PERIPHERAL MECHANISMS OF INFLAMMATORY PAIN WITH PARTICULAR REFERENCE TO TRPV1, BURN INJURY AND THE ACTIONS OF CERTAIN GASEOUS GENERAL ANAESTHETICS

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by

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ABSTRACT

1. The literature relating to peripheral mechanisms of inflammatory pain is reviewed.

2. To investigate the role of the transient receptor potential vanilloid type 1 (TRPV1) ion channel in spinal nociceptive processing after burn injury, the candidate studied scalding-type burn injury-induced activation of extracellular signal-regulated kinase 1/2 (ERK1/2) in the spinal dorsal horn of wild-type (WT) and TRPV1 knock-out (KO) mice. Activation of ERK1/2 in the spinal dorsal horn is a recognised marker for spinal nociceptive processing. At 5 minutes after severe scalding injury to WT mouse hind-paw, a substantial number of phosphorylated ERK1/2 (pERK1/2) immunopositive neurons were found in the ipsilateral dorsal horn. At 1 hour post-injury, the number of pERK1/2-labelled neurons remained substantially the same. However, at 3 hours post-injury, a further increase in the number of labelled neurons was found on the ipsilateral side, while a remarkable increase in the number of labelled neurons on the contralateral side resulted in there being no significant difference between the extent of the labelling on both sides. By 6 hours post-injury, the number of labelled neurons was reduced on both sides without there being significant difference between the two sides. A similar pattern of severe scalding injury-induced activation of ERK1/2 in spinal dorsal horn neurons over the same time-course was found in TRPV1 KO mice, except that the extent to which ERK1/2 was activated in the ipsilateral dorsal horn at 5 minutes post-injury was significantly greater in WT animals when compared to TRPV1 null animals. This difference in activation of ERK1/2 in spinal dorsal horn neurons was abolished within 1 hour after injury, demonstrating for the first time that TRPV1 is not essential for the maintenance of ongoing spinal nociceptive processing in inflammatory pain conditions in mouse resulting from at least certain types of severe burