contralateral DRG at any time point post-injury studied (naïve: 0.00 ± 0.00 , Mdn=0.00, $n=4$, $p=1$; sham: 0.88 ± 0.30 , Mdn=1.14, $n=4$, $p=0.617$; 5 min: 1.91 ± 1.01 , Mdn=1.5, $n=4$, $p=0.280$; 30 min: 1.34 ± 1.34 , Mdn=0.00, $n=4$, $p=0.449$; 1 hour: 3.23 ± 2.14 , Mdn=2.16, $n=4$, $p=0.701$; GzLM; Figure 13.10). Interestingly, most neurons describing positive nuclear staining for pS10H3 were small-sized although few larger cells also show this epigenetic modification (Figure 13.11).

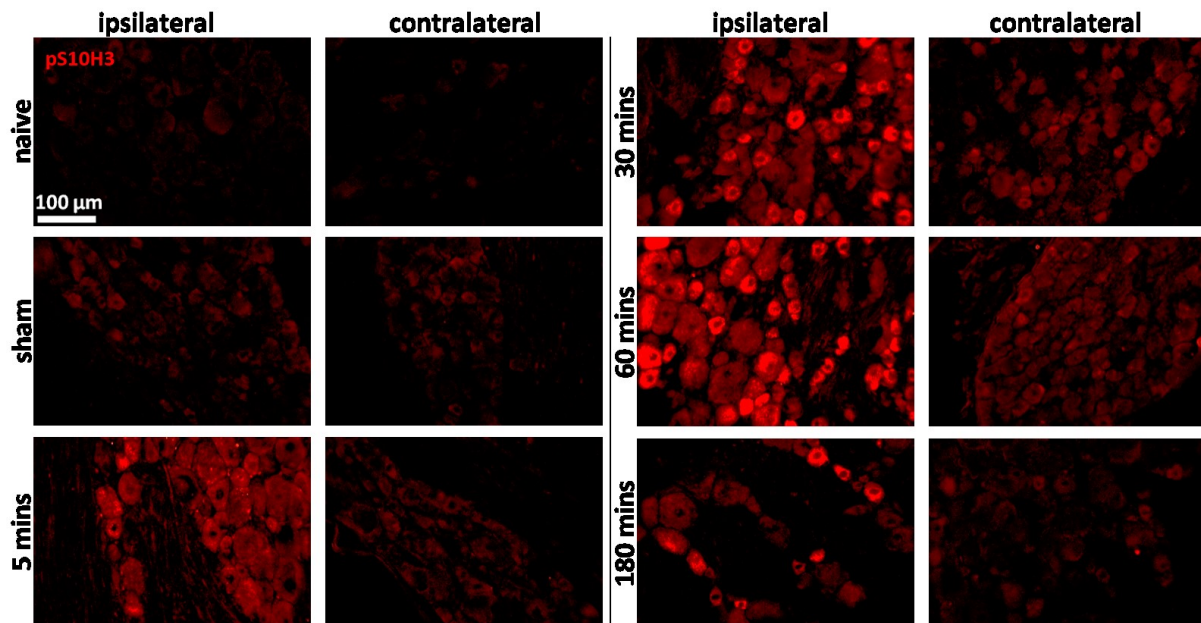


Figure 13.9. **Time course of pS10H3 induction in L4 and L5 DRG after burn injury model.** In all pictures red is for pS10H3. The experimental groups (n=4) were naive animals (naive), 37°C water for 2 minutes and 5 minutes post-injury period (sham), 60°C water for 2 minutes and 5 minutes post-injury period (5 mins), 60°C water for 2 minutes and 30 minutes post-injury period (30 mins), 60°C water for 2 minutes and 60 minutes post-injury period (60 mins) and 60°C water for 2 minutes and 180 minutes post-injury period (180 mins). Scale bar equals 100μm.

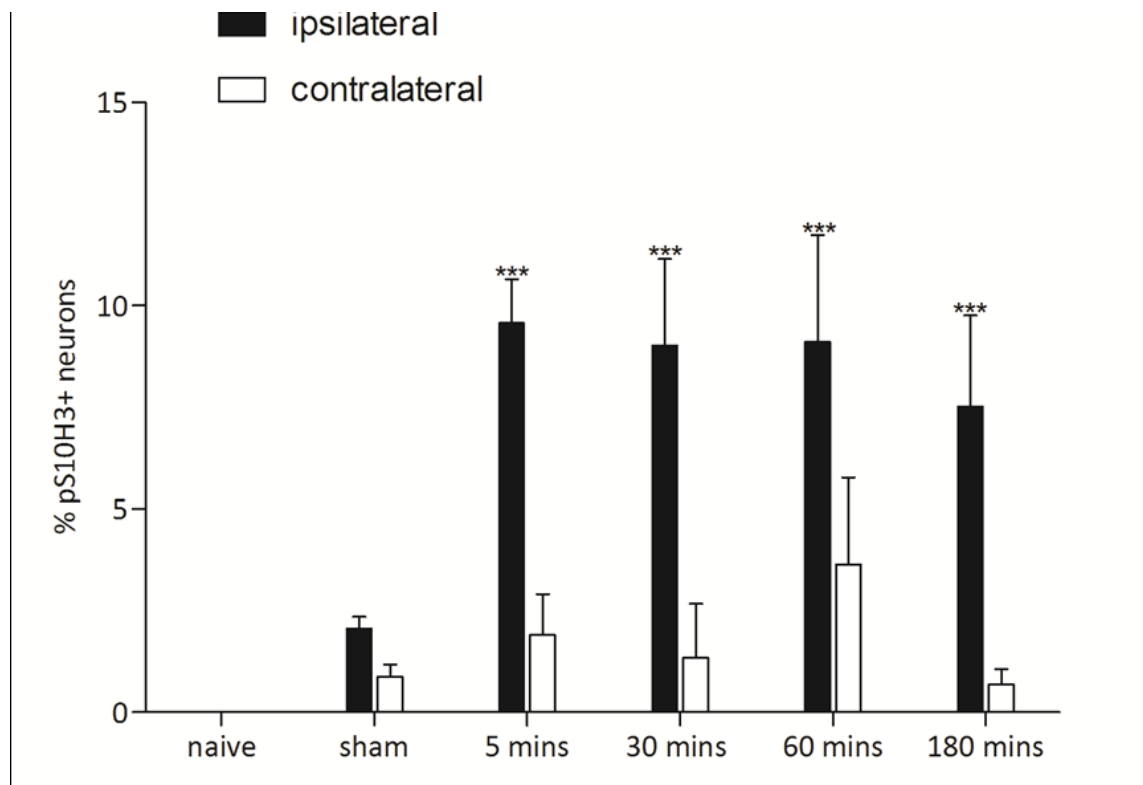


Figure 13.10. **Percentage of pS10H3 nuclear staining in DRG of the burn-injury model.** Data is presented as mean±SEM. The experimental groups (n=4) are naive animals (naive), 37°C water for 2 minutes and 5 minutes post-injury period (sham), 60°C water for 2 minutes and 5 minutes post-injury period (5 mins), 60°C water for 2 minutes and 30 minutes post-injury period (30 mins), 60°C water for 2 minutes and 60 minutes post-injury period (60 mins) and 60°C water for 2 minutes and 180 minutes post-injury period (180 mins). *** p<0.001 vs. naive ipsilateral pS10H3.

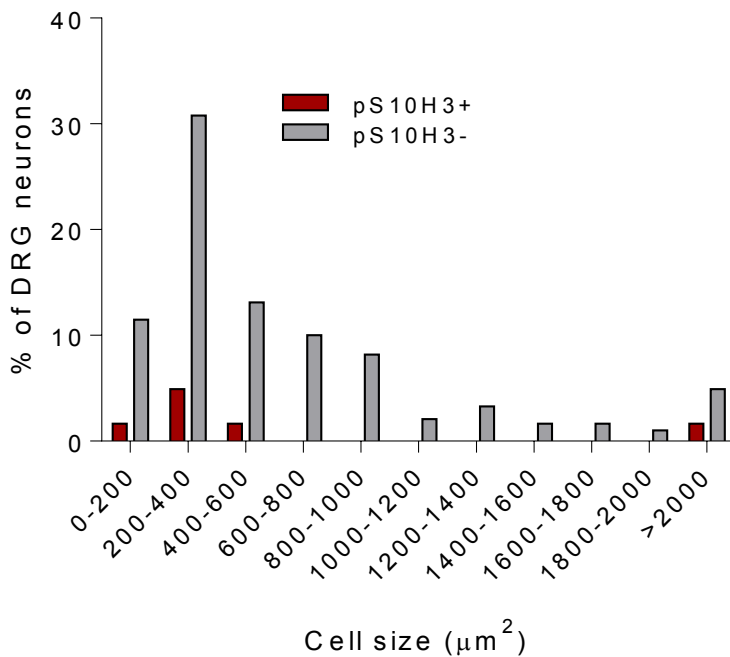


Figure 13.11. **Cell size distribution of pS10H3 positive neurons in DRG following the burn-injury model.** Data obtained from ipsilateral DRG from 5 minutes after burn injury. The majority of DRG neurons positive for pS10H3 are small-sized except few larger cells.

13.4.5 Sub-cellular distribution of pS10H3 in DRG

Histone H3 is a nuclear protein and, as stated previously (see above in this chapter), pS10H3 immunopositive staining in the spinal cord section was also positive for DAPI. Hence, the subcellular pattern of pS10H3 in the DRG was assessed.

At 5 minutes following induction of the burn-injury, pS10H3 positive staining was present in co-expression with DAPI but no restricted to that pattern (Figure 13.12). However, this subcellular distribution pattern was not present within cultured primary sensory neurons following noxious heat-stress stimulation (Figure 13.13). There, pS10H3 positive staining was not present in the control cultures but some immunopositive labelling was observed after the stimulation, which was exclusively localised in the nuclear compartment. These findings, together with electron microscopy of sections cut from ipsilateral DRGs of burn-injured animals which

showed that labelling outside the nuclei was not consistently associated to any structure in the cytoplasm (see Supplementary Figure 20.2), prompted us to restrict our studies to the nuclear staining.

For validation purposes, the statistical analysis of DRG sections after induction of the burn injury model was done with both the percentage of cells with positive nuclear staining (see analysis in the section above, Figure 13.10) and the percentage of total positively stained cells (see Supplementary Figure 20.3). In both occasions, a significant increase in the percentage of pS10H3 positive cells was observed in the ipsilateral DRG after the burn injury induction.

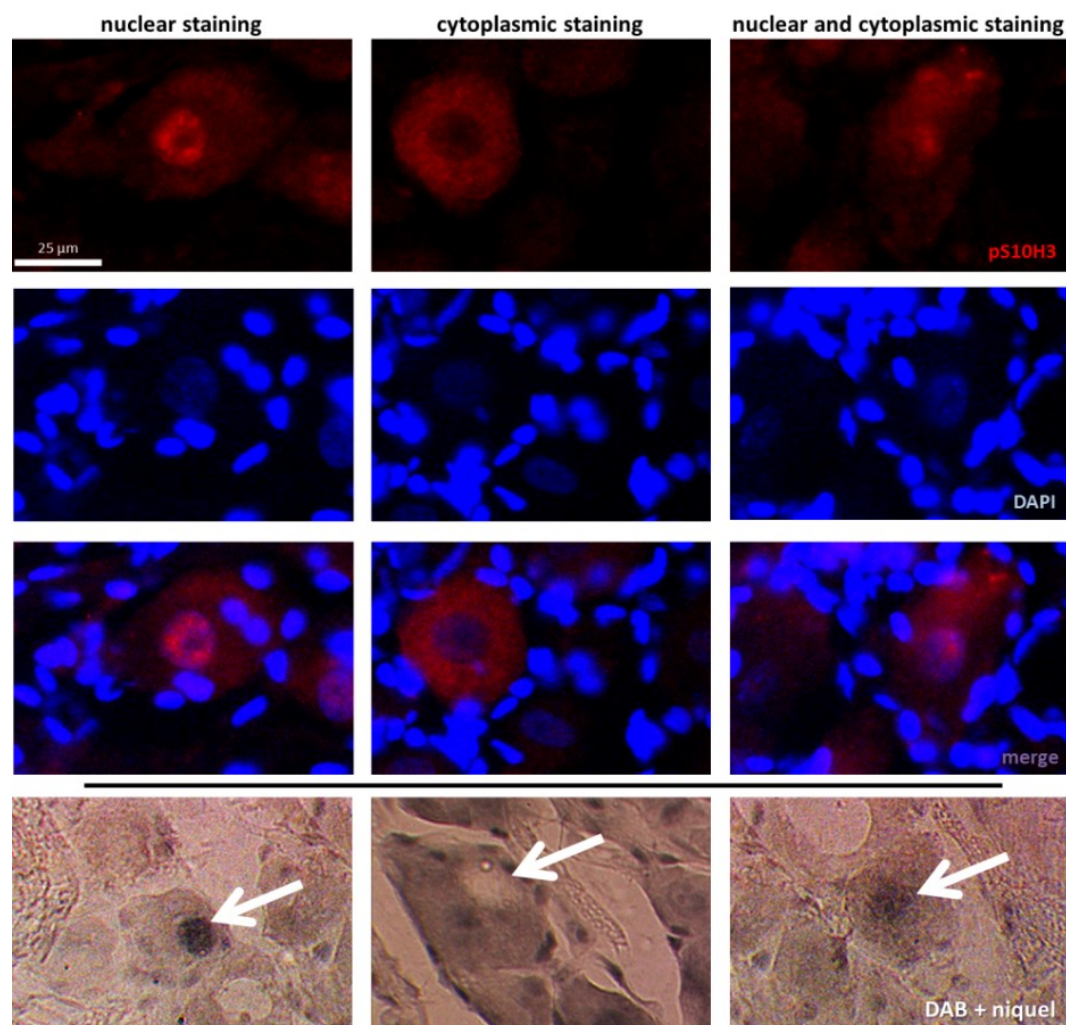


Figure 13.12. **Different patterns of pS10H3 staining at subcellular level.** Animals from the 5' group (60°C water for 2 minutes and 5 minutes post-injury period). Both techniques IF and IHC (white arrows pointing at the nucleus) were performed to stain pS10H3. Each column represents a different pattern: a) nuclear staining, b) cytoplasmic staining and c) both nuclear and cytoplasmic staining. The scale bar equals 25μm.

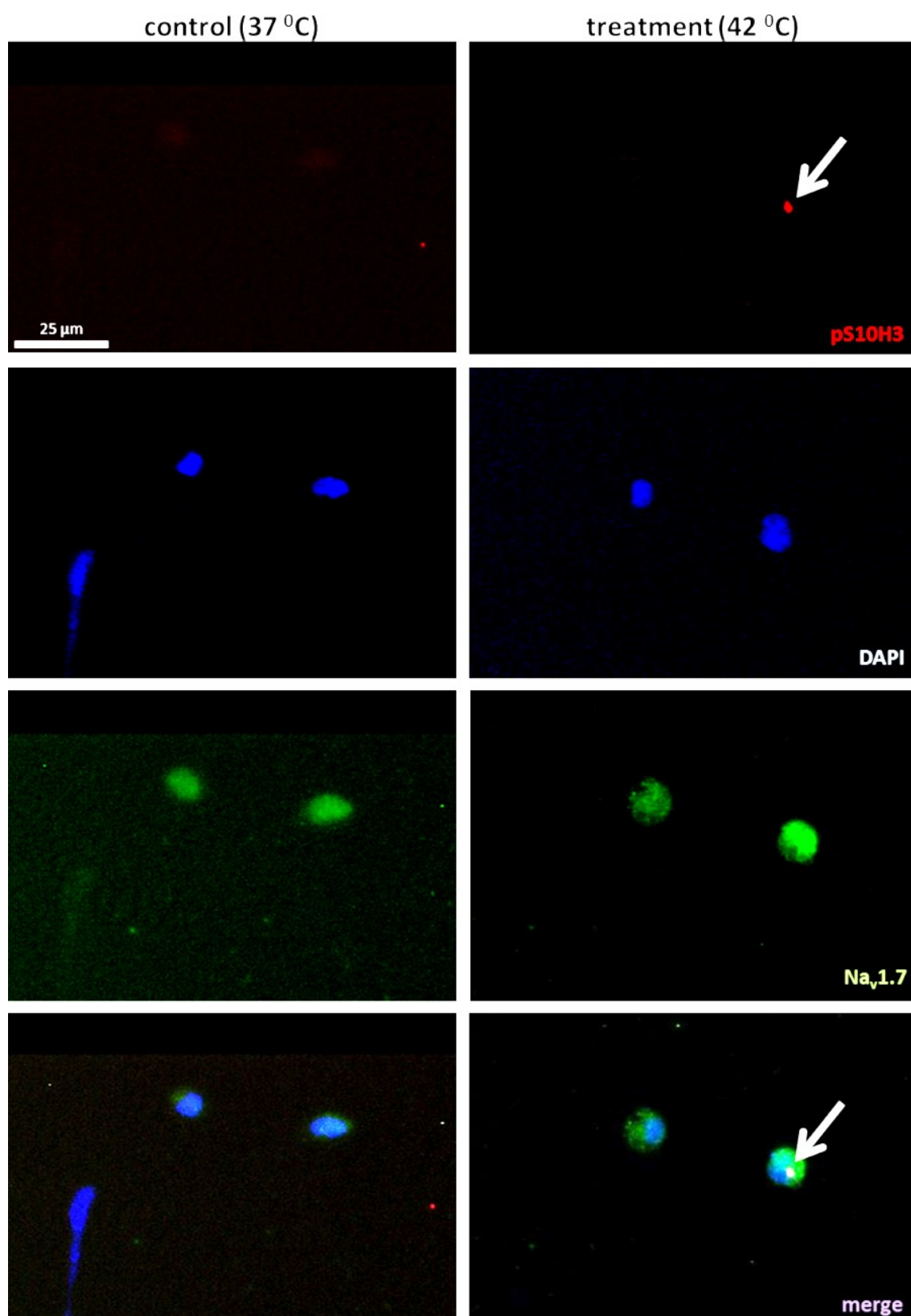


Figure 13.13. **pS10H3 staining is localized at nuclear compartment after the *in vitro* noxious heat stress model.** The treatment consisted in 10 minutes incubation at 42°C and a recovery period of 30 minutes. The scale bar equals 25μm. Note that pS10H3 expression (white arrow) also co-localizes with Na_v1.7 positive cells.

13.4.6 pCREB co-expression with pS10H3-immunopositive nuclei in the DRG following burn injury

Ipsilateral DRG sections from 5 minutes survival period after induction of burn injury were further assessed for both pCREB and pS10H3 positive immunostaining.

Around 50% (52.56 ± 13.51 , $n=4$) cells with nuclei immunopositive for pCREB also expressed immunolabelling positive for pS10H3 in the nuclear compartment whereas ~85% (86.67 ± 8.16 , $n=4$) of pS10H3 positive nuclei were also pCREB positives (Figure 13.14). However, while pS10H3 was only apparent in cells that visually described as neurons, pCREB was detected in both neurons and satellite cells within the DRG (Figure 13.14).

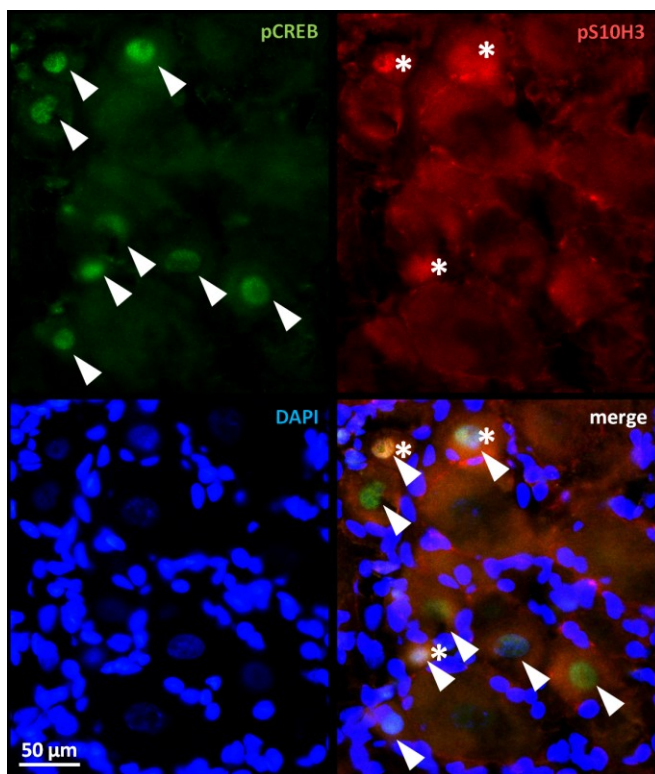


Figure 13.14. **pS10H3 and pCREB co-expression in the ipsilateral DRG burn-injury model.** DRG of animals from the 5 minutes group (60°C water for 2 minutes and 5 minutes post-injury period) were stained for pS10H3 (red) and pCREB (green). Asterisk indicate neurons with positive nuclei for pS10H3 and arrowheads point neurons with nuclei positive for pCREB. The scale bar equals 100μm.

13.4.7 Co-expression of pS10H3 positive neurons with Nav1.7 in the DRG after induction of the burn injury model

As mentioned earlier, Nav1.7 has an important role in the development of heat hypersensitivity after burn injury (see Chapter 12 and (Shields *et al.*, 2012)). Hence, its expression was further assessed in relationship to pS10H3.

The statistical analysis of immunostained DRG sections revealed a significant ($p < 0.001$, GzLM) increase of ~75% (5 min: $75 \pm 10.21\%$, Mdn=75, $n=4$; naïve: 0 ± 0 , Mdn=0, $n=4$) pS10H3 positive neurons that were also Nav1.7 positive at 5 minutes post-injury in the ipsilateral side (Figure 13.15 and Figure 13.16). These co-expression was maintained up to 3 hours following induction of the burn injury model (30 min: 67.21 ± 12.40 , Mdn=62.99, $n=4$, $p=0.638$; 1 hour: 61.98 ± 14.58 , Mdn=66.15, $n=4$, $p=0.432$; 3 hours: 81.55 ± 16.16 , Mdn=96.43, $n=4$, $p=0.693$; GzLM when compared with number of neurons in 5 minutes group; Figure 13.16)

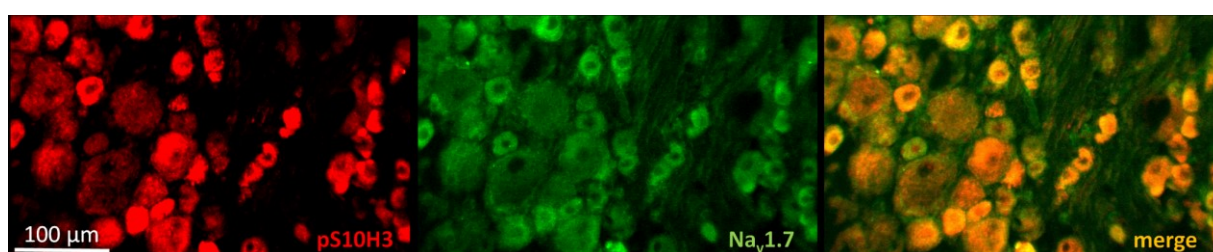


Figure 13.15. pS10H3 and Nav1.7 co-expression in the ipsilateral DRG burn-injury model. DRG of animals from the 5 minutes group (60°C water for 2 minutes and 5 minutes post-injury period) were stained for pS10H3 (red) and Nav1.7 (green). The scale bar equals 100µm.

On the other hand, the number of DRG neurons positive for Nav1.7 that also described pS10H3 positive staining was significantly increase ($p=0.001$, GzLM) in the ipsilateral at 5 minutes post-burn to ~20% (5 min: 20.24 ± 7.31 , Mdn=15.07, $n=4$; naïve: 0 ± 0 , Mdn=0, $n=4$). This increased co-expression of around 20% was maintained in the ipsilateral side up to one hour post-thermal injury (30 min: 20.98 ± 5.16 , Mdn=19.53, $n=4$, $p=0.905$; 1 hour: 20.51 ± 6.65 , Mdn=21.72, $n=4$,

p=0.966; GzLM vs. 5 minutes). However, a 50% reduction on the numbers of neurons positive for Nav1.7 that were also positive for pS10H3 was observed after 3 hours, the longest survival time studied, of the burn injury model (10.85 ± 2.61 , Mdn=12.30, n=4, p=0.089 vs. naïve, p=0.135 vs. 5 minutes, GzLM).

The compared statistical analysis between the percentage of pS10H3 positive neurons from the total number of cells counted and the percentage of cells immunolabelled for both from the total number of neurons counted did not show any significant difference at any end point studied (numbers not shown, Figure 13.17).

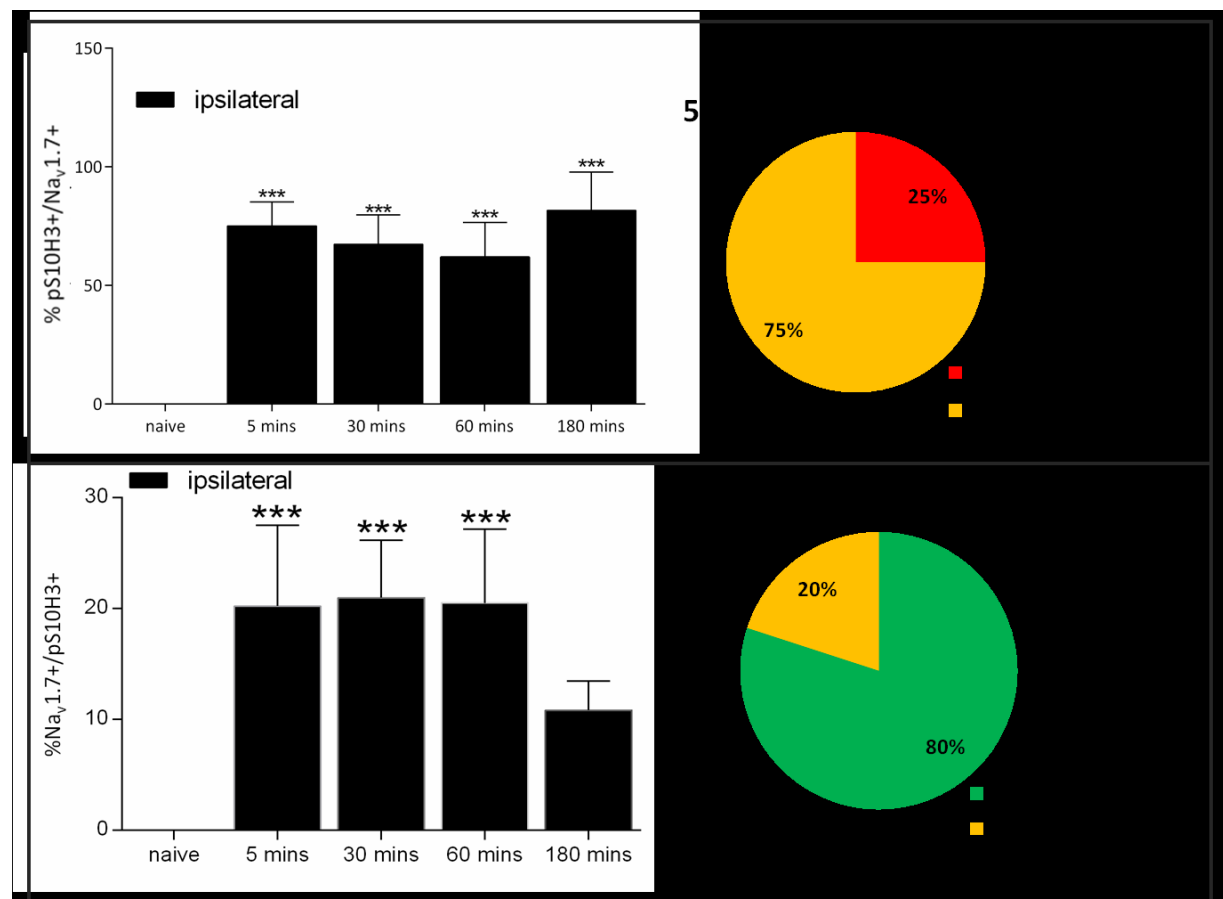


Figure 13.16. Co-expression between Nav1.7 and pS10H3 in L4 and L5 ipsilateral DRG after burn injury model. The experimental groups (n=4) were naive animals (naive), 60°C water for 2 minutes and 5 minutes post-injury period (5 mins) and 60°C water for 2 minutes and 180 minutes post-injury period (180 mins). **A)** graph showing pS10H3+ neurons that are also Nav1.7+. Data is presented as mean±SEM. *** p<0.001 vs. naive ipsilateral; pie chart showing the proportion of pS10H3+ neurons co-expressing with Nav1.7 after a burn injury **B)** graph showing Nav1.7 + neurons that are also pS10H3+. Data is presented as mean±SEM. *** p<0.001 vs. naive ipsilateral; pie chart showing the proportion of Nav1.7 + neurons co-expressing with pS10H3 after a burn injury.

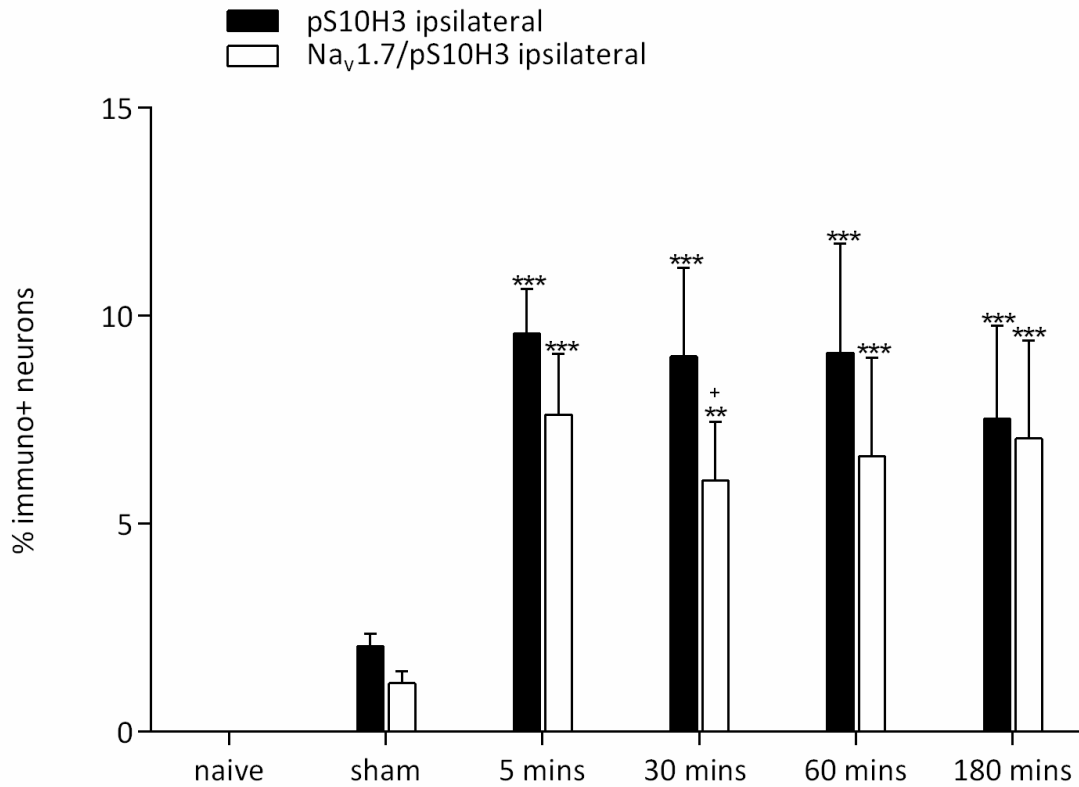


Figure 13.17. **Percentage of pS10H3+ neuronal nuclei and co-expression with Na_v1.7 in the ipsilateral DRG of the burn-injury model.** The data is presented as mean±SEM. The experimental groups (n=4) are naive animals (naive), 37°C water for 2 minutes and 5 minutes post-injury period (sham), 60°C water for 2 minutes and 5 minutes post-injury period (5 mins), 60°C water for 2 minutes and 30 minutes post-injury period (30 mins), 60°C water for 2 minutes and 60 minutes post-injury period (60 mins) and 60°C water for 2 minutes and 180 minutes post-injury period (180 mins). *** p<0.001 vs. naive ipsilateral pS10H3; + p<0.05 vs. 5' ipsilateral pS10H3.

13.5 Discussion

Here, it is shown for the first time that pS10H3 expression is increased following a peripheral burn injury both in a group of primary sensory neurons and a sub-population of superficial spinal dorsal horn neurons though the dynamics of up-regulation are different in the two types of neurons. Hence, could be used as a novel nociceptive marker.

13.5.1 After the induction of the scalding type burn injury model, pS10H3 is up-regulated in spinal cord neurons

The phosphorylation of this histone residue, pS10H3, appears in the nucleus of a sub-population of spinal cord neurons following the induction of the scalding type burn injury model (White *et al.*, 2011b). The up-regulation of pS10H3 is detected as early as 5-minutes post-injury, with an expression value that is maintained until one hour after the noxious stimulation. Most of these neurons are situated in the laminae I and II. This, together with the proximity to primary sensory terminals (Nav1.7 positive fibres), suggests that, at least, a great number of them are second order neurons, a population of cells that are crucial for the nociceptive response.

pERK1/2, a well-known marker of nociceptive processing at the spinal cord (Ji *et al.*, 1999; Ji *et al.*, 2003; Gao & Ji, 2009; White *et al.*, 2011a), is thought to be involved in the phosphorylation of S10H3 (Thomson *et al.*, 1999a; Soloaga *et al.*, 2003; Arthur, 2008). This PTM has been observed in neurons from the CNS involved different functions such as memory formation and addictions (Bilang-Bleuel *et al.*, 2005; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008; Crow *et al.*, 2015). Similarly, a high number of neurons expressing pERK1/2 were also positive for pS10H3 in spinal cord following this noxious input. However, just around 50% of

those expressing pS10H3 were also positive for pERK1/2. This disparity can be explained by two means or a combination of them: first that S10H3 can also be phosphorylated by other cascades of signalling kinases such as pp38 (Arthur, 2008). Nonetheless, previous reports show that p38 is mainly phosphorylated in spinal cord microglia following other peripheral inflammatory models (Hua *et al.*, 2005). Secondly, this could be due to different kinetics of ERK1/2 activation and S10H3 phosphorylation. Indeed, pERK1/2 peaks at 5 minutes after the burn and slowly decreases while the increase of pS10H3 is maintained up until 1 hour of the peripheral stimulation.

Following different kinds of stimulation, pS10H3 leads to transcription of different IEG, including c-Fos (Mahadevan *et al.*, 1991; Barratt *et al.*, 1994; Cheung *et al.*, 2000a; Cheung *et al.*, 2000b; Lo *et al.*, 2000; Nowak & Corces, 2000; Zhong *et al.*, 2000; Salvador *et al.*, 2001; Saccani *et al.*, 2002; Prigent & Dimitrov, 2003; Dyson *et al.*, 2005; Dong & Bode, 2006), an established marker of nociception in neurons of the spinal cord (Hunt *et al.*, 1987; Williams *et al.*, 1990; Dubner & Ruda, 1992; Todd *et al.*, 2002; Gao & Ji, 2009; White *et al.*, 2011a). Accordingly, around 70% of c-Fos positive neurons were pS10H3 positive while a similar percentage of pS10H3 positive neurons were also positive for c-Fos. This suggests that there could be a functional link between pS10H3 and c-Fos following this scalding type burn injury model.

13.5.2 pS10H3 is also up-regulated in DRG neurons following the peripheral burn injury

Following the burn injury, there is a nociceptive-specific increase of pS10H3 in DRG neurons as early as 5 minutes of the noxious stimulation. This change remains

present throughout the 3 hours of the experiment. This epigenetic mark is mainly found in small-sized DRG neurons, regarded as nociceptors. However, it is not only localised in the nucleus if not also in the cytoplasm. Although histone proteins have been detected extracellularly following activation of different immune cells (Watson *et al.*, 1995; Gabler *et al.*, 2003; Ogawa *et al.*, 2003; Gabler *et al.*, 2004; Gilthorpe *et al.*, 2013), this extracellular accumulation seems to be associated with apoptosis (Gabler *et al.*, 2004). This same epigenetic tag has been observed extracellularly within hippocampal neurons, in a process associated with neuronal dysfunction and neurodegeneration due to the pathology of the Alzheimer's disease (Ogawa *et al.*, 2003). Additionally, following the *in vitro* model of heat stress there is an up-regulation of pS10H3, similar to the one observed at heat shock genes from fruit flies after a heat stress (Nowak & Corces, 2000), that is confined to the nuclear compartment. Furthermore, the percentage of neurons with nuclei positive for pS10H3 was similar (~10%) to that observed of pCREB, while the percentage of total positive cells for pS10H3 was much greater (~50%). Together, these findings suggest that the cells that show pS10H3 outside the nuclei within the *in vivo* model might be in a state of cellular stress that could be due to agents released within the ganglia or in the injured tissues.

pCREB is a transcription factor that is phosphorylated by the same kinases that lead to pS10H3 (Ji *et al.*, 2003; Kawasaki *et al.*, 2004; Bie *et al.*, 2011; Li *et al.*, 2014). Similarly to pS10H3, pCREB is implicated in transcription activation of different genes following cellular stimulation (Thomson *et al.*, 1999b; Strahl & Allis, 2000). Accordingly, there is a high number of pS10H3 positive cells that also show pCREB. However, only half of the cells that are positive for pCREB are also positive for pS10H3. This indicates that phosphorylation of CREB is a more general process that

affects more cells following burn injury, which is in favour with the hypothesis that pS10H3 is a limiting step regulating transcriptional activation of a small sub-population of genes in neurons (Cheung *et al.*, 2000b; Bilang-Bleuel *et al.*, 2005; Keum *et al.*, 2013). Furthermore, these two phosphorylation events showed different kinetics: while pS10H3 is up-regulated at 5 minutes and remains elevated until, at least, three hours of the stimulation, pCREB peaks at 5 minutes and rapidly returns to basal numbers until 3-hour post-injury where peaks again (see previous chapter). Thus, although these two are dynamics markers of nociception in DRG, they are differently regulated.

Nav1.7 is important to develop heat hyperalgesia following burn injury (Shields *et al.*, 2012). Furthermore, the expression of this channel is up-regulated following a noxious stimulation, as assessed in the previous chapter with the burn injury model (Chapter 12) and after other noxious stimuli (Gould *et al.*, 1998; Black *et al.*, 2004; Gould *et al.*, 2004; Yeomans *et al.*, 2005; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Dib-Hajj *et al.*, 2010; Dib-Hajj *et al.*, 2013). Accordingly, around 75% of pS10H3 positive neurons were also Nav1.7 positive. However, the number of Nav1.7 neurons that were also positive to pS10H3 was much lower (~20%) and declined at later time points. This discrepancy could be because only the Nav1.7 positive neurons with their receptive field in the hind leg get activated by the noxious input, hence showing phosphorylation of histone H3 at serine 10. Furthermore, the decrease at later time points correlates with the up-regulation of Nav1.7 (see previous chapter), indicating that while the number of neurons positive for Nav1.7 is increased, the number of pS10H3 remains the same. Thus, the number of primary sensory neurons that are activated by the peripheral injury remains the same despite

the increase in Nav1.7. Most possibly these neurons express Nav1.7 de novo and are recruited at later times, which could not be assessed in this model.

13.5.3 Conclusion and future work

In this chapter, I have shown for the first time that phosphorylation of histone H3 at serine 10 (pS10H3) is a nociceptive-specific event in neurons both in the superficial spinal cord and DRG following a burn injury.

In the spinal cord, this neuronal population are in close apposition with terminals from primary sensory neurons (assessed by Nav1.7 expression), which suggest that at least most of them are second order neurons. Moreover, pS10H3 exhibits a high degree of co-localisation with pERK1/2 and c-Fos expression. Thus, a functional link can be postulated between these molecules. Hence, future work needs to be done to further characterise the types of the cells that express this PTM, and if other types of noxious stimulation also induce it as well as the functional consequences of its blockade for nociception.

At DRG, pS10H3 is mainly localised in nociceptors as well as showing some overlap with pCREB. Nonetheless, histones containing this PTM have been localised outside the nuclear compartment which, although described previously by others, is an issue that needs to be further assessed. Thus, more research is needed to study the profile and dynamics of pS10H3-positive DRG neurons following this and other noxious inputs as well as the potential blockade of this event for analgesic purposes.

14 Nociceptive-specific induction of pS10H3 in superficial spinal cord neurons

14.1 Background

At present, persistent/prolonged pain is still not efficiently treated (Breivik *et al.*, 2006) and further research is needed to elucidate its mechanism and evolution, as well as potential analgesic strategies. It is, in part, the result of a dysfunctional process of neuroplasticity known as central sensitization (Ji *et al.*, 1999; Julius & Basbaum, 2001; Ji *et al.*, 2003; Marchand, 2008; Hunt, 2009; Browne *et al.*, 2011; Steeds, 2013).

Central sensitization refers to the induction of long-lasting increased excitability of neurons from the CNS, although it is especially important at the superficial DHSC, the major place where primary afferents from nociceptors terminate and synapse with second-order neurons (Ji *et al.*, 1999; Hunt & Mantyh, 2001; Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; Yoshimura & Furue, 2006; Marchand, 2008; Hunt, 2009; Todd, 2010; Woolf, 2011; Steeds, 2013). Central sensitization depends on activated NMDA due to a prolonged release of glutamate (Zou *et al.*, 2000; Ji & Woolf, 2001; Marchand, 2008) and is considered a form of long-term potentiation (LTP) (Bliss & Lomo, 1973; Ji & Woolf, 2001; Ji *et al.*, 2003; Costigan *et al.*, 2009; Hunt, 2009; Bie *et al.*, 2011; Woolf, 2011).

The phosphorylation of MAPK, such as pERK1/2, is involved in the generation of LTP (Ji *et al.*, 1999; Ji & Woolf, 2001; Ji *et al.*, 2003). They are rapidly induced on superficial spinal dorsal horn following different types of noxious stimulation (heat, cold or mechanical) in an intensity-dependent manner (Ji *et al.*, 1999; Ji & Woolf, 2001; Polgar *et al.*, 2007; Black *et al.*, 2008; Gao & Ji, 2009; White *et al.*, 2011a; White *et al.*, 2011b). MAPK alter neuronal excitability by modulating the activity of different proteins such as sodium and potassium channels, synaptic vesicle trafficking and others (Black *et al.*, 2008; Ji *et al.*, 2009; Dib-Hajj *et al.*, 2010;

Stambouliau *et al.*, 2010). Moreover, they are important regulators of gene expression, although their role on this is not completely understood (Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; Kawasaki *et al.*, 2004).

Epigenetic mechanisms, such as post-translational modification (PTM) of histones, play an important role in the development and maintenance of different pain states by regulating the accessibility of the transcriptional machinery to the DNA (Geranton *et al.*, 2007; Chiechio *et al.*, 2009; Bai *et al.*, 2010; Zhang *et al.*, 2011; Denk & McMahon, 2012; Geranton, 2012; Liu *et al.*, 2012; Crow *et al.*, 2013; Denk *et al.*, 2013; Rahn *et al.*, 2013; Bai *et al.*, 2015; Chen *et al.*, 2015; Crow *et al.*, 2015; Geranton & Tochiki, 2015b; a). Phosphorylation of histone H3 at serine 10 (pS10H3) is a transcriptionally permissive PTM associated to a subset of IEG (Dong & Bode, 2006; Keum *et al.*, 2013) and implicated on memory formation (Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008; Sarantis *et al.*, 2012; Rotllant *et al.*, 2013; Crow *et al.*, 2015). The pathways that induce this PTM are not fully comprehended. The up-regulation of pS10H3 at hippocampal neurons seems to be a NMDA receptor-dependent process, and implicates MAPK pathways (ERKs). However, MAPK cannot directly phosphorylate histone H3 at this residue (Saccani *et al.*, 2002), and the downstream kinases MSK1/2 are suggested as the principal effectors (Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008; Mackowiak *et al.*, 2013). Nonetheless, and at exception of previous chapter's findings following a peripheral burn injury, the implication of pS10H3 at spinal neurons following an injury has never been investigated.

14.2 Aims

The **aims** of this chapter are:

- To investigate the potential role of pS10H3 in spinal cord neurons following different noxious stimuli other than burn injury.

And if that is the case:

- To assess if this up-regulation of pS10H3 at spinal cord neurons is MAPK-dependent.
- To assess if the induction of this PTM mark is also and NMDA receptor-dependent process.

14.3 Material and Methods

No difference in the up-regulation of pS10H3 in the spinal cord was found between Sprague-Dawley and Wistar rats (data not shown). Hence, both strains were used: Sprague-Dawley for *in vivo* procedures while Wistar for *in vitro* preparations.

14.3.1 *In vivo* capsaicin injection

Under sodium pentobarbital anaesthesia, rodents received an intraplantar injection of capsaicin (25µL of 3mg/mL dissolved in 10% Tween 80; Sigma, M2028) into one of the hind limbs, followed by two-minutes gentle stroke. After corresponding post-injection times (5 or 30 minutes, n=13 and n=3 respectively), rats were terminally anaesthetised and either transcardially-perfused and tissues processed for immunostaining or their necks were dislocated and tissues processed for western blotting. Naïve (n=13) and saline (n=3) injected animals were used as negative and sham controls respectively.

Following perfusion, L4 and L5 spinal cord segment were cryoprotected, transversally sectioned at 10µm using a cryostat and thaw-mounted on Superfrost Plus microscope slides. Sections were then processed for **immunostaining** (n=3 for each group, including control, sham, 5-minutes capsaicin injection and 30 minutes capsaicin injection). Briefly, slides were incubated overnight at 4°C using primary antibodies against pS10H3 (Santa Cruz, SC8656R, [1:750]), pERK1/2 (Neuromics, RA15002, [1:1500]), TRPV1 (Neuromics, GP141000, [1:1500]) and MAP2 (Abcam, AB5392, [1:5000]). All sections were revealed with normal immunofluorescence except pS10H3 that was further amplified using the TSA procedure. Corresponding negative and positive controls were done to validate the specificity of the procedures.

Finally, immunopositive cells were assessed, microphotographs taken, and statistical differences calculated using the GzLM and regarded as significant at $p < 0.05$.

For **western blotting**, ipsi- and contra-lateral dorsal horn of fresh L4 and L5 spinal cord segments from control animals ($n=4$) or rats subjected to capsaicin with 5 minutes post-injury period ($n=4$) were collected and processed for total histone extraction (Epigentek, OP-0006-100) as suggested by the manufacturer. Resulting samples were then separated by electrophoresis using NuPAGE Novex 12% Bis-Tris Protein Gels (Invitrogen, NP0342BOX), electrotransferred to a PVDF membranes and immunolabelled against pS10H3 (1:500) and β actin (Millipore, MAB1522, [1:2000]). After their reaction with chemiluminescence, membrane was stripped and reacted again against pan-histone H3 (Millipore, 07-690, [1:4000]). Finally, densitometry values were obtained, normalised, and used to assess the ratios for each animal (intensity in the ipsilateral side divided by the contralateral). Statistical differences were assessed using the independent t -test and regarded as significant at $p < 0.05$. For further control, a duplicate gel was run in parallel inverting the order of immunoreactions. Additionally, resulting histone extracts from a control and a capsaicin stimulated animal were run in duplicate using the EpiQuik Histone H3 Modification Multiplex Assay Kit (Epigentek, P-3100-96) as described by the manufacturer to compare amounts of different histone H3 modifications.

To increase the amount of histone in the samples, ipsi- and contra-lateral dorsal horn of fresh L4 and L5 spinal cord segments from animals treated equally were combined: controls rodents ($n=2 \times 3$) or subjected to capsaicin with 5 minutes post-injury period ($n=2 \times 3$). Following, resulting extracts were processed for western blotting as explained above.

14.3.2 *In vitro* capsaicin application

This experiment was performed with the help of Dr. Péter Sántha, from University of Szeged, and the complete procedure is explained in the resulting paper (accepted for publication at *Scientific Reports*). Briefly, rats were terminally anaesthetised with sodium pentobarbital and decapitated. Then, the lumbar vertebral column was exposed and spinal cord ejected by injecting artificial cerebrospinal fluid (aCSF). Following, a razor blade was used to cut the lumbar cord and maintained in gassed aCSF at 37°C. After a resting period of 30 minutes, slides were either a) exposed to capsaicin (10µM, n=9) or b) pre-incubated with SB-747651A (10µM) for 20 minutes and then with both capsaicin and SB-747651A (n=8). A control group (n=9) was also performed in which slides were only incubated in vehicle. After 3 hours, spines were fixed with PFA solution and processed for immunostaining as explained above. Resulting sections were reacted against pS10H3 and IB4 (Molecular Probes, 121411, [1:1,000]).

14.3.3 Noxious thermal and mechanical *in vivo* models

Cold (4°C) or hot (45°C) stimuli were produced by immersing one hind paw in water at corresponding temperature for periods of 10 seconds with 10-second gaps in between for a total of 2 minutes (6 immersions in total). The final post-injury period was 5 minutes. Following treatment, animals (n=3 for each treatment) were processed for immunostaining as explained above. Additionally, skin preparation from the hind paw, together with baseline and capsaicin injected animals, were also stained for H&E.

Mechanical stimulation (n=3) was performed with the help of Dr. Botton Gaal, from University of Debrecen, and the complete procedure is explained in the resulting

paper (accepted for publication at *Scientific Reports*). Briefly, one hind paw of anaesthetised animals was exposed to dorso-plantar compression (6N/cm²) for periods of 10 seconds with 10-second gaps in between for a total of 2 minutes. After 5 minutes of the initial procedure, rats were also processed for immunostaining (see above).

14.3.4 *In vivo* electrical stimulation

Electrical stimulation of the saphenous nerve was done with the help of Dr. Anna Andreou. Following anaesthesia, animals were cannulated for mechanical ventilation and then nerve from one of the hind paws exposed. Nerve was then placed onto a bipolar platinum wire electrode and subjected to electrical stimulation. The trains of stimulation consisted on: a) ten trains of two hundred pulses each of 30V amplitude and 2ms width at 10Hz frequency (n=3), or b) a continuous pulse of 15 Volts (0.5Hz and 0.1ms) for 5 minutes. At the end of the experiments, rats were terminally anaesthetised and processed for immunostaining as described above.

14.3.5 *In vitro* electrical stimulation

This procedure was performed with the help of Dr. Peter Szucs, from University of Debrecen, and the complete procedure is explained in the resulting paper (already accepted at *Scientific Reports*). Briefly, lumbar spinal cord was dissected with unilateral L4 and L5 DRG attached, glued to a golden plate and transferred to a recording chamber. Stimulation was carried out through suctioning of DRG with an electrode inserted in a borosilicate glass capillary. In all animals (n=8) stimulation consisted on four pulses of 500µA amplitude and 1ms width at 2Hz for 15 seconds with 15 seconds gap in between. Half of those rats (n=4) received a bath application of D-APV (50µM) 20 minutes prior and during electrical stimulation. Following

corresponding protocol, preparations were left to recover for 3 minutes and processed for immunostaining as explained above.

14.4 Results

In the previous chapter (Chapter 13), pS10H3 was validated as a marker of neuronal activation following a burn injury in both DHSC and DRG. Here we studied its expression in DHSC under different pain models to further assess its induction after noxious stimuli.

Initially the capsaicin injection model was performed to analyse the induction of pS10H3 post-inflammatory response. This model was also used to characterize the cell type expressing this PTM. To do so, western blotting, immunostaining and *in vitro* pharmacology were performed. Next, pS10H3 expression was assessed after exposure to noxious stimulation without the induction of tissue damage (noxious heat, cold or mechanical stimulation), and following electrical stimulation of the peripheral process of primary sensory neurons. Finally, we studied the different PTM present in histone H3 together with pS10H3 following capsaicin injection with the aim to further decipher the histone code after a noxious stimulation.

14.4.1 pS10H3 expression in spinal cord after capsaicin injection

It is well known that capsaicin injection induces an inflammatory response (Jancso *et al.*, 1967) with a subsequent sensitisation of both primary sensory neurons and DHSC neurons (Simone *et al.*, 1989a; Simone *et al.*, 1989b). Hence, we studied this model to test if a painful inflammatory stimulation alone was capable to induce pS10H3 in the DHSC.

Western blotting analysis from DHSC histone extracts at 5 minutes post-intraplantar injection of capsaicin showed up-regulation of pS10H3 in the ipsilateral DHSC when compared to naïve animals (capsaicin: 1.58 ± 0.28 , $n=4$; naive: 1.18 ± 0.07 , $n=4$).

However, it failed to reach statistical significance ($p=0.081$, Student's *t* test, Figure 14.1 B). Nevertheless, western blotting from DHSC total protein extracts from animals pooled together (two animals equally treated combined in each lane), did show a significant increase on the expression of pS10H3 in the ipsilateral DHSC after capsaicin injection (naïve: 0.92 ± 0.04 , $n=3$; capsaicin injection: 3.29 ± 0.79 , $n=3$; $p=0.04$, student's *t*-test, Figure 14.1 A).

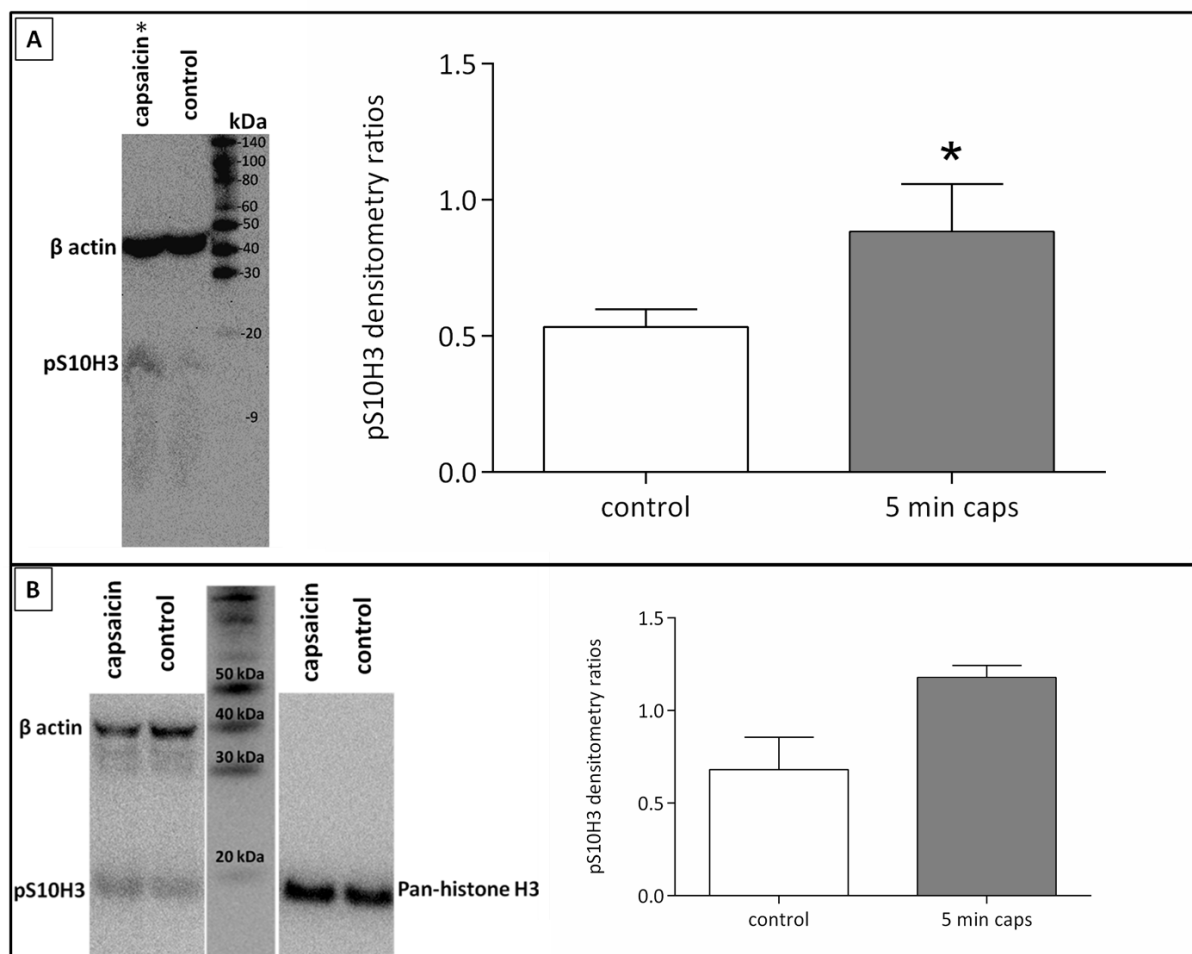


Figure 14.1. **pS10H3 increase in the DHSC after capsaicin injection.** All animals were treated by s.c. capsaicin (25 μ L of 3 mg/mL) injection in the hind paw with 2-minutes stroke and 5-minutes post-injection period. **A)** Representative western blot of DHSC from protein extracts of ipsi- and contra-lateral sides of two combined animals ($n=3$) after capsaicin injection; graph shows the normalized densitometry ratios. Data is presented as mean+SEM. Student's *T* test was performed with normalized densitometry ratios between the contra- and the ipsi-lateral sides of each animal. * $p=0.03$. **B)** Representative western blot of DHSC total histone extracts from 2 animals. First stained for pS10H3 (left), stripped and then reacted for pan-histone H3 (right; graph shows the normalized densitometry ratios ($n=4$). Data is presented as mean+SEM. Student's *T* test was performed with normalized densitometry ratios between the contra- and the ipsi-lateral sides of each animal. The increase did not reach significance, with $p=0.081$.

Immunodetection of nuclei expressing pS10H3 in spinal cord sections also revealed a significant up-regulation on the number of positive neurons in the ipsilateral DHSC at 5 minutes of the capsaicin injection (naïve: 3.33 ± 0.88 , Mdn=3.00, n=3; capsaicin injection: 78.33 ± 27.79 , Mdn= 64, n=3; $p < 0.001$, GzLM; Figure 14.2 and Figure 14.3). This increase in the ipsilateral DHSC persisted 30 minutes after the injection (80.00 ± 10.44 , Mdn= 78.00, n=3; $p < 0.001$, GzLM, Figure 14.2 and Figure 14.3). No up-regulation was observed in the ipsilateral DHSC of saline injected animals (2.00 ± 1.00 , Mdn= 3.00, n=3; $p = 0.914$, GzLM; Figure 14.2 and Figure 14.3) or in any contralateral DHSC studied within these experiments (naïve: 2.00 ± 2.00 , Mdn=0.00, n=3, $p = 0.921$; sham: 0.00 ± 0.00 , Mdn=0.00, n=3, $p = 0.803$; 5 min: 2.00 ± 1.15 , Mdn=2, n=3, $p = 0.921$; 30 min: 0.00 ± 0.00 , Mdn=0.00, n=3, $p = 0.803$; GzLM).

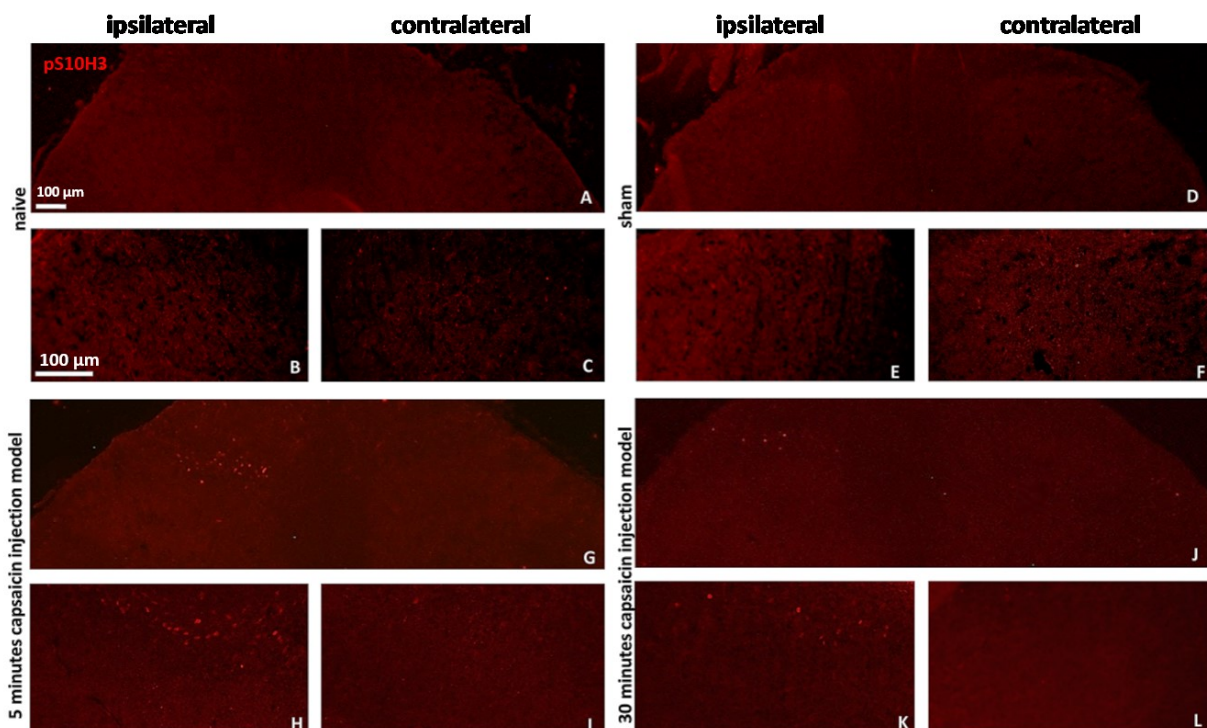


Figure 14.2. Time course of pS10H3 induction in the DHSC after capsaicin injection model. In all pictures, red is for pS10H3. The scale bars equal 100µm. Pictures A, D, G and J were taken at the same magnification (5x) and pictures B, C, E, F, H, I, K and L at a higher magnification (20x). The experimental groups (n=3 each) were: naïve animals (naïve), s.c 25µL saline injection in the hind paw and 5 minutes post-injection period (sham), s.c. capsaicin (25µL of 3 mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period (5 minutes), and s.c. capsaicin (25µL of 3 mg/mL) injection in the hind paw with 2 minutes stroke and 30 minutes post-injection period (30 minutes).

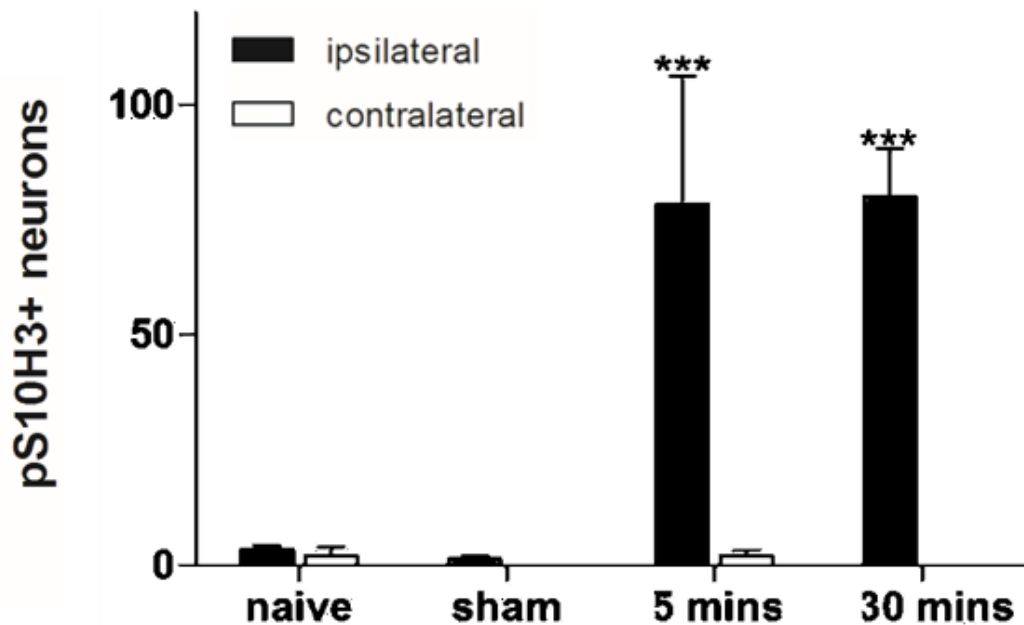


Figure 14.3. Immunolabelled pS10H3 neurons in the DHSC of the capsaicin model. The data is presented as mean+SEM. Experimental groups (n=3) were naive animals (naive), s.c. capsaicin (25 μ L of 3 mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period (5 minutes), and s.c. capsaicin (25 μ L of 3 mg/mL) injection in the hind paw with 2 minutes stroke and 30 minutes post-injection period (30 minutes). *** $p < 0.001$ vs. naive ipsilateral.

14.4.2 pERK1/2 positive immunolabelling in the capsaicin injection and its comparison to pS10H3 after a capsaicin injection

Similar as for the burn-injury model (Chapter 13), the immunopositive staining for pS10H3 was compared with the expression pattern of pERK1/2. Therefore, spinal cord sections were also immunolabeled for pERK1/2 (Figure 14.4).

Up-regulation of pERK1/2 was also detected in the ipsilateral DHSC within 5 minutes of the capsaicin injection (5 min: 73.67 ± 12.33 , Mdn=86.00, n=3; naïve: 3.67 ± 0.33 , Mdn=4.00, n=3; $p < 0.001$, GzLM) with similar values as observed for pS10H3 ($p = 0.727$, GzLM). Nonetheless, the number of pERK1/2 positive cells after 30 minutes of the neurotoxin injection returned to baseline numbers (30 min: 8.67 ± 2.73 ,

Mdn=7.00, n=3, p=0.338, GzLM) while the number for pS10H3 remained elevated (Figure 14.4), thus indicating different kinetics of activation for both markers.

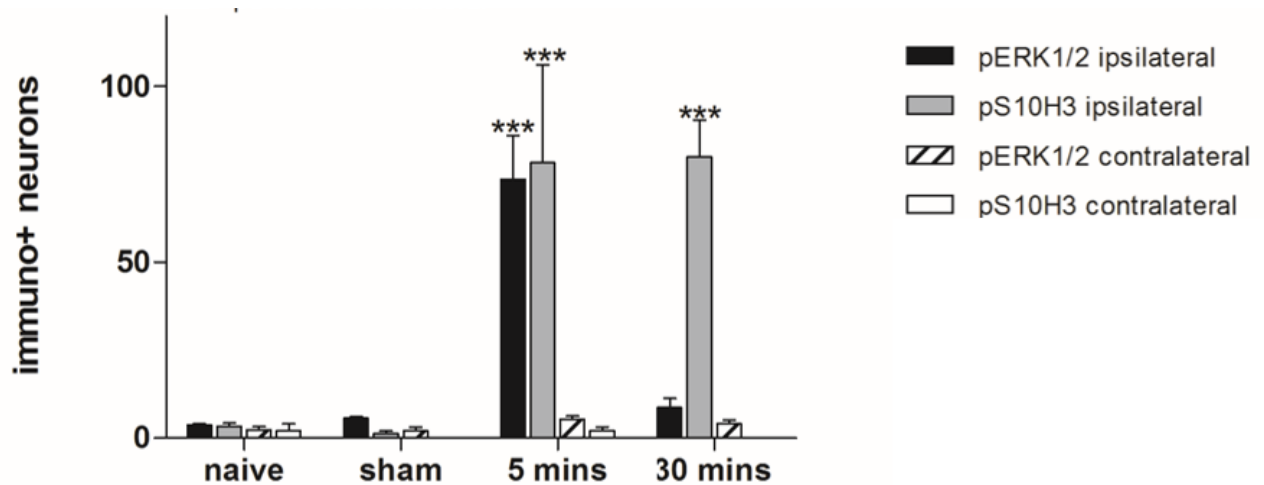


Figure 14.4. **immunolabelled pERK1/2 and pS10H3 neurons in the DHSC of the capsaicin model.** The data is presented as mean+SEM. The experimental groups (n=3) were naive animals (naive), s.c. capsaicin (25 μ L of 3 mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period (5 minutes), and s.c. capsaicin (25 μ L of 3 mg/mL) injection in the hind paw with 2 minutes stroke and 30 minutes post-injection period (30 minutes). *** p<0.001 vs. naive ipsilateral pERK1/2.

As seen for pS10H3 (see above), no increase of pERK1/2 was detected in the ipsilateral DHSC after saline injection (sham: 5.67 ± 0.33 , Mdn=6.00, n=3, p=0.701, GzLM) or in any contralateral DHSC analysed (naïve: 2.33 ± 0.88 , Mdn= 2.00, n=3, p=0.798; sham: 1.33 ± 0.67 , Mdn=2.00, n=3, p=0.655; 5 min: 5.33 ± 0.88 , Mdn=5.00, n=3, p=0.749; 30 min: 4.00 ± 1.15 , Mdn=4.00, n=3, p=0.949; GzLM).

14.4.3 Proximity of pS10H3 positive nuclei to TRPV1-expressing fibres in the spinal cord

Capsaicin activates TRPV1 in a selective and specific manner (Caterina *et al.*, 1997). Hence, we studied if the neurons with nuclei positive for pS10H3 following capsaicin injections were in close apposition to TRPV1-expressing terminals. To do so, spinal cord sections of animals after 5 minutes capsaicin injection were immunolabelled for TRPV1, pS10H3 and with the neuron specific anti-microtubule-

associated protein 2 (MAP2). The last one, MAP2, was used to visualise the cytoplasm of the neurons (Figure 14.5). Analysis by visual assessment revealed that ~74% ($74.43 \pm 0.98\%$, $n=3$) of the neurons with pS10H3 positive nuclei were in close proximity to TRPV1 immunopositive fibres (Figure 14.5).

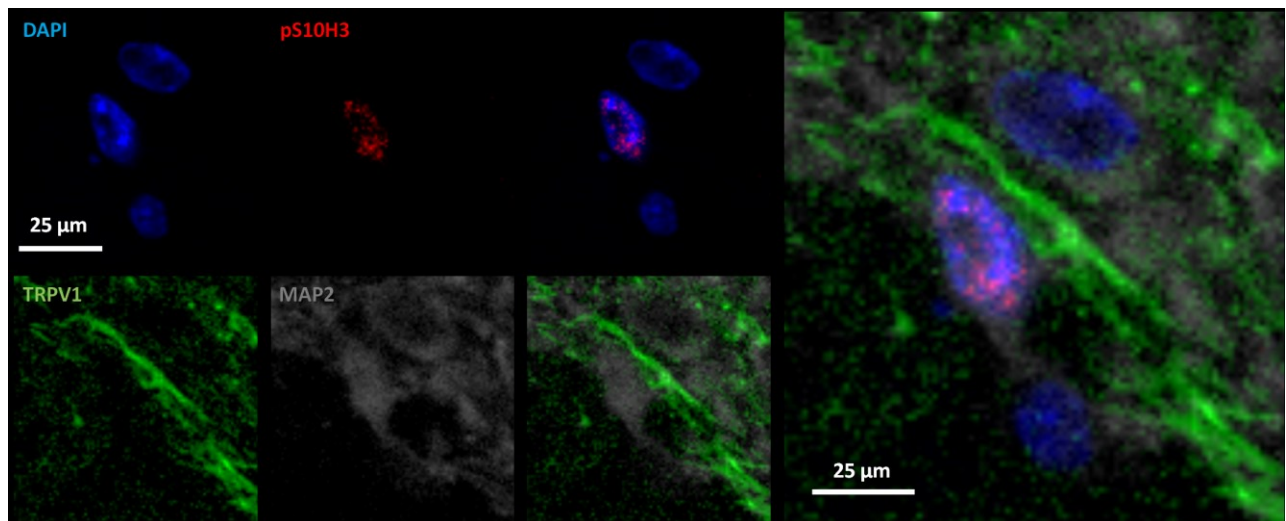


Figure 14.5. **Proximity of TRPV1 fibres to pS10H3 positive nuclei in the DHSC at 5 minutes post-injection of capsaicin in the hind paw.** Images were taken with confocal microscopy. Colours of the images were amended digitally to a better visibility. Blue is the nuclear marker DAPI, green shows the TRPV1-fibres projected by the primary order neurons, red is pS10H3 and grey the neuron specific cytoplasmic marker MAP2. Scale bars equal 25µm. Note that pS10H3 is restricted to the nucleus and that TRPV1 fibres terminate in close contact to the cytoplasm of the pS10H3 positive neuron.

14.4.4 Expression pathway involved in S10H3 phosphorylation in the spinal cord neurons using an *in vitro* model of capsaicin application

The application of capsaicin into spinal cord slides (*in vitro*) also up-regulated the number of pS10H3 positive nuclei in the superficial dorsal horn (sham-vehicle: 4.09 ± 1.47 , $n=9$; capsaicin: 19.80 ± 1.90 , $n=9$; $p < 0.001$, ANOVA with Bonferroni post-hoc test; Figure 14.6) in a similar fashion as described for the *in vivo* model (see above). IB4 co-expression confirmed that neurons with nuclei positive for pS10H3

were mainly localised in the superficial laminae, similarly as seen *in vivo* (Chapter 13).

Next, slides were pre-incubated with SB747651A dihydrochloride (10 μ M for 20 minutes), a blocker of MSK1 (Naqvi *et al.*, 2012), before the addition of capsaicin. Data from this study showed a significant reduction of neurons labelled with pS10H3 in comparison with the up-regulation after capsaicin application alone (SB747651A: 0.76 ± 0.28 , $n=8$, $p<0.001$, ANOVA with Bonferroni post-hoc test; Figure 14.6).

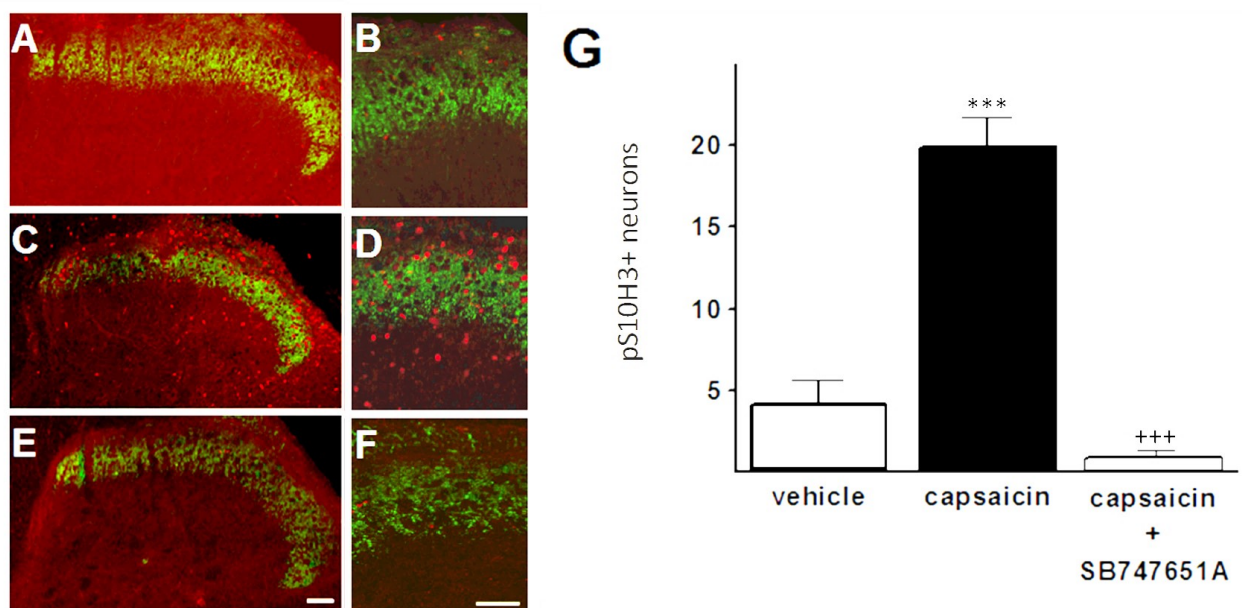


Figure 14.6. MSK1/2 blockade inhibits capsaicin-induced increase of pS10H3 in the DHSC. A-F) Confocal microscopic images of sections cut from *in vitro* spinal cord preparations exposed to vehicle (A and B), 10mM capsaicin (C and D) or 10mM capsaicin together with 10mM SB 747651A, an inhibitor of MSK1 (E and F). Sections were immunoreacted against pS10H3 (red) and against IB4 lectin (green) to distinguish the ventral border of lamina II. Scale bars equal 50 μ m. **G)** bar chart corresponding to the data analysis ($n=9$; ANOVA followed by the Bonferroni post-hoc test). *** $p<0.001$ vs. vehicle; +++ $p<0.001$ vs. capsaicin. Note that pS10H3 positive neurons are mainly localised in lamina I and II.

14.4.5 pS10H3 expression in the spinal cord after exposure to noxious stimuli without causing tissue damage

It is well known that noxious heat ($>42^{\circ}\text{C}$) also activates TRPV1 (Caterina *et al.*, 1997). Hence, we studied if it had an effect on the expression of pS10H3 in the

spinal cord as a model of noxious stimulation alone, without the induction of tissue damage. To prevent the tissue destruction, the stimulation was done by exposing the hind paw to 44°C water a total of 6 times for a period of 10 seconds each and with 10-second gap between stimulations. After this noxious activation, no up-regulation of pS10H3 was detected in either ipsilateral (ipsilateral: 4.33 ± 1.2 , Mdn=5, n=3; control: 3.33 ± 0.88 , Mdn=3, n=3; $p=0.689$, GzLM, Figure 14.7) or contralateral DHSC (contralateral: 2.00 ± 2.00 , Mdn=0, n=3, $p=0.183$, GzLM). However, a significant increase of pERK1/2 was observed after the noxious heat in the ipsilateral DHSC (control: 3.67 ± 0.33 , Mdn=4, n=3; ipsilateral: 20.33 ± 6.33 , Mdn=14, n=3, $p<0.001$; contralateral: 0.00 ± 0.00 , Mdn=0, n=3, $p=0.143$; GzLM, Figure 14.7).

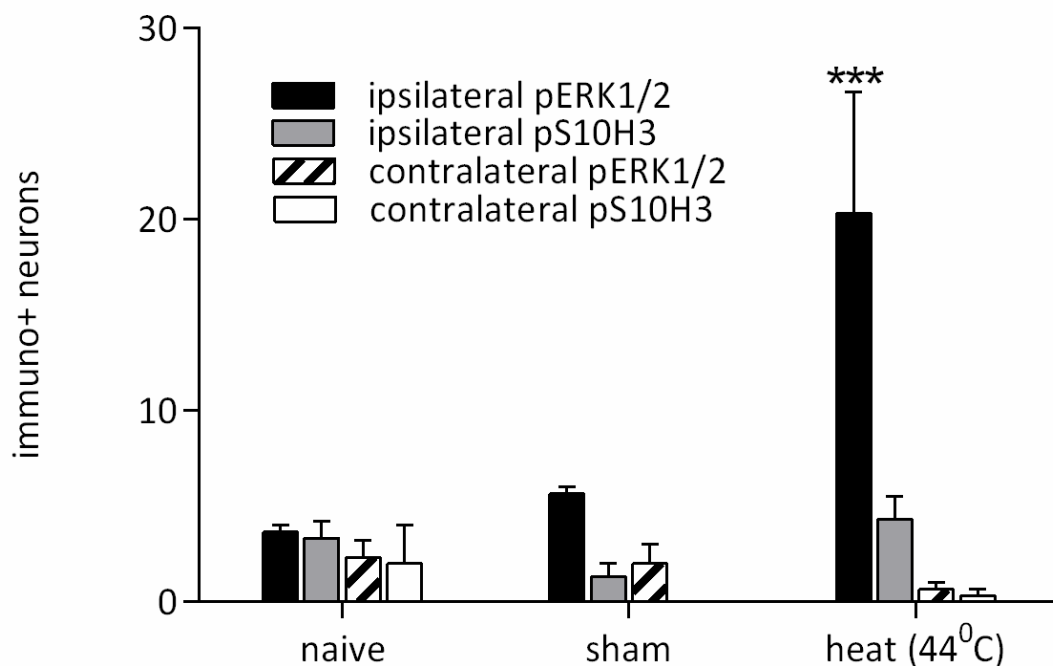


Figure 14.7. **immunolabelled pERK1/2 and pS10H3 neurons in the superficial DHSC after noxious heat stimulation (~44°C).** The data is presented as mean+SEM. The experimental groups (n=3 each) were naive animals (naive), 37°C water for 2 minutes and 5 minutes post-injury period (sham); and 6 times 44°C water for 10 seconds with 10 seconds gaps in-between [heat (44°C)]. *** $p<0.001$ vs. naive pERK1/2 ipsilateral

To further validate that noxious stimulation by itself does not induce up-regulation of pS10H3 in DHSC, a similar stimulation protocol was used with cold. Here, cold water

(icy water, $\sim 4^{\circ}\text{C}$) was used to expose the hind paw in a similar patten as the noxious heat stimulation (6 expositions for 10 seconds, elapsed by 10 seconds rest). In this experimental set-up, neither pS10H3 or pERK1/2 expressed a significant increase in the ipsilateral (pS10H3: 1.00 ± 1.00 , Mdn=0, n=3; $p=0.358$; pERK1/2: 3.33 ± 2.85 , Mdn=1, n=3, $p=0.894$; GzLM) or contralateral DHSC (pS10H3: 0.00 ± 0.00 , Mdn=0, n=3, $p=0.183$; pERK1/2: 0.00 ± 0.00 , Mdn=0, n=3, $p=0.143$; GzLM; Figure 14.8).

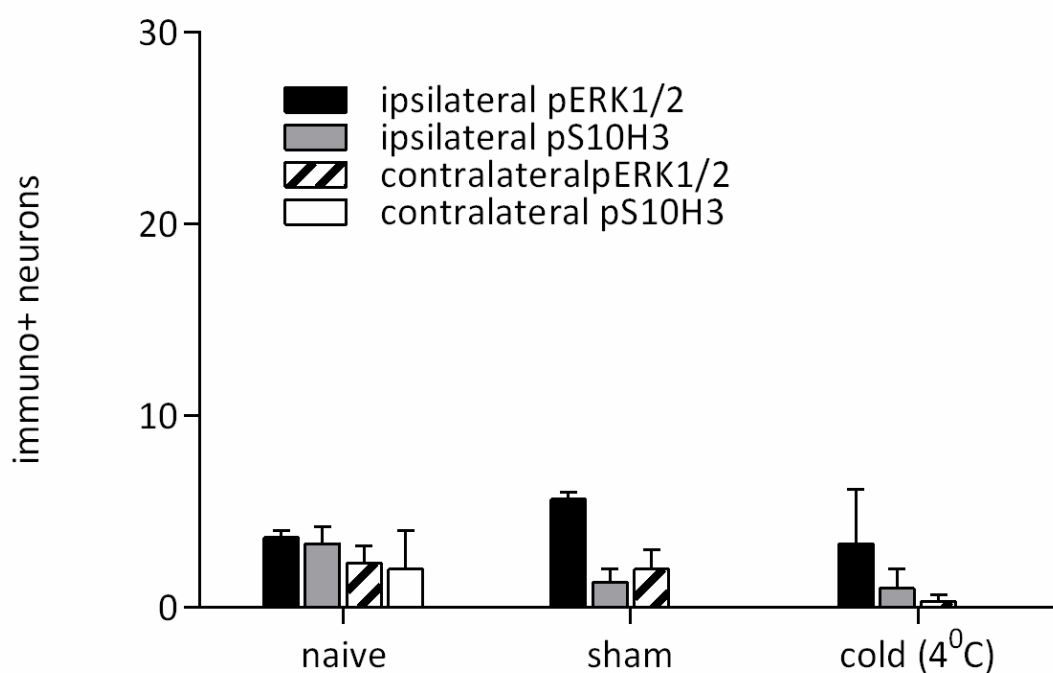


Figure 14.8. **immunolabelled pERK1/2 and pS10H3 neurons in the DHSC after noxious cold stimulation.** The data is presented as mean $\pm$ SEM. The experimental groups (n=3 each) were naive animals (naive), 37°C water for 2 minutes and 5 minutes post-injury period (sham); and 6 times 4°C water for 10 seconds with 10 seconds gaps in-between [Cold (4°C)].

The analysis of the hind paw skin sections stained for H&E revealed that, similarly as the skin of naïve controls, the skin of animals after noxious cold stimulation did not show any sign of damage (Figure 14.9) while noxious heat stimulation produced some signs of liquid infiltration in the dermis although the destruction was much less dramatic than the observed in the skin of animals subdued to capsaicin injection, used here as positive controls (Figure 14.9).

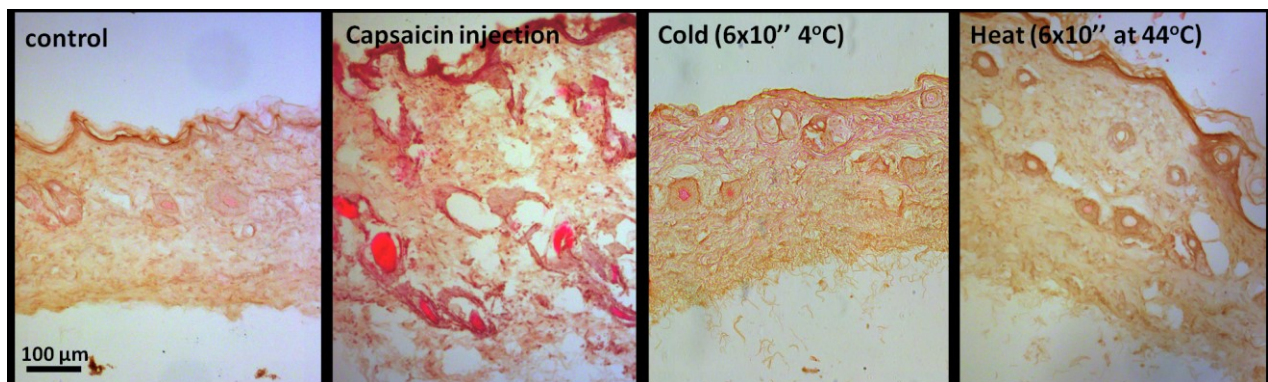


Figure 14.9. **Histopathology of the skin after different treatments.** Microphotographs of haematoxylin and eosin stained skin sections from ipsilateral hind paw of a naive animal (control), a s.c. capsaicin (25 μ L of 3 mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period (capsaicin injection); an animal from 4°C water hind paw immersion for 6 periods of 10 seconds separated with 10 seconds rest each, and 5 minutes post-treatment period (cold); and an animal from 44°C water for 6 periods of 10 seconds separated with 10 seconds rest each, and 5 minutes post-treatment period (heat). Scale bar equals 100 μ m. Note that in “Capsaicin injection” there is a massive damage of epidermis and dermis. A mild damage of the intermediate layers is present in the “heat”, while it does not appear in the “control” and “cold”.

Further, a model of noxious pressure, done in collaboration with another lab (Department of Anatomy, Histology and Embryology, University of Debrecen, Hungary), was also used to assess the expression of these markers. Thus, spinal cord of mechanically stimulated (pressure of 60mN/mm²) and control rats were send over for sectioning and immunolabelling. Similarly to noxious cold and noxious heat, pressure did not up-regulate pS10H3 in either the ipsilateral (ipsilateral: 4.33 $\pm$ 0.06, Mdn=5, n=3, p=0.281, GzLM) or the contralateral side of the DHSC (contralateral: 2.33 $\pm$ 0.88, Mdn=2, n=3, p=0.859; GzLM). However, a significant increase in the number of pERK1/2 positive neurons was detected in the ipsilateral DHSC after the noxious pressure (ipsilateral: 23.00 $\pm$ 2.88, Mdn=23, n=3; p<0.001; contralateral: 3.33 $\pm$ 0.88, Mdn=3, n=3, p=0.156; GzLM; Figure 14.10).

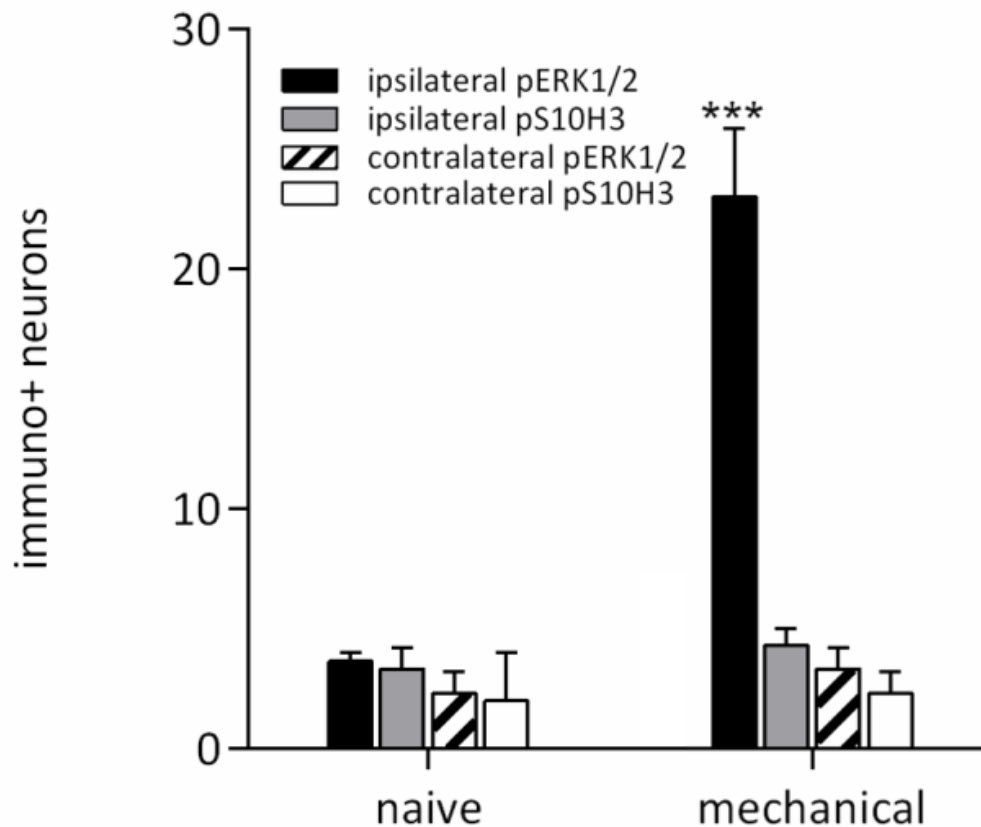


Figure 14.10. **immunolabelled pERK1/2 and pS10H3 neurons in the DHSC after the model of noxious pressure.** The data is presented as mean $\pm$ SEM. The experimental groups (n=3) were naive animals (naïve) and hind paw mechanically stimulated by a pressure of 60mN/mm² (mechanical). *** p<0.001 vs. naive pERK1/2 ipsilateral.

14.4.6 pS10H3 expression in spinal cord after electrical stimulation

Inflammation, through inflammatory mediators, activates primary sensory neurons (Jancso *et al.*, 1967; Petho & Reeh, 2012), which results in afferent barrage into spinal cord and expression of signalling molecules in those second order neurons (Ji *et al.*, 1999). Hence, we studied the ability of afferent barrages to induce pS10H3 in DHSC. To do so, high frequency electrical stimulation (10Hz) was applied in the saphenous nerve, primary sensory fibres that innervate the hind paw, for five minutes.

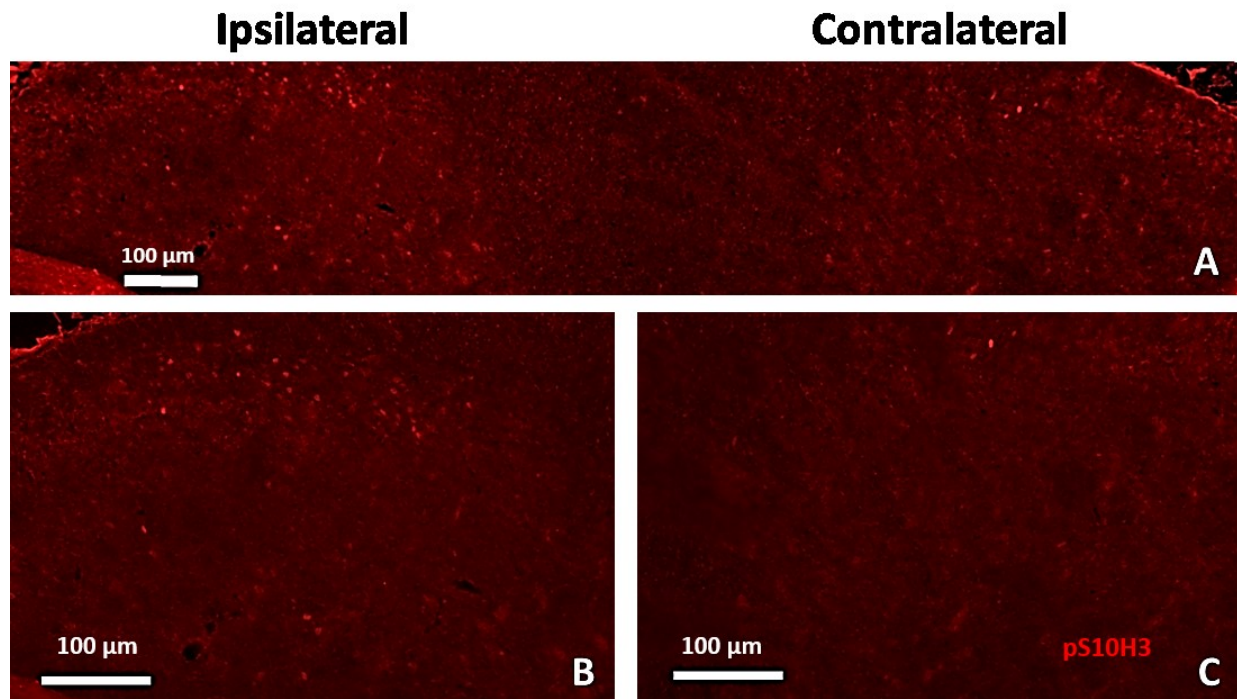


Figure 14.11. **pS10H3 induction in the DHSC after high electrical stimulation.** Red shows pS10H3 positive staining. The scale bars equal 100µm. Pictures A was taken at a lower magnification (5x) than pictures B and C (20x). The stimulation of the saphenous nerve by high frequency electrical stimulation (10 seconds stimulation at 10Hz and 10 seconds rest for up to 5 minutes) induced an increased expression of pS10H3 in neurons of the ipsilateral side mainly in the superficial laminae, although a minor population appeared at deeper layers.

Visual assessment of the staining showed an activation of pS10H3 in the superficial laminae of the spinal cord, together with few cells also positive for pS10H3 in deeper laminae of the ipsilateral side (Figure 14.11). This activation resulted in a significant up-regulation of pS10H3 positive neurons in the ipsilateral DHSC (control: 2.67 ± 1.45 , Mdn=3, n=3; ipsilateral: 72.33 ± 8.41 , Mdn=66, n=3; $p < 0.001$, GzLM; figure 3.12) that was not present in the contralateral side (contralateral: 6.67 ± 3.48 , Mdn=6, n=3, $p = 0.313$, GzLM, Figure 14.12).

In addition, the number of neurons positive for pERK1/2 was significantly increased in both ipsi- (control: 5.33 ± 1.86 , Mdn=4, n=3; ipsilateral: 93.33 ± 6.39 , Mdn=92, n=3; $p < 0.001$, GzLM) and contra-lateral (contralateral: 16.67 ± 1.86 , Mdn=18, n=3, $p = 0.004$, GzLM) DHSC after high frequency electrical stimulation (Figure 14.12).

However, this up-regulation was significantly higher in the ipsilateral side than in the contralateral ($p<0.001$, GzLM; Figure 14.12).

When the electrical stimulation was done at a low frequency to only stimulate myelinated primary afferent fibres, no increase on pS10H3 positive neurons was detected in either the ipsilateral or contralateral sides of the spinal cord (ipsilateral: 2.33 ± 1.2 , Mdn=3, $n=3$, $p=0.933$; contralateral: 1.67 ± 0.88 , Mdn=2, $n=3$, $p=0.801$; GzLM; figure 3.12). Further, a significant up-regulation of pERK1/2 was still detected in the ipsilateral DHSC after this low frequency electrical stimulation (ipsilateral: 14.00 ± 1.00 , Mdn=15, $n=3$, $p=0.029$, GzLM, Figure 14.12) although its numbers were significantly smaller than those observed after high frequency stimulation ($p<0.001$, GzLM).

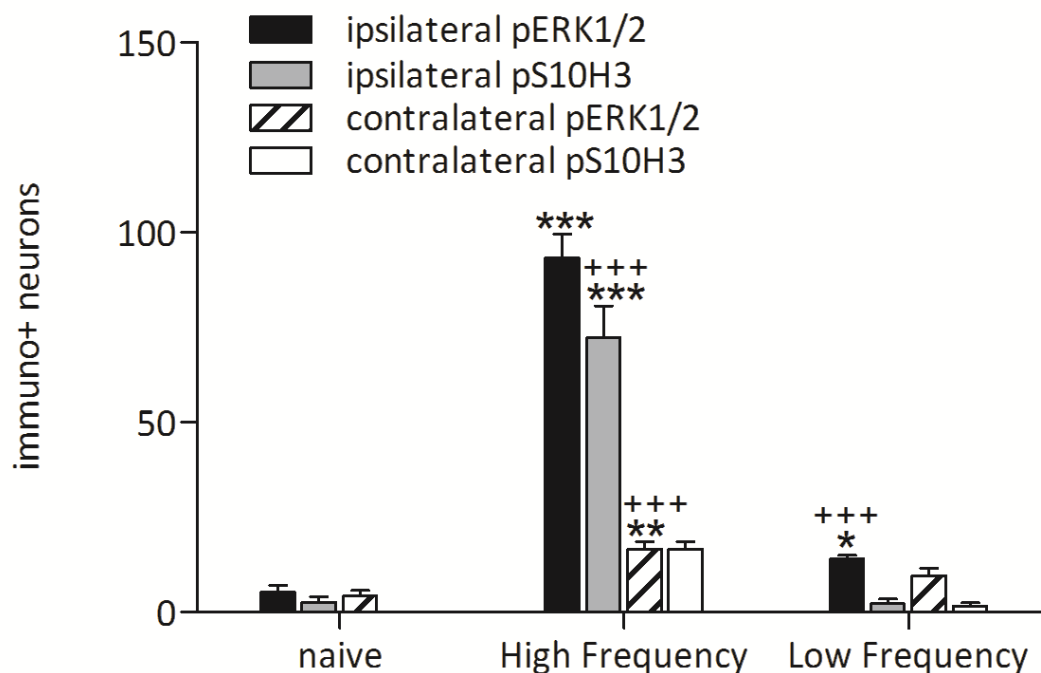


Figure 14.12. **immunolabelled pS10H3 and pERK1/2 neurons in the DHSC following electrical stimulation of the saphenous nerve.** The data is presented as mean+SEM. The experimental groups ($n=3$ each) were naive animals (naive), electrical stimulation of the saphenous nerve by 10 seconds at 10Hz and 10 seconds rest for up to 5 minutes (High Frequency), and continuous electrical stimulation at 0.1Hz for 5 minutes (Low Frequency). * $p<0.05$ vs. naive ipsilateral pERK1/2; ** $p<0.01$ vs. naive ipsilateral pERK1/2; *** $p<0.001$ vs. naive ipsilateral pERK1/2; +++ $p<0.001$ vs. High Frequency ipsilateral pERK1/2.

Following these findings and based on the higher degree of co-expression between pS10H3 and pERK1/2 (see above within this chapter and Chapter 13), it was decided to study the effect of blocking NMDA receptor, the activation of which results in ERK1/2 activation, (Ji *et al.*, 1999)) using the NMDA receptor blocker, (2R)-amino-5-phosphonovaleric acid (D-APV), after electrical stimulation on an *in vitro* set-up (Szucs *et al.*, 2009). Hence, a collaborator induced the electrical stimulation on L4-L5 in intact spinal cord preparations (*in vitro*) via suction of L4-L5 dorsal roots in the absence or presence of D-APV.

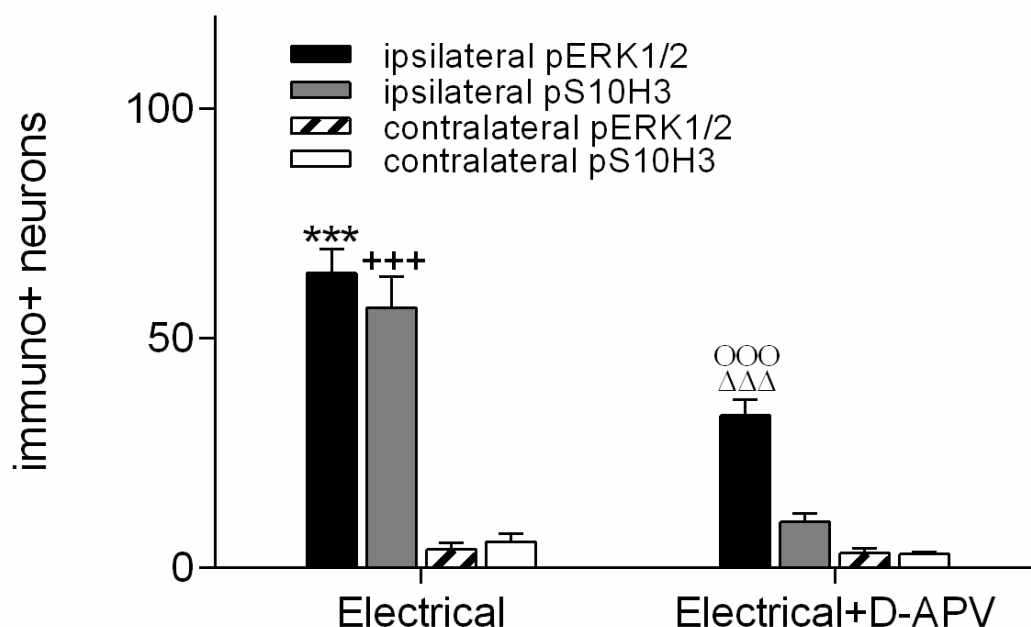


Figure 14.13. **immunolabelled pS10H3 and pERK1/2 neurons in spinal cord sections following an *in vitro* model of electrical stimulation.** The data is presented as mean+SEM. The experimental groups spinal cords sections electrically stimulated (Electrical; see Material and Methods sections for details) and sections equally stimulated but in presence of D-APV, a blocker of NMDA receptors (Electrical+D-APV). *** $p < 0.001$ vs. Electrical contralateral pERK1/2; +++ $p < 0.001$ vs. Electrical contralateral pS10H3; OOO $p < 0.001$ vs. Electrical+D-APV contralateral pERK1/2; $\Delta\Delta\Delta$ $p < 0.001$ vs. Electrical ipsilateral pERK1/2.

In agreement with the data from electrical stimulation *in vivo*, pS10H3 expressing neurons were increased in the ipsilateral DHSC after the electrical stimulation (ipsilateral: 56.75 ± 6.70 , Mdn=57.5, $n=4$; contralateral: 5.75 ± 1.80 , Mdn=4.50, $n=4$;

$p < 0.001$, GzLM). Similarly, an increase on pERK1/2 was detected in the ipsilateral DHSC (ipsilateral: 64.25 ± 5.30 , Mdn=63, $n=4$; contralateral: 4.00 ± 1.41 , Mdn=3, $n=4$; $p < 0.001$, GzLM, Figure 14.13). Nonetheless, a complete loss of pS10H3 was detected in the ipsilateral DHSC when D-APV was present during the *in vitro* electrical stimulation (ipsilateral: 10.00 ± 1.96 , Mdn=9.5, $n=4$, contralateral: 3.00 ± 0.41 , Mdn=3, $n=4$, $p=0.097$; GzLM,). Although up-regulation of pERK1/2 in the ipsilateral DHSC after electrical stimulation with the presence of D-APV were still significant (ipsilateral: 33.25 ± 3.42 , Mdn=31.5, $n=4$; contralateral: 3.25 ± 1.11 , Mdn=3, $n=4$; $p < 0.001$, GzLM), there was a reduction of ~50% when compared with the number without D-APV ($p < 0.001$, GzLM, Figure 14.13).

14.4.7 Different PTM on histone H3 at DHSC tissues after 5 minutes of a noxious stimulation

With the purpose of identify other PTM and decipher the histone code present in neurons from DHSC after an inflammatory response, histone extracts of 5-minutes capsaicin and naïve animals (same ones used for western blotting –see above) were run in duplicates on a multiplex assay to check for differential histone H3 modifications (Figure 14.14).

The study of the percentage of relative change of each PTM (Figure 14.14 A) showed an increase on: mono- and tri-methylation of lysine 27 (mK27H3 and 3mK27H3) and lysine 79 (mK79H3 and 3mK79H3), di-methylation of lysine 36 (2mK36H3), acetylation of lysine 14 (aK14H3) and lysine 56 (aK56H3) and phosphorylation of serine 28 (pS28H3). However, the kit failed to detect the increase on pS10H3, even showing a decrease in its percentage of relative change.

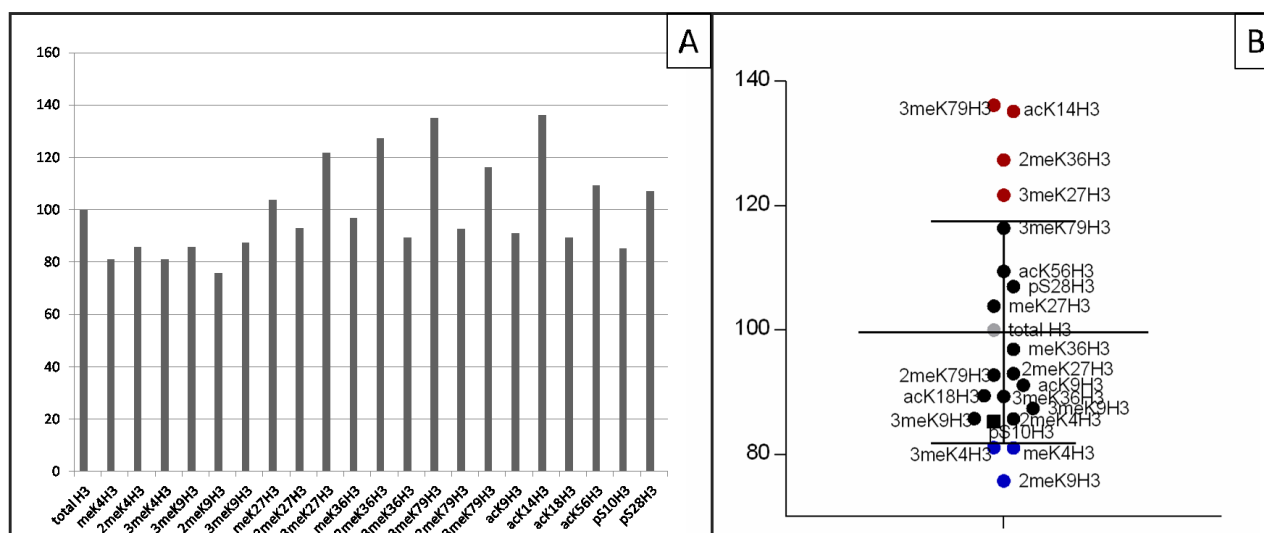


Figure 14.14. **Differences on histone H3 PTM between ipsilateral DHSC of capsaicin injected animals and controls.** Animals either treated with an s.c. capsaicin (25 μ L of 3 mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period or kept in their cages until the end of experiment (control). Samples were processed in duplicate with the EpiQuik Histone H3 Modification Multiplex Assay Kit (Epigentek). **A)** Percentage of relative change of each histone H3 PTM between control and capsaicin samples; if the bar reaches 100 means that there is an increase after stimulation for that specific PTM. **B)** Plotted percentages with mean $\pm$ StandardDeviation. The values outside this range were regarded as significant.

Other PTM exhibited a decrease on their percentage of relative change: mono-, di- and tri-methylation of lysine 4 (mK4H3, 2mK4H3 and 3mK4H3) and lysine 9 (mK9H3, 2mK9H3 and 3mK9H3), di-methylation of lysine 27 (2mK27H3) and lysine 79 (2mK79H3), mono- and tri-methylation of lysine 36 (mK36H3 and 3mK36H3) and acetylation of lysine 9 (aK9H3) and lysine 18 (aK18H3).

Finally, these percentages were plotted together in a graph with the mean $\pm$ Standard Deviation (Figure 14.14 B). The values outside this range were considered outliers and thus regarded as significantly different from the percentage of relative change of total histone H3. For this analysis, I consider that 3mK79H3, aK14H3, 2mK36H3 and 3mK27H3 were up-regulated after 5 minutes of the capsaicin injection in the DHSC while mK4H3, 3mK9H3 and 2mK9H3 were down-regulated following this noxious stimulation.

14.5 Discussion

These findings describe pS10H3 as a novel epigenetic marker occurring in a sub-population of superficial DHSC following inflammation induced by capsaicin and after electrical stimulation with high frequency of C-fibres. This up-regulation was observed both *in vivo* and *in vitro*. In contrast, low frequency electrical stimulation or brief noxious stimulations that do not induce tissue damage failed to up-regulate this epigenetic covalent modification.

14.5.1 Capsaicin application induces pS10H3 at the superficial DHSC

As observed after the scalding type burn injury model (see previous chapter), there is an up-regulation of pS10H3 at the superficial DHSC following a subcutaneous injection of capsaicin at hind paw's plantar surface of Sprague-Dawley rats.

Furthermore, the dynamics of S10H3 phosphorylation and activation of ERK1/2, a MAPK implicated on nociception and signalling molecule implicated in S10H3 phosphorylation (Chadee *et al.*, 1999; Cheung *et al.*, 2000b; Ji & Woolf, 2001; Davie, 2003; Black *et al.*, 2008; White *et al.*, 2011a; White *et al.*, 2011b; Edelmayer *et al.*, 2014), are also different. The increase of pS10H3 expression is detected as early as 5 minutes and persists after half an hour (the last time point studied) and pERK1/2 also peaks at 5 minutes of the simulation. However, pERK1/2 is de-phosphorylated much quicker than pS10H3. This difference suggests differential kinetics between the markers. Importantly, pERK1/2 data is similar at what Ji and collaborators observed in 1999 (Ji *et al.*, 1999). In their study, capsaicin produced an activation of this MAPK at 2-5 minutes after its injection that gradually returned to basal values (Ji *et al.*, 1999).

As observed both *in vivo* and *in vitro*, the sub-population of neurons with pS10H3-positive nuclei are distributed in the superficial layers, where fibres from primary nociceptive neurons terminate. Importantly, most of these positive neurons are seen in close proximity to endings of those primary neurons. These findings are similar to those observed with the burn injury model in the previous chapter (see previous chapter). Hence, it is reasonable to assume that, at least, a great proportion of them are second order neurons.

MSK1/2 are situated downstream of MAPK (De Cesare *et al.*, 1998; Deak *et al.*, 1998; Davie, 2003; Dong & Bode, 2006), and are considered the main *writers* of pS10H3 following the activation of those (Thomson *et al.*, 1999a; Clayton *et al.*, 2000; Davie, 2003; Soloaga *et al.*, 2003; Dong & Bode, 2006). Accordingly, blocking MSK1 completely prevented the phosphorylation of S10H3 following capsaicin application. Thus, confirming the functional link between MSK1 and pS10H3.

The fact that immunostaining data revealed a much greater up-regulation than western blot is a consequence of the tissue heterogeneity of the spinal cord (Denk *et al.*, 2013; Bai *et al.*, 2015). It is not only composed by neurons, there are other cells in which this epigenetic mark could be potentially activated either by the same kinases, or by others such as Aurora family (Prigent & Dimitrov, 2003; Soloaga *et al.*, 2003) in a smaller amount and that is not detected by immunostaining. Thus, from a protein extract from the dorsal horn, in which all proteins from all cell types are combined, is more difficult to assess a difference that is only present in this small population of neurons from the superficial layers of the spinal cord. Accordingly, the western blot from total histone extracts showed a much smaller difference due to “background pS10H3”.

14.5.2 Brief noxious stimuli do not induce pS10H3 in the spinal cord

Repeated brief stimulation with noxious temperature or pressure, with minimal or no tissue damage, failed to induce this epigenetic mark on the spinal cord. However, these types of noxious stimuli were able to up-regulate pERK1/2. This differential expression suggests that there are two populations of pERK1/2-positive neurons in response to either tissue injury/inflammation or brief noxious stimulation.

Similarly as Ji and collaborators, intense mechanical punctuate and hot stimulus induced pERK in the superficial DHSC at ~5 minutes of the application to the hind paw (Ji *et al.*, 1999). However, cold stimulus, which also increased pERK in their investigation (Ji *et al.*, 1999), did not caused such peak in the experiments described here. This discrepancy could be a consequence of the weaker activation caused by cold that did not reach significance here. Although Ji *et al.* do not report the number of positive cells following noxious heat, cold or pressure (Ji *et al.*, 1999), the images shown seem to describe a milder up-regulation after cold than the other two. Additionally, cold noxious stimulation is thought to only activate around 15% of the fibres, less than noxious heat stimulation (Simone & Kajander, 1997; Julius & Basbaum, 2001).

Furthermore, skin preparation showed intact skin structure after cold stimulus while some minor damage was observed following both mechanical and noxious heat stimulation. Hence, it is also possible that the cold protocol applied here was not successful enough inducing a noxious stimulation.

14.5.3 Induction of pS10H3 at superficial DHSC following noxious stimulation is a NMDA receptor-dependent process

The fact that A δ - and C-fibres stimulation induced this PTM while A β -fibre stimulation failed, although A β -fibres stimulation also activates different cells in the spinal cord, indicates that pS10H3 is a noxious specific event. Furthermore, pS10H3-positive neurons are restricted to a similar pattern as observed with the other types of noxious stimuli (see above). However, some few neurons in deeper laminae seem to get activated following the electrical input. These neurons could correspond to wide dynamic range (WDR) neurons, which normally respond to both innocuous and nociceptive stimuli (Polgar *et al.*, 2007; Marchand, 2008; Hunt, 2009; Todd, 2010; Steeds, 2013; Zhang *et al.*, 2014), that got recruited via polysynaptic pathways because of this intense electrical stimulation, although further research is required to confirm this suggestion.

The capsaicin-induced ERK activation is known to be a NMDA-dependent event (Ji *et al.*, 1999). Likewise, pS10H3 is also a NMDA-dependent process in rat pyramidal neurons (Mackowiak *et al.*, 2013). Accordingly, NMDA receptor blockade *prior* to the induction of electrical barrages activating A δ - and C-fibres (noxious stimulation) totally prevented the up-regulation of pS10H3 in the spinal cord neurons. Therefore, a functional link is proposed between NMDA receptors and pS10H3, most possibly throughout pERK1/2. Thus, pS10H3 seems to be a marker present in sensitised second order neurons following tissue injury/inflammation.

While NMDA receptor blockade totally inhibited the stimulus-evoked induction of pS10H3, the inhibition was partial for pERK1/2. This is in accordance with Ji's paper and supports the view that ERK can be recruited by other mechanism (Ji *et al.*, 1999).

14.5.4 Deciphering the epigenetic signature of superficial DHSC neurons following inflammation

pS10H3 regulates the transcription of IEG, an event that has also been observed on hippocampal neurons (Chadee *et al.*, 1999; Cheung *et al.*, 2000b; Davie, 2003; Bilang-Bleuel *et al.*, 2005; Chandramohan *et al.*, 2008; Sarantis *et al.*, 2012; Keum *et al.*, 2013). However, different histone modifications can occur simultaneously and interact dynamically between them to deliver a specific functional output (Cheung *et al.*, 2000a; Strahl & Allis, 2000; Rando, 2012). This “crosstalk” can affect recruitment and binding of other factors as well as jointly interfere with nucleosome compaction (Rea *et al.*, 2000; Strahl & Allis, 2000; Geranton & Tochiki, 2015b). Accordingly, a multiplex assay was performed to identify other PTM and decipher the histone code present in neurons from DHSC after an inflammatory response (capsaicin injection). Different epigenetic modifications were significantly up-regulated following this model: aK14H3, 2mK36H3, 3mK27H3, 3mK79H3. Acetylation of histone H3 at lysine 14 (**aK14H3**) is a PTM commonly found together with pS10H3. When present in the same histone, pS10H3 and aK14H3 act as regulated binding platforms for nuclear factors or increasing the stability of such bindings (Cheung *et al.*, 2000a; Cheung *et al.*, 2000b; Lo *et al.*, 2000; Strahl & Allis, 2000; Kim *et al.*, 2012; Ng & Cheung, 2016). Interestingly, both phosphorylation and acetylation neutralise positive charges of histone proteins and weaken the affinity between them and the DNA, which results in chromatin relaxation and facilitates the access of the transcriptional machinery (Cheung *et al.*, 2000b; Geranton & Tochiki, 2015b). Similarly, methylation of histone H3 at lysine 36 (**mK36H3**) has been observed in some brain areas following different learning paradigms, together with pS10H3 and aK14H3, and associated to promoters of pro-memory formation genes, including *bdnf*, *zif268* and *homer1* (Kim

et al., 2012; Sando *et al.*, 2012; Rahn *et al.*, 2013; Geranton & Tochiki, 2015b). Trimethylation of histone H3 at lysine 79 (**3mK79H3**) has also been detected at transcribed regions of active genes of yeast, fruit flies, mice and human and has been implicated in different processes such as regulation of telomeric silencing, cellular development (including generation of cortical projection neurons), cell-cycle checkpoint, DNA repair, and transcriptional regulation (Buttner *et al.*, 2010; Farooq *et al.*, 2016). Furthermore, methylation of this residue has also been indirectly implicated in spinal muscular atrophy through malfunction of the *reader* of this modification, the protein survival motor neuron (SMN) (Sabra *et al.*, 2013). On the contrary, tri-methylation of histone H3 at lysine 27 (**3mK27H3**) is a mark related to facultative heterochromatin (Borrelli *et al.*, 2008; Geranton & Tochiki, 2015b; Guiretti *et al.*, 2016), which suggest that this noxious model is also inducing silencing of some other genes.

Other PTM were significantly down-regulated in response to the capsaicin application: mK4H3 and 3mK4H3, and 2mK9H3. Methylation of lysine 9 from histone H3 (**mK9H3**, either mono-, di- or tri-methylated) is associated to transcriptionally inactive areas, mainly constitutive heterochromatin (Jenuwein & Allis, 2001; Guiretti *et al.*, 2016). It facilitates the association of heterochromatin protein 1 (HP1), which has greater affinity once more methyl groups are added to this lysine (Meaney & Ferguson-Smith, 2010). Furthermore, mK9H3 has a potential inhibitory role on pS10H3 (Cheung *et al.*, 2000a) and vice versa, in a mechanism referred to as the *methyl/phos switch*: the HP1 protein binds to mK9H3 to promote gene condensation, however this binding is regulated by the adjacent pS10H3 (Rea *et al.*, 2000; Jenuwein & Allis, 2001; Prigent & Dimitrov, 2003; Borrelli *et al.*, 2008; Rando, 2012; Keum *et al.*, 2013; Chen *et al.*, 2015). Similarly, this methylation is mutually exclusive

to acetylation of the same amino acid, aK9H3 (Rea *et al.*, 2000; Agalioti *et al.*, 2002). For example: in studies using PC12 cells, the application of NGF and subsequent induction of the δ -opioid receptor correlates with aK9H3 (and reversal of 3mK9H3) (Agalioti *et al.*, 2002; Geranton, 2012). Therefore, it is not surprising to find a decrease of this modification in a sample where pS10H3 and aK14H3 were up-regulated. Interestingly, methylation of this residue has indirectly been linked to pain through mutations in PRDM12, a transcriptional regulator needed to recruit a methyltransferase that selectively demethylates histone H3 at lysine 9. Missense mutations on PRDM12 led to defects on nociceptors' development and thus causes Congenital Insensitivity to Pain (CIP) (Chen *et al.*, 2015). On the contrary, methylation of histone H3 at lysine 4 (**mK4H3**) seems to be facilitated if the histone tail is previously acetylated, for example at aK14H3, leading to addition of more methyl groups, **3mK4H3**. (Jenuwein & Allis, 2001). Accordingly, these two covalent modifications (aK14H3 and 3mK4H3) had been observed in combination at promoters of genes actively transcribed (Jenuwein & Allis, 2001; Borrelli *et al.*, 2008; Geranton & Tochiki, 2015b; Guiretti *et al.*, 2016). Hence, the decrease of this methylation is a striking finding.

Importantly, pS10H3, the epigenetic mark of interest, was not found significantly up-regulated following the capsaicin injection, which contradicts previous data from immunostaining and western blotting (see above). This could be due to the small number of cells that express this modification. The histone extracts used to perform this experiment contained a mixed population of different cells, and DNA methylation and histone modification patterns are known to be very cell-type specific (Thomson *et al.*, 2001; Lefebvre *et al.*, 2002; Denk & McMahon, 2012; Geranton, 2012). Accordingly, acetylation of different histone H3 residues has been regarded as a

more general process and not limited to neurons (Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2008; Sarantis *et al.*, 2012). Even some researchers suggest that pS10H3, more sparsely present, could be serving as a rate-limiting step as well as a signal integrator for chromatin remodelling (Cheung *et al.*, 2000b; Bilang-Bleuel *et al.*, 2005; Keum *et al.*, 2013). pS10H3 could be facilitating fast and directed recruitment and activation of the transcriptional machinery to selected promoters, including proteins with HAT activity (Saccani *et al.*, 2002). Thus, phosphorylation seems to be affecting a minor population of nucleosomes that then help shift the balance of HAT and HDAC activity within those genes (Thomson *et al.*, 2001; Bilang-Bleuel *et al.*, 2005). For example: retinoid agonist stimulation of mammalian cultures is able to induce fast pS10H3 at the promoter of the mRAR β 2 gene although the induction of acetylation remains unchanged (Lefebvre *et al.*, 2002).

14.5.5 Conclusion and future work

The data presented here, together with the previous chapter, identifies pS10H3 in a sub-population of superficial DHSC following primary nociceptor activation by burn injury, capsaicin injection or high frequency electrical activation of corresponding nociceptive fibres. This dynamic up-regulation is an NMDA receptor-dependent process that occurs in apparently second order neurons. The major pathway leading to this epigenetic modification could be through pERK1/2, which then activates MSK1 as the principal kinase *writing* pS10H3. In contrast, repeated brief stimulation with noxious temperature (heat or cold) or pressure, with minimal or no tissue damage, did not induce this epigenetic mark on the superficial DHSC neurons.

Hence, pS10H3 could be considered as a novel mark present in sensitised second order neurons following tissue injury/inflammation.

Central sensitization describes an activity-dependent process that results in increased excitability of spinal cord neurons, which, in part, is a consequence of transcriptional changes in these neurons (Ji *et al.*, 2003). pS10H3 seems to regulate the transcriptional activation of a subset of IEG, including c-fos, c-jun , c-myc, cox-2 and zif-268 (Chadee *et al.*, 1999; Cheung *et al.*, 2000b; Davie, 2003; Bilang-Bleuel *et al.*, 2005; Chandramohan *et al.*, 2008; Sarantis *et al.*, 2012; Keum *et al.*, 2013), implicated in processes of neuronal plasticity. Hence, pS10H3 in the superficial DHSC after noxious stimulations could be regulating the expression of some of these genes and thus contributing to acute pain hypersensitivity and the development of persistent pain. Therefore, future research should aim to study those transcriptional changes and the implication of pS10H3, among other histone PTMs, on them as well as testing its blockade as a novel therapeutic target.

15 Nociceptive-specific induction of pS10H3 in DRG neurons

15.1 Background

At present, persistent pain represents an unmet medical need (Breivik *et al.*, 2006). Partially, it is the result of a dysfunctional process of increased responsiveness known as peripheral sensitization in which the sensory fibres get their activity tuned-up (Hunt & Mantyh, 2001; Ji *et al.*, 2003; Marchand, 2008; Costigan *et al.*, 2009; Steeds, 2013). Peripheral sensitization is a use-dependent plasticity process that involves intracellular signalling pathways and leads to altered production, transport and membrane insertion of different channels (Ji *et al.*, 2003; Costigan *et al.*, 2009; White *et al.*, 2011a).

Nociceptors are the sensory fibres that respond to noxious stimuli. They connect peripheral tissues, where terminate as free endings, with the spinal cord (Iggo, 1960; Woolf *et al.*, 2004; Patel, 2010; Woolf, 2011; Steeds, 2013). Their soma is collected together at dorsal root ganglia (DRG), pseudounipolar neurons with an axon that is divided in two branches: one towards the periphery and the other centrally directed that synapses with second order neurons at the superficial spinal cord (Hunt & Mantyh, 2001; Julius & Basbaum, 2001). Most nociceptors are able to respond to different stimulation modalities, such as noxious mechanical, thermal ($>42^{\circ}\text{C}$ and $<10^{\circ}\text{C}$) or chemical (Hunt & Mantyh, 2001; Woolf *et al.*, 2004; Marchand, 2008).

The vanilloid receptor TRPV1 is present in a great proportion of small- to medium-size primary neurons (all C fibres and some A δ). It is a nonselective cation channel that can be activated by noxious heat (threshold $\sim 44^{\circ}\text{C}$), capsaicin and acidic pHs, among others (Caterina *et al.*, 1997; Julius & Basbaum, 2001; Zhuang *et al.*, 2004; Coutaux *et al.*, 2005; Hunt, 2009; Nagy *et al.*, 2014). Interestingly, the expression of TRPV1 at nociceptors increase following peripheral inflammation, which contributes to the enhanced sensitivity (Woolf *et al.*, 2004; Green *et al.*, 2013). Contrarily, cold

noxious stimulus (4⁰C) is sensed by other mechanism involving the generation of depolarizations by blocking the maintenance of the Na⁺ and K⁺ balance or by actively promoting influx of Ca²⁺ and/or Na⁺ (Simone & Kajander, 1997; Julius & Basbaum, 2001). The transmission of noxious mechanical stress, on the contrary, is still not completely understood and seems to involved different candidates such as BNC1 (also known as MDEG and ASIC2), TRPV4, Piezo2 or G-protein coupled receptors (e.g. P2Y) (Julius & Basbaum, 2001; Raouf *et al.*, 2012; Ranade *et al.*, 2014; Viatchenko-Karpinski & Gu, 2016).

Epigenetic mechanisms, such as histones' PTM, play an important role in pain development by controlling the accessibility of the DNA to be replicated and thus are able to govern gene transcription (Geranton *et al.*, 2007; Chiechio *et al.*, 2009; Bai *et al.*, 2010; Zhang *et al.*, 2011; Denk & McMahon, 2012; Geranton, 2012; Liu *et al.*, 2012; Crow *et al.*, 2013; Denk *et al.*, 2013; Rahn *et al.*, 2013; Bai *et al.*, 2015; Chen *et al.*, 2015; Crow *et al.*, 2015; Geranton & Tochiki, 2015b; a). Phosphorylation of histone H3 at serine 10 (pS10H3) is a transcriptionally permissive PTM associated to a subset of IEG (Dong & Bode, 2006; Keum *et al.*, 2013). This epigenetic covalent modification has previously been observed in neurons of the CNS, where is implicated in processes of memory consolidation (Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008; Sarantis *et al.*, 2012; Rotllant *et al.*, 2013; Crow *et al.*, 2015). Although not completely understood, the pathway leading to pS10H3 in neurons seems to be NMDA receptor-dependent, implicates MAPK pathways (ERKs) and corresponding downstream kinases as the effectors *writing* this modification (Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008; Mackowiak *et al.*, 2013). Interestingly, some of those effector kinases seem to also be responsible for

phosphorylation of CREB (pCREB) (Xing *et al.*, 1996; De Cesare *et al.*, 1998; Deak *et al.*, 1998; Thomson *et al.*, 1999b; Lo *et al.*, 2000; Soloaga *et al.*, 2003), a cellular transcription factor implicated on pain processing (Ji *et al.*, 2003; Kawasaki *et al.*, 2004; Bie *et al.*, 2011; Li *et al.*, 2014). Due to these facts and the similarities between memory formation and other use-dependence processes such as peripheral sensitization (Ji *et al.*, 1999; Julius & Basbaum, 2001; Ji *et al.*, 2003; Marchand, 2008; Hunt, 2009; Denk & McMahon, 2012; Geranton, 2012; Rahn *et al.*, 2013; Steeds, 2013; Geranton & Tochiki, 2015b), pS10H3 seems a good candidate neuronal epigenetic modification contributing to nociceptors enhanced sensitivity by noxious stimulation.

15.2 Aims

The **aims** of this chapter are:

- To investigate whether pS10H3 get up-regulated in DRG neurons following different noxious stimuli other than burn injury.

And if so:

- To assess if the induction of this PTM mark is a stimulus-dependent process
- To assess the effector kinase responsible to this up-regulation of pS10H3 at DRG neurons.

15.3 Material and Methods

Most of the experiments done here are the same as described in the precedent chapter (Chapter 12) but referred to the collection of DRG instead of spinal cord. Therefore, a short description is given here.

15.3.1 *In vivo* capsaicin injection

Sodium pentobarbital-anaesthetised rodents received an intraplantar injection of capsaicin (25µL of 3mg/mL dissolved in 10% Tween 80; Sigma, M2028) and two minutes of gentle stroke. Following either 5 or 30 minutes (n=13 and n=3 respectively), rats were terminally anaesthetised and either transcardially-perfused and tissues processed for immunostaining or decapitated and tissues processed for western blotting. Naïve (n=13) and saline (n=3) injected animals were also used as negative and sham controls respectively.

Following perfusion, L4 and L5 DRG were sectioned and processed for **immunostaining** (n=3 for each group, including control, sham, 5 minutes capsaicin injection and 30 minutes capsaicin injection). Slides were incubated overnight at 4°C using primary antibodies against pS10H3 (Santa Cruz, SC8656R, [1:750]), pCREB (Cell Signalling, 9198, [1:5000]) and TRPV1 (Neuromics, GP141000, [1:1500]), and revealed using standard immunofluorescence technique except for pS10H3 that was further amplified using Tyramide Signal Amplification. To validate the technique, corresponding negative and positive controls were done in parallel. Statistical differences were calculated from the resulting microphotographs using the GzLM, and regarded as significant at $p<0.05$.

For **western blotting**, ipsi- and contra-lateral L4 and L5 DRG from animals subjected to either capsaicin injection with 5 minutes post-injury period or controls

were combined in pairs (2 animals with same treatment, $n=3 \times 2$ in each group) to increase the amount of histone in the corresponding protein extracts. Resulting samples were then separated by electrophoresis using NuPAGE Novex 12% Bis-Tris Protein Gels (Invitrogen, NP0342BOX), electrotransferred to a PVDF membranes, immunolabelled against pS10H3 (1:500) and β actin (Millipore, MAB1522, [1:2000]), reacted with chemiluminescence, stripped and reacted again against pan-histone H3 (Millipore, 07-690, [1:4000]). The obtained densitometry values were normalised and used to assess the ratios for each animal (intensity in the ipsilateral side divided by the contralateral). Statistical differences were assessed using the independent *t*-test and regarded as significant at $p < 0.05$. For further validation, a duplicate gel was run in parallel inverting the order of immunoreactions.

To increase the amount of histone in the samples, ipsi- and contra-lateral L4 and L5 DRG from control ($n=4$) or 5 minutes capsaicin animals ($n=4$) were collected and processed for total histone extraction (Epigentek, OP-0006-100) as suggested by the manufacturer, and resulting extracts were processed for western blotting as explained above.

15.3.2 *In vitro* capsaicin application

This procedure was performed with the help of Miss Sara Beattie, as part of her BSc project and under my laboratory supervision. Rats maintained in baseline conditions were terminally anaesthetised, decapitated and DRG collected for culturing ($n=9$). Once cultures were established, capsaicin was used as activator of TRPV1, capsazepine (Sigma, C191) as blocker of TRPV1 activation and SB747651A dihydrochloride (Tocris, 4630) as blocker of MSK1 activity.

Group 1: half of the cultured DRG dishes of 3 animals (n=3) were treated with 5 μ M of capsaicin in extracellular buffer for 5 minutes. And the other half used as controls (extracellular buffer). Following corresponding procedure, cultures were scraped and mixed with 300 μ L RIPA buffer for immunoprecipitation and posterior western blot, except one of each that was fixed and processed for immunostaining as explained above.

Group 2: the cultured DRG of other 3 animals (n=3) were pre-treated with 5 μ M capsazepine in extracellular buffer for 2 minutes before the incubation with 5 μ M of capsaicin and 5 μ M capsazepine in extracellular buffer for 5 minutes, while half of the wells from each animal were only treated with capsazepine (corresponding controls). Cultures were finally processed for immunostaining.

Group three: half of the cultured wells of 3 additional animals (n=3) were pre-treated with 3 μ M of SB747651A dihydrochloride in extracellular buffer for two minutes and incubated with 5 μ M of capsaicin together with 3 μ M of SB747651A dihydrochloride in extracellular buffer for 5 minutes. The corresponding treatment control (the other half of the wells) consisted on incubating only with the MSK1/2 inhibitor. At the end of the procedure, cultures were processed for immunostaining.

For immunoprecipitation, samples from group one were incubated overnight at 4°C with the pS10H3 primary antibody (2 μ g per 500 μ g of protein extract) and with A/G Plus Agarose (Santa Cruz, sc-2003) to isolate and concentrate the protein of interest from the sample. Resulting extracts were used to perform western blot as stated above. Due to lack of housekeeping proteins as a consequence of immunoprecipitation, band corresponding to the immunoglobulin G was used to standardize the ratios. Student's T-test was performed with normalized densitometry ratios.

15.3.3 Noxious thermal and mechanical *in vivo* models

Cold (4°C) or hot (45°C) stimuli were produced by six immersions of one hind paw in water at corresponding temperature for periods of 10 seconds with 10-second gaps. The final post-injury period was 5 minutes. Following treatment, animals (n=3 for each treatment) were processed for immunostaining as explained above.

Mechanical stimulation (n=3) was performed with the help of Dr. Boton Gaal, from University of Debrecen, and the complete procedure is explained in the resulting paper (already accepted at *Scientific Reports*). Briefly, one hind paw of anaesthetised animals was exposed to dorso-plantar compression (6N/cm²) for periods of 10 seconds with 10-second gaps in between for a total of 2 minutes. After 5 minutes of the initial procedure, rats were also processed for immunostaining (see above).

15.4 Results

In chapter 13 I argued that pS10H3 could be a novel marker of activity following burn injury in DHSC and DRG, and in chapter 12 we studied its expression in DHSC following capsaicin injection and other noxious stimuli.

Here, in this chapter, the expression of pS10H3 is assessed in DRG following capsaicin injection and some other noxious stimuli. The increase of pS10H3 in DRG after exposure to this neurotoxin was studied using western blotting and immunostaining, and using both *in vivo* and *in vitro* set-ups. Further, I evaluated pS10H3 co-expression with TRPV1 and its relationship with TRPV1, pCREB and MSK1/2.

15.4.1 pS10H3 expression in L4-L5 DRG following capsaicin injection

Histone total extracts from ipsi- and contra-lateral DRG of animals under baseline conditions (control) and 5 minutes following a capsaicin injection in the hind paw were used for western blotting against pS10H3. In agreement with previous data from DHSC samples (Chapter 12), a positive band was detected at ~15KDa corresponding to pS10H3 or total histone H3 (Figure 15.1). The study of the resulting densitometry ratios revealed a significant up-regulation of pS10H3 in the ipsilateral DRG within 5 minutes of the capsaicin injection (capsaicin: 2.42 ± 0.5 , $n=4$; control: 1.01 ± 0.07 , $n=4$; $p=0.03$, student's t-test; Figure 15.1 B). However, this increase failed to achieve a statistical meaning ($p=0.15$, student's t-test) on western blotting analysis when using total protein extracts of DRG from animals pooled together (two in each lane; control: 1.04 ± 0.04 , $n=3$; capsaicin: 1.53 ± 0.27 , $n=3$; $p=0.149$, student's t-test).

Next, L4 and L5 DRG sections from animals under control conditions, animals injected with saline in the hind paw (sham) and after 5 minutes or 30 minutes of a capsaicin injection were immunolabelled against pS10H3 to further validate this finding. A group of neurons positive for pS10H3 was detected in the ipsilateral DRG after 5 minutes of the capsaicin injection, which remained following 30 minutes of the injection (Figure 15.2). Statistical analysis of the percentage of neurons with positive nuclei for pS10H3 from the total number of neurons counted revealed a significant increase in the ipsilateral DRG after 5-minute post-injury (control: 0.00 ± 0.00 , Mdn=0, n=3; 5 min: 14.83 ± 5.88 , Mdn=13.54, n=3; $p < 0.001$, GzLM; Figure 15.3). At 30-minute post-injection, the up-regulation of pS10H3 positive nuclei in the ipsilateral side was still considerable (30 min: 9.55 ± 2.93 , Mdn=8.7, n=3, $p < 0.001$, GzLM) in spite of being significantly minor to that assessed after 5 minutes ($p = 0.019$, GzLM). No significant difference on the percentage of neurons with nuclei positive for pS10H3 was observed in the ipsilateral DRG of sham-injured animals (sham: 0.37 ± 0.37 , Mdn=0, n=3, $p = 0.868$, GzLM) or in the contralateral DRG of any group of animals studied (naïve: 0.00 ± 0.00 , Mdn=0, n=3, $p = 1$; sham: 0.51 ± 0.25 , Mdn=0.71, n=3, $p = 0.821$; 5 min: 0.46 ± 0.23 , Mdn=0.68, n=3, $p = 0.838$; 30 min: 1.57 ± 0.51 , Mdn=1.7, n=3, $p = 0.483$; GzLM; Figure 15.3).

Similar to the burn-injury studies (Chapter 13), the same analysis was done with the percentage of cells with positive staining for pS10H3, not only nuclear, revealing a similar pattern as described above (data not shown).

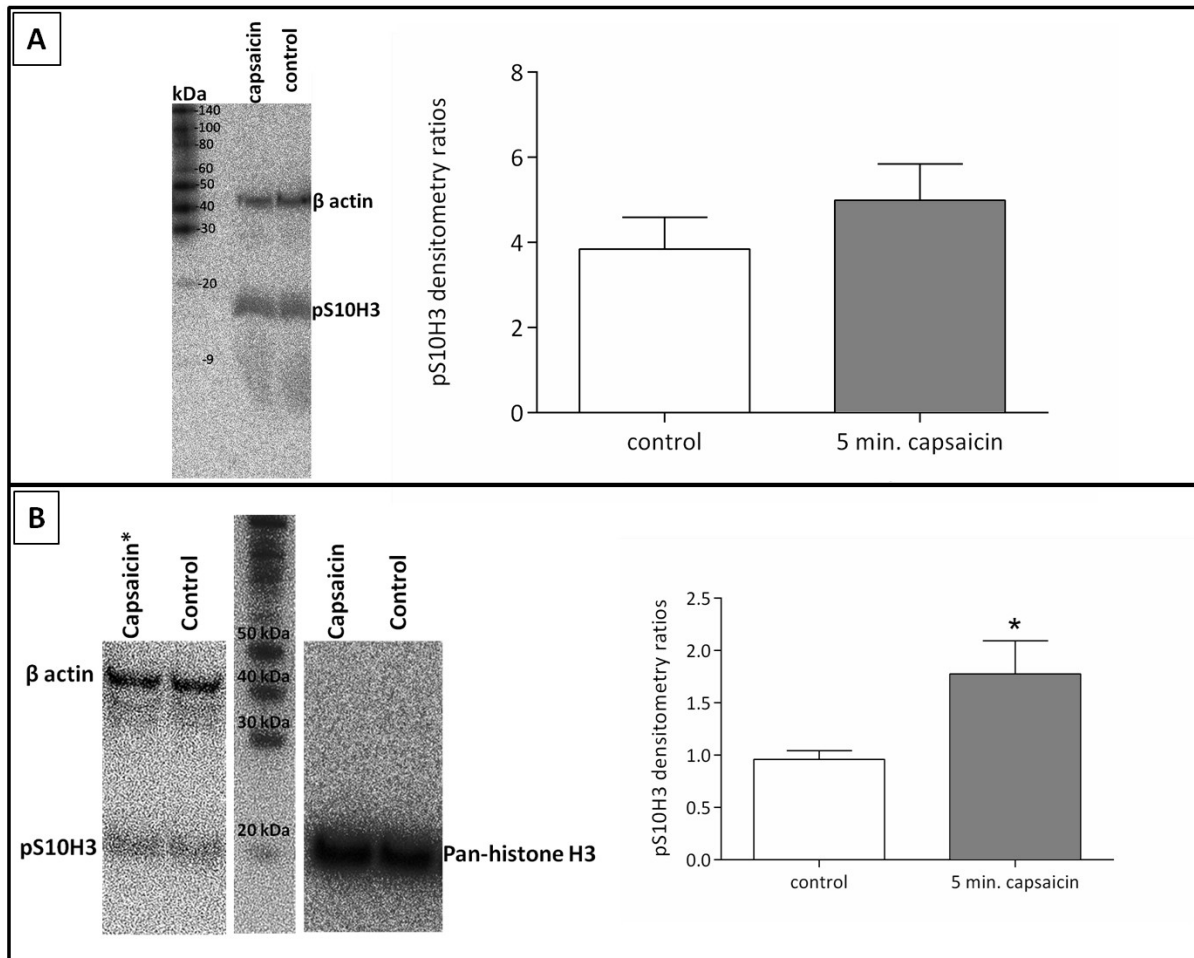


Figure 15.1. **pS10H3 increases in the ipsilateral DRG after capsaicin injection.** All animals were treated by s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period. **A)** Representative western blot of DRG from protein extracts of ipsi- and contra-lateral sides of two combined animals (n=3) after capsaicin injection; graph shows the normalized densitometry ratios. Data is presented as mean+SEM. T test was performed with normalized densitometry ratios. Note that the increase did not reach significance, with p=0.12. **B)** Representative western blot of histone extracts from ipsi- and contra-lateral sides (n=4). First stained for pS10H3 (left), stripped and then reacted for pan-histone H3 (right); graph shows the normalized densitometry ratios. Data is presented as mean+SEM. T test was performed with normalized densitometry ratios. * p<0.05

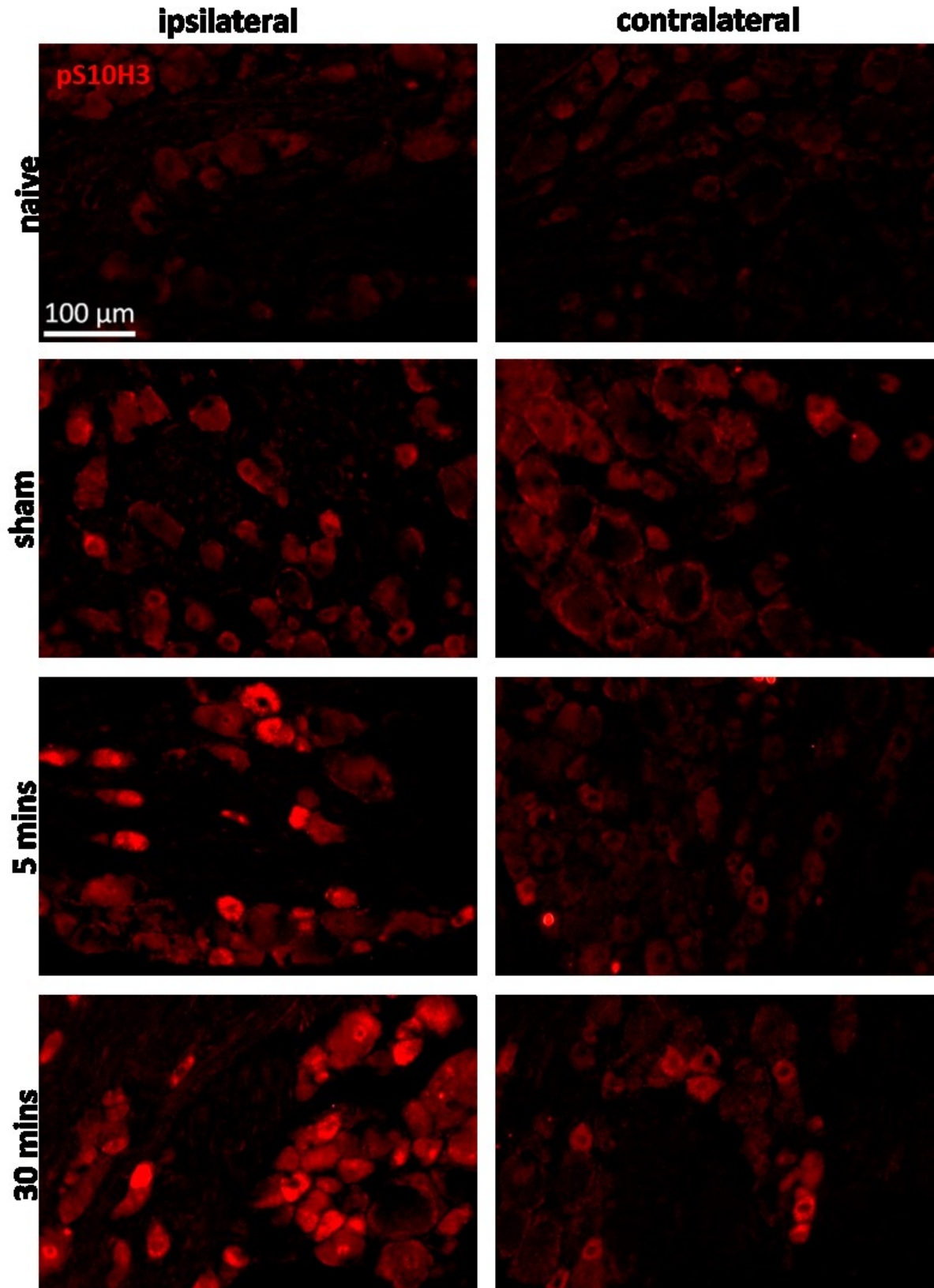


Figure 15.2. Time course of pS10H3 induction in L4 and L5 DRG after capsaicin injection model. In all pictures, red is for pS10H3. The scale bar equals 100μm. The experimental groups (n=3 each) were naive animals (naive), s.c 25μL saline injection in the hind paw and 5 minutes post-injection period (sham), s.c. capsaicin (25μL of 3mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period (5 minutes), and s.c. capsaicin (25μL of 3mg/mL) injection in the hind paw with 2 minutes stroke and 30 minutes post-injection period (30 minutes).

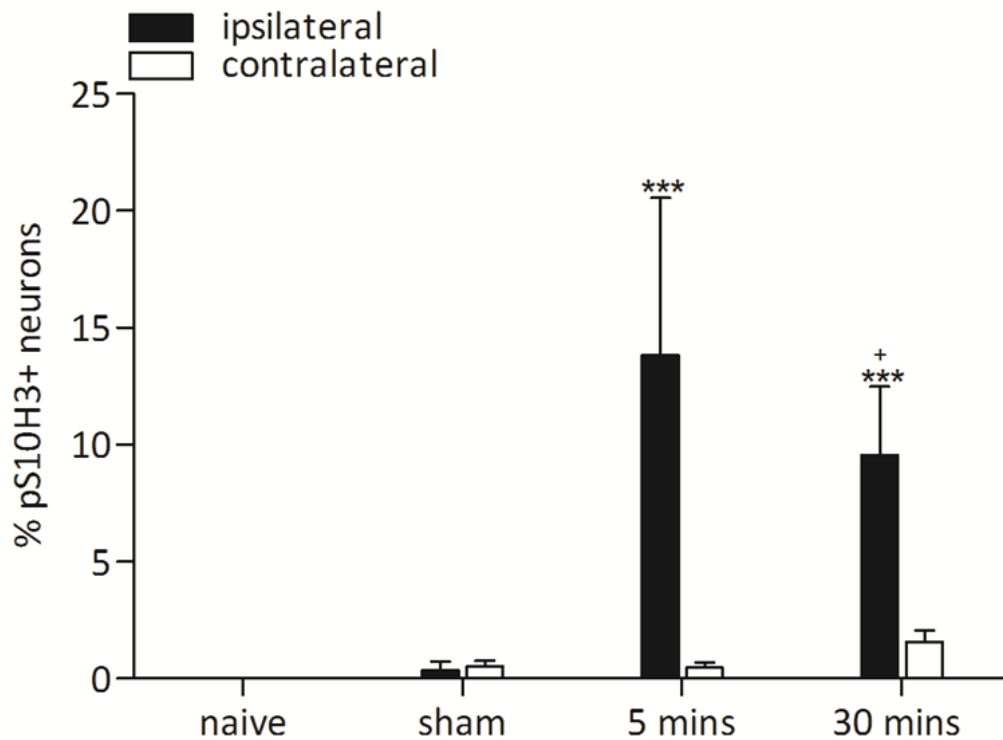


Figure 15.3. **Percentage of pS10H3 nuclear staining in DRG of the capsaicin injection model.** The data is presented as mean+SEM. The experimental groups (n=3 each) were naive animals (naive), s.c 25 μ L saline injection in the hind paw and 5 minutes post-injection period (sham), s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period (5 minutes), and s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 30 minutes post-injection period (30 minutes). *** p<0.001 vs. naive ipsilateral pS10H3; + p<0.05 vs. 5' ipsilateral pS10H3.

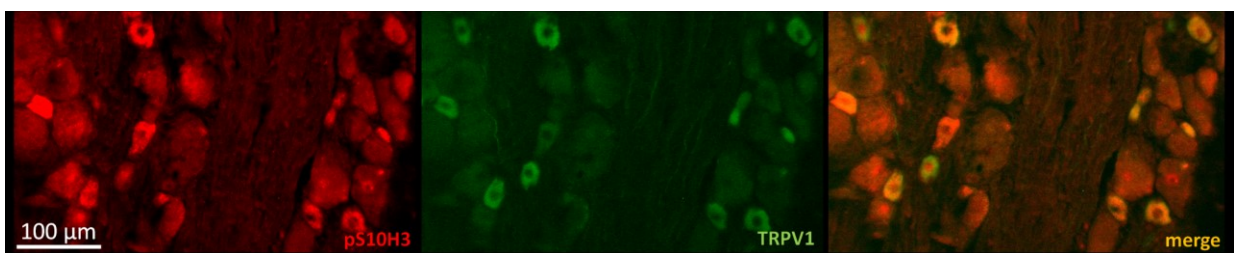


Figure 15.4. **pS10H3 and TRPV1 co-expression in the ipsilateral DRG capsaicin injection.** DRG of animals from the 5 minutes group (s.c. capsaicin injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period) were stained for pS10H3 (red) and TRPV1 (green). The scale bar equals 100 μ m.

15.4.2 pS10H3 positive nuclei co-expressing with TRPV1 in the L4 and L5 DRG following capsaicin injection

Due to the fact that capsaicin selectively and specifically activates TRPV1 (Caterina *et al.*, 1997), we next studied whether neurons with pS10H3 positive nuclei were also immunopositive for TRPV1 (Figure 15.4). The statistical analysis of the double-immunolabelled DRG section at 5 minutes following the capsaicin injection revealed a significant ($p < 0.001$, GzLM) increase in the ipsilateral DRG of ~70% (control: $0.00 \pm 0.00\%$, $n=3$; 5 min: $78.81 \pm 6.14\%$, $n=3$) neurons with positive nuclei for pS10H3 that were also TRPV1 positive (Figure 15.5 A and B). After 30 minutes, the co-expression was partially reduced ($p=0.003$ vs. control, $p=0.386$ vs. 5 min) to ~60% (30 min: $60.44 \pm 2.1\%$, $n=3$).

On the other hand, the percentage of TRPV1 positive neurons that were also pS10H3 was also significantly increased following 5 minutes of the capsaicin injection to ~30% (control: $0.00 \pm 0.00\%$, $n=3$; 5 min: $32.00 \pm 14.60\%$, $n=3$; $p=0.05$, GzLM). However, a 50% reduction was observed at 30-minutes post-injection (30 min: $16.73 \pm 5.2\%$, $n=3$, $p=0.348$, GzLM, Figure 15.5 C and D).

I also compared the percentage of cells with positive nuclei for pS10H3 with the percentage of cells immunopositive for both pS10H3 and TRPV1. No statistical difference between their expression patterns was detected at any time point studied (Figure 15.6).

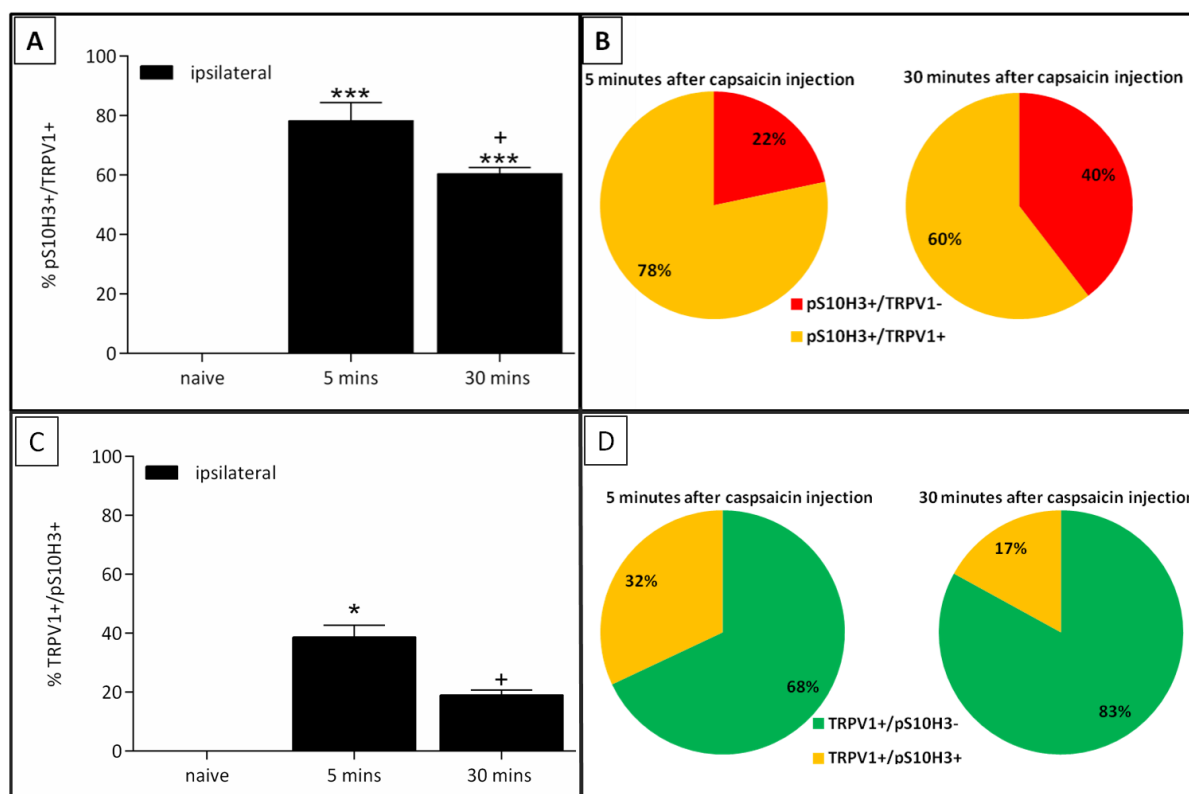


Figure 15.5. **Co-expression between TRPV1 and pS10H3 in L4 and L5 ipsilateral DRG after capsaicin injection.** The experimental groups (n=3 each) were naive animals (naive), s.c. 25 μ L saline injection in the hind paw and 5 minutes post-injection period (sham), s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period (5 minutes), and s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 30 minutes post-injection period (30 minutes). **A)** graph showing pS10H3+ neurons that are also TRPV1+. Data is presented as mean+SEM. *** p<0.001 vs. naive ipsilateral; + p<0.05 vs. ipsilateral 5 mins. **B)** pie chart showing the proportion of pS10H3+ neurons co-expressing with TRPV1 after a capsaicin injection. **D)** graph showing TRPV1+ neurons that are also pS10H3+. Data is presented as mean+SEM. * p<0.05 vs. naive ipsilateral; + p<0.05 vs. ipsilateral 5 mins. **E)** pie chart showing the proportion of TRPV1+ neurons co-expressing with pS10H3 after a capsaicin injection.

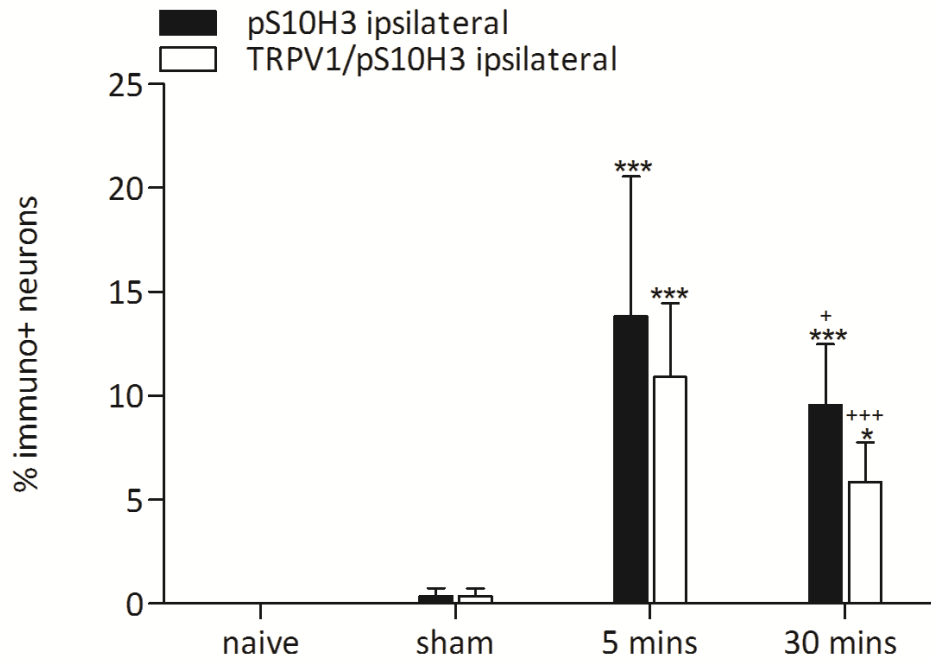


Figure 15.6. **Percentage of pS10H3+ neuronal nuclei and co-expression with TRPV1 in the ipsilateral DRG of the capsaicin injection model.** The data is presented as mean+SEM. The experimental groups (n=3 each) were naive animals (naive), s.c. 25 μ L saline injection in the hind paw and 5 minutes post-injection period (sham), s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period (5 minutes), and s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 30 minutes post-injection period (30 minutes). * p<0.05 vs. naive ipsilateral pS10H3; *** p<0.001 vs. naive ipsilateral pS10H3; + p<0.05 vs. 5' ipsilateral pS10H3; +++ p<0.001 vs. 5' ipsilateral pS10H3.

15.4.3 pCREB expression in the L4-L5 DRG following an injection of capsaicin

To study the expression of pCREB after this noxious model, L4 and L5 DRG sections of control, sham, 5 minutes and 30 minutes after capsaicin injection were immunolabelled against pCREB.

A group of cells, neurons and satellite cells, were detected with positive nuclei for pCREB in the ipsilateral DRG after 5 and 30 minutes post-injection (Figure 15.7). Statistical study of the percentage of neurons positive for pCREB revealed a significant up-regulation in the ipsilateral DRG at 5 minutes (naïve: 0.55 $\pm$ 0.55, Mdn=0, n=3; 5 min: 11.82 $\pm$ 4.12, Mdn=13.45, n=3; p=0.001, GzLM) and 30 minutes

(30 min: 18.07 ± 4.64 , Mdn=20.56, $n=3$, $p<0.001$, GzLM) following injection of this neurotoxin (Figure 15.8). This increase however, was significantly higher at 30-minutes post-injury than at 5 minutes ($p=0.05$, GzLM; Figure 15.8).

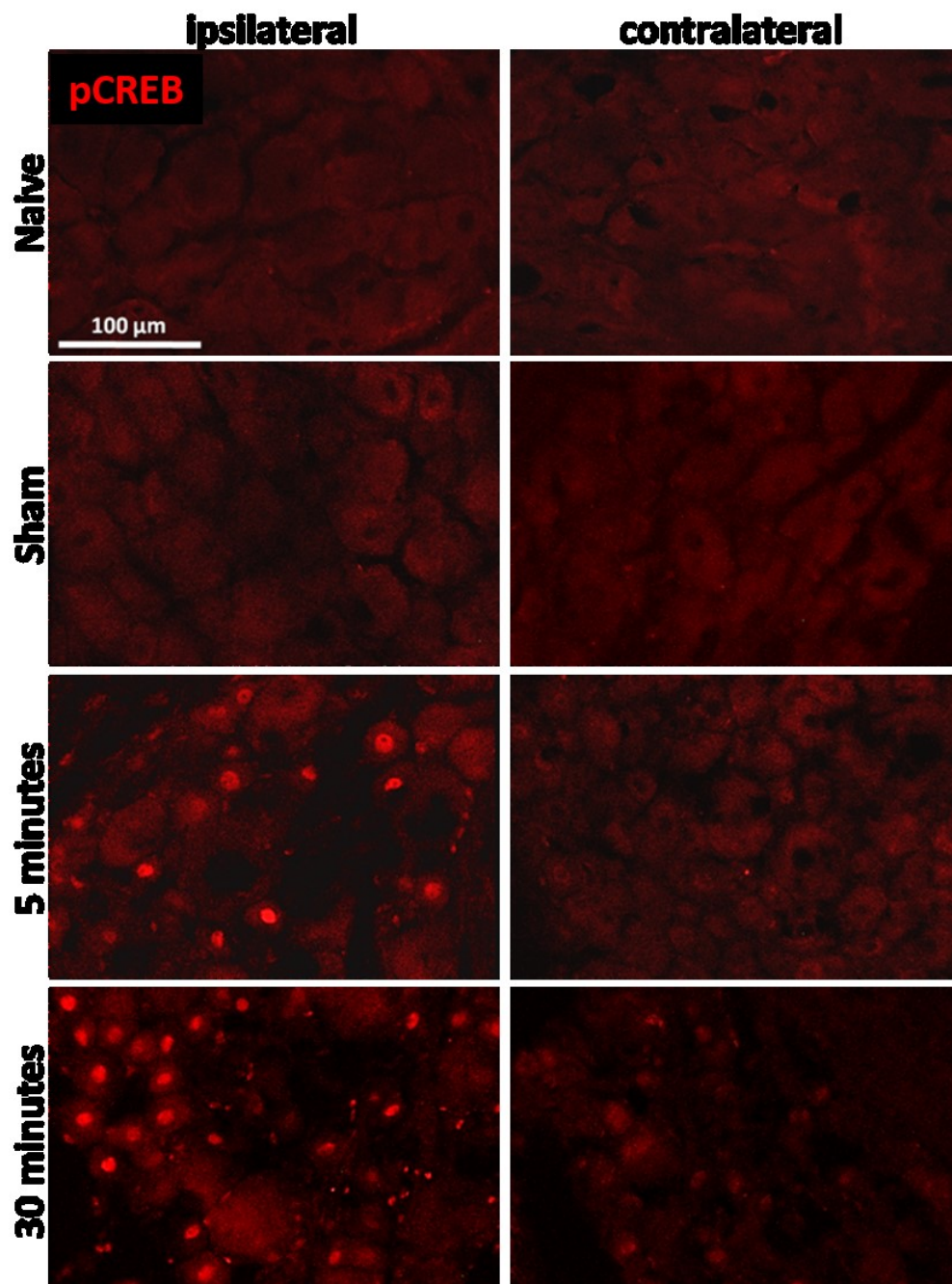


Figure 15.7. Time course of pCREB induction in DRG after capsaicin injection. In all pictures red is for pCREB. The scale bar equals 100 μ m. The experimental groups ($n=3$) were naive animals (naive), s.c 25 μ L saline injection in the hind paw and 5 minutes post-injection period (sham), s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period (5 minutes), and s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 30 minutes post-injection period (30 minutes). Note that both neurons and satellite cells appear stained for pCREB in the ipsilateral following the neurotoxin injection.

An statistical increase was also detected in the contralateral DRG following capsaicin injection (5 min: 7.66 ± 3.83 , Mdn=11.43, $p=0.029$; 30 min: 7.16 ± 3.09 , Mdn=4.82, $p=0.042$; GzLM), with no difference through time ($p=0.879$, GzLM) and significantly lower than the numbers assessed in the ipsilateral sides (5 min: $p=0.043$; 30 min: $p=0.001$; GzLM; Figure 15.8).

Further, no up-regulation was detected in the sham animals at neither ipsilateral (0.57 ± 0.57 , Mdn=0, $n=3$, $p=0.993$, GzLM) or contralateral DRG (0.93 ± 0.22 , Mdn=0.65, $n=3$, GzLM).

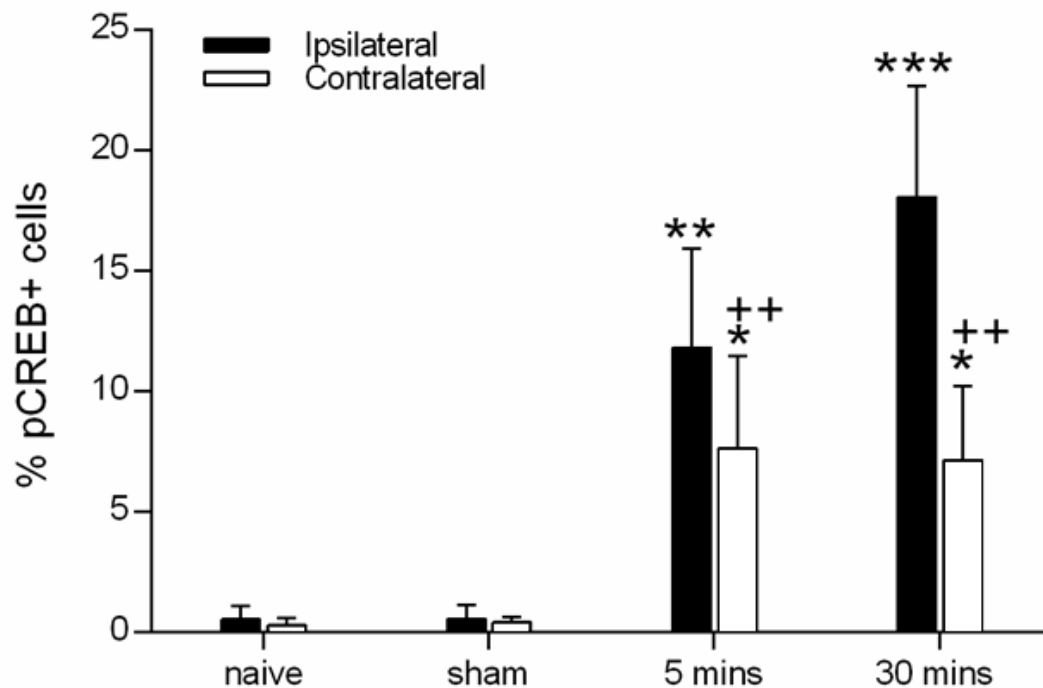


Figure 15.8. **Percentage of pS10H3 nuclear staining in DRG of the burn-injury model.** The data is presented as mean $\pm$ SEM. The experimental groups ($n=3$) were naive animals (naive), s.c. 25 μ L saline injection in the hind paw and 5 minutes post-injection period (sham), s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period (5 minutes), and s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 30 minutes post-injection period (30 minutes). * $p<0.05$ vs. naive ipsilateral pCREB; ** $p<0.01$ vs. naive ipsilateral pCREB; *** $p<0.001$ vs. naive ipsilateral pCREB; ++ $p<0.01$ vs. 30 mins ipsilateral pCREB.

15.4.4 pCREB positive nuclei are expressed in TRPV1-expressing neurons in the L4-L5 DRG following capsaicin injection

Similarly as for pS10H3 (see above), it was decided to assess whether there was co-expression between pCREB and TRPV1. Hence, DRG sections were double-stained against TRPV1 and pCREB (Figure 15.9).

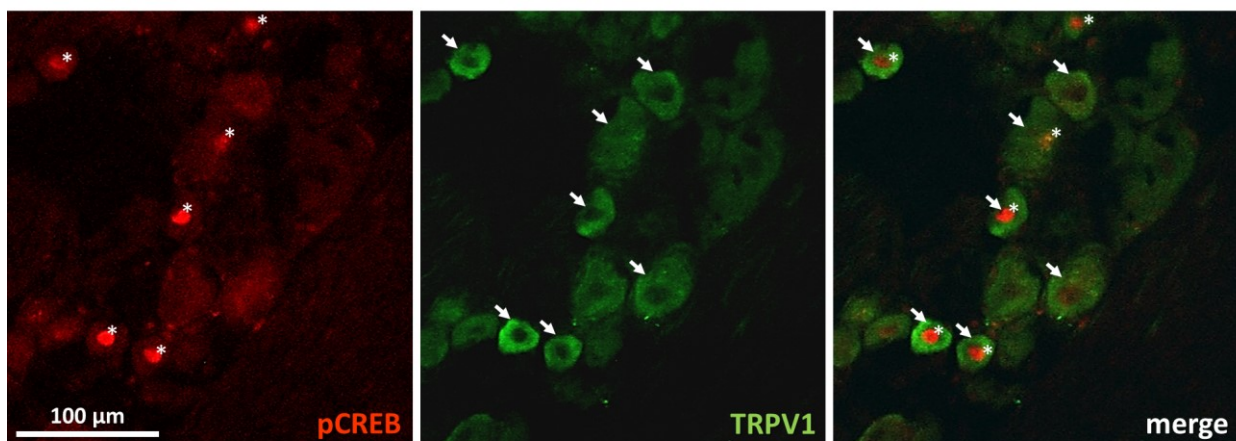


Figure 15.9. **pCREB and TRPV1 co-expression in the ipsilateral DRG following capsaicin injection.** DRG of animals from the 5 minutes group (s.c. capsaicin injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period, n=3) were stained for pCREB (red), asterisks; and TRPV1 (green), arrows. The scale bar equals 100μm. pCREB is present intra-nuclear.

A significant increase ($p < 0.001$, GzLM) in the ipsilateral DRG of ~80% (naïve: $0.00 \pm 0.00\%$, n=3; 5 min: $83.3 \pm 16.67\%$, n=3) of the neurons positives for pCREB and also for TRPV1 was statistically significant at 5 minutes following the capsaicin injection (Figure 15.10). This co-expression remained unchanged ($p = 0.143$, GzLM) once 30 minutes after capsaicin injection were elapsed (30 min: $58.12 \pm 19.72\%$, n=3, GzLM; Figure 15.10). On the other hand, around 50% of the cells expressing TRPV1 were also positive for pCREB at both 5 minutes and 30 minutes following capsaicin injection (5 min: $46.3 \pm 4.16\%$; 30 min: $54 \pm 3.46\%$; n=3, $p = 0.366$, GzLM).

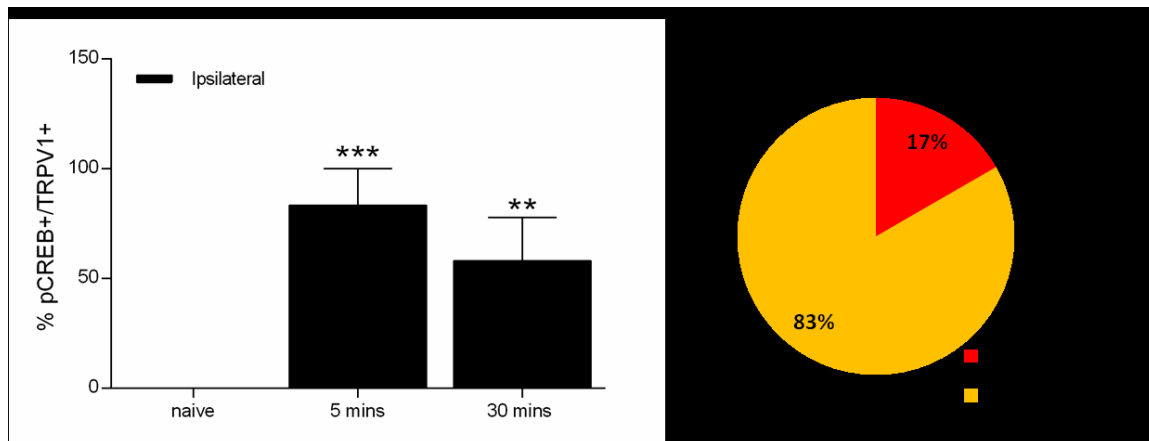


Figure 15.10. Co-expression between TRPV1 and pCREB in L4 and L5 ipsilateral DRG after capsaicin injection. The experimental groups (n=3) were naive animals (naive), s.c 25 μ L saline injection in the hind paw and 5 minutes post-injection period (sham), s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period (5 minutes), and s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 30 minutes post-injection period (30 minutes). The graph shows pCREB+ neurons that are also TRPV1+. Data is presented as mean+SEM. ** p<0.01 vs. naive ipsilateral; *** p<0.001 vs. naive ipsilateral. Pie chart is showing the proportion of pCREB+ neurons co-expressing with TRPV1 after capsaicin injection. pCREB co-expresses with TRPV1 in a 83.3 $\pm$ 16.67%.

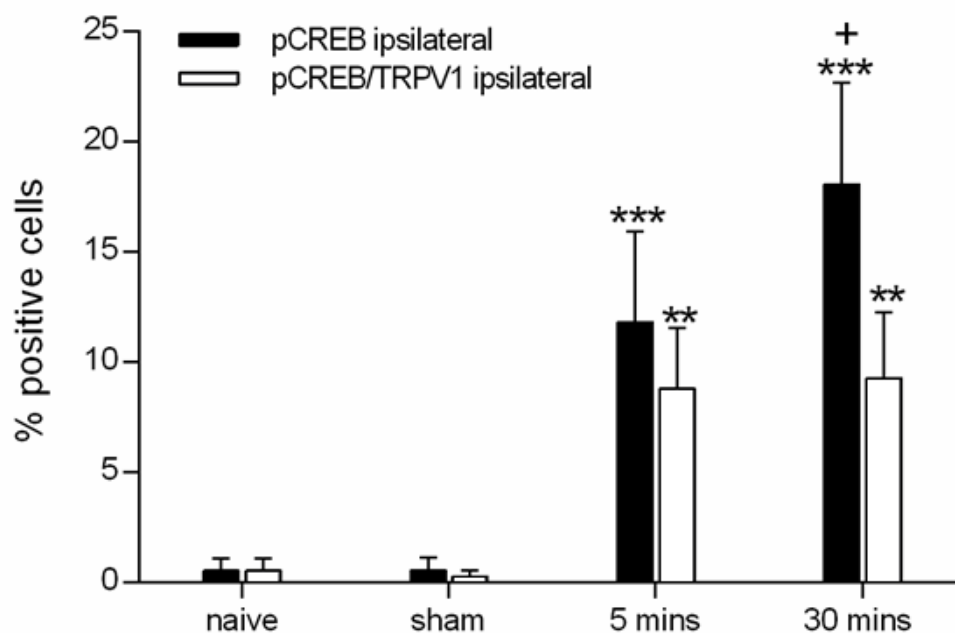


Figure 15.11. Percentage of pCREB+ neuronal nuclei and coexpression with TRPV1 in the ipsilateral DRG of capsaicin injection. The data is presented as mean $\pm$ SEM. The experimental groups (n=3) were naive animals (naive), s.c 25 μ L saline injection in the hind paw and 5 minutes post-injection period (sham), s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 5 minutes post-injection period (5 minutes), and s.c. capsaicin (25 μ L of 3mg/mL) injection in the hind paw with 2 minutes stroke and 30 minutes post-injection period (30 minutes). * p<0.05 vs. naive ipsilateral pCREB; ** p<0.01 vs. naive ipsilateral pCREB; *** p<0.001 vs. naive ipsilateral pCREB; + p<0.05 vs. 5' ipsilateral pCREB.

The comparative study of 1) the percentage of pCREB positive nuclei of the total number of cells versus 2) the percentage of pCREB positive nuclei with TRPV1 immunopositive labelling of the total number of cells counted did not show any difference statistically significant between both patterns (Figure 15.11).

15.4.5 pCREB co-expression with pS10H3 immunolabelled nuclei in DRG neurons following capsaicin injection

Ipsilateral DRG sections from 5 minutes post- capsaicin injection animals were also double-immunolabelled against pCREB and pS10H3. Around 80% ($83.33 \pm 16.67\%$, $n=3$) of pS10H3 positive nuclei also exhibited pCREB staining within their nuclei whereas $\sim 25\%$ ($24.52 \pm 2.49\%$, $n=3$) of the pCREB positive neuronal nuclei had immunopositive nuclear staining for pS10H3 (Figure 15.12).

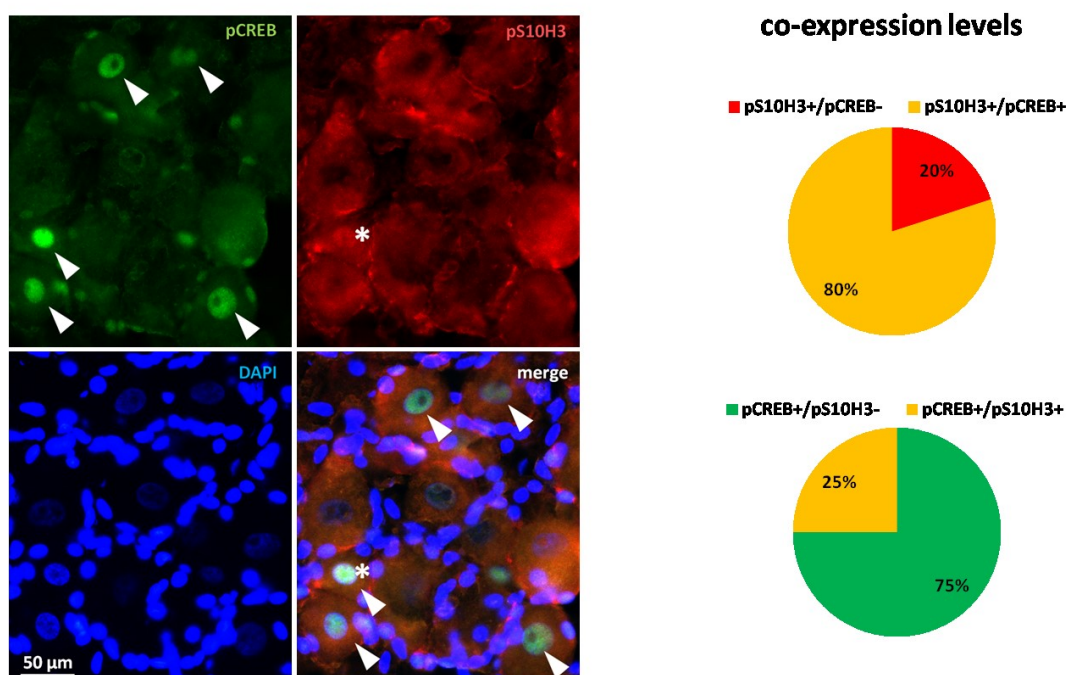


Figure 15.12. Co-expression between pCREB and pS10H3 in L4 and L5 ipsilateral DRG after capsaicin injection. DRG sections of animals ($n=3$) from the group that received a s.c. capsaicin injection ($25\mu\text{L}$ of 3mg/mL) in the hind paw, 2 minutes stroke and 5 minutes post-injection period were reacted against pCREB (green) and pS10H3 (red). DAPI (blue) was used as a nuclear marker. Arrowheads (Δ) point to nuclei positive for pCREB and asterisk (*) marks nuclear staining positive for pS10H3. Note that both markers co-express in the nuclear compartment. The first pie chart is showing the proportion of pCREB neurons co-expressing with pS10H3 after a capsaicin injection

($24.52 \pm 2.49\%$) and the second pie chart shows the proportion of pS10H3+ neurons that co-express with pCREB following 5 minutes of a capsaicin injection ($83.33 \pm 16.67\%$).

15.4.6 pS10H3 expression in cultured primary sensory neurons after capsaicin incubation

To study the expression of pS10H3 in an *in vitro* set-up and after a noxious stimulation, cultures of primary sensory neurons (derived from DRG) were either maintained in extracellular buffer (controls) or capsaicin was added together with the extracellular buffer for 5 minutes (capsaicin). Protein extracts were then immunoprecipitated against pS10H3 and used for western blotting. The immunoblotted membranes showed a positive band for pS10H3 at ~15KDa, together with other bands corresponding to the different combinations of agarose-antibody-pS10H3; for example, at ~80KDa corresponding to the antibody alone, which was used for normalisation (Figure 15.13).

The statistical analysis of the densitometry ratios failed to show an increase on the expression of pS10H3 after immersion with capsaicin, although a tendency (control: 0.78 ± 0.11 , $n=3$; capsaicin: 1.13 ± 0.08 , $n=3$; $p=0.1$, student's t-test, Figure 15.13) was observed. Nonetheless, the study of the cultured primary sensory neurons after immunolabelling against pS10H3 revealed a significant increase on the percentage of cells exhibiting nuclear positive staining after the immersion of the neurotoxin (control: 21.46 ± 10.38 , $Mdn=30.15$, $n=3$; capsaicin: 41.54 ± 3.21 , $Mdn=40.26$, $n=3$; $p<0.05$, Mann-Whitney U test; Figure 15.14 and Figure 15.15).

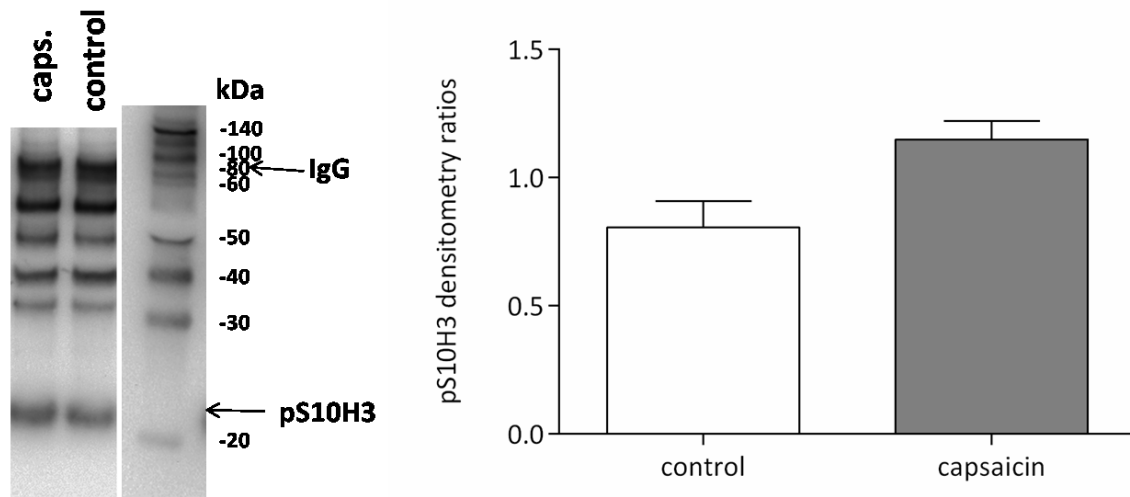


Figure 15.13. **pS10H3 in cultured primary sensory neurons during control conditions and after capsaicin injection.** Representative western blot of immunoprecipitated pS10H3 extracts from capsaicin (caps., cultures incubated with 5 μ M of capsaicin in extracellular buffer for 5 minutes) and control cultures; graph shows normalized densitometry ratios. Data is presented as mean+SEM. Student's T-test was performed with normalized densitometry ratios. The increase did not reach significance, with $p=0.1$

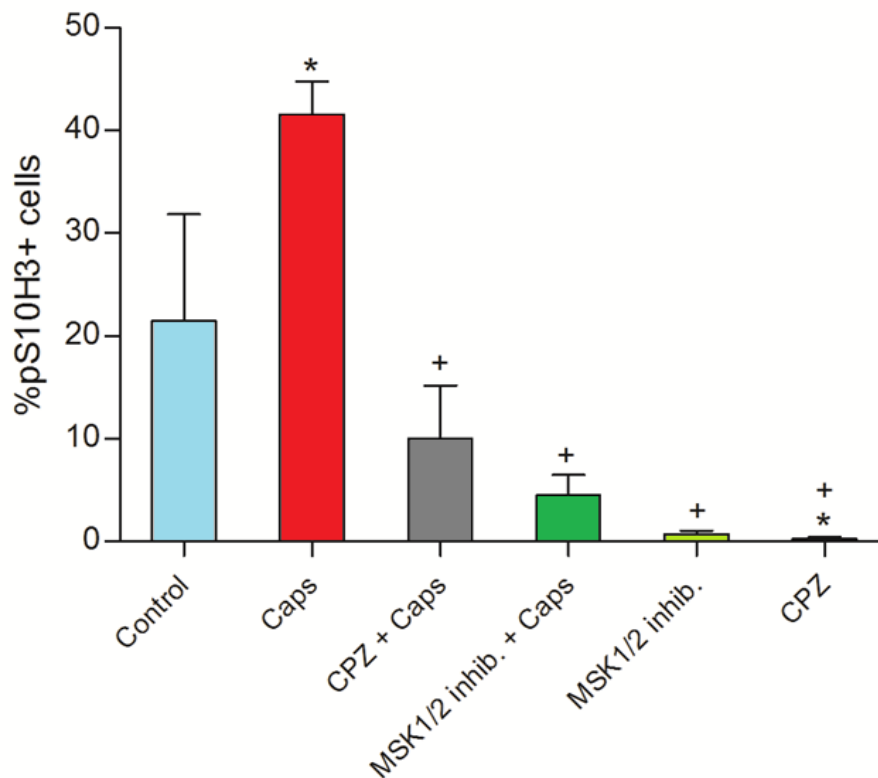


Figure 15.14. **Immunolabelled pS10H3 cultured primary sensory neurons after different treatments.** Data is presented as mean+SEM. The experimental groups ($n=3$ each) were: control sides (**Control**) and cultures incubated with 5 μ M of capsaicin in extracellular buffer for 5 minutes (**Caps**); 5 μ M of capsazepine in extracellular buffer for 5 minutes (**CPZ**), and 5 minutes pre-incubated with 5 μ M of capsazepine in extracellular buffer following 5 minutes incubation with 5 μ M of capsaicin in extracellular buffer (**CPZ + Caps**); 3 μ M of SB747651A dihydrochloride in extracellular buffer for 5 minutes (**MSK1/2 inhib.**), and 5 minutes pre-incubated with 3 μ M of SB747651A dihydrochloride in extracellular buffer and then 5 minutes incubation with 5 μ M of capsaicin in extracellular buffer (**MSK1/2 inhib. + Caps**). Data was evaluated with Mann-Whitney. * $p<0.05$ vs. Control; + $p<0.05$ vs. Caps.

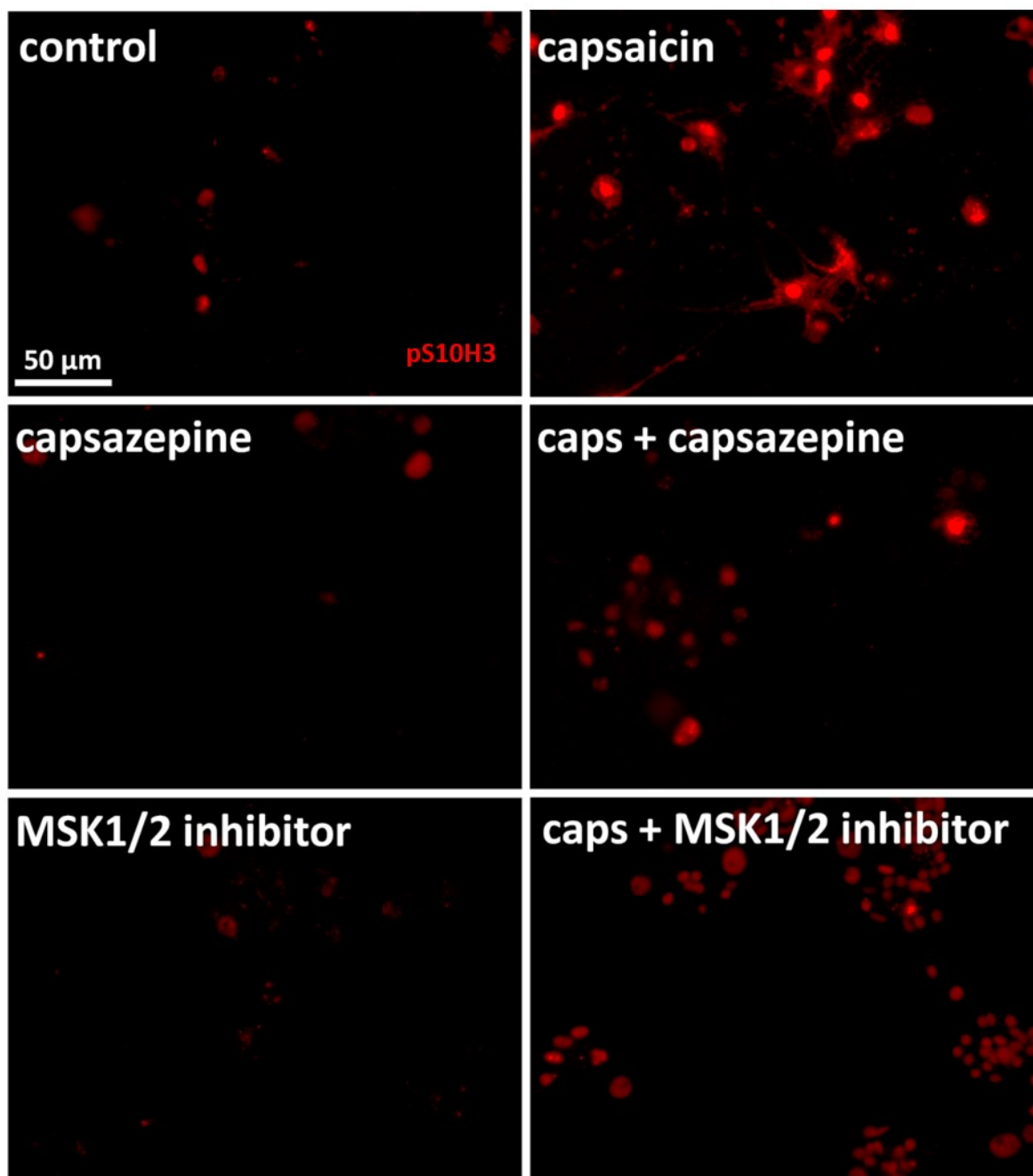


Figure 15.15. **Representative images of immunolabeled pS10H3 cultured primary sensory neurons after different treatments.** The experimental groups (n=3 each) were: control sides (**control**) and cultures incubated with 5μM of capsaicin in extracellular buffer for 5 minutes (**capsaicin**); 5μM of capsazepine in extracellular buffer for 5 minutes (**capsazepine**), and 5 minutes pre-incubated with 5μM of capsazepine in extracellular buffer following 5 minutes incubation with 5μM of capsaicin in extracellular buffer (**caps + capsazepine**); 3μM of SB747651A dihydrochloride in extracellular buffer for 5 minutes (**MSK1/2 inhibitor**), and 5 minutes pre-incubated with 3μM of SB747651A dihydrochloride in extracellular buffer and then 5 minutes incubation with 5 μM of capsaicin in extracellular buffer (**caps + MSK1/2 inhibitor**). The scale bar equals 50μm.

Next, cultures were pre-incubated with either capsazepine, a specific blocker of TRPV1 (Caterina *et al.*, 1997; Liu & Simon, 1997), or with SB747651A, the blocker of

MSK1/2 (Naqvi *et al.*, 2012), before the addition of capsaicin (CPZ+Caps and MSK1/2 inhib+Caps, respectively). The purpose of this experiment was to assess interactions within those components and to elucidate the putative pathway leading to pS10H3. As controls, cultured cells were only incubated with either capsazepine (CPZ) or the MSK1/2 blocker (MSK1/2 inhib).

On the one hand, the presence of MSK1/2 blocker before and during the immersion of capsaicin produced a significant reduction in the percentage of cells with pS10H3 positive nuclei when compared with the number observed in the cultures only treated with capsaicin (MSK1/2 inhib: 0.72 ± 0.32 , Mdn=0.53, n=3, p=0.05; MSK1/2 inhib+Caps: 4.49 ± 2.00 , Mdn=2.85, n=3, p=0.05; Mann-Whitney U test; Figure 15.14 and Figure 15.15). On the other hand, when capsazepine was present in the culture before and during the application of capsaicin, a reduction in the corresponding number of pS10H3 positive nuclei was also observed (CPZ: 0.25 ± 0.18 , Mdn=0.15, n=3, p=0.05; CPZ+Caps: 10.00 ± 5.2 , Mdn=12.5, n=3, p=0.05; Mann-Whitney U test) with a similar extend than the one found after the application of MSK1/2 inhibitor (MSK1/2 inhib vs. CPZ: p=0.275, MSK1/2 inhib+Caps vs. CPZ+Caps: p=0.513; Mann-Whitney U test; Figure 15.14 and Figure 15.15). Further, the application of capsazepine by itself to the intracellular buffer produced a reduction of pS10H3 positive cells significantly different from that observed under control condition (p=0.05, Mann-Whitney U test), indicating that some basal expression of pS10H3 could be present under control culturing conditions throughout the activation of TRPV1.

15.4.7 Co-expression of pS10H3 and TRPV1 in cultured primary sensory neurons following capsaicin incubation

Closer analysis of the pS10H3 positive staining in cultured primary sensory neurons after immersion in capsaicin showed positive immunolabelling only present at the nuclear compartment (figure 4.16 A, B, D and F). The co-expression analysis revealed that ~35% ($37 \pm 7.12\%$, $n=3$) of TRPV1 positive neurons also exhibited positive signal for pS10H3 under control conditions (figure 4.17) while this percentage was significantly increased ($p < 0.001$, GzLM) to ~75% ($74 \pm 12.32\%$, $n=3$) once the culturing were activated by capsaicin addition (Figure 15.16 and Figure 15.17). Moreover, nearly half ($46 \pm 8.87\%$, $n=3$) of the cells that were pS10H3 positive were also TRPV1 positives under control conditions whereas this percentage was also significantly increased ($p = 0.011$, GzLM) to ~60% ($60 \pm 7.54\%$, $n=3$) after immersion with capsaicin (Figure 15.17).

All these data together could also be indicating that there is a basal expression of pS10H3 under cultured control condition but independently to the activation of TRPV1.

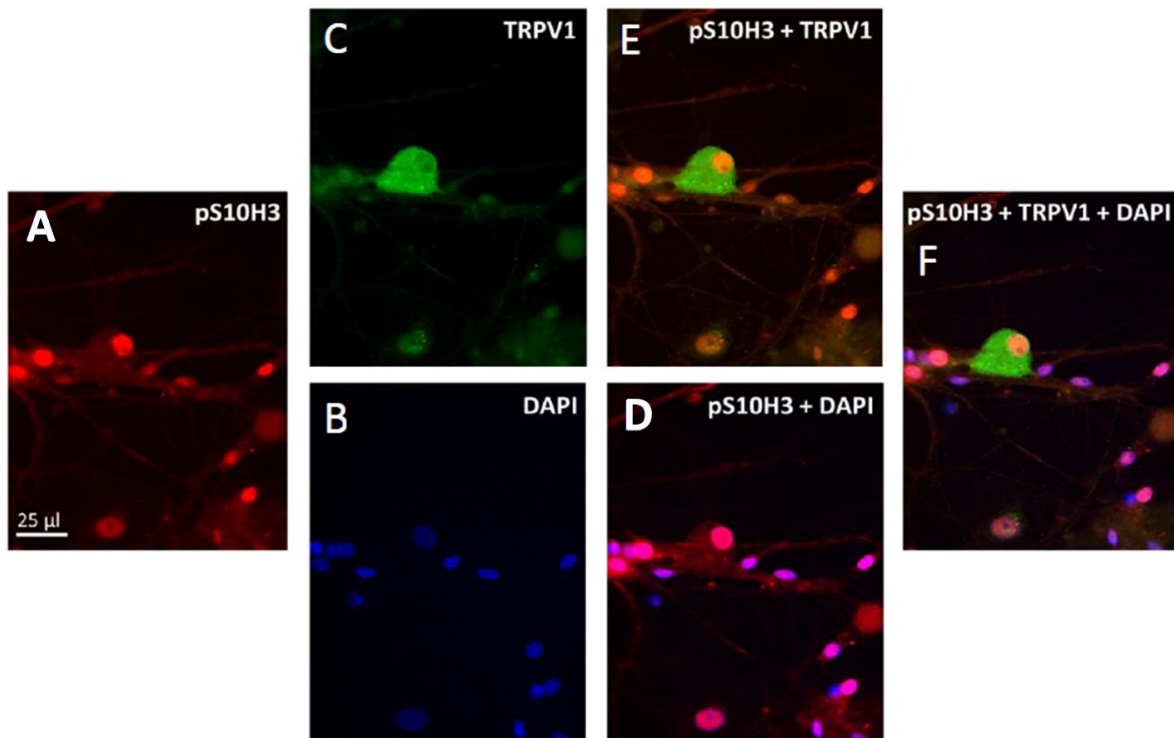


Figure 15.16. **Sub-cellular distribution of pS10H3 and its co-expression with TRPV1 in capsaicin stimulated cultures of primary sensory neurons.** Cultures incubated with 5 μ M of capsaicin in extracellular buffer for 5 minutes were stained for pS10H3 (red), TRPV1 (green) and nuclei (DAPI; in blue). The scale bar equals 25 μ m. pS10H3 is present intra-nuclear while TRPV1 is observed at the membrane surface and cytoplasm.

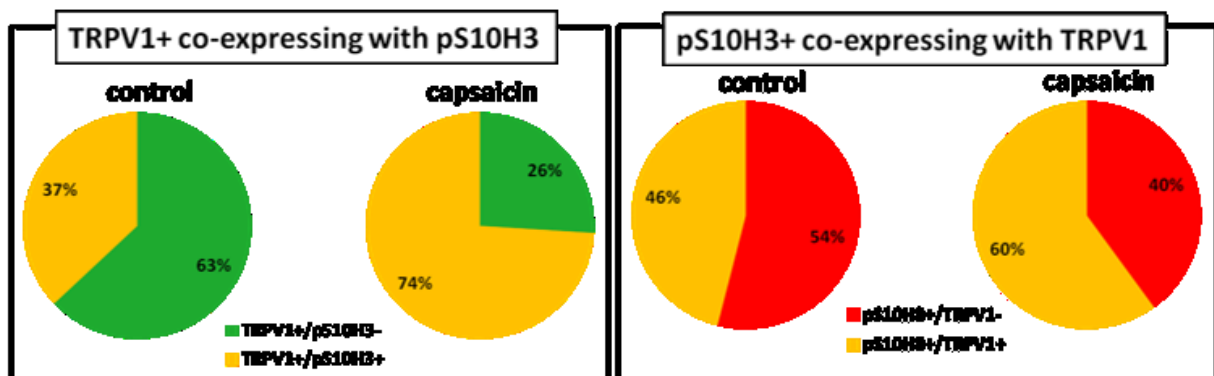


Figure 15.17. **Co-expression between TRPV1 and pS10H3 in cultured primary sensory neurons increases after capsaicin activation.** TRPV1+ neurons in the capsaicin group (n=3; 5 μ M of capsaicin in extracellular buffer for 5 minutes) exhibited a higher co-expression with pS10H3 than control group (n=3); from 37 $\pm$ 7.12% to 74 $\pm$ 12.32%. Likewise, there is a higher proportion of pS10H3+ neurons co-expressing TRPV1 in the capsaicin than control group, from 46 $\pm$ 8.87% to 60 $\pm$ 7.54%.

15.4.8 pS10H3 expression in L4-L5 DRG after exposure to noxious stimuli without causing tissue damage

Similar as done before in the spinal cord (Chapter 12), I studied if noxious heat (a model of noxious stimulation without induction of tissue damage) had an effect on the expression of pS10H3 in the DRG.

After this noxious model, no increase on the percentage of neurons with nuclei positives for pS10H3 was detected in either ipsilateral (control: 0.00 ± 0.00 , Mdn=0, n=3; ipsilateral-heat: 2.11 ± 0.07 , Mdn=2.13, n=3; $p=0.066$, GzLM) or contralateral DRG (contralateral-heat: 0.83 ± 0.32 , Mdn=1.11, n=3, $p=0.185$, GzLM, Figure 15.18).

Next, I studied two more models of noxious activation without the induction of tissue damage: noxious cold, exposing the hind paw to $\sim 4^{\circ}\text{C}$ water or mechanical noxious stimulation, pressure applied on the hind paw (600 mN/mm^2). The statistical analysis of the percentage of positive nuclei for pS10H3 did not show an increase in either ipsi- or contra-lateral DRG after the noxious cold stimulation (ipsilateral-cold: 0.96 ± 0.96 , Mdn=0, n=3, $p=0.126$; contralateral-cold: 0.67 ± 0.67 , Mdn=0, n=3, $p=0.284$; GzLM). Similarly, no up-regulation was detected after the mechanical noxious model in either ipsilateral (ipsilateral-mechanical: 0.46 ± 0.29 , Mdn=0.38, n=3, $p=0.146$, GzLM) or contralateral DRG (contralateral-mechanical: 0.75 ± 0.38 , Mdn=1.11, n=3, $p=0.360$, GzLM, Figure 15.18).

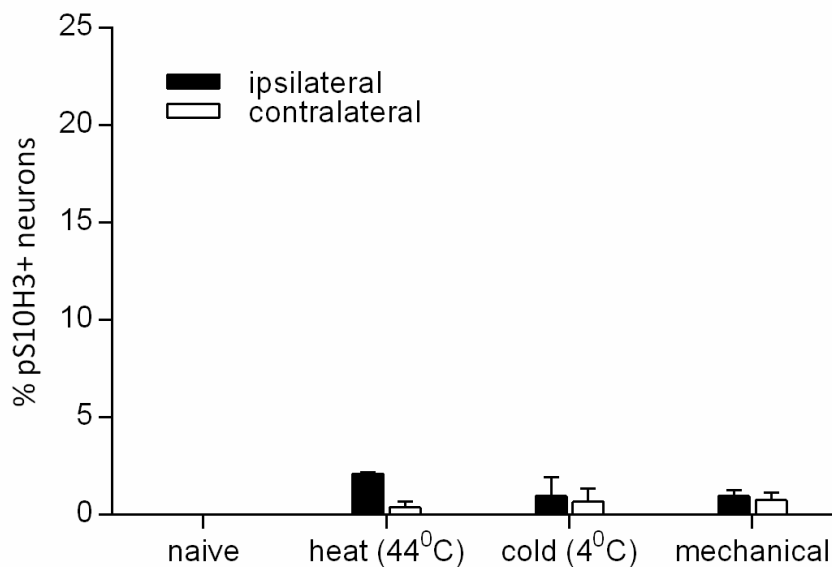


Figure 15.18. **Percentage of pS10H3 nuclear staining in DRG following different noxious models.** Data is presented as mean+SEM. The experimental groups (n=3) were naive animals (naive), 6 times 44°C water for 10 seconds with 10 seconds gaps in-between [heat (44°C)], 6 times 4°C water for 10 seconds with 10 seconds gaps in-between [Cold (4°C)] and hind paw mechanically stimulated by a pressure of 60mN/mm² (mechanical). No significant differences were observed.

Despite that no up-regulation in the percentage of pS10H3 positive staining was detected, it has to be mentioned that the seldom positive staining detected was always localised in the nucleus (Figure 15.19).

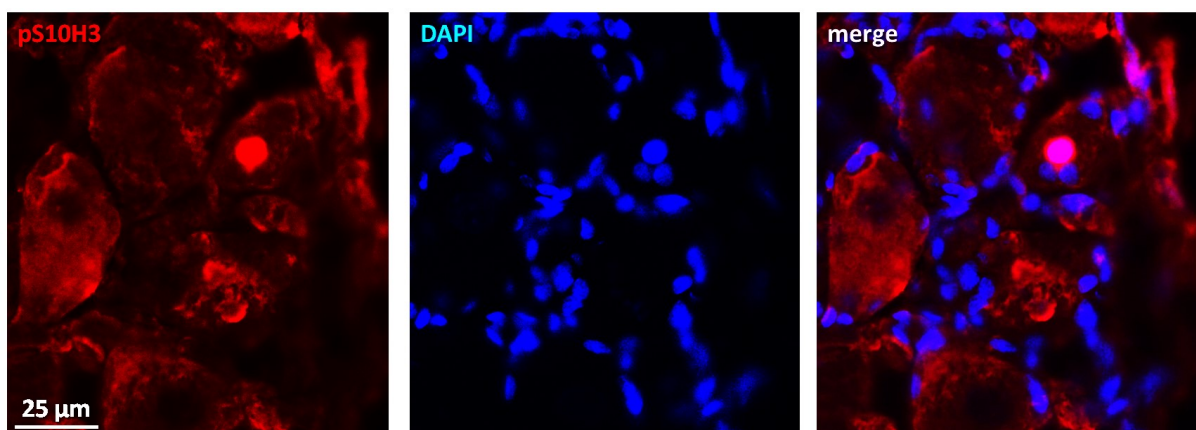


Figure 15.19. **Sub-cellular distribution of pS10H3 following noxious heat stimulation.** Images were taken from animals stimulated by immersing one of the hind paws 6 times at 44°C water for 10 seconds with 10-seconds gaps in-between. Despite not being a significant increase on the number of pS10H3 following this noxious model, the pS10H3 positive staining was exclusively localised in the cellular compartment. Scale bar equals 25μm.

15.5 Discussion

Results presented in this chapter show that pS10H3 is a dynamically inducible epigenetic mark occurring in DRG neurons following inflammation induced by capsaicin, as observed both *in vivo* and *in vitro*. This process affects nociceptors through activation of TRPV1, which induces intracellular pathways of kinases that lead to activation of MSK1 and the subsequent phosphorylation of this histone H3 tail. Similarly, this cascade of events (pathway) is also activating, at least partially, pCREB, which is also associated to transcriptional activation in pain states (Ji *et al.*, 2003; Kawasaki *et al.*, 2004; Bie *et al.*, 2011; Li *et al.*, 2014). On the contrary, brief noxious stimulations without inducing tissue damage failed to up-regulate this epigenetic covalent modification.

15.5.1 pS10H3 at DRG neurons following capsaicin is TRPV1-specific

Following a capsaicin application, pS10H3 is up-regulated in DRG neurons as observed by western blotting and immunofluorescence in both *in vivo* and *in vitro* experiments. The up-regulation peaks at 5 minutes of the simulation and decreases at later times. A great proportion of cells that responded after 5 minutes, express TRPV1, the channel selectively activated by capsaicin (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). Accordingly, the blockade of this channel prior to the application of capsaicin prevented the up-regulation of pS10H3 in cultured DRG neurons. These data indicate that capsaicin-induced up-regulation of pS10H3 is a TRPV1-dependent event, which agrees with the fact that pS10H3 was not induced in either rats subjected to saline injection or animals under baseline conditions. In contrast, a certain expression of pS10H3 is observed in cultured DRG neurons at

control conditions. This basal expression observed *in vitro* could be due to the NGF present in the media, which has been shown to induce this epigenetic mark in PC12 cells (Deak *et al.*, 1998). Similarly, other growth factors had been shown to induce pS10H3 in different cell type using other pathways. For example: FSH (follicle-stimulating hormone) induces pS10H3 in granulosa cells via PKA in a process also associated to a small subset of genes (Salvador *et al.*, 2001). In accordance, the percentages of pS10H3 positive cells that expressed TRPV1 under control conditions and after capsaicin application did not increase much while the percentage of TRPV1-positive cells that were also pS10H3 increased substantially, supporting a TRPV1-independent basal expression of pS10H3 in the *in vitro* setup. As other researchers suggest, the epigenetic code of a process is dynamic and specific for each cell type (Gutierrez & Hnilica, 1967; Salvador *et al.*, 2001; Thomson *et al.*, 2001; Lefebvre *et al.*, 2002; Bilang-Bleuel *et al.*, 2005; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008; Meaney & Ferguson-Smith, 2010; Zhang *et al.*, 2011; Geranton, 2012). Consequently, the dynamics *in vivo* of pS10H3 following this stimulation are different as observed in the burn injury model (Chapter 13) or at the superficial DHSC following capsaicin (Chapter 12). Hence, the increase of pS10H3 is also tissue- and stimulus-specific.

Western blot from total protein extracts of animals combined (injected with capsaicin) failed to show a significant increase. This could be due to DRG cell heterogeneity (Denk *et al.*, 2013; Bai *et al.*, 2015). Within DRG there are other cell types than neurons, such as satellite cells that can have basal expression of histone phosphorylation (due to other kinases such Aurora family (Prigent & Dimitrov, 2003; Soloaga *et al.*, 2003)), which could be masking any increase. Moreover, from all the neuronal bodies found in a DRG, only those TRPV1-expressing cells the receptive

field of which is at the hind leg injected with capsaicin will respond. Accordingly, only around 30% of the TRPV1-expressing neurons exhibited positive signal for pS10H3 at 5 minutes of the stimulation, which accounts for a small portion of the neurons. In agreement with this, immunofluorescence data shows a maximal peak of around 10%.

At 30 minutes of the stimulation at the experiment *in vivo*, there is a significant reduction of the pS10H3-expressing cells that had TRPV1, suggesting that some of the cells activated at that time had been recruited in a TRPV1-independent manner. (Ji *et al.*, 1999; Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; Marchand, 2008; Hunt, 2009; Woolf, 2011; Steeds, 2013). Similarly, the number of TRPV1-positive neurons that also show staining for pS10H3 at 30 minutes diminished which could be a consequence of the decrease of pS10H3 and/or an increased production of TRPV1.

15.5.2 The up-regulation of pS10H3 following capsaicin is mediated by MSK1

MSK1, MSK2 and RSK2 are three kinases appointed as *writers* of pS10H3 (De Cesare *et al.*, 1998; Deak *et al.*, 1998; Davie, 2003; Soloaga *et al.*, 2003; Dong & Bode, 2006). MSK1 is the principal writer in response to ERK pathway activation (Thomson *et al.*, 1999a; Clayton *et al.*, 2000; Davie, 2003; Soloaga *et al.*, 2003; Dong & Bode, 2006). The induction of pS10H3 in neurons following learning processes seems to be regulated by AMPA and NMDA receptors, and implicates ERKs and MSK1/2 as the effectors (Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008). Accordingly, the blockade of MSK1 *in vitro* prevented the increase of pS10H3 on cultured DRG neurons

following a capsaicin application. Therefore, the main writer of this epigenetic mark in this context is MSK1.

15.5.3 The induction of pCREB by capsaicin is partially regulated by other kinases

Either MSK1, MSK2 and RSK2 contain two kinase domains, which allow them to phosphorylate both pS10H3 and pCREB (Xing *et al.*, 1996; De Cesare *et al.*, 1998; Deak *et al.*, 1998; Thomson *et al.*, 1999b; Lo *et al.*, 2000; Soloaga *et al.*, 2003). In agreement with this, a great number of pS10H3-expressing cells also exhibit positivity for pCREB. On the other hand, only around a fourth of the cells that expressed pCREB were also pS10H3-positive and around 80% of those pCREB-expressing neurons were also TRPV1 positive. This suggests that CREB phosphorylation is also a TRPV1-specific event although is, at least in part, achieved by other kinases than MSK1.

Interestingly, the *in vivo* model shows that pCREB gets activated following 5 minutes of the capsaicin injection and increases further at 30 minutes. Nonetheless, certain up-regulation is also detected at corresponding contralateral sides. This could be a consequence of circulating inflammatory mediators that can directly or indirectly activate pCREB in distant neurons. Small diameter DRG neurons respond to cytokines such as IL-1 β or TNF- α by activating MAPK p38 (Ji & Woolf, 2001; Edelmayer *et al.*, 2014) with MSK2, and not MSK1 or RSK2, as the main effector kinase activated by pp38 (Thomson *et al.*, 1999a; Clayton *et al.*, 2000; Davie, 2003; Soloaga *et al.*, 2003; Dong & Bode, 2006). Moreover, cytokine activations also seem to be able to induce pS10H3 through IKK α in a process directed to certain NF- κ B-dependent promoters (Yamamoto *et al.*, 2003; Yu *et al.*, 2015). Accordingly, only

around half of TRPV1-positive cells exhibited pCREB after the injection of capsaicin, suggesting that at least half of the cell were recruited by a process independent of TRPV1 activation.

Furthermore, pCREB was also observed at neuronal nuclei of satellite cells. Although these cells were not analysed in this work, this indicates that satellite cells play an active role during nociceptive processing (Ohara *et al.*, 2009). The induction of pCREB within these cells could be a consequence of MSK2 activation via pp38, a MAPK that has been observed in glial cells (Ji *et al.*, 2002b; Geranton *et al.*, 2010; White *et al.*, 2011a). This could be an interesting topic for future studies.

15.5.4 Brief noxious stimuli do not induce pS10H3 in DRG neurons

As observed at the spinal cord, repeated brief stimulation with noxious temperature or pressure did not induce pS10H3 in DRG. Thus, it is suggested that a more sustained noxious stimulation is required to induce pS10H3 in DRG neurons. Nonetheless, the absence of pS10H3 does not necessarily mean that these cells were not activated by the noxious stimuli. Nociceptors could have been stimulated and even sensitized by different pathways of activation such as MAPK (Hunt & Mantyh, 2001; Ji *et al.*, 2003; Marchand, 2008; Costigan *et al.*, 2009; Steeds, 2013), but no changes in gene expression, lead by pS10H3, were induced.

Interestingly, pS10H3 was restricted to the nuclear compartment in the few neurons that express it following these noxious stimuli, similarly as observed after the *in vitro* application of capsaicin. Hence, the extracellular accumulation only appears in severe models *in vivo* and seems linked to cellular stress (Gabler *et al.*, 2004) as

observed in different immune cells (Watson *et al.*, 1995; Gabler *et al.*, 2003; Gabler *et al.*, 2004; Gilthorpe *et al.*, 2013) and hippocampal neurons (Ogawa *et al.*, 2003).

15.5.5 Conclusion and future work

The data presented here, together with those described in chapter 13, identifies phosphorylation of histone H3 at serine 10 (pS10H3) in a sub-population of small-sized DRG neurons following noxious peripheral stimulation by burn injury and capsaicin injection. This dynamic increase is stimulus-specific and selectively affects the nociceptors directly activated by the noxious input. The activation of these nociceptors leads to phosphorylation of MSK1 as the principal kinase *writing* pS10H3, a pathway also responsible, at least partially, to the phosphorylation of CREB. In contrast, repeated brief stimulation with noxious temperature (heat or cold) or pressure, with minimal or no tissue damage, did not induce this epigenetic PTM. Therefore, pS10H3 is regarded as a novel marker present in sensitised primary neurons following tissue injury/inflammation.

Peripheral sensitization describes an activity-dependent process restricted to the injury side that involves intracellular signalling pathways and results in increased activation of the cells involved, leading to altered production, transport and membrane insertion of different channels (Ji *et al.*, 2003; Costigan *et al.*, 2009; White *et al.*, 2011a). pS10H3 seems to regulate the transcriptional activation of a subset of IEG, including c-fos, c-jun, c-myc, cox-2 and zif-268 (Chadee *et al.*, 1999; Cheung *et al.*, 2000b; Davie, 2003; Bilang-Bleuel *et al.*, 2005; Chandramohan *et al.*, 2008; Sarantis *et al.*, 2012; Keum *et al.*, 2013), implicated in this use-dependent process. Hence, pS10H3 at nociceptors following noxious stimulations could be regulating their expression and thus contributing to pain progression. Therefore, future studies

are needed to assess the implication of pS10H3 in such transcriptional changes as well as its behavioural consequences for nociceptive processing.

16 Blocking pS10H3, via MSK1/2-

**deletion, impairs the development of
hypersensitivity after carrageenan
injection**

16.1 Background

At present, persistent/prolonged pain is still not efficiently treated and further research is needed (Breivik *et al.*, 2006; Laycock *et al.*, 2013). The transition from acute to persistent pain is a consequence of neuroplasticity (Ji *et al.*, 1999; Julius & Basbaum, 2001; Ji *et al.*, 2003; Marchand, 2008; Hunt, 2009; Browne *et al.*, 2011; Steeds, 2013). Processes of peripheral and central sensitization, disinhibition, wind-up, hyperalgesia and allodynia are events that involve this use-dependent plasticity, which emphasise the flexibility of the nervous system to adapt in response to tissue injury (Ji & Woolf, 2001; Ji *et al.*, 2003; Woolf *et al.*, 2004; Hunt, 2009; Steeds, 2013; Edelmayer *et al.*, 2014). These changes are driven by cascades of intracellular transducers in response to the inputs received from the injury site and can lead to altered gene expression (Dubner & Ruda, 1992; Zou *et al.*, 2000; Ji & Woolf, 2001; Ji *et al.*, 2003; Woolf *et al.*, 2004). The phosphorylation of MAPK, such as the extracellular signal-regulated kinases 1 and 2 (pERK1/2), are involved in those events of neuroplasticity (Ji *et al.*, 1999; Ji & Woolf, 2001; Ji *et al.*, 2003; Polgar *et al.*, 2007; Black *et al.*, 2008; Gao & Ji, 2009; White *et al.*, 2011a; White *et al.*, 2011b). MAPK modify neuronal excitability by non-transcriptional (altering membrane excitability) and transcriptional means (modifying gene expression) (Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; Kawasaki *et al.*, 2004; Black *et al.*, 2008; Ji *et al.*, 2009; Dib-Hajj *et al.*, 2010; Stambouliau *et al.*, 2010).

Epigenetic modifications, such as PTMs of histone tails, play a key role in the development and maintenance of different pain states by regulating the accessibility of the transcriptional machinery to the DNA (Geranton *et al.*, 2007; Chiechio *et al.*, 2009; Bai *et al.*, 2010; Zhang *et al.*, 2011; Denk & McMahon, 2012; Geranton, 2012; Liu *et al.*, 2012; Crow *et al.*, 2013; Denk *et al.*, 2013; Rahn *et al.*, 2013; Bai *et al.*,

2015; Chen *et al.*, 2015; Crow *et al.*, 2015; Geranton & Tochiki, 2015b; a). After induction of different pain modalities (neuropathic, inflammatory and visceral), the acetylation of histones (e.g. acetylation of lysine 9 or lysine 14 at histone H3, aK9H3/aK14H3) gets up-regulated in neuronal and non-neuronal cells throughout the nociceptive pathway (Zhang *et al.*, 2011; Denk & McMahon, 2012; Bai *et al.*, 2015; Geranton & Tochiki, 2015a). Altering the acetylation of histones, by using inhibitors of deacetylation or other means (KO studies), has shown analgesic effects within different animal noxious models (Chiechio *et al.*, 2009; Bai *et al.*, 2010; Zhang *et al.*, 2011; Denk & McMahon, 2012; Denk *et al.*, 2013; Bai *et al.*, 2015; Geranton & Tochiki, 2015a).

Phosphorylation of histone H3 at serine 10 (pS10H3) is a transcriptionally permissive PTM associated to a subset of IEG, such as c-fos or c-jun (Dong & Bode, 2006; Keum *et al.*, 2013). In neurons, pS10H3 is implicated on memory formation through a NMDA receptor-dependent process and via MAPK (ERKs), with MSK1/2 as the main writers of this epigenetic mark (Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008; Sarantis *et al.*, 2012; Mackowiak *et al.*, 2013; Rotllant *et al.*, 2013; Crow *et al.*, 2015). When present in the same histone tail, there is a functional coupling between pS10H3 and aK14H3 (Cheung *et al.*, 2000a; Cheung *et al.*, 2000b; Lo *et al.*, 2000; Strahl & Allis, 2000; Kim *et al.*, 2012; Ng & Cheung, 2016). Both types of covalent modifications (acetylation and phosphorylation) act neutralising positive charges from histone proteins. This weakens the affinity between them and the DNA, inducing chromatin relaxation and facilitating the access of the transcriptional machinery (Cheung *et al.*, 2000b; Geranton & Tochiki, 2015b). It has even been suggested that pS10H3 could be acting as a rate-limiting step, being required for the subsequent acetylation of

histones (Thomson *et al.*, 1999b; Cheung *et al.*, 2000b; Lo *et al.*, 2000; Strahl & Allis, 2000; Agaloti *et al.*, 2002; Yamamoto *et al.*, 2003; Lo *et al.*, 2005).

Taking all together, and considering the up-regulation of pS10H3-positive neurons observed in previous chapters of this thesis following different noxious stimuli (see above), this epigenetic modification is postulated as a key event during nociceptive processing and a feasible target for analgesic purposes following inflammation/peripheral injury.

16.2 Aims

The **aims** of this chapter are:

- To investigate whether pS10H3 get up-regulated following a peripheral injection of carrageenan.
- To investigate the link between this PTM and the expression of c-Fos, a prototypical IEG and an established marker of cell activation following noxious stimulation.
- To assess the effect of ablating MSK1/2, the principal writers of pS10H3, on the lay down of this modification and the subsequent expression of c-Fos.
- To assess behavioural consequences of ablating MSK1/2 following carrageenan.

16.3 Material and Methods

Altogether, 2 Sprague-Dawley rats, 19 WT mice (strain C57BL/6) and 19 MSK1/2^{-/-} mice (with C57BL/6 background) (Soloaga *et al.*, 2003; Arthur, 2008) were used within this chapter to test the induction of pS10H3 in DRG and spinal neurons and the behavioural consequences of its blockade following carrageenan injection.

16.3.1 Carrageenan injection in Sprague-Dawley rats

Two urethane-anaesthetised Sprague-Dawley rats were subjected to an intraplantar injection of 0.1mL of 1.5% λ -carrageenan (Sigma) and to a post-injury period of either 30 minutes (n=1) or one hour (n=1). During development of the inflammation, oedema was assessed by measuring the increase of paw circumference. At the end of corresponding post-injury period, rats were overdosed with sodium pentobarbital and tissues fixed with PFA by transcardial perfusion. After fixation, L4 and L5 DRG from ipsi- and contra-lateral sides were collected together with corresponding spinal cord sections, post-fixed, cryoprotected, transversally cut at 10 μ m using a cryostat, and mounted in Sperfrost Plus slides. The final sections were **immunostained** against pS10H3 (Santa Cruz, SC8656R, [1:750]), using the tyramide signal amplification protocol, and pERK1/2 (Neuromics, RA15002, [1:1500]) with standard immunofluorescence. The technique was validated by performing negative and positive controls in parallel.

16.3.1.1 Carrageenan injection in WT and MSK1/2^{-/-} mice

Anaesthetised WT mice received an intraplantar injection of either saline (n=2) or 25 μ L of 1.5% λ -carrageenan (n=4). Similar number of MSK1/2^{-/-} mice were treated equally as the WT (n=2, sham; n=4, carrageenan). In all cases, animals were monitored and subjected to a post-injury period of 3 hours, at the end of which

lumbar spinal cord portions were processed as above for **immunostaining** and reacted against pS10H3 and c-Fos (Santa Cruz, sc-52, [1:1000]).

For **behavioural studies**, WT and MSK1/2^{-/-} mice were previously trained and acclimated to the corresponding testing environment. The day of the experiment, mice were lightly anaesthetised with ether and received the intraplantar injection of carrageenan. Mechanical allodynia was tested using von Fray hairs: series of successive von Frey hairs (from 2 to 0.008 g, Bioseb) were applied to the plantar surface of the hind paw (25µL of 1.5% λ-carrageenan; n=6, WT; n=6, MSK1/2^{-/-}) until their bending for 3 seconds or until the animal's withdrawal response and corresponding 50% hind paw withdrawal value was calculated. And heat hyperalgesia was assessed by:

1. Hot plate test: animals that received the intraplantar injection of carrageenan (25µL of 1.5% λ-carrageenan; n=4, WT; n=4, MSK1/2^{-/-}) placed on a hot plate at 52°C and time until they exhibited pain-related behaviour recorded (with a maximal exposure of 20 to prevent injury).
2. Hargreaves test: mice (n=3, WT; n=3, MSK1/2^{-/-}) subjected to a high-intensity beam of light directed to the hind leg that received the injection (50µL of 1.5% λ-carrageenan), and paw withdrawal latencies measured.

In all cases, mice were behaviourally assessed before the injection and at 30 minutes, 1 hour, 2 hours and 3 hours after carrageenan's application. Statistical differences were assessed by two-way ANOVA followed by Bonferroni's post-test, with differences regarded as significant at p<0.05.

16.4 Results

As established previously (Chapters 13 and 12), the number of neurons with nuclei positive for pS10H3 in the DRG and DHSC increases following an extensive noxious stimulation such as scalding-type burn injury, capsaicin injection or high electrical stimulation. This phosphorylation is, at least partially, carried out by MSK1/2 which in turn is activated by MAPK kinases, such as pERK1/2 and pp38, because of the noxious stimuli (Cheung *et al.*, 2000a; Chandramohan *et al.*, 2007; Arthur, 2008; Gutierrez-Mecinas *et al.*, 2011). This PTM is required for the transcriptional activation of different genes, including IEG such as *c-fos*, and thus leading to activation of superior levels within the nociceptive pathway. In this chapter, I tested if nociceptive activation can indeed be reduced by disrupting this cascade of events. To do so, carrageenan injection, a well-known inducer of long term nociceptive activation (van Arman *et al.*, 1970; Fehrenbacher *et al.*, 2012), was performed on Sprague-Dawley rats. These animals were initially tested at different time points to confirm the increase of pS10H3 following carrageenan injection. Then, mice genetically modified to not express MSK1/2 (MSK1/2^{-/-}, KO) and corresponding mice control (WT) were used for behavioural experiments following carrageenan injection to evaluate possible mechanical and thermal differences.

16.4.1 pS10H3 expression in spinal cord and DRG of Sprague-Dawley rats following a carrageenan injection

L4 and L5 spinal cord sections from animals injected with carrageenan (25µL of 1.5% carrageenan dilution) and subjected to a post-injury period of 30 minutes or 1 hour (n=1 for each time point) were immunolabelled against pS10H3 and pERK1/2. A group of neurons positive for pS10H3 (30 min: 24, 1 hour: 22) was detected at the

inner portion of the laminae I, in the ipsilateral DHSC (Figure 16.1). A similar increase of pERK1/2 positive neurons (30 min: 29, 1 hour: 14) was detected in the same area of the ipsilateral DHSC. On the contrary, no relevant up-regulation was detected in the contralateral side with either pS10H3 (30 min: 0, 1 hour: 1) or pERK1/2 (30 min: 3, 1 hour: 8).

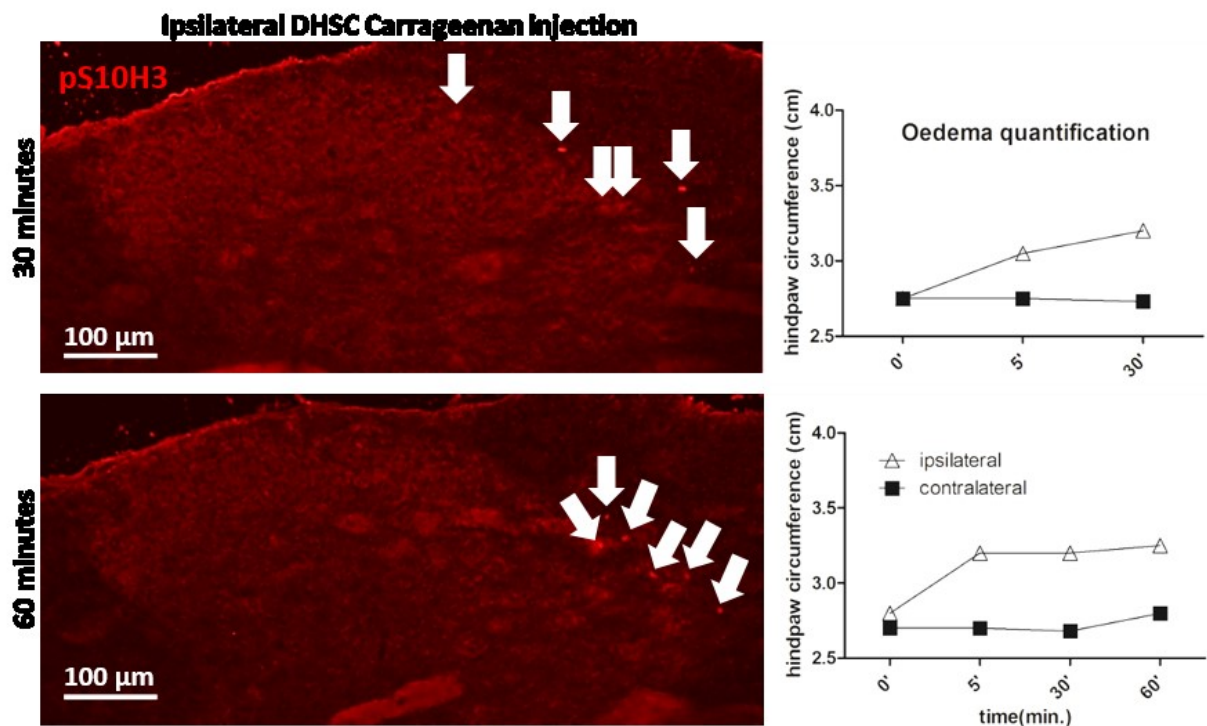


Figure 16.1. **Carrageenan injection induces pS10H3 positive neurons in the DHSC.** Images show pS10H3 immunospositive staining in the ipsilateral DHSC of animals (n=1) injected with carrageenan (25 μ L of 1.5% carrageenan dilution) and subjected to a post-injury period of 30 minutes (first row) or 1 hour (second row). Graphs show increase of hind paw circumference (oedema development) in the ipsilateral (white triangles) and contralateral (black squares) sides of corresponding animals. Scale bars equal 100 μ m. Note that the positive staining (arrows) is localised in the inner portion of lamina I. No positive cells were detected at the contralateral side (no images shown).

Following, L4 and L5 DRG sections from the same animals were immunoreacted against pS10H3. Here, no increase of pS10H3 positive neuronal nuclei was detected at any time point and in either ipsi- or contra-lateral sides (Figure 16.2).

Oedema development was also monitored throughout the experimental procedure (Figure 16.1). An increase on the ipsilateral hind paw circumference was detected in both animals (30 min: from 2.75cm before the injection to 3.2cm after 30 minutes, 1

hour: from 2.8cm before the injection to 3.2cm one hour after) that was not observed in the contralateral hind paws (30 min: from 2.75cm to 2.75cm, 1 hour: from 2.7cm to 2.8cm).

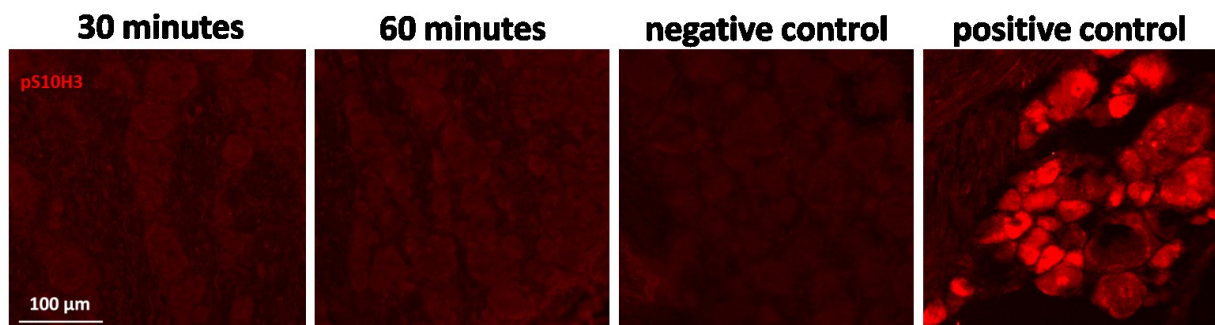


Figure 16.2. **Carrageenan injection does not induce pS10H3 in DRG.** Images show pS10H3 immunopositive staining in the ipsilateral DHSC of animals (n=1) injected with carrageenan (25μL of 1.5% carrageenan dilution) and subjected to a post-injury period of 30 minutes or 1 hour. In the negative control no primary antibody was added, and for positive control same staining was applied in parallel in DRG of animals subjected to a capsaicin injection. Scale bar equals 100μm. Note that no positive staining is observed in animals subjected to carrageenan injection, while it was present (mainly nuclear but also cytoplasmic) in the positive control.

16.4.2 pS10H3 expression in spinal cord of WT mice following a carrageenan injection

Once established that carrageenan injection increases pS10H3 expression in rat spinal cord neurons, its expression was assessed in WT mice tissues.

L4 and L5 spinal cord sections of WT mice subjected to an injection of either saline (sham injury, n=2) or carrageenan (25μL of 1.5% carrageenan dilution, n=4) were immunolabelled against pS10H3 and c-Fos (Figure 16.3). A population of pS10H3 positive neurons was observed in the superficial layers of the ipsilateral DHSC of animals subjected to carrageenan that was not present in the animals treated with saline. Nonetheless, positive cells in the white matter surrounding the laminae were observed, possibly correspondingly to glial cells (red staining in Figure 16.3). Similarly, an increase on c-Fos positive cells in the superficial laminae of the

ipsilateral dorsal horn was observed at 3-hours post-injection of carrageenan that did not appear when animals were treated with saline (green staining in Figure 16.3).

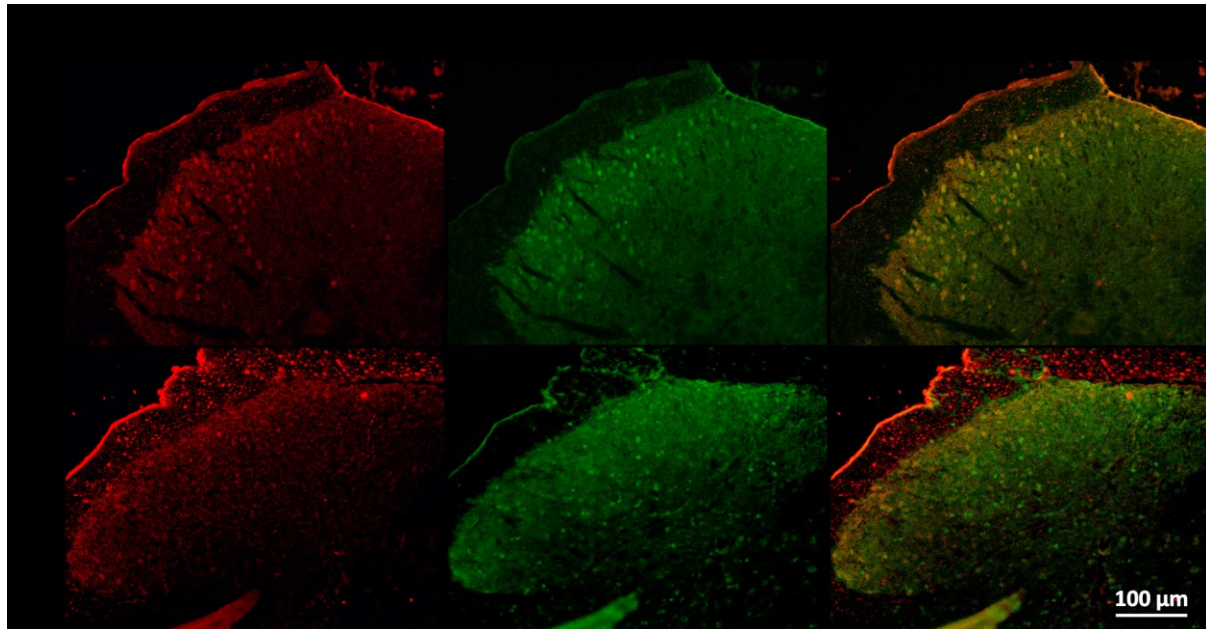


Figure 16.3. **Carrageenan injection induces pS10H3 and c-Fos positive neurons in the DHSC of WT mice.** Images show immunopositive staining for pS10H3 (red) and c-Fos (green) at the ipsilateral DHSC of WT mice injected with carrageenan (25 μ L of 1.5% carrageenan dilution, first row, n=4) or saline (25 μ L, second row, n=2) and subjected to a post-injury period of 3 hours. Scale bar equals 100 μ m. Note that the positive neurons are localised in the lamina I and only appears in the animals subjected to carrageenan injection. There is a high degree of co-expression between pS10H3 and c-Fos (~80%). Note also that some glial cells also appear pS10H3+, but not c-Fos.

Statistical analysis of the number of pS10H3 positive neurons revealed a significant increase in the ipsilateral DHSC of carrageenan injected animals when compared with animals injected with saline (saline: 4.5 ± 1.5 , Mdn=4.5, n=2; carrageenan: 44.5 ± 2.33 , Mdn=43.5, n=4; $p < 0.001$, GzLM, Figure 16.4) that was not observed on the contralateral side (saline: 5.5 ± 1.5 , Mdn=5.5, n=2; carrageenan: 4 ± 1.08 , Mdn=3.5, n=4; $p = 0.582$, GzLM).

The number of c-Fos positive cells was also significantly increased in the ipsilateral DHSC 3 hours following after carrageenan injection (saline: 6.5 ± 0.5 , Mdn=6.5, n=2; carrageenan: 61.5 ± 4.37 , Mdn=61.5, n=4; $p < 0.001$, GzLM, Figure 16.4). No c-Fos

increase was detected in the contralateral DHSC (saline: 8 ± 1 , Mdn=8, n=2; carrageenan: 7.75 ± 0.49 , Mdn=7.5, n=4; $p=0.199$, GzLM).

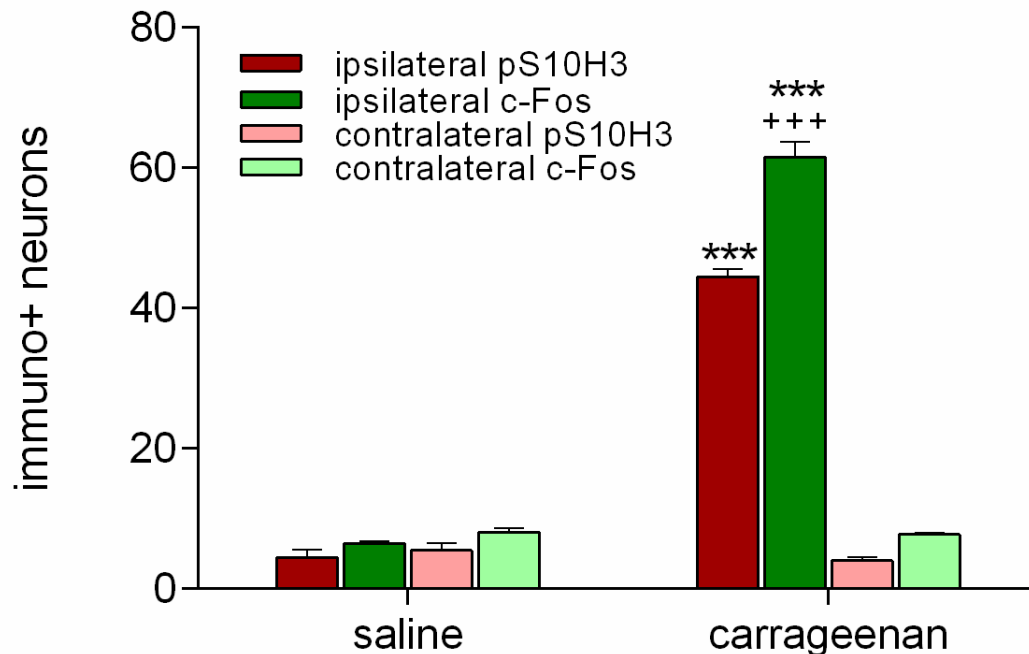


Figure 16.4. **Carrageenan increases pS10H3 and c-Fos positive neurons in the DHSC of WT mice.** This graph shows the number of immunopositive neurons for pS10H3 (red) and c-Fos (green) counted in the ipsilateral and contralateral DHSC of WT mice injected with carrageenan (25 μ L of 1.5% carrageenan dilution, n=4) or saline (25 μ L, second row, n=2) and subjected to a post-injury period of 3 hours. *** $p<0.001$ vs. ipsilateral pS10H3 saline; +++ $p<0.001$ vs. ipsilateral pS10H3 carrageenan.

Analysis of the co-expression values of both markers following 3 hours of the carrageenan injection (n=4) revealed that around 80% ($83.21 \pm 3.79\%$) of the pS10H3 positive neurons also expressed c-Fos whereas $78.32 \pm 3.13\%$ of the c-Fos positive cells were also positive for pS10H3.

16.4.3 pS10H3 expression in spinal cord of MSK1/2^{-/-} mice following a carrageenan injection

The same experimental design was also applied on the MSK1/2^{-/-} mice (KO mice): subjected to saline (n=2) or carrageenan (n=4) injection followed by a post-injury period of 3 hours.

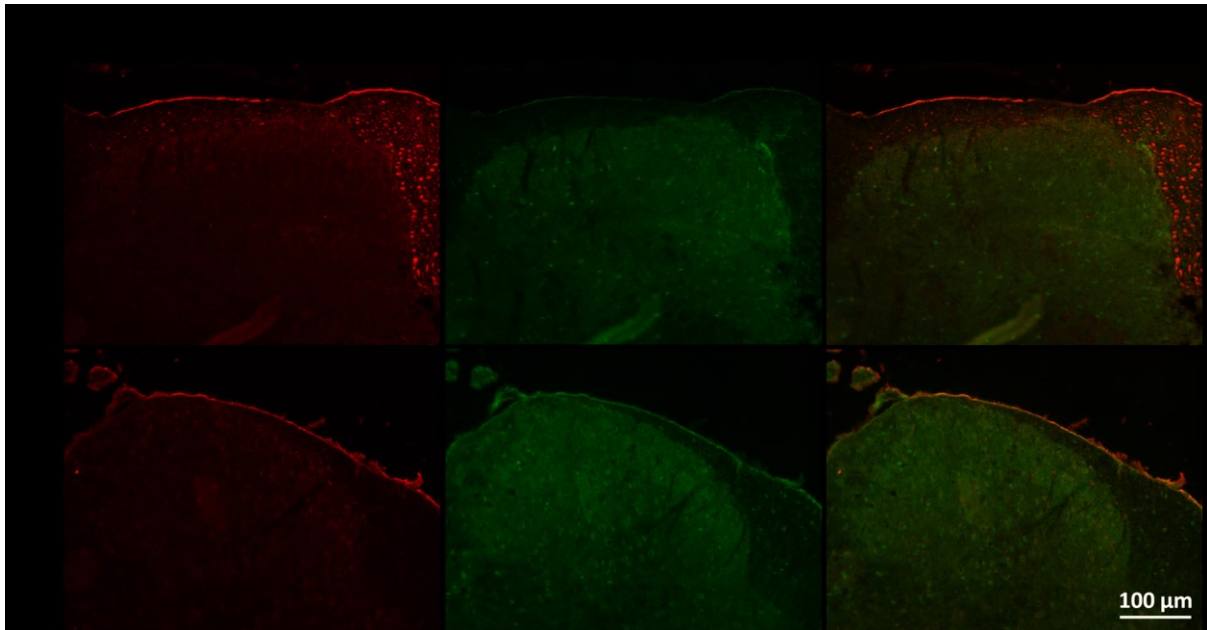


Figure 16.5. **Carrageenan injection does not induce pS10H3 or c-Fos in the DHSC of KO mice.** Images show immunopositive staining for pS10H3 (red) and c-Fos (green) at the ipsilateral DHSC of KO mice (MSK1/2^{-/-}) injected with carrageenan (25μL of 1.5% carrageenan dilution, first row, n=4) or saline (25μL, second row, n=2) and subjected to a post-injury period of 3 hours. Scale bar equals 100μm. Note that no positive neurons are observed after any treatment although some glial cells can be pS10H3, but not c-Fos, positive in a similar fashion as observed in the WT mice.

Sections from L4 and L5 spinal cord of these animals were immunoreacted against pS10H3 and c-Fos (Figure 16.5). No positive neurons for pS10H3 or c-Fos were detected in any side, ipsi- or contra-lateral, and after either treatment. Only some pS10H3 glial cells were detected in some animals studied (red staining in Figure 16.5). Accordingly, the statistical analysis did not show any increase of pS10H3 positive neurons in the animals injected with carrageenan in relation to the numbers observed in the saline-injected animals in either ipsi- (saline: 2.5 ± 0.5 , Mdn=2.5, n=2; carrageenan: 1.25 ± 0.75 , Mdn=1, n=4, $p=0.646$, GzLM) or contra-lateral sides (saline: 2.5 ± 0.5 , Mdn=2.5, n=2; carrageenan: 0.25 ± 0.25 , Mdn=0.00, n=4; $p=0.408$, GzLM, Figure 16.6). Likewise, no significant up-regulation of c-Fos was detected in the ipsilateral (saline: 5 ± 0 , Mdn=5, n=2; carrageenan: 5.75 ± 2.75 , Mdn=4.5, n=4; $p=0.783$, GzLM) or contralateral side (saline: 3.5 ± 0.5 , Mdn=3.5, n=2; carrageenan: 2 ± 1.22 , Mdn=1.5, n=4, $p=0.582$, GzLM, Figure 16.6).

Thus, while carrageenan induced pS10H3 and c-Fos in WT mice (see above) the up-regulation of these markers was silenced in the MSK1/2 KO mice.

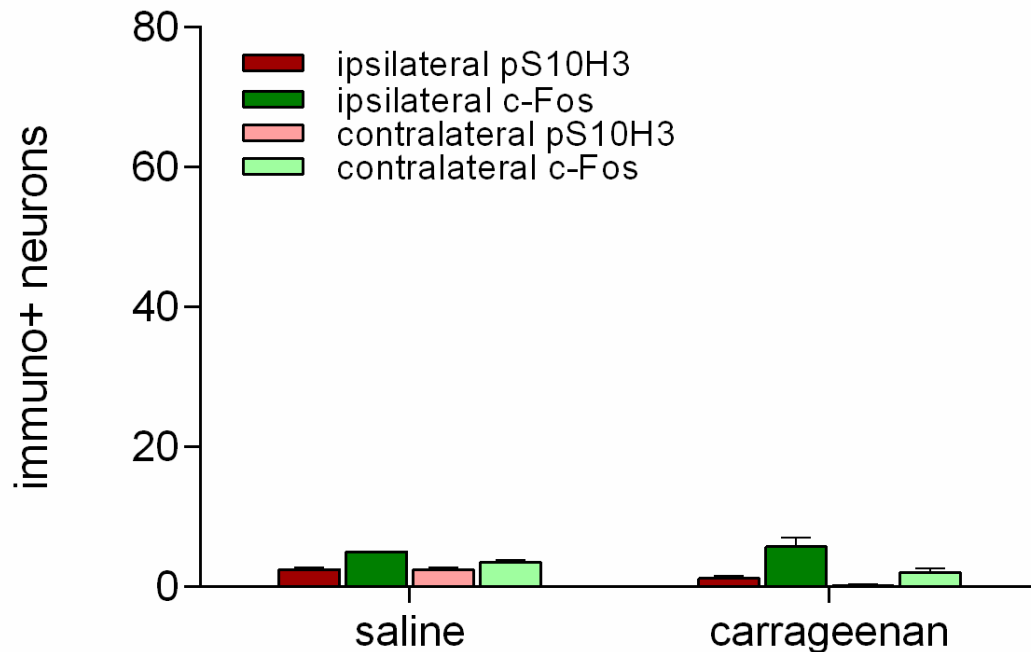


Figure 16.6. **No significant increase of either pS10H3 or c-Fos is observed in the DHSC of KO mice following carrageenan injection.** This graph shows the number of immunopositive neurons for pS10H3 (red) and c-Fos (green) counted in the ipsilateral and contralateral DHSC of KO mice (MSK1/2^{-/-}) injected with carrageenan (25μL of 1.5% carrageenan dilution, n=4) or saline (25μL, second row, n=2) and subjected to a post-injury period of 3 hours.

16.4.4 Mechanical allodynia in WT and MSK1/2^{-/-} mice following a carrageenan injection

Here we studied the effect of blocking pS10H3 on the development of persistent pain. To do so, **Von Frey hairs** (Chung *et al.*, 1992) were used to assess the development of hypersensitivity to mechanical stimulation (mechanical allodynia) following a carrageenan injection (25μL of 1.5% carrageenan dilution) in WT mice and in comparison with MSK1/2^{-/-} mice, to which the “writers” of the phosphorylation of interest were not present (Thomson *et al.*, 1999a; Soloaga *et al.*, 2003; Arthur, 2008).

In agreement with previous studies (van Arman *et al.*, 1970), carrageenan rapidly induced mechanical allodynia in WT animals, characterised by a reduction of the 50% hind leg withdrawal threshold that persisted until the end of the 3 hours study (0 min-basal value-: 0.86 ± 0.07 , $n=6$; 30 min: 0.17 ± 0.08 , $n=6$, $p < 0.001$ vs. basal; 1 hour: 0.06 ± 0.01 , $n=6$, $p < 0.001$ vs. basal; 2 hours: 0.03 ± 0.003 , $n=6$, $p < 0.001$ vs. basal; 3 hours: 0.04 ± 0.01 , $n=6$, $p < 0.001$ vs. basal; multiple ANOVA followed by Tukey's test, Figure 16.7).

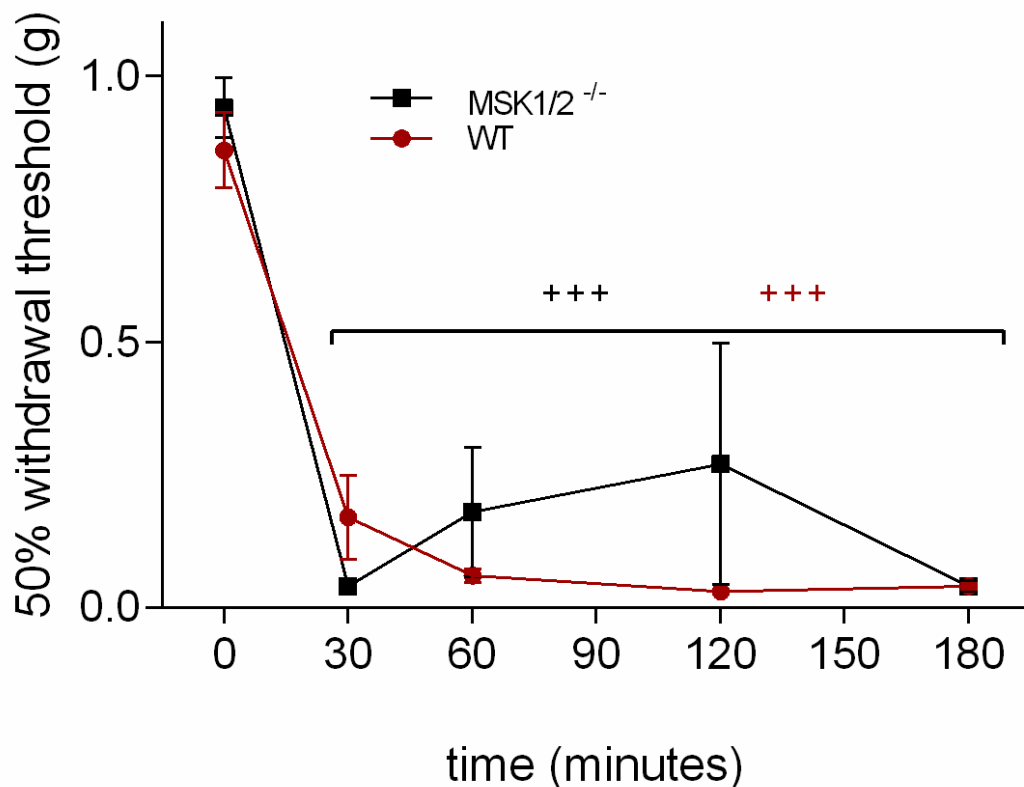


Figure 16.7. **Mechanical allodynia develops normally in the absence of MSK1/2.** Time course of hind paw 50% mechanical withdrawal threshold using Von Frey hairs following and injection of carrageenan. WT mice ($n=6$) are indicated with red circles and MSK1/2^{-/-} mice ($n=6$) with black squares. Note that in both groups, MSK1/2^{-/-} and WT mice, mechanical allodynia developed similarly. +++ $p < 0.001$ vs. 0 minutes of the corresponding mice group.

Similar findings were observed in the KO mice where mechanical allodynia also developed (0 min-basal value-: 0.94 ± 0.06 , $n=6$; 30 min: 0.04 ± 0.003 , $n=6$, $p < 0.001$ vs. basal; 1 hour: 0.18 ± 0.12 , $n=6$, $p < 0.001$ vs. basal; 2 hours: 0.27 ± 0.23 , $n=6$,

$p < 0.001$ vs. basal; 3 hours: 0.04 ± 0.01 , $n=6$, $p < 0.001$ vs. basal; multiple ANOVA followed by Tukey's test, Figure 16.7).

Despite the fact that KO mice showed some signs of recovery at 1 hour and 2 hours post carrageenan injection, no significant differences on the response to mechanical stimuli were observed between WT and KO mice (WT vs. KO mice; 0 min: $p=0.999$, 30 min: $p=0.979$, 1 hour: $p=0.985$, 2 hours: $p=0.561$, 3 hours: $p=1$; multiple ANOVA followed by Tukey's test, Figure 16.7).

16.4.5 Heat hyperalgesia does not develop in $MSK1/2^{-/-}$ mice following carrageenan injection

Finally, the development of hypersensitivity to noxious thermal stimulation (heat hyperalgesia) was also assessed in the carrageenan model. Animals injected with carrageenan (25 μ L of 1.5% carrageenan dilution) were subjected to the **hot plate test** (Eddy & Leimbach, 1953). The withdrawal latency was gradually reduced in WT mice, reaching significance at 1 hour post-carrageenan injection and lasting until the end of the experiment at 3 hours (0 min-basal value-: 10.15 ± 0.87 , $n=4$; 30 min: 10.5 ± 2.6 , $n=4$, $p=0.466$ vs. basal; 1 hour: 8.25 ± 0.97 , $n=4$, $p=0.03$ vs. basal; 2 hours: 5.99 ± 1.09 , $n=4$, $p < 0.001$ vs. basal; 3 hours: 3.83 ± 0.87 , $n=4$, $p < 0.001$ vs. basal; multiple ANOVA followed by Tukey's test, Figure 16.8). In contrast, no reduction of withdrawal latency was observed in the KO mice. Instead, there was a gradual increase that reached significance at 3 hours post-injection of carrageenan (0 min-basal value-: 9.32 ± 0.53 , $n=4$; 30 min: 12.32 ± 0.94 , $n=4$, $p=0.782$ vs. basal; 1 hour: 13.88 ± 1.16 , $n=4$, $p=0.261$ vs. basal; 2 hours: 15.07 ± 0.67 , $n=4$, $p=0.067$ vs. basal; 3 hours: 16.13 ± 1.29 , $n=4$, $p=0.016$ vs. basal; multiple ANOVA followed by Tukey's test, Figure 16.8). Accordingly, the response to noxious heat stimulation was

significantly different between WT and KO mice following a carrageenan injection (WT vs. KO mice; 0 min: $p=1$, 30 min: $p=0.651$, 1 hour: $p<0.001$, 2 hours: $p<0.001$, 3 hours: $p<0.001$; multiple ANOVA followed by Tukey's test, Figure 16.8).

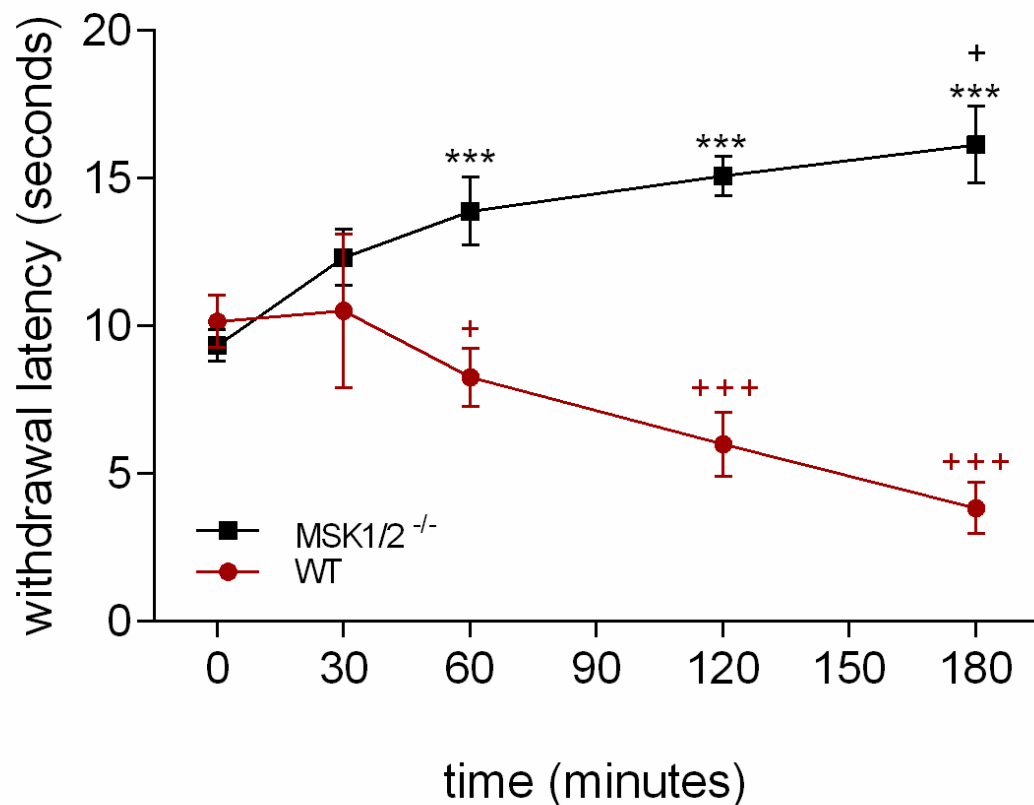


Figure 16.8. Carrageenan injection fails to develop heat hyperalgesia in the absence of MSK1/2; studied using the hot plate test. Time course of withdrawal latency of the hind paw on the hot plate following an injection of carrageenan. WT mice (n=4) are indicated with red circles and MSK1/2^{-/-} mice (n=4) with black squares. Note that MSK1/2^{-/-} mice developed heat hypoalgesia whereas WT mice showed heat hyperalgesia. + $p<0.05$ vs. 0 minutes of the corresponding mice group; +++ $p<0.001$ vs. 0 minutes of the corresponding mice group; *** $p<0.001$ vs. WT value at the same time point.

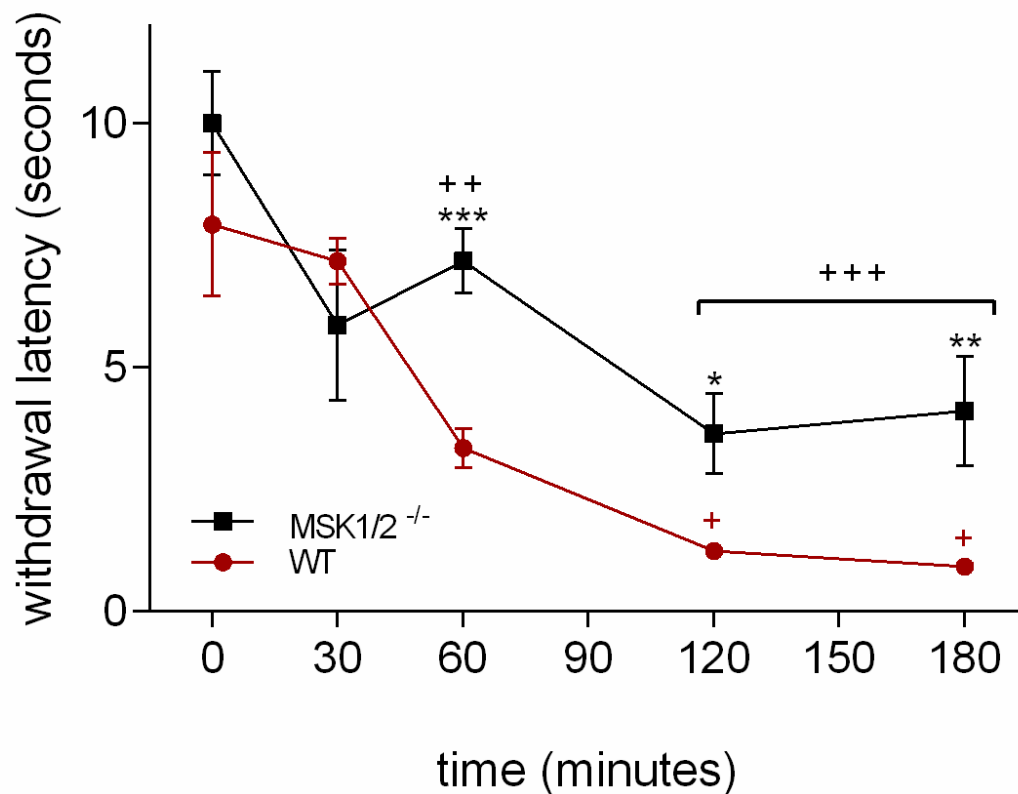


Figure 16.9. **Carrageenan injection fails to develop heat hyperalgesia in the absence of MSK1/2; studied using the Hargreaves Test.** Time course of withdrawal latency of the hind paw on the Hargreaves assay following and injection of carrageenan. WT mice (n=3) are indicated with red circles and MSK1/2^{-/-} mice (n=3) with black squares. + p<0.05 vs. 0 minutes of the corresponding mice group; ++ p<0.01 vs. 0 minutes of the corresponding mice group; +++ p<0.001 vs. 0 minutes of the corresponding mice group; * p<0.05 vs. WT value at the same time point; ** p<0.01 vs. WT value at the same time point; *** p<0.001 vs. WT value at the same time point.

To further validate these findings, a second behavioural model was carried out: **Hargreaves assay** (Hargreaves *et al.*, 1988). This test was used after an injection of carrageenan (50µL of 1.5% carrageenan dilution) in WT and KO mice. Similarly as observed in the hot plate test, a gradual reduction of the withdrawal latency was observed in WT mice. This reduction reached significance at 2 hours post-injection and persisted until the end of the experiment (0 min-basal value-: 7.93±1.48, n=3; 30 min: 7.18±0.47, n=3, p=1 vs. basal; 1 hour: 3.35±0.4, n=3, p=0.113 vs. basal; 2 hours: 1.24±0.04, n=3, p=0.001 vs. basal; 3 hours: 0.92±0.05, n=3, p=0.001 vs. basal; multiple ANOVA followed by Tukey's test, Figure 16.9). A similar trend on

withdrawal latency was assessed in KO mice, where significance was achieved at 1 hour post-injection of carrageenan (0 min-basal value-: 10 ± 1.06 , $n=3$; 30 min: 5.86 ± 1.54 , $n=3$, $p=0.101$ vs. basal; 1 hour: 7.17 ± 0.66 , $n=3$, $p=0.005$ vs. basal; 2 hours: 3.64 ± 0.83 , $n=3$, $p<0.001$ vs. basal; 3 hours: 4.1 ± 1.12 , $n=3$, $p<0.001$ vs. basal; multiple ANOVA followed by Tukey's test, Figure 16.9). Nonetheless, the reduction in withdrawal latency was less steep in this last group, thus exhibiting significant differences on response to noxious heat stimulation between WT and KO mice (WT vs. KO mice; 0 min: $p=0.853$, 30 min: $p=0.986$, 1 hour: $p<0.001$, 2 hours: $p=0.041$, 3 hours: $p=0.002$; multiple ANOVA followed by Tukey's test, Figure 16.9).

16.5 Discussion

The data presented in this chapter, identifies pS10H3 in a sub-population of superficial DHSC neurons following intraplantar injection of carrageenan in both Sprague-Dawley rats and WT mice. Similarly, pERK1/2 gets up-regulated in the spinal cord at 30 minutes and 1 hour after this inflammation model. At 3 hours of such noxious stimulus, pS10H3 is still up-regulated co-expressing with c-Fos, while this induction of pS10H3 and subsequent transcription of c-Fos is disrupted in animals lacking MSK1/2. Additionally, the lack of MSK1/2 prevented the development of heat hyperalgesia.

16.5.1 Carrageenan injection up-regulates pS10H3 in superficial DHSC

There is an up-regulation of spinal pS10H3 by intraplantar carrageenan in Sprague-Dawley rats, as observed by immunostaining after 30 and 60 minutes. The expression of pERK1/2 get also increased in a similar fashion. Interestingly, the number of positive neurons for both was quite constant although more animals are needed to assess statistical differences. The increase in pERK1/2 is similar as initially observed by Galan and collaborators in 2002 (Galan *et al.*, 2002). They described a spinal peak of pERK1/2 at 30 minutes of the carrageenan induction, which returned to basal values at 90 minutes of the stimulation in both mice and rats (Galan *et al.*, 2002). Nonetheless, Cho and colleagues mentioned that the expression of pERK1/2 was already basal by one hour of the carrageenan injection in Sprague-Dawley rats (Cho *et al.*, 2015). In any case, the increase of pERK1/2 seems to be a carrageenan-dependent event. The activation of ERK1/2 can lead to pS10H3 throughout MSK1/2 in PC12 cells and hippocampal neurons (Deak *et al.*,

1998; Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008). This, together with the up-regulation of pS10H3 observed in a sub-population of peripheral DHSC neurons after peripheral burn injury and capsaicin injection (Chapters 13 and 12) and the data presented here, suggests that pS10H3 is a nociceptive-specific activation and, at least partially, pERK1/2-dependent.

On the contrary, no up-regulation of pS10H3 was detected in DRG neurons following either 30 minutes or 1 hour of the carrageenan injection. This could be because this model is not able to increase pS10H3, although this possibility is unlikely to be true due to the presence of this epigenetic mark following different models of inflammation and/or injury (see previous chapters). Another possibility is that pS10H3 was initially up-regulated but reduced by 30 minutes of the injection, which could be a consequence of the expression of the mitogen-activated protein kinase phosphatase-1 (MKP1). MKP1 is a phosphatase able to remove this epigenetic mark (*eraser*). It is a nuclear protein encoded by an IEG the expression of which is governed by pS10H3 itself (Pages, 2009). In human endothelial cells, MKP1 seems to act as a temporal transcriptional repressor/regulator: stimulating with thrombin and VEGF (vascular endothelial growth factor) induces MKP1 as early as 10 minutes after, with a maximal peak at 1 hour, which modulates the expression of inflammatory genes (Kinney *et al.*, 2009).

Furthermore, the increase on paw thickness observed by the carrageenan injection confirms that the model successfully induced inflammation, which was similar as described previously by other researchers (Galan *et al.*, 2002; Fehrenbacher *et al.*, 2012; Pritchard *et al.*, 2016).

Taking all together, the carrageenan injection is a feasible model to study the induction of pS10H3 and the consequences of its blockade for nociceptive processing in rodents.

16.5.2 pS10H3 at superficial spinal neurons following carrageenan injection is MSK1/2-dependent and is associated with the expression of c-Fos

After 3 hours of the peripheral injection of carrageenan, there is an up-regulation of pS10H3 in superficial DHSC neurons of mice. The fact that this stimulus-dependent activation is observed at such longer time-points supports the involvement of pS10H3 in the development of prolonged pain, and probably in processes such as central sensitization. The phosphorylation of histone H3 at this residue leads to the expression of c-Fos (Mahadevan *et al.*, 1991; Barratt *et al.*, 1994; Thomson *et al.*, 1999a; Cheung *et al.*, 2000a; Cheung *et al.*, 2000b; Clayton *et al.*, 2000; Nowak & Corces, 2000; Prigent & Dimitrov, 2003; Gutierrez-Mecinas *et al.*, 2011; Keum *et al.*, 2013), which is encoded by an IEG and is an established marker for nociceptive processing in spinal neurons (Hunt *et al.*, 1987; Todd *et al.*, 2002; Gao & Ji, 2009; White *et al.*, 2011a). Accordingly, there is great proportion of neurons that co-express these markers.

MSK1/2 are protein kinases activated downstream of MAPK such as pERK1/2 (De Cesare *et al.*, 1998; Deak *et al.*, 1998; Davie, 2003; Dong & Bode, 2006) and considered the main effectors laying down pS10H3 in neurons (Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008; Mackowiak *et al.*, 2013). Consistently, deleting MSK1/2 completely prevented the S10H3 phosphorylation, similarly as observed in the isolated cords where MSK1/2

was blocked prior to the capsaicin application (Chapter 12). Therefore, MSK1/2 are suggested as the main effector kinases (*writers*) phosphorylating S10H3 in superficial DHSC neurons following inflammation/tissue injury. Additionally, the deletion of MSK1/2 also prevented the expression of c-Fos. Hence, confirming the functional correlation between pS10H3 and the subsequent expression of IEG such as c-Fos also exist at neurons of the superficial DHSC.

16.5.3 Deletion of MSK1/2 prevents the development of heat hyperalgesia following carrageenan injection

Heat hyperalgesia development is impaired in the MSK1/2^{-/-} mice, while mechanical allodynia developed normally after carrageenan injection. If the absence of MSK1/2 at superficial spinal neurons is solely responsible of the heat hypoalgesia after carrageenan injection, it suggests that the up-regulation of pS10H3 affects neurons that respond exclusively to noxious heat. This dissociation between thermal and mechanical hypersensitivity have been observed several times within the literature and has been attributed to either the activation of different sub-populations within the spinal cord and DRG cell types and/or the activation of different intracellular pathways (Zou *et al.*, 2000; Ji *et al.*, 2002b; Gould *et al.*, 2004; Priest *et al.*, 2005; Geranton *et al.*, 2008; Todd, 2010; White *et al.*, 2011a; Shields *et al.*, 2012; Li *et al.*, 2014). Thermal sensitization seems to rely mainly into peripheral sensitization while mechanical sensitivity seems much more complex and involves central sensitization via depression of the serotonergic pathway (Ji *et al.*, 2002b; Geranton *et al.*, 2008). Moreover, non-peptidergic nociceptors seem to function mainly to transmit noxious mechanical stimuli, while peptidergic C-fibres are responsible for noxious thermal stimuli (Todd, 2010). Neurons that express Nav1.7 together with Nav1.8 seem

important to process noxious mechanical responses while the Nav1.7-positive neurons that do not express Nav1.8 are important for noxious thermosensation (Nassar *et al.*, 2004; Eijkelkamp *et al.*, 2012; Shields *et al.*, 2012; Minett *et al.*, 2015). Additionally, some researcher suggest that mechanical hyperalgesia is processed via PKC in a NMDA-independent manner (Zou *et al.*, 2000; Nozaki-Taguchi & Yaksh, 2002). Pre-treatment with a somatostatin receptor activator before the injection of bee venom in the hind paw of rodents prevented the induction of thermal hyperalgesia, while mechanical hypersensitivity still developed (Li *et al.*, 2014). Rhizotomy of the dorsal horn was able to prevent mechanical allodynia, but not thermal hyperalgesia, within the neuropathic model of spinal nerve ligation in rodents (Obata *et al.*, 2004; White *et al.*, 2011a), in a process that also seems to involve activation of ERK within spinal cord microglia (Katsura *et al.*, 2006; White *et al.*, 2011a). Ablation of non-peptidergic nociceptive C afferent fibres in mouse selectively inhibits sensitivity to noxious mechanical stimuli, but not noxious thermal stimuli (Todd, 2010). Within Nav1.7 cKO mice, only heat hyperalgesia was impaired after either CFA injection or burn injury while mechanical hypersensitivity remained intact (Minett *et al.*, 2012; Shields *et al.*, 2012). Therefore, this suggests that different sets of superficial DHSC neurons get activated, inducing pS10H3, in a nociceptive-modality specific modality, regulating heat hyperalgesia.

The reported lack of heat hyperalgesia shown here may point at MSK1/2 as potential therapeutic targets for managing inflammatory pain. However, MSK1/2 are found in a variety of cells and, through activation of CREB and phosphorylation of histones, are involved in the regulation of different processes such as the immune and inflammatory responses (Ananieva *et al.*, 2008; Bertelsen *et al.*, 2011; MacKenzie *et al.*, 2013) and learning and memory formation (Chwang *et al.*, 2006; Arthur, 2008;

Gutierrez-Mecinas *et al.*, 2011), hence introducing potential side effects. To avoid this issue, research should focus on compounds acting on the *readers* of the different epigenetic marks, rather than on the *writers*, since they have a greater situation-specific control (Denk & McMahon, 2012; Keum *et al.*, 2013). For example: targeting 14-3-3 ϵ instead of MSK1/2 to control pain development (Keum *et al.*, 2013).

16.5.4 Conclusion and future work

Here, pS10H3 was identified in a population of superficial DHSC following an injection of carrageenan into a hind leg of rodents. pS10H3 is a permissive epigenetic mark associated with the transcription of certain IEG, including c-fos, c-jun, arc (activity-regulated cytoskeleton-associated protein gene) and ptgs2 (prostaglandin synthase 2) among others (Chandramohan *et al.*, 2007; Reul *et al.*, 2009), the product of which participate in processes of neuronal sensitization (Bramham *et al.*, 2008; Keum *et al.*, 2013) and regulate the transcription of secondary response genes (SRG) (Zhang *et al.*, 2002). Ablation of MSK1/2, the principal kinases responsible for producing this modification (the writers), not only prevented the induction of pS10H3 and subsequent transcriptional activation of c-Fos, it also resulted in impaired development of heat hyperalgesia. Despite interactions with other epigenetic modifications, pS10H3 is suggested to serve as a rate-limiting step as well as a signal integrator for chromatin remodelling and is limited to neurons (Cheung *et al.*, 2000b; Bilang-Bleuel *et al.*, 2005; Chwang *et al.*, 2006; Chandramohan *et al.*, 2008; Sarantis *et al.*, 2012; Keum *et al.*, 2013). Although affecting a small number of nucleosomes, pS10H3 could be facilitating fast directed recruitment and activation of the transcriptional machinery to some gene promoters shifting the balance of genes transcribed (Thomson *et al.*, 2001; Lefebvre *et al.*,

2002; Saccani *et al.*, 2002; Bilang-Bleuel *et al.*, 2005) and thus contributing to the development and maintenance of persistent pain. Therefore, this data together with previous chapters, points at pS10H3 as a crucial event for nociceptive processing.

17 $\text{Na}_v1.7$ blockade by protoxin II

reduces the neuronal nociceptive

activation by a burn injury

17.1 Background

At present, opioids are the principal drugs used for pain control in burn care facilities (Carrougher *et al.*, 2003; Wiechman Askay *et al.*, 2009; Phoenix-Society, 2011). However, pain associated to burn injuries, a common feature of these type of injuries (Ulmer, 1997; Carrougher *et al.*, 2003; Phoenix-Society, 2011), is being undertreated; the strategies used are not sufficient for the management of most burn pain (Ptacek *et al.*, 1995; Ulmer, 1997; Dauber *et al.*, 2002; Demling, 2008; Wiechman Askay & Patterson, 2008). Accordingly, burn injury-associated pain is one of the principal concerns of burn survivors (Dauber *et al.*, 2002; Sandoval *et al.*, 2014), even perceived as equally important as the resumption of normal function (Sandoval *et al.*, 2014).

Nav1.7 is principally expressed in small diameter DRG neurons (Toledo-Aral *et al.*, 1997; Nassar *et al.*, 2004; Dib-Hajj *et al.*, 2007; Minett *et al.*, 2012; Dib-Hajj *et al.*, 2013), mainly nociceptors (A δ and C fibres) (Toledo-Aral *et al.*, 1997; Djouhri *et al.*, 2003; Nassar *et al.*, 2004; Dib-Hajj *et al.*, 2007; Black *et al.*, 2012; Dib-Hajj *et al.*, 2013), where co-expresses highly with Nav1.8 (Nassar *et al.*, 2004; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Minett *et al.*, 2012; Dib-Hajj *et al.*, 2013). Nav1.7 is present at the soma and peripheral and centrally directed fibres of these neurons (Toledo-Aral *et al.*, 1997; Dib-Hajj *et al.*, 2007; Black *et al.*, 2012; Minett *et al.*, 2012; Dib-Hajj *et al.*, 2013). Nav1.7 generates fast activation and inactivation currents with a slow-repriming from the inactivated states, which allows it to act as a threshold channel for firing action potentials (Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Dib-Hajj *et al.*, 2010; Black *et al.*, 2012; Hoeijmakers *et al.*, 2012; Dib-Hajj *et al.*, 2013; Zhang *et al.*, 2013).

Studies in humans point at mutations in the gene SCN9A, which encodes for Nav1.7, as causative of altered pain perception (Yang *et al.*, 2004; Cox *et al.*, 2006; Fertleman *et al.*, 2006; Dib-Hajj *et al.*, 2007; Nilsen *et al.*, 2009; Dib-Hajj *et al.*, 2010; Raouf *et al.*, 2010; Hoeijmakers *et al.*, 2012; Crow *et al.*, 2013; Ramirez *et al.*, 2014). Gain-of-function mutations are found in cases of inherited primary erythromelalgia, paroxysmal extreme pain disorder, idiopathic small fibre neuropathy and distal limb pain (Cummins *et al.*, 2004; Yang *et al.*, 2004; Fertleman *et al.*, 2006; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Dib-Hajj *et al.*, 2010; Raouf *et al.*, 2010; Eijkelkamp *et al.*, 2012; Hoeijmakers *et al.*, 2012; Dib-Hajj *et al.*, 2013; Huang *et al.*, 2014b); while Nav1.7 loss-of-function mutations lead to Congenital Insensitivity to Pain (Cox *et al.*, 2006; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Nilsen *et al.*, 2009; Raouf *et al.*, 2010; Weiss *et al.*, 2011; Eijkelkamp *et al.*, 2012; Hoeijmakers *et al.*, 2012; Ramirez *et al.*, 2014).

Importantly, recent findings in animal models show that Nav1.7 has a key role in burn-injury associated pain (Black *et al.*, 2012; Hoeijmakers *et al.*, 2012; Shields *et al.*, 2012). Shields and colleagues showed that Nav1.7-cKO mice failed to develop heat hyperalgesia following a peripheral burn injury, while it developed normally in control animals (wild type mice from the same strain background) (Shields *et al.*, 2012). Moreover, this also correlated with the lack of c-Fos induction. Therefore, Nav1.7 is indeed needed to develop heat hypersensitivity and could be a feasible target to control pain in burn patients (Shields *et al.*, 2012).

Different natural toxins have been tested to specifically block Nav1.7 with variable grades of success (Middleton *et al.*, 2002; Cummins *et al.*, 2004; Schmalhofer *et al.*, 2008; Clare, 2010; Eijkelkamp *et al.*, 2012; Dib-Hajj *et al.*, 2013; Yang *et al.*, 2013; Zhang *et al.*, 2013). However, most of them have high molecular weights, lack

isoform specificity and/or have narrow therapeutic windows (Williams *et al.*, 2007; England, 2008; McGowan *et al.*, 2009; Clare, 2010; Bregman *et al.*, 2011; Eijkelkamp *et al.*, 2012; Macsari *et al.*, 2012; Crow *et al.*, 2013; Yang *et al.*, 2013). Currently, there are different scientific groups and pharmaceutical companies testing state-dependent and isoform-specific Nav1.7 blockers for lumbosacral radiculopathy and trigeminal neuralgia (Eijkelkamp *et al.*, 2012; Biocentury, 2016) or to treat inherited erythromelalgia (Chowdhury *et al.*, 2011; Goldberg *et al.*, 2012; Crow *et al.*, 2013; Xenon, 2015). Interestingly, these drugs, already within clinical phases for affections, could potentially be effective to treat burn injury-associated pain.

Within this chapter, the aim is to study the implications of Nav1.7 blockade as a potential antinociceptive strategy following the scalding type burn injury. To do so, the expression of pS10H3, a novel nociceptive epigenetic marker of neuronal activation validated within this thesis (Chapters 12, 13,14 and 15) will be used as a marker of nociceptive activation.

17.2Aims

The **aims** of this chapter are:

- To study if $\text{Na}_v1.7$ blockade could affect the development of burn injury-nociceptive activation.
- To study whether $\text{Na}_v1.7$ contributes to oedema development or not and if its blockade has any effect on it.

17.3 Material and Methods

Protoxin II (ProTxII) is a venom toxin which is 100-fold times more specific to block Nav1.7 than any other sodium channel (Schmalhofer *et al.*, 2008). It has an IC₅₀ of 0.3nM for this sodium channel (Schmalhofer *et al.*, 2008), is resistant to protease activity, peaks after 15 minutes of administration and has a plasma half-life time of 40 minutes (Kikuchi *et al.*, 2015). Although the affinity to plasma proteins is still not known, its fast clearance indicates that it is excreted via renal rout (Kikuchi *et al.*, 2015). Previous studies have shown that Nav1.7 is implicated in the development of heat hyperalgesia in burn injury (Shields *et al.*, 2012). As discussed, we found an up-regulation of Nav1.7 in primary sensory neurons 3 hours after burn injury (Chapter 12). Thus, it was decided to study whether blocking Nav1.7 could be a feasible approach to block the development of burn injury-nociceptive activation. For ethical reasons, the development of pain was assessed by studying markers of nociceptive activation of neurons in rats which were subjected to the scalding-type burn (White *et al.*, 2011b). The assessment was done 60 minutes after the burn injury (60 minutes, n=3).

ProTxII (0.1mg/Kg, Tocris) was injected intraperitoneally either 15 minutes after (ProTxII-after, n=3) or 15 minutes prior to the injury (ProTxII-before, n=3). Due to the unpredictability of burn injuries, the group ProTxII-after is the one with a real interest as a potential therapeutic approach. Nonetheless, the group ProTxII-before was done to assess if blocking Nav1.7 prior to the scalding-type burn injury can prevent nociceptive activation of neurons. It was selected the dose of 0.1mg/Kg for administration because it is the highest dose tolerable by the rats, in with the mortal plasma concentration is 3μM (Schmalhofer *et al.*, 2008; Kikuchi *et al.*, 2015).

Morphine is an opioid analgesic with therapeutic efficacy to treat burn injured patients (Furman *et al.*, 1990; Saxe *et al.*, 2001; Donnerer & Liebmann, 2012). Hence, morphine was used as a positive control. Morphine sulphate (3mg/Kg, Sigma) was also injected intraperitoneally 15 minutes before (morphine-before, n=3) or after (morphine-after, n=3) the induction of the burn injury. As a further control, the study included a group of naive animals (naïve, n=3). As shown in previous chapters, saline injection fails to induce nociceptive-activation. Hence, no sham control group was included to reduce the number of animals used. After one hour of corresponding treatment, rats were terminally anaesthetised and transcardially-perfused with PFA. Following perfusion, L4-L5 DRG and lumbar spinal cord portion from ipsi- and contra-lateral side of each animal were collected, cryoprotected, transversally sectioned at 10µm using a cryostat and thaw-mounted on Superfrost Plus microscope slides. Sections were then processed for **immunostaining**. Briefly, slides were incubated overnight at 4⁰C using primary antibodies against pS10H3 (Santa Cruz, SC8656R, [1:750]) and pERK1/2 (Neuromics, RA15002, [1:1500]). Immunoreactivity for pERK1/2 was revealed with standard immunofluorescence and pS10H3 staining was further amplified using the Tyramide Signal Amplification procedure. Finally, the number of immunopositive cells was assessed, microphotographs taken, and statistical differences determined by the GzLM method. Differences were regarded as significant at $p<0.05$. Corresponding negative and positive controls were done in parallel to validate the technique. During this procedure, **oedema development** was assessed in all groups by measuring the increase of paw circumference at all time points (0 minutes, 5 minutes, 30 minutes and 60 minutes). One-way ANOVA followed by Bonferroni's post-test was used to assess statistical differences, which were regarded as significant at $p<0.05$.

17.4 Results

17.4.1 ProTxII reduces the nociceptive activation in the DHSC in burn injury

In agreement with previous results (Chapter 13), statistical analysis revealed a significant increase in the number of neurons with nuclei positive for pS10H3 in the ipsilateral DHSC at the 60 minutes post-burn injury time point (60 min: 30.00 ± 1.73 , Mdn=30, n=3; control: 2.33 ± 0.88 , Mdn=2, n=3; $p < 0.001$, GzLM; Figure 17.1). Intraperitoneal injection of ProTxII at 15-minutes post-injury significantly reduced the number of neurons exhibiting pS10H3 expression in the ipsilateral DHSC (ProTxII-after: 11.33 ± 1.20 , Mdn=12, n=3, $p < 0.001$, GzLM). When ProTxII was injected 15 minutes before the burn injury, the number of pS10H3 positive nuclei in the ipsilateral DHSC was also reduced (ProTxII-before: 12.33 ± 1.45 , Mdn=12, n=3, $p < 0.001$, GzLM). This reduction was like that found in the ProTxII-after group ($p = 0.491$, GzLM, Figure 17.1).

Injection of morphine 15 minutes after the induction of the burn injury also significantly reduced the number neurons exhibiting pS10H3 immunopositive nuclei (morphine-after: 0.33 ± 2.40 , Mdn=9, n=3, $p < 0.001$, GzLM; Figure 17.1).

The number of neurons exhibiting pS10H3 immunopositive nuclei in the morphine-before group was even more reduced (morphine-before: 1.66 ± 0.88 , Mdn=2, n=3, $p = 0.646$, GzLM; Figure 17.1). In fact, the number of activated neurons found in the morphine-after group was not significantly different from those found in naïve animals. Thus, the injection of morphine before the burn injury prevented the up-regulation of pS10H3 expression.

Statistical analysis of the number of pS10H3 positive nuclei in the contralateral DHSC did not reveal any significant difference between any group studied (naïve: 2.00 ± 2.00 , Mdn=0, n=3, $p=0.819$; 60 min: 2.00 ± 1.00 , Mdn=1, n=3, $p=0.819$; morphine-before: 0.67 ± 0.33 , Mdn=1, n=3, $p=0.251$; ProTxII-before: 0.00 ± 0.00 , Mdn=0, n=3, $p=0.108$; morphine-after: 0.00 ± 0.00 , Mdn=0, n=3, $p=0.108$; ProTxII-after: 0.00 ± 0.00 , Mdn=0, n=3, $p=0.108$; GzLM; Figure 17.1).

These data together indicated that blocking $\text{Nav}1.7$ with an intraperitoneal injection of ProTxII (0.1mg/Kg) following a burn injury can significantly reduce nociceptive activation of neurons in the DHSC and the efficacy of this approach is comparable to that produced by an injection of 3mg/Kg of morphine sulphate.

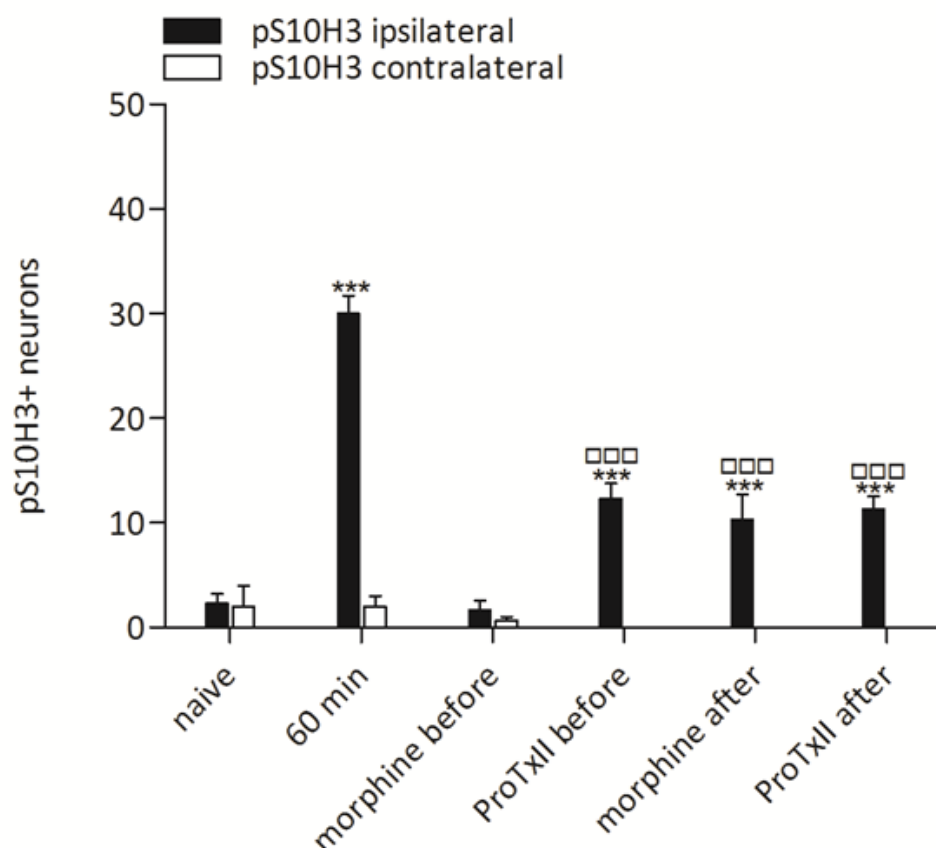


Figure 17.1. **The number of neurons exhibiting pS10H3 immunopositive nuclei in DHSC in burn-injured animals after different treatments: ProTxII and morphine significantly reduce activation of neurons.** Data is presented as mean+SEM. The experimental groups are naive animals (naive), 60°C water for 2 minutes and 60 minutes post-injury period (60 min), and 60°C water for 2 minutes and 60 minutes post-injury period with and injection of either ProTxII or morphine 15 minutes before or after the burn injury (morphine before, ProTxII before, morphine after and ProTxII after). *** $p < 0.001$ vs. naive ipsilateral pS10H3; □□□ $p < 0.001$ vs. 60' before pS10H3.

I also assessed nociceptive activation of DHSC neurons by studying the number of neurons expressing pERK1/2. A significant increase was observed in the ipsilateral DHSC one hour after the burn-injury (naïve: 3.67 ± 0.33 , Mdn=4, n=3; 60 min: 44.67 ± 2.73 , Mdn=43, n=3; $p < 0.001$, GzLM; Figure 17.2). The number of neurons exhibiting pERK1/2 immunopositivity in the ipsilateral DHSC of animals treated with ProTxII after and before the injury (ProTxII-before: 29.33 ± 5.93 , Mdn=32, n=3; ProTxII-after: 28.33 ± 5.55 , Mdn=30, n=3) was significantly reduced (ProTxII-before: $p < 0.001$ vs. naïve, $p < 0.001$ vs. 60 min; ProTxII-after: $p < 0.001$ vs. naïve, $p < 0.001$ vs. 60 min; GzLM). There was no significant difference in the effect of ProTxII in the two groups of animals ($p = 0.785$, GzLM).

Intraperitoneal injection of morphine 15 minutes following the burn injury also reduced the number of neurons exhibiting immunopositivity for pERK1/2 (morphine-after: 17.33 ± 2.40 , Mdn=16, n=3, $p < 0.001$, GzLM; Figure 17.2).

Similarly as observed for the number of pS10H3-expressing neurons, the number of pERK1/2 positive neurons in the animals injected with morphine before the induction of the burn injury was also reduced to a number found in naïve (morphine-before: 7.33 ± 1.86 , Mdn=6, n=3, $p = 0.318$, GzLM). Hence, this reduction was significantly greater than the reduction detected in the morphine-after group ($p = 0.006$, GzLM).

No significant change in the number of pERK1/2 was detected in the contralateral DHSC in any group injected with ProTxII or morphine at 15 minutes after or before the burn injury (morphine-before: 0.67 ± 0.33 , Mdn=1, n=3, $p = 0.414$; ProTxII-before: 2.33 ± 1.45 , Mdn=2, n=3, $p = 0.717$; morphine-after: 0.67 ± 0.67 , Mdn=0, n=3, $p = 0.414$; ProTxII-after: 1.67 ± 1.67 , Mdn=0, n=3, $p = 0.586$; GzLM).

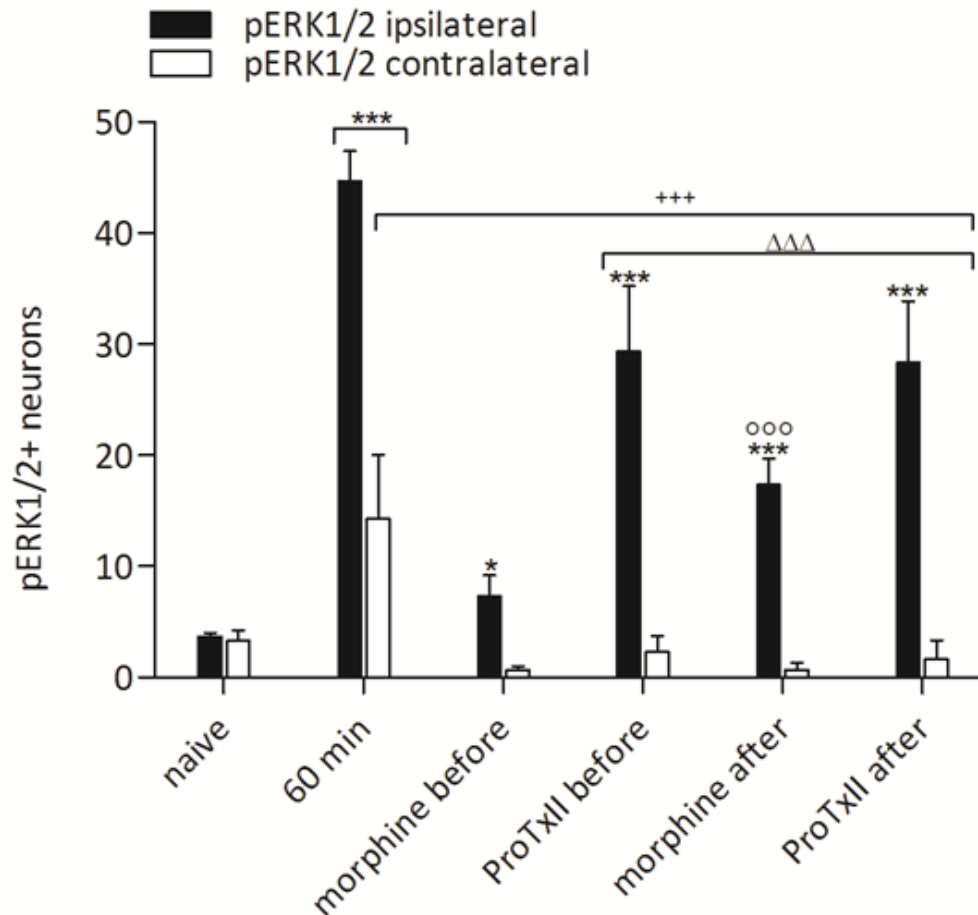


Figure 17.2. **Number of pERK1/2 immunopositive neurons in DHSC in burn-injury animals after different treatments: ProTxII and morphine reduce the nociceptive signal.** Data is presented as mean±SEM. The experimental groups are naive animals (naive), 60°C water for 2 minutes and 60 minutes post-injury period (60 min), and 60°C water for 2 minutes and 60 minutes post-injury period with and injection of either ProTxII or morphine 15 minutes before or after the burn injury (morphine before, ProTxII before, morphine after and ProTxII after). * p<0.05 vs. naive ipsilateral pERK1/2; *** p<0.001 vs. naive ipsilateral pERK1/2; +++ p<0.001 vs. 60' ipsilateral pERK1/2. ΔΔΔ p<0.001 vs. morphine before pERK1/2; ooo p<0.001 vs. ProTxII before pERK1/2.

17.4.2 ProTxII blocks pS10H3 at DRG neurons in burn injury

DRG sections from all the experimental groups described above were reacted with the anti-pS10H3 antibody and the number of neurons with positive nuclear staining for pS10H3 was established.

In agreement with previous findings (Chapter 13), a significant increase in the number of neurons positive for pS10H3 was found in the ipsilateral DRG at 60 minutes post-burn injury (control: 0.00 ± 0.00 , Mdn=0, n=3; 60 minutes: 11.68 ± 0.74 ,

Mdn=11.81, n=3; $p<0.001$, GzLM; Figure 17.3). Importantly, both ProTxII and morphine, injected either before or after the injury, significantly reduced the number of pS10H3 immunopositive neurons in the ipsilateral DRG. The number of pS10H3 immunopositive cells in any of the ProTxII or morphine treated groups was not significantly different from the number of pS10H3 immunopositive neurons found in naïve animals (morphine-before: 0.75 ± 0.75 , Mdn=0, n=3, $p=0.471$; ProTxII-before: 0.54 ± 0.33 , Mdn=0.5, n=3, $p=0.601$; morphine-after: 0.23 ± 0.23 , Mdn=0, n=3, $p=0.823$; ProTxII-after: 0.56 ± 0.32 , Mdn=0.56, n=3, $p=0.590$; GzLM).

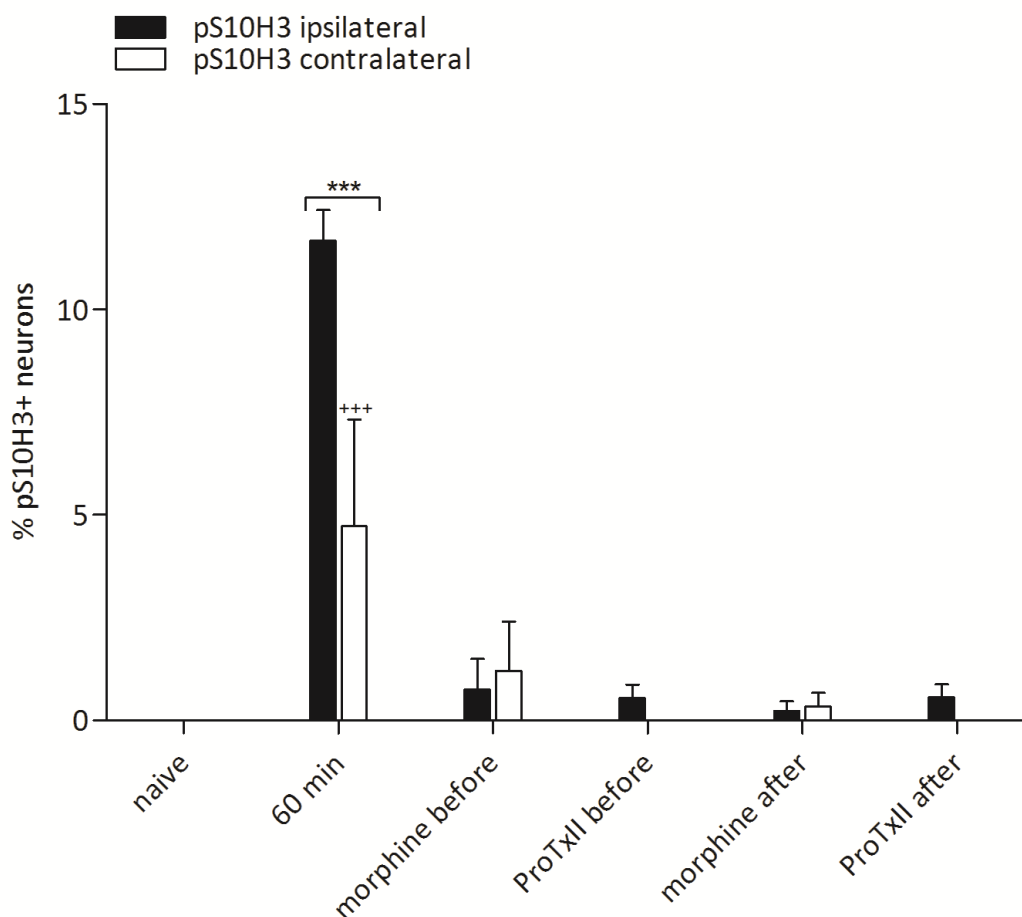


Figure 17.3. **Percentage of DRG neurons with nuclei positive for pS10H3 in the burn-injury model after different treatments.** Data is presented as mean+SEM. The experimental groups are naive animals (naive), 60°C water for 2 minutes and 60 minutes post-injury period (60 min), and 60°C water for 2 minutes and 60 minutes post-injury period with and injection of either ProTxII or morphine 15 minutes before or after the burn injury (morphine before, ProTxII before, morphine after and ProTxII after). *** $p<0.001$ vs. naive ipsilateral pS10H3; +++ $p<0.001$ vs. 60' ipsilateral pS10H3.

No statistical difference on the number of pS10H3 positive nuclei were detected in the contralateral DRG (morphine-before: 1.21 ± 1.21 , Mdn=0, n=3, p=0.246; ProTxII-before: 0.00 ± 0.00 , Mdn=0, n=3, p=1; morphine-after: 0.34 ± 0.34 , Mdn=0, n=3, p=0.746; ProTxII-after: 0.00 ± 0.00 , Mdn=0, n=3, p=1; GzLM, Figure 17.3).

These data together indicated that injecting either ProTxII or morphine either before or after burn injury can reduce transcriptional changes in primary sensory neurons.

Similar analysis was done by counting the total number of positively stained neurons, which in addition to their nuclei also exhibited immunopositivity in their cytoplasm (see further description at Supplementary Figure 20.4).

17.4.3 Morphine injected before the burn injury partially reduced the increase on the circumference of scalded hind paws

As described previously (Chapter 12), there was a significant increase in the hind paw circumference at the ipsilateral side 1 hour after the induction of the burn injury when compared to the contralateral hind paw (ipsilateral: 3.55 ± 0.17 , n=5; contralateral: 2.98 ± 0.58 , n=5, p=0.001, one-way ANOVA post-hoc Bonferroni's test; Figure 17.4).

ProTxII injection, either before or after the burn-injury, did not affect the increase in the circumference of the ipsilateral hind paw at 60 minutes post-injury (ProTxII-before: 3.60 ± 0.12 , n=3, p<0.001 vs ipsilateral 0' and p=0.778 vs ipsilateral 60'; ProTxII-after: 3.78 ± 0.11 , n=3, p<0.001 vs ipsilateral 0' and p=0.183 vs ipsilateral 60'; one-way ANOVA post-hoc Bonferroni's test; Figure 17.4 A).

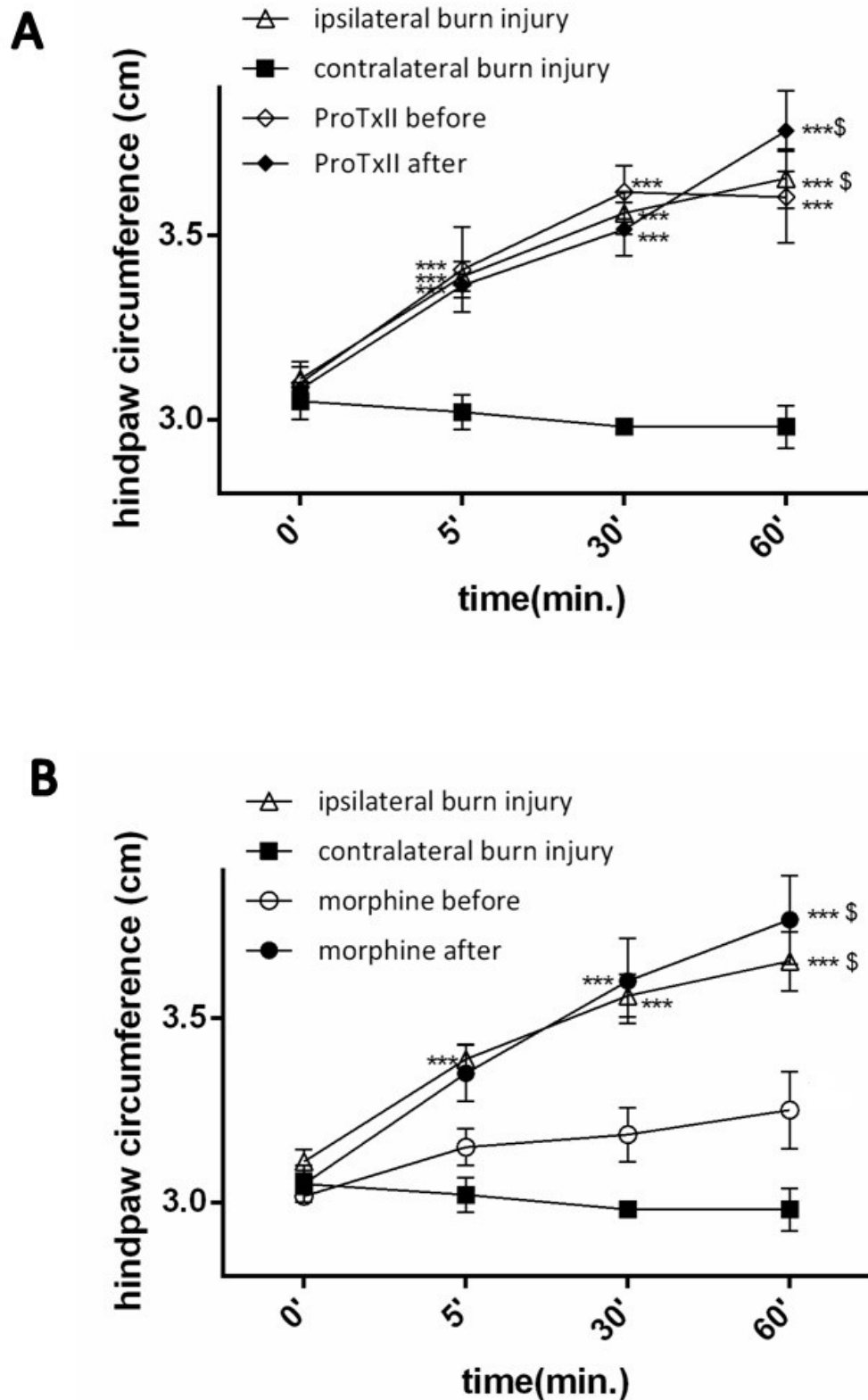


Figure 17.4. **Effect of different treatments on oedema development: morphine injection before burn-injury partially reduces its advancement.** Both ipsi- and contra- lateral hind paw of 60°C water for 2 minutes and 60 minutes post-injury period (burn injury) are compared with either injection of ProTxII (**A**) or morphine (**B**) 15 minutes before or after burn injury. Data is presented as mean $\pm$ SEM. *** $p < 0.001$ vs. ipsilateral 0'; \$ $p < 0.05$ vs. ipsilateral 5'.

A similar lack of effect was observed in animals injected with morphine after the burn-injury: a significant increase in the circumference of the ipsilateral hind paw (morphine-after: 3.77 ± 0.12 , $n=3$, $p<0.001$, one-way ANOVA post-hoc Bonferroni's test) which was not significantly different from the value found in animals 1 hour after the burn-injury with no treatment ($p=0.221$, one-way ANOVA post-hoc Bonferroni's test; Figure 17.4 B).

In contrast, morphine injection 15 minutes before the noxious stimulation was able to block the increase in the circumference of the ipsilateral hind paw as assessed at one hour post-injury (3.25 ± 0.10 , $n=3$, $p=0.252$, one-way ANOVA post-hoc Bonferroni's test; Figure 17.4). These findings indicate that the burn injury-induced oedema formation has a significant neurogenic component and that Nav1.7 does not contribute to that neurogenic inflammatory process.

17.5 Discussion

17.5.1 $\text{Na}_v1.7$ blockade has an antinociceptive effect in burn injury

An injection of ProTxII either before or after was able to reduce the number of spinal neurons activated by a peripheral burn injury, as assessed by both pS10H3 and pERK1/2. The reduction in the number of pS10H3-positive neurons by ProTxII was greater within DRG neurons than spinal neurons, highlighting its peripheral mechanism of action. ProTxII preferentially targets $\text{Na}_v1.7$, present at primary sensory neurons, to which is 100-fold times more specific than for other VGSC (Schmalhofer *et al.*, 2008). This reduced activation suggests that $\text{Na}_v1.7$ has an important role in mediating nociceptive signalling following burn injury, which agrees with Shields and colleagues' paper, where they showed that $\text{Na}_v1.7$ is required to develop heat hyperalgesia after burn injury (Shields *et al.*, 2012). If this sodium channel indeed acts as a threshold for firing action potentials (Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Dib-Hajj *et al.*, 2010; Black *et al.*, 2012; Hoeijmakers *et al.*, 2012; Dib-Hajj *et al.*, 2013; Zhang *et al.*, 2013) and since it is circumscribed to the PNS (Toledo-Aral *et al.*, 1997; Morinville *et al.*, 2007; Dib-Hajj *et al.*, 2010; Black *et al.*, 2012; Dib-Hajj *et al.*, 2013; Minett *et al.*, 2015), its inhibition may be a feasible target to ameliorate pain after burn injuries.

Although ProTxII has a limited ability to cross the blood-nerve barrier (Middleton *et al.*, 2002; Schmalhofer *et al.*, 2008; Clare, 2010; Eijkelkamp *et al.*, 2012), it could inhibit the nociceptive activation of spinal neurons caused by this burn injury model. This could be explained because of the severity of the injury caused: sensory fibres are normally isolated by the perineurium, which prevents the access of large and/or

hydrophobic molecules; however, following an inflammation, this barrier can be disrupted allowing access of peptides to the nerve endings (Coutaux *et al.*, 2005), allowing the access of ProTxII. This could also explain why the effect was greater in DRG neurons than on the spinal secondary neurons, which may also receive innervations from primary sensory neurons situated away from the site of injury by a process of secondary hyperalgesia (Ji *et al.*, 1999; Ji & Woolf, 2001; Julius & Basbaum, 2001; Ji *et al.*, 2003; Marchand, 2008; Hunt, 2009; Woolf, 2011; Steeds, 2013). In support of this, Schmalhofer's group showed specific inhibition of C-fibres activity of isolated rat saphenous nerves preparations that were previously desheated, but not when were intact (Schmalhofer *et al.*, 2008). Therefore; ProTxII can block the nociceptive activation caused by severe burn injuries by selectively tackling Nav1.7 within nociceptors whose receptive field is at the site of injury. Although some researchers claim that Nav1.7 inhibitors should tackle both peripheral and central terminals to obtain clinically relevant effects (Dib-Hajj *et al.*, 2013), the data presented here suggests that inhibiting peripherally-situated Nav1.7 channels causes significant impairment of the burn-injury associated nociceptive activation.

Morphine, injected here at a dosage known to produce significant peripheral antinociception (Olivier *et al.*, 1991; Desroches *et al.*, 2014), also caused a reduction in the number of neurons activated equivalent to that observed by the injection of ProTxII. However, morphine, as an opioid, has different side effects including: sedation and sleeping alterations, respiratory distress, delirium, nausea, low mood, constipation or hypotension (Ptacek *et al.*, 1995; Raymond *et al.*, 2004; Demling, 2008; Wiechman Askay & Patterson, 2008; Wiechman Askay *et al.*, 2009; Phoenix-Society, 2011). Furthermore, opioids alone are an incomplete approach for burn injury since they may become less effective through time and usage (Furman *et al.*,

1990; Wiechman Askay & Patterson, 2008; Wiechman Askay *et al.*, 2009) and lead to opioid resistances (Choiniere *et al.*, 1991; Malenfant *et al.*, 1996; Shakespeare, 1998; Dauber *et al.*, 2002; Carrougner *et al.*, 2003; Schneider *et al.*, 2006; Pavoni *et al.*, 2010; Browne *et al.*, 2011). Thus, ProTxII seems a more efficient drug, specifically directed to the primary nociceptors and without inducing the side effects reported by opioids.

17.5.2 Morphine prevents the development of oedema in burn injury

Oedema developed normally in all the treatment groups except in the animals that received an injection of morphine before the burn injury. This suggest that Nav1.7 does not contribute to the oedema's formation following a burn injury, while opioids seem to interact with this inflammatory process. The anti-inflammatory effects of peripherally acting opioids have been reported previously in the literature (Planas *et al.*, 1995; Sacerdote *et al.*, 1996; Coutaux *et al.*, 2005; Romero *et al.*, 2005; Walwyn *et al.*, 2007; Moshourab & Stein, 2012; Spahn *et al.*, 2013; Stein & Kuchler, 2013). Opioid receptors are not only found at DRG neurons, they are also present within different immune and epidermal cells (Salemi *et al.*, 2005; Sharp, 2006; Bigliardi *et al.*, 2009; Stein & Kuchler, 2013). Nonetheless, at such early stages after an injury, their effect is not likely to involve the immune system (Stein & Kuchler, 2013). Opioids can decrease the response of DRG neurons to an inflammatory process, which consequently causes a decreased of neuropeptides release (such as substance P and CGRP) from their peripheral terminals and consequently causes vasoconstriction (Walwyn *et al.*, 2007; Moshourab & Stein, 2012; Spahn *et al.*, 2013;

Stein & Kuchler, 2013). Hence, oedema development has a neurogenic component in a process not regulated by Nav1.7.

Although these findings suggest that peripherally acting opioids such as morphine can have a promising therapeutic value for modulating tissue inflammation, targeting this pathophysiological process should only be affronted when dysregulated or chronic (Stein & Kuchler, 2013; Tabas & Glass, 2013). Therefore, this effect accounts as another unwanted side effect of the use of morphine.

17.5.3 Conclusion and future work

The findings presented here point at ProTxII as a potential therapeutic approach. Nonetheless, this peptide can affect the muscle tone and impair the locomotor function, and metabolises within few hours of its administration (Middleton *et al.*, 2002; Schmalhofer *et al.*, 2008; Clare, 2010; Eijkelkamp *et al.*, 2012), all aspects that cannot be assessed within this model. Furthermore, ProTxII was only administered at the highest dose tolerable for rats (0.1mg/Kg), with the aim to assess if its application has any effect, and only analysed by immunostaining. Therefore, more research is needed to assess those effects and validate its actions in a dose-dependent manner. To further investigate, electrophysiological recordings from both *in vitro* (acutely dissociated cells) and *in vivo* (anaesthetised animals) following this burn injury model should be used to assess different concentrations of ProTxII or other Nav1.7 blockers as potential antinociceptive approaches.

Interestingly, the structure of this channel is extremely conserved between rodents and humans, highlighting the importance of animal studies to assess the activity and regulation of Nav1.7 and the translational characteristics of those studies to humans (Brochu *et al.*, 2006; Dib-Hajj *et al.*, 2013; Gingras *et al.*, 2014).

Additionally, it could be interesting to test the combinatorial effect of both opioids such as morphine, and Nav1.7 blockers, like ProTxII. Similarly, and in accordance with the knowledge gained within the previous chapters of this thesis, this potential therapeutic approach could be combined with the so called “epigenetic drugs”. By these means, the side effects due to opioids could be reduced in an opioid sparing effect (Sharar *et al.*, 1998; Linneman *et al.*, 2000; Han *et al.*, 2007; Demling, 2008; Wiechman Askay *et al.*, 2009) and a better therapeutic treatment for burn pain could be achieved.

At present, there are different scientific groups and pharmaceutical companies testing Nav1.7 blockers in both animal studies and human trials. Convergence Pharmaceutical (a subsidiary of Biogen) is testing a Nav1.7 inhibitor, currently in phase two for lumbosacral radiculopathy and trigeminal neuralgia (Eijkelkamp *et al.*, 2012; Biocentury, 2016). Xenon Pharmaceuticals also published some pilot data with an oral Nav1.7 antagonist to treat IEM (Chowdhury *et al.*, 2011; Goldberg *et al.*, 2012; Crow *et al.*, 2013; Xenon, 2015). Importantly, some of these drugs clinically used for certain pathophysiologies could potentially be effective to treat others, such as burn injury-associated pain. Carbamazepine, for example, selectively acts on fast-activating VGSC by shifting their response to more negative potentials and has shown efficacy on PEPD, although ineffective for IEM (Theile & Cummins, 2011; Eijkelkamp *et al.*, 2012). CDA54, a compound similarly acting as anticonvulsants and antiarrhythmic, is another state-dependent sodium blocker (affects Nav1.7 and Nav1.8) with great oral bioavailability (Shao *et al.*, 2005; Brochu *et al.*, 2006). This molecule has shown analgesic properties (around 45% less pain), with no cardiac effect, in an animal model of neuropathic pain (Shao *et al.*, 2005; Brochu *et al.*, 2006). Another example is Lacosamide, a peptide that selectively enhances slow

inactivation, and which has shown efficacy as anticonvulsant. Lacosamide is not selective to Nav1.7, it also affects Nav1.8 and Nav1.3. Interestingly, since the main VGSC affected by Lacosamide are preferentially expressed in DRG neurons, it still has a potential therapeutic use despite lacking specificity for Nav1.7 (Eijkelkamp *et al.*, 2012; Dib-Hajj *et al.*, 2013).

Once I finished the experimental period of my PhD, a scientific research proposal was being written using the data presented in this chapter as preliminary findings. In there, Lacosamide will be tested for transdermal application in healthy human volunteers as a potential analgesic strategy for severe burn injuries.

18 Summary of results and conclusions

Here the major findings of the thesis are described, first distributed within the different chapters of results in which have been introduced and then in a conclusion integrating them all. Additionally, some new discoveries in the field, possible problems to consider and final remarks are included.

18.1 Highlights of each chapter

18.1.1 **Nav1.7 is highly express within small-size DRG neurons and gets up-regulated after burn injury**

The first chapter of results (Chapter 12) demonstrated that the scalding type burn injury model effectively induces a partial thickness, second degree, burn in the hind paw of Sprague-Dawley rats. An injury that consequently induced the activation of pERK1/2 at superficial DHSC neurons and pCREB at DRG neurons, both considered dynamic markers of nociceptive activation. Additionally, the expression of Nav1.7 was also assessed revealing certain up-regulation after 3 hours of the burn injury, which implicates Nav1.7 in pain processing following burn injuries.

The study of Nav1.7 distribution using a validated antibody showed that Nav1.7 appears in the soma and axonal compartment, both peripherally and centrally directed branches, of around 40% DRG neurons, of which the majority are small- and middle-sized nociceptors (400-600 μm^2). These findings are in accordance with

previous reports (Toledo-Aral *et al.*, 1997; Nassar *et al.*, 2004; Dib-Hajj *et al.*, 2007; Black *et al.*, 2012; Dib-Hajj *et al.*, 2013) and suggest a role for Nav1.7 on neuronal firing, action potential propagation (Toledo-Aral *et al.*, 1997; Dib-Hajj *et al.*, 2007; Black *et al.*, 2012; Minett *et al.*, 2012; Dib-Hajj *et al.*, 2013) as well as regulating neurotransmitter release within presynaptic boutons at the superficial spinal cord (Toledo-Aral *et al.*, 1997; Dib-Hajj *et al.*, 2007; Black *et al.*, 2012; Minett *et al.*, 2012; Dib-Hajj *et al.*, 2013). The immunostaining and western blot studies revealed an increase in the number of Nav1.7-expressing DRG neurons at 3-hour post burn injury. This suggests that Nav1.7 has an important role in burn-injury associated nociception, an effect previously observed by others (Gould *et al.*, 1998; Black *et al.*, 2004; Gould *et al.*, 2004; Yeomans *et al.*, 2005; Dib-Hajj *et al.*, 2007; Drenth & Waxman, 2007; Dib-Hajj *et al.*, 2010; Dib-Hajj *et al.*, 2013). This overexpression points at Nav1.7 as a key regulator of nociception following the scalding-type burn injury model, which is in accordance with Shields' paper where it was described that this channels is crucial to develop heat hyperalgesia following burn injury (Shields *et al.*, 2012).

Following the induction of burn injury (White *et al.*, 2011b), the histopathological analyses of skin samples showed the formation of oedema and the loss of structure of the skin. This, together with the macroscopic appearance of the paw, show the induction of a second degree burn by this scalding-type burn injury model in Sprague-Dawley rats (Moritz, 1947; WHO, 2007; Abdullahi *et al.*, 2014). The percentage of TBSA and the localisation of this burn injury count as great advantages for this injury model.

After the induction of this scalding type burn injury, there is a rapid and transient nociceptive-specific phosphorylation of the MAPK ERK1/2 (pERK1/2), a well-known

marker of nociceptive processing at the spinal cord (Ji *et al.*, 1999; Ji *et al.*, 2003; Gao & Ji, 2009; White *et al.*, 2011a), in a sub-population of superficial DHSC neurons, a finding that is supported by previous research (Ji *et al.*, 1999; Ji & Woolf, 2001; Polgar *et al.*, 2007; Black *et al.*, 2008; Gao & Ji, 2009; White *et al.*, 2011a; White *et al.*, 2011b). Therefore, the burn injury model induced a nociceptive activation within neurons of the superficial spinal cord.

The phosphorylation of CREB (pCREB), a transduction factor downstream of MAPK (Thomson *et al.*, 1999b; Strahl & Allis, 2000; Ji *et al.*, 2003; Kawasaki *et al.*, 2004; Bie *et al.*, 2011; Li *et al.*, 2014), was observed in both DRG neurons and glial cells in a nociceptive-specific manner as early as 5 minutes' post-injury. This sub-populations rapidly returned to basal numbers and peaked its expression again at 3-hours post-injury. Most neurons where this marker was induced also expressed Nav1.7 (around 80%) and thus were regarded as nociceptors. Hence, DRG neurons were also nociceptive-activated following the burn injury model.

18.1.2 After the scalding type burn injury model, pS10H3 is up-regulated in superficial spinal cord and DRG neurons.

The second chapter of results (Chapter 13) describes the rapid and transient induction of pS10H3 in DRG and superficial spinal neurons by the scalding type burn injury. This epigenetic mark appears in the nuclei of a sub-population of superficial spinal cord neurons, at least most of them considered second order neurons, following the scalding type burn injury model (White *et al.*, 2011b). It peaks at 5 minutes and remains elevated until one hour after the burn injury. Studies of co-expression suggest that, similarly to hippocampal neurons (Bilang-Bleuel *et al.*, 2005; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008; Crow *et al.*, 2015),

this epigenetic tagging is regulated via the upstream kinases pERK1/2 and also points at pS10H3 as a permissive mark regulating IEG expression such as c-Fos (Mahadevan *et al.*, 1991; Barratt *et al.*, 1994; Cheung *et al.*, 2000a; Cheung *et al.*, 2000b; Lo *et al.*, 2000; Nowak & Corces, 2000; Zhong *et al.*, 2000; Salvador *et al.*, 2001; Saccani *et al.*, 2002; Prigent & Dimitrov, 2003; Dyson *et al.*, 2005; Dong & Bode, 2006).

Similarly, the number of pS10H3 positive DRG neurons gets up-regulated as early as 5 minutes after the injury, although remains elevated after 3 hours. However, this epigenetic tagging is not only circumscribed to the nuclear compartment and can also be found at the cytoplasm. This ectopic localisation of histones has been observed previously and seems associated to cellular stress (Watson *et al.*, 1995; Gabler *et al.*, 2003; Gabler *et al.*, 2004; Gilthorpe *et al.*, 2013) and/or processes of mitotic catastrophe (Ogawa *et al.*, 2003). The transcription factor pCREB, implicated in nociceptive processing that seems to be activated by the same kinases that lead to pS10H3 (Ji *et al.*, 2003; Kawasaki *et al.*, 2004; Bie *et al.*, 2011; Li *et al.*, 2014), co-expresses highly with the neurons showing pS10H3. Most of these neurons were also positive for Nav1.7 and therefore considered primary sensory neurons.

18.1.3 pS10H3 identifies nociceptive-specifically activated superficial DHSC neurons

In accordance to the second chapter of results, the third (chapter 12) describes an up-regulation of pS10H3 in a sub-population of superficial DHSC neurons following other noxious models. A subcutaneous injection of capsaicin induced an increase of pS10H3 that was detected at 5 minutes and persisted after half an hour. Electrical stimulation of C-fibres strength was also able to induce this epigenetic tagging within

superficial spinal neurons. However, brief noxious stimuli (noxious temperature or pressure), which did not produce significant tissue damage, failed to induce this epigenetic mark on the spinal cord. Studies using pharmacological blockers demonstrated that this epigenetic tagging is activated via NMDA-receptors, and laid down via MSK1 as the main effector kinase.

Additionally, other epigenetic modifications were found up-regulated (aK14H3, mK36H3, 3mK79H3 and 3mK27H3) or down-regulated (1-, 2- and 3- mK9H3; mK4H3 and 3mK4H3) within dorsal horn tissues after 5 minutes of the capsaicin injection. Studying these other epigenetic tags can help to elucidate the “histone code” and the consequences of such combination of tags for nociceptive processing.

18.1.4 pS10H3 identifies nociceptive-specifically activated DRG neurons

The fourth chapter of results (Chapter 15) showed that pS10H3 was also dynamically up-regulated within a population of small-sized DRG neurons after capsaicin application, which peaked at 5 minutes of the stimulation to decrease at later times. This up-regulation is a TRPV1-dependent event and the main writer of this epigenetic tag in this context was also MSK1/2. The analysis of pCREB also showed that its activation depends on TRPV1, although it was, at least in part, achieved by other kinases than MSK1/2. Brief noxious stimuli (noxious temperature or pressure), which did not produce significant tissue damage, also failed to induce this histone covalent modification on DRG neurons.

18.1.5 Targeting pS10H3 via MSK1/2 prevents the development of heat hyperalgesia

The fifth chapter of results (Chapter 16) showed that pS10H3 also get up-regulated in a nociceptive-specific manner at superficial spinal neurons following an intraplantar injection of carrageenan in a process, at least partially, pERK1/2-dependent. An elevated percentage of co-expression was observed between c-Fos and pS10H3 suggesting that this epigenetic tag leads to the expression of this IEG (Hunt *et al.*, 1987; Mahadevan *et al.*, 1991; Barratt *et al.*, 1994; Thomson *et al.*, 1999a; Cheung *et al.*, 2000a; Cheung *et al.*, 2000b; Clayton *et al.*, 2000; Nowak & Corces, 2000; Todd *et al.*, 2002; Prigent & Dimitrov, 2003; Gao & Ji, 2009; Gutierrez-Mecinas *et al.*, 2011; White *et al.*, 2011a; Keum *et al.*, 2013).

Deletion of MSK1/2 (assessed using MSK1/2^{-/-} mice) completely prevented the expression of pS10H3 following carrageenan. Thus, further pointing at MSK1/2 as the main effector kinases (*writers*) mediating this epigenetic modification following inflammation/tissue injury. Within this KO mice, the expression of c-Fos was also prevented, which confirmed the need of pS10H3 to induce the expression of this IEG. Behavioural studies also showed that the development of heat hyperalgesia was impaired in the MSK1/2^{-/-} mice following carrageenan injection, while mechanical allodynia developed similarly as WT mice.

18.1.6 Nav1.7 blockade has an antinociceptive effect in burn injury

Within the last chapter of results of this thesis (Chapter 17), it was demonstrated that blocking Nav1.7 via an injection of ProTxII (1mg/Kg), a selective venom toxin (Schmalhofer *et al.*, 2008), reduced the number of DRG and spinal neurons

activated by a peripheral burn injury in a similar effect to the injection of morphine. Nonetheless when morphine was injected prior to the burn injury oedema did not develop, which indicates that morphine impaired the neurogenic component of the inflammatory process and that this was independent to Nav1.7. Therefore, these findings confirm that Nav1.7 is an important channel for nociceptive processing following burn injury (Shields *et al.*, 2012) and that its blockade, here using ProTxII, seems a more efficient antinociceptive strategy with fewer side effect than opioids.

18.2 Conclusion

In conclusion, two main findings have been elucidated within this thesis: first, pS10H3, a novel epigenetic modification, has been established as a key molecular event for possible gene processing within DRG and superficial spinal cord neurons following peripheral inflammation/tissue injury. And secondly, the feasibility of blocking the VGSC Nav1.7, mainly expressed within primary sensory neurons, as an analgesic strategy following burn injury has been reported.

Here, it is shown for the first time that the post-translational epigenetic modification pS10H3 is rapid and dynamically up-regulated in a subpopulation of both DRG and spinal cord neurons following noxious stimulation in a specific pattern for each stimulus. Within superficial DHSC neurons, burn injury, capsaicin or carrageenan injection and high electrical stimulation of C-fibres nociceptors specifically up-regulated pS10H3 at nuclei of, at least, a great number of second order neuron. These observations were also corroborated by the *in vitro* models of capsaicin application and repetitive electrical stimulation of nociceptors. At DRG neurons, pS10H3 was also seen up-regulated following burn injury and capsaicin injection, which was further validated by the *in vitro* models of capsaicin application and

exposure to heat stress. In contrast, low frequency electrical stimulation or brief noxious stimulations without inducing tissue damage failed to up-regulate this epigenetic tagging. The characterisation of the cells expressing pS10H3 after noxious stimuli revealed that this epigenetic tagging is due to the activation of TRPV1 and/or the NMDA receptor, which induce intracellular pathways of kinases, possibly via pERK1/2, leading to the activation of the downstream kinases MSK1/2 (Figure 18.1). These effector kinases, mainly MSK1, are responsible for this covalent tagging of histone H3 at serine 10, which triggers chromatin changes that activate transcription of certain genes, such as the IEG c-Fos (Figure 18.1). This cascade is also activating, at least partially, the pCREB transcription factor, implicated in pain processing (Ji *et al.*, 2003; Kawasaki *et al.*, 2004; Bie *et al.*, 2011; Li *et al.*, 2014). Disrupting this epigenetic tagging from happening via blockade of MSK1/2, the main effectors, prevented the development of heat hyperalgesia following a peripheral noxious stimulation. Therefore, pS10H3 seems to regulate the neuronal nociceptive gene processing and could be used as a novel nociceptive marker of inflammation/tissue injury in both DRG and superficial spinal cord neurons.

The distribution of the sodium channel Nav1.7, a channel implicated in the processing of burn injury (Shields *et al.*, 2012), has been studied throughout DRG. An up-regulation on Nav1.7 was observed at 3-hours post-injury and its blockade via ProTxII reduced the nociceptive activation. Although further studies are needed, these findings demonstrate the importance of Nav1.7 for burn injury nociceptive activation and point at its blockade as a feasible target for a better management of burn injury associated pain. Accordingly, new studies on this fields, using electrophysiological recordings in animal models or studies involving human volunteers are in progress.

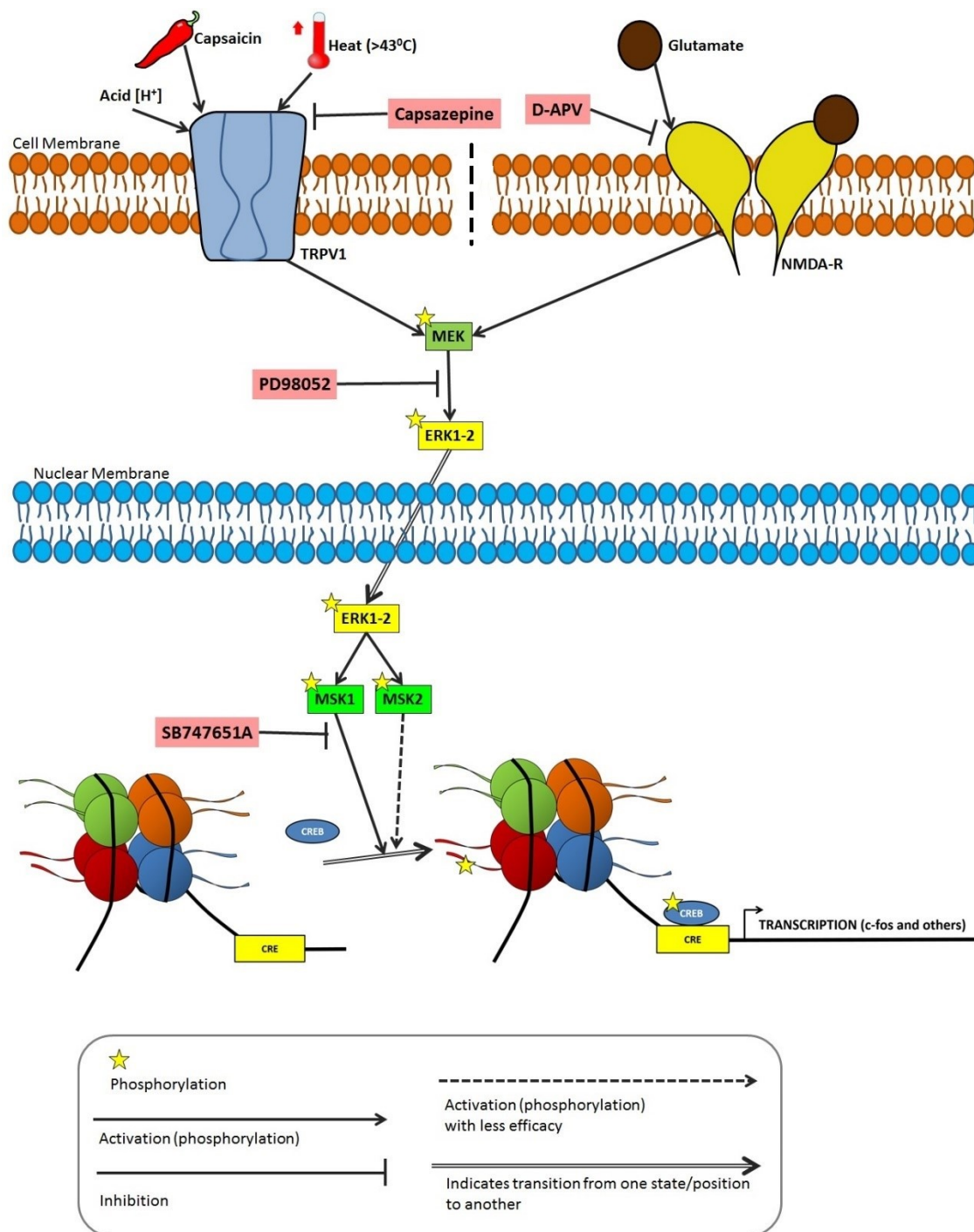


Figure 18.1. **Diagram of neuronal activation leading to pS10H3 following peripheral inflammation/tissue injury.** Phosphorylation at serine 10 of histone H3 (red histone in the drawing) triggers gene activation, including the IEG c-Fos. This epigenetic tagging is NMDA receptor- and TRPV1-dependent, mediated via pERK1/2 as the main MAPK, and laid down by the effector kinases MSK1 (mainly) and MSK2. CRE: cAMP response element; CREB: cAMP response element-binding protein; D-APV: 2-amino-5-phosphonopentanoic acid; ERK: extracellular signal-regulated kinase; MEK: mitogen-activated protein kinase kinase; MSK: mitogen- and stress-activated protein kinase; NMDA-R: N-methyl-D-aspartate receptor; TRPV1: transient receptor potential cation channel subfamily V member 1.

18.3 Data independently validated

During the writing up of this thesis and while the manuscript regarding pS10H3 in spinal cord neurons following painful stimuli was under revision at *Scientific Reports* (already accepted for publication), Tochiki et al. published a paper in *Pain* with quite similar data (Tochiki *et al.*, 2016). They described a rapid and transient increase of pS10H3 in superficial spinal cord neurons following nociceptive activation by formalin and capsaicin injection. Tochiki's paper also showed that pS10H3 is an event downstream of pERK1/2 and MSK1 that leads to gene expression (c-Fos and Zif268), and that targeting this histone modification via MSK1-blockade inhibited formalin-induced nociceptive behaviour. Although this information was not considered for discussion during this thesis, a short revision is given here.

In the formalin model, Tochiki and collaborators detected an increase of pS10H3 at 5 minutes of the injection, with a maximal peak at 30 minutes, which gradually returned to baseline numbers by 4 hours of the stimulation. This pattern is in accordance with the rapid increase that we detected following other noxious models. Nonetheless, the 30 minutes' peak that they show was not observed in any of the models analysed here, which suggest that this rapid and transient pS10H3 increase is stimulus specific. They also confirmed the presence of pS10H3 in this sub-population of neurons at one hour of a capsaicin injection model, which complements the up-regulation that we observed at 5 minutes and 30 minutes' time points. However, they do not include naïve animals (animals without any treatment) and their sham controls consisted in rats subjected to anaesthesia but that did not receive any injection because, as they argue, that could trigger certain inflammation that could potentially activate the pathway studied. On the contrary, we included both naïve and sham (injected with saline) animals. By doing so we concluded that, despite the possible

activation induced by saline, this sham stimulation was not enough to significantly up-regulate this pathway.

The characterisation of this pS10H3-positive neuronal population, assessed at 30 minutes of the formalin injection showed that around half of them also expressed pERK, which is like what we observed at 5 minutes of the burn injury. Nonetheless, they described that only around 45% of the pERK-expressing cells also showed pS10H3 up-regulation while we quantified a much greater number, of around 84%. From the co-expression with c-Fos at 1 hour of the formalin injection, they showed that around 60% of the pS10H3-expressing cells also had c-Fos while around 17% of the c-Fos-positive neurons were pS10H3, while we described values of 72% and 66% respectively as assessed at 30 minutes of the burn injury model. These subtle discrepancies could be explained because of the different noxious stimuli (formalin *versus* burn injury, which seems to be much more severe), the use of different antibodies (their pS10H3 and c-Fos antibodies are from different companies to the ones that we used) or due to performing the analysis at different time points. As they also point out, these phosphorylation events seem to peak at different time points which could be masking greater percentages of co-expression.

Additionally, they looked at the presence of neurokinin receptor 1 (NK1) within the small population, but crucial for nociceptive processing (Hunt & Mantyh, 2001; Khasabov *et al.*, 2002; Suzuki *et al.*, 2002; Todd *et al.*, 2002; Todd, 2010), of pS10H3-positive cells and demonstrated that the expression of pS10H3 is partially modulated by descending serotonergic circuits following the formalin test. We did not perform this interesting analysis. Nonetheless, taking in account that we observed a greater co-expression of c-Fos with pS10H3 and that a great majority of neurons with NK1 receptor express c-Fos when activated by noxious stimuli (Todd *et al.*,

2002), it could be assumed that certain co-expression between pS10H3 and NK1 also happens in the noxious models that we studied.

Their strategy to stop the phosphorylation of this histone H3 tail was blocking MSK1 with an intrathecal injection of SB727651A prior to the noxious stimulation. In agreement to our MSK1/2^{-/-} KO mice studies after carrageenan injection, they showed that this approach impaired the up-regulation of pS10H3 and prevented the development of formalin-induced nociceptive behaviour. During our experiments, a collaborator of us also attempted to use this drug to block MSK1 activity *in vivo*. However, serious side effects were observed within those animals and it was decided to limit its application to *in vitro* studies. Thus, although MSK1 seems a good target, much caution is needed due to its narrow therapeutic window and ubiquitous expression (Chwang *et al.*, 2006; Ananieva *et al.*, 2008; Arthur, 2008; Bertelsen *et al.*, 2011; Gutierrez-Mecinas *et al.*, 2011; MacKenzie *et al.*, 2013).

Therefore, Tochicki's paper (Tochiki *et al.*, 2016) together with the discoveries explained within this thesis and partially presented in our published paper (already accepted for publication) suggest that a population of superficial dorsal horn neurons get rapidly marked with pS10H3 following peripheral inflammatory/tissue injury as a downstream event of pERK and MSK1, and that this epigenetic tagging could have a key role in the altered gene regulation and subsequent behavioural changes.

18.4 Problems to consider when studying epigenetics

The interpretation of observed epigenetic marks is not a simple process (Meaney & Ferguson-Smith, 2010; Rando, 2012; Geranton & Tochiki, 2015b; Guiretti *et al.*,

2016). Observed global changes of certain epigenetic modification may or may not involve the state of such modification in a certain gene. Thus, the functional regulatory effect of that epigenetic factor can only be determined if observed on relevant gene/s (Nowak & Corces, 2000; Zhang *et al.*, 2011; Geranton, 2012; Denk *et al.*, 2013; Bai *et al.*, 2015; Geranton & Tochiki, 2015b; a; Guiretti *et al.*, 2016).

Some genes can appear in a poised states where marks regarded as activating and repressive coexist waiting for further cues (Geranton, 2012; Geranton & Tochiki, 2015b). The epigenetic state of a certain gene is the result of multiple modifications which can also affect the expression of other genes (Kim *et al.*, 2012; Geranton & Tochiki, 2015a). Further, certain combination of epigenetic marks can be read by different sets of proteins and lead to completely different outputs (Strahl & Allis, 2000; Monsey *et al.*, 2011). Thus, although some specific epigenetic modifications or combination of them seem to correlate with a certain functional outcome, this will depend on the surrounding interactions (Strahl & Allis, 2000; Monsey *et al.*, 2011; Keum *et al.*, 2013; Geranton & Tochiki, 2015a).

Furthermore, studies *in vitro* can be misleading, although useful as an initial approach (Rando, 2012). Some interactions between a certain mark and regulatory protein can be observed in different assays but do not occur or, are not meaningful, in the *in vivo* scenario (Rando, 2012). However, there also are some limitations when studying epigenetic modifications *in vivo*. Biological samples contain a mixed population of different cells, referred as tissue heterogeneity, (Denk *et al.*, 2013; Bai *et al.*, 2015) and DNA methylation and histone modification patterns are known to be cell-type specific (Thomson *et al.*, 2001; Lefebvre *et al.*, 2002; Denk & McMahon, 2012; Geranton, 2012).

Further, epigenetic processing is dynamic and reversible even in adulthood (Geranton, 2012; Geranton & Tochiki, 2015b; a), can change through time (Crow *et al.*, 2013; Geranton & Tochiki, 2015b), be transient or enduring (Geranton, 2012) and even be affected by age (Gutierrez & Hnilica, 1967; Geranton & Tochiki, 2015b). Different epigenetic factors can interact between each other and from this crosstalk leading to different sets of genes regulated at different times and after different processes (Cheung *et al.*, 2000b; Lo *et al.*, 2000; Strahl & Allis, 2000; Meaney & Ferguson-Smith, 2010; Zhang *et al.*, 2011; Denk & McMahon, 2012). Additionally, some correlation can mistakenly be assumed as causal-consequence reactions (Meaney & Ferguson-Smith, 2010).

Different organisms favour the use of different mechanisms, e.g. different kinases, to achieve the same epigenetic mark (Cheung *et al.*, 2000a; Geranton, 2012; Seok *et al.*, 2013). Similarly, those processes can vary within cell, tissue type an organism (Gutierrez & Hnilica, 1967; Salvador *et al.*, 2001; Thomson *et al.*, 2001; Lefebvre *et al.*, 2002; Bilanz-Bleuel *et al.*, 2005; Chandramohan *et al.*, 2007; Chandramohan *et al.*, 2008; Meaney & Ferguson-Smith, 2010; Zhang *et al.*, 2011; Geranton, 2012).

Even more problematic is the study in humans of the transcriptional changes happening during pain development at certain tissues such as spinal cord and DRG (Denk & McMahon, 2012). Premature extrapolation of epigenetic findings to humans can be misleading and induce to risk messaging (Juengst *et al.*, 2014).

Despite some encouraging results on the systematic administration of drugs targeting epigenetic modifications to treat nociception, it is still unclear how these drugs can affect the other cells of the organism that also possess epigenetic machinery. Different non-histone proteins are susceptible to acetylation and methylation, which means that they also are potential substrates for HDACs and

demethylases (Bai *et al.*, 2015). Methylation can also affect RNA (Bai *et al.*, 2015). HDACi can also deacetylate at lysine sites of other cellular components, inducing an effect by other mechanism rather than by targeting the epigenetic process (Geranton, 2012; Crow *et al.*, 2013; Crow *et al.*, 2015; Geranton & Tochiki, 2015a). For example, the HDACi valproate can also induce inhibition of sodium channels, important in pain processing (Geranton, 2012). Even local applications can be challenging since non-neuronal or non-sensory neurons can also be targeted (Bai *et al.*, 2015; Geranton & Tochiki, 2015b), thus the aim should be to achieve greater selectivity and tissue-specificity (Crow *et al.*, 2013; Geranton & Tochiki, 2015b; a). To reduce this effect, it is suggested to use Class IIa HDAC instead of Class I since they have a more restricted pattern and are able to shuttle between the nucleus and the cytoplasm (Bai *et al.*, 2010; Denk & McMahon, 2012; Kim *et al.*, 2012; Sando *et al.*, 2012; Crow *et al.*, 2013). Notwithstanding, the effect of HDAC inhibition and their toxicity have still not been assessed at long-term (Denk & McMahon, 2012; Geranton, 2012).

Another line of investigation could be the search for compounds that act on the *readers* of the different epigenetic marks, rather than on the *writers*, since they have a greater situation-specific control (Denk & McMahon, 2012; Keum *et al.*, 2013). For example: targeting 14-3-3 ϵ instead of the writer kinases (MSK1 and MSK2) and thus control pain development (Keum *et al.*, 2013).

A common problem with immunochemical studies of different PTMs is the occlusion of epitope to antibody recognition: when different PTMs are presented in close proximity they could affect each other's recognitions by antibodies raised against one of those PTMs (Clayton *et al.*, 2000; Nowak & Corces, 2000; Thomson *et al.*, 2001; Davie, 2003; Rotllant *et al.*, 2013; Ng & Cheung, 2016). Detection of a

phosphorylated site by a certain antibody could be prevented by acetylation of nearby lysines in the same way as a phosphorylation can block the recognition of the acetylated residues. For example: an antibody that detects acetylation of both K9 and K14 in histone H3 is not able to bind if S10 was phosphorylated in the same tail (Davie, 2003). During mass spectrometry studies, the phosphorylation of certain residues is also susceptible to get dissociated (Ng & Cheung, 2016).

Moreover, it is still not clearly understood how all the epigenetic *writers*, *readers*, *erasers* and associated proteins are selectively recruited to a small subset of promoters during interphase and not to the rest of them (Strahl & Allis, 2000; Davie, 2003).

18.5 Final remarks and future of epigenetic research

If epigenetics, and particularly neuroepigenetics, is a field that still is in its infancy, the sub-discipline of neuroepigenetics in the context of nociception (potentially named nocineuroepigenetics) remains in its embryonic stages.

The epigenome, by their ability to integrate different signalling pathways, to regulate the consequent readout of genes and due to its reversibility, could potentially be a key control point to obtain an optimal treatment for different pathological states, including pain development (Cheung *et al.*, 2000b; Kim *et al.*, 2012; Sweatt, 2013; Geranton & Tochiki, 2015a). If the epigenome of a process of disease is completely understood at the implicated tissues and at different time points, we can then target the specific genes that are being activated due to those processes or even attempt to modify the epigenetic landscape itself (Strahl & Allis, 2000; Kim *et al.*, 2012; Crow *et al.*, 2013; Geranton & Tochiki, 2015b; Roadmap Epigenomics *et al.*, 2015). If succeeding on this, we will be able to interpret the link between the gene profile and

an outside experience such as an injury, and thus obtain a better treatment of pain (Geranton, 2012).

Furthermore, epigenetic discoveries in this field can take advantage from drugs already clinically used to target epigenetics for the treatment of other pathologies (e.g. valproate, an HDACi already been used as anticonvulsant), which might potentially be useful to study and/or manage pain stages (Geranton, 2012).

At present, some databases are being created, such as the integrative analysis of human epigenomes established by the Roadmap Epigenomics Consortium in 2015, to establish maps of epigenomic modifications for an increasing number of cell types and under different environmental conditions (Denk & McMahon, 2012; Sweatt, 2013; Roadmap Epigenomics *et al.*, 2015). This could potentially help us elucidate the epigenetic landscape of an injury, such as burns. However, these are dynamic processes, which highlights the need for these databases to be extended to time lines of events for specific processes such as pain development following burn injury.

Within the new techniques that we can count on, some are appointed as crucial to advance in our field. The study of the nuclear architecture, visualize specific locus and the different chromosomal interactions in real time by computational and imaging techniques will help to elucidate the functional requirements of specific cells (Rajapakse & Groudine, 2011; Luger *et al.*, 2012; Sweatt, 2013; Collepardo-Guevara *et al.*, 2015; Guiretti *et al.*, 2016; Scholes & Weinzierl, 2016), such as the neurons that describe pS10H3 following a peripheral injury. Further, techniques such as flow cytometry that helps us to sort out different cells within a tissue (for example separating neurons from glia), and thus avoid tissue heterogeneity (Denk *et al.*, 2013), together with ChIP-Seq (combining chromatin immunoprecipitation with gene

sequencing) will keep neuroscientist busy for years to come and certainly help to reach new milestones in pain treatment.

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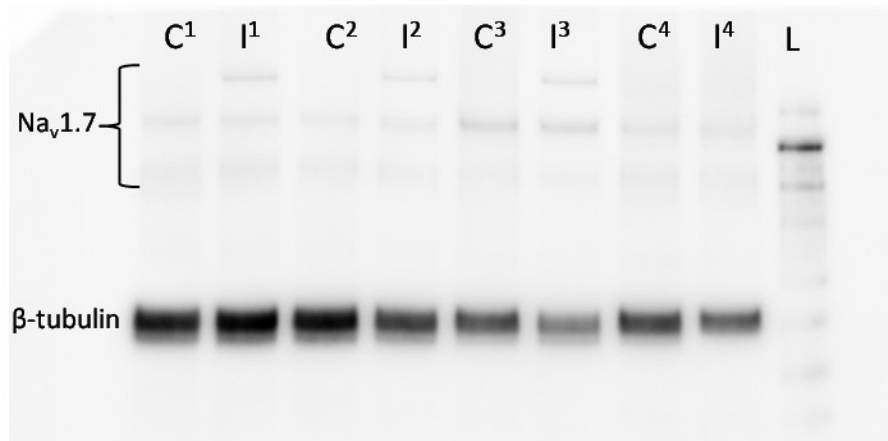
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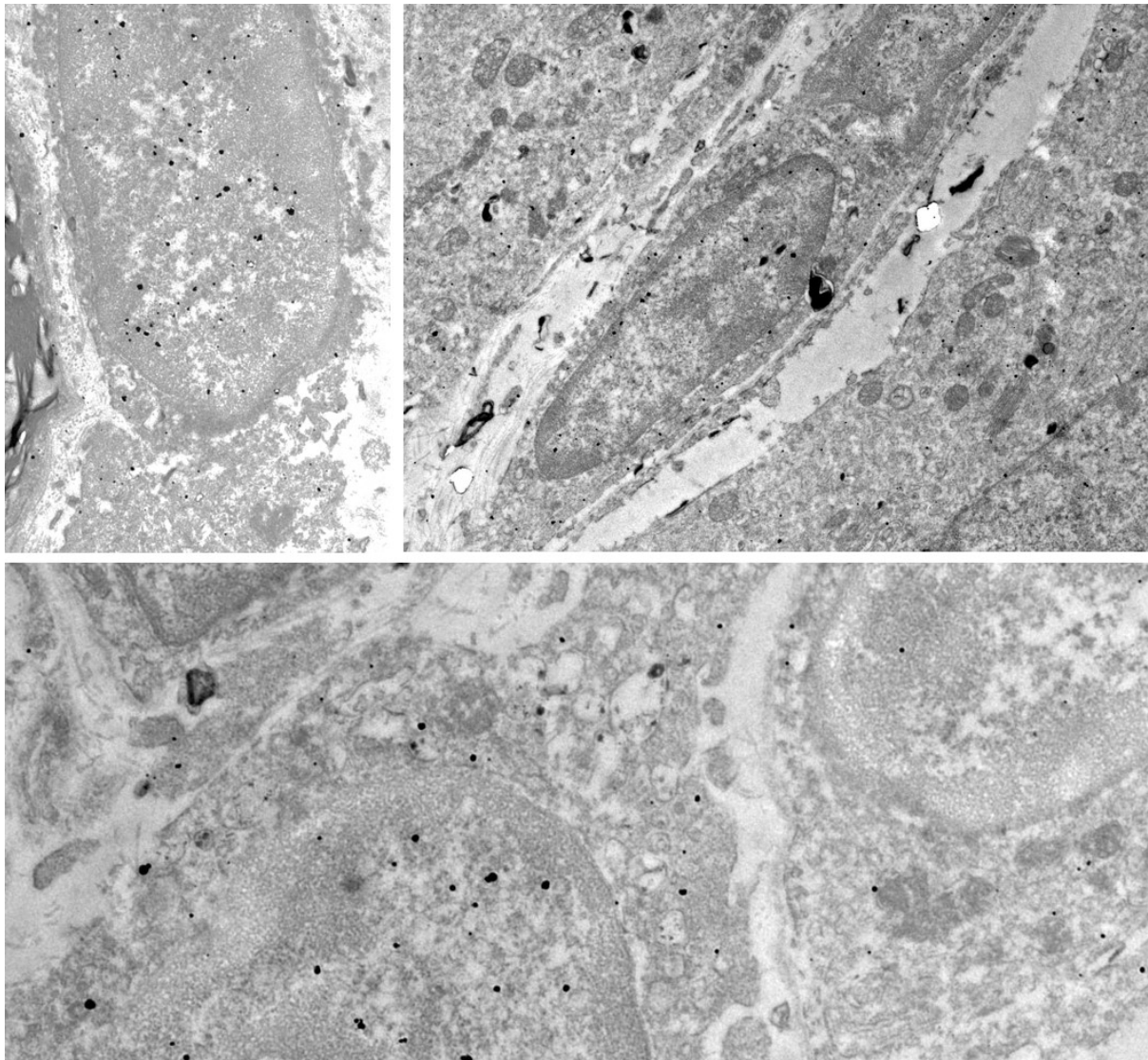
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20 Appendix

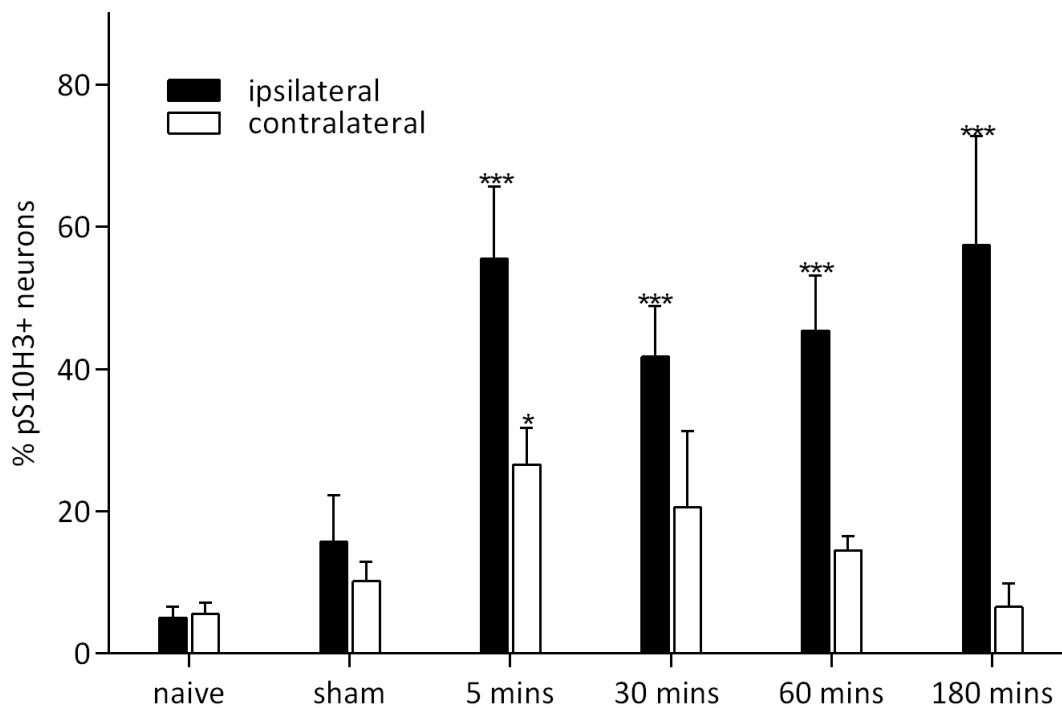
20.1 Supplementary figures



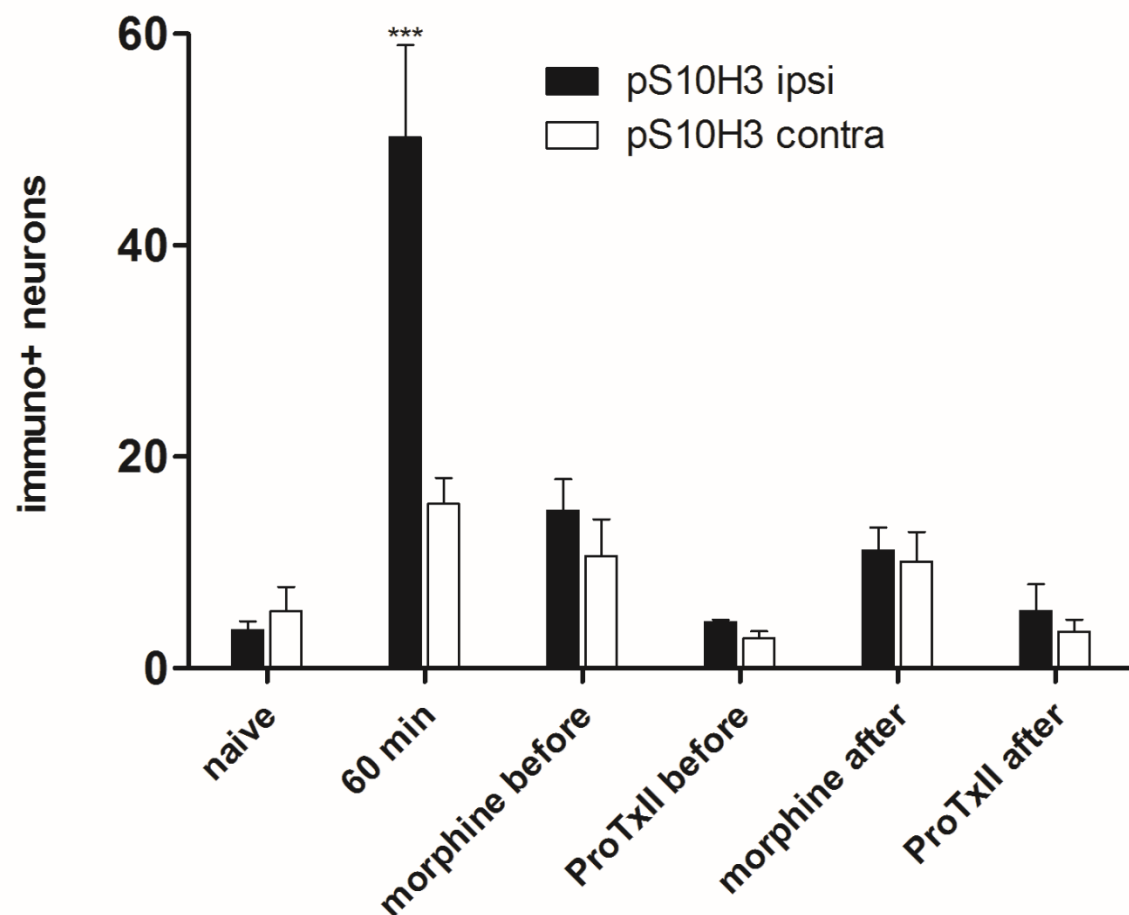
Supplementary Figure 20.1. **DRG western blot for Na_v1.7 and β Tubulin at 5 minutes post-burn injury.** Lanes from left to right: contralateral animal 1, ipsilateral animal 1, contralateral animal 2, ipsilateral animal 2, contralateral animal 3, ipsilateral animal 3, contralateral animal 4, ipsilateral animal 4, biotinilated ladder. At the 3-hours western blot, two bands (~130 and ~210 KDa) were detected for Na_v1.7. However, a higher band also appeared in the ipsilateral sides in three of the four animals. This could be due to any PTM (phosphorylation or glycosylation) (Rush *et al.*, 2007; Stamboulia *et al.*, 2010; Laedermann *et al.*, 2013), or covalent union of regulatory β subunit (Pertin *et al.*, 2005; Lopez-Santiago *et al.*, 2006; Rush *et al.*, 2007; Patino & Isom, 2010; Eijkelkamp *et al.*, 2012). However, since this band was not present in all animals at 5-minutes post-injury and did not appear in the ipsilateral side of animals after 3 hours, was not included in the analysis.



Supplementary Figure 20.2. **Sub-cellular distribution of pS10H3 staining at DRG, as seen by electronic microscopy.** DRG of an animal from 5-minutes group (2 minutes burn injury and 5-minutes post-injury period) was processed for electronic microscopy and immunostained against pS10H3. Black dots indicate positive staining. Note that pS10H3 is also localised outside the nucleus but not attached to any specific structure. The neurons showing pS10H3 outside the nuclei seem to correspond to cells dying because of the injury and thus do not directly participate in the nociceptive transmission (Watson *et al.*, 1995; Gabler *et al.*, 2003; Gabler *et al.*, 2004; Gilthorpe *et al.*, 2013).



Supplementary Figure 20.3. **Percentage of pS10H3 positive neurons at DRG of the burn-injury model.** Data is presented as mean $\pm$ SEM. The experimental groups (n=4) are naive animals (naive), 37°C water for 2 minutes and 5-minutes post-injury period (sham), 60°C water for 2 minutes and 5-minutes post-injury period (5 mins), 60°C water for 2 minutes and 30 minutes post-injury period (30 mins), 60°C water for 2 minutes and 60 minutes post-injury period (60 mins) and 60°C water for 2 minutes and 180 minutes post-injury period (180 mins). *** $p < 0.001$ vs. naive ipsilateral pS10H3, * $p < 0.05$ vs. naive ipsilateral pS10H3. Note that similarly as observed when assessing the nuclear staining (figure 2.10), there is a nociceptive-specific increase of pS10H3 in DRG neurons as early as 5 minutes of the noxious stimulation. However, the increase of total staining is around 50% while of around 10% when only considering nuclear staining.



Supplementary Figure 20.4. **Percentage of DRG neurons with positive staining for pS10H3 in the burn-injury model after different treatments.** Here, I was considering the number of cell with staining positive for pS10H3, not only localised at the nuclei. Data is presented as mean+SEM. The experimental groups are naive animals (naive), 60°C water for 2 minutes and 60-minutes post-injury period (60 min), and 60°C water for 2 minutes and 60-minutes post-injury period with and injection of either ProTxII or morphine 15 minutes before or after the burn injury (morphine before, ProTxII before, morphine after and ProTxII after). *** $p < 0.001$ vs. naive ipsilateral pS10H3.

Supplementary Figure 20.5. ←**Signalling pathways linking extracellular stimuli to modified gene expression.** pS10H3 integrates different pathways, recruits other proteins and serves as a docking site to direct gene expression. AMPA-R: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, AP1: activator protein 1, ASK: apoptosis signal-regulating kinase, ATF: activating transcription factor, ATP: adenosine triphosphate, BDNF: brain-derived neurotrophic factor, CaM: calmodulin, CaMkinase: Ca²⁺/calmodulin-dependent protein kinase, cAMP: cyclic adenosine monophosphate, CBP/p300: CREB-binding protein with the related p300 protein, cdk9: cyclin-dependent kinase 9, CRE: cAMP-response element, CREB: cAMP response element-binding protein, DAG: diacylglycerol *aka* diglyceride, eIF4E: eukaryotic synthesis initiation factor 4E, Elk: ETS (E26 transformation-specific) domain-containing protein, ERK: extracellular signal-regulated kinases, FSH: follicle stimulating hormone, FYN: proto-oncogen tyrosine-protein kinase fyn, Gcn5: general control of amino acid 5 HAT, GDP: guanosine diphosphate, GPCR: G protein-coupled receptor, GR: glucocorticoid receptor, GRB2: growth factor receptor-bound protein 2, GTP: guanosine-5'-triphosphate, HAT: histone acetyltransferase, HDAC: histone deacetylase, IκB: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, IκBα: IκB kinase-α *aka* IKK1, IL-1: interleukin-1, JNK: Jun amino-terminal kinase, MAP2K: MAPK kinase, MAP3K: MAP2K kinase, MAPK: mitogen-activated protein kinase, MeCP2: methyl-CpG-binding protein 2, MEK: MAPK/ERK kinase, MEKK: MEK kinase, MKK: mitogen-activated protein kinase kinase, MKP-1: MAPK phosphatase 1, MLK: mixed lineage kinase, MLTK: MLK-like mitogen-activated protein, MNK: MAP kinase-interacting kinase, MSK: mitogen and stress activated protein kinase, Mos: proto-oncogen ser/thr-protein kinase mos, NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells, NMDA-R: N-methyl-D-aspartic acid receptor, p38: p38 MAPK, Pi3kinase: phosphoinositide 3-kinase, PIP₂: phosphatidylinositol 4,5-bisphosphate, PIP₃: phosphatidylinositol (3,4,5)-triphosphate, PKA: protein kinase A, PKC: protein kinase C, PLC: phospholipase C, PP1: protein phosphatase 1, Rac1: Ras-related C3 botulinum toxin substrate 1 (small signalling GTPase), Raf: RAF proto-oncogen ser/thr-protein kinase *aka* proto-oncogen c-RAF, RasGRP: Ras guanyl-releasing protein, RE1: neuron-restrictive silencer, RNAPol II: RNA polymerase II, RSK: ribosomal s6 kinase, RTK: receptor tyrosine kinase, SAPK: stress-activated protein kinase, SEK: SAPK/ERK kinase, SIN3A: paired amphipathic helix protein Sin3a, SRE: serum response element, Shc: SHC-transforming protein, SIE: sis-inducible element, Spt: suppressor of Ty protein, SRF: serum response factor, SWI-SNF: switch/sucrose non-fermentable, TAB: TAK1-binding protein, Taf: TBP associated factor, TAK1: transforming growth factor beta-activated kinase 1, TBP: TATA-binding protein, TFIID: transcription factor II D, TGFβ: transforming growth factor beta, TNFα: tumor necrosis factor alpha, TPA: 12-o-tetradecanoylphorbol-13-acetate, UV light: ultraviolet light.

Info combined from (Xing *et al.*, 1996; Deak *et al.*, 1998; Thomson *et al.*, 1999b; Cheung *et al.*, 2000b; Clayton *et al.*, 2000; Lo *et al.*, 2000; Salvador *et al.*, 2001; Agaloti *et al.*, 2002; Sacconi *et al.*, 2002; Davie, 2003; Choi *et al.*, 2005; Lo *et al.*, 2005; Dong & Bode, 2006; Geranton *et al.*, 2007; Borrelli *et al.*, 2008; Chandramohan *et al.*, 2008; Chiechio *et al.*, 2009; Pages, 2009; Sando *et al.*, 2012; Keum *et al.*, 2013; Rahn *et al.*, 2013).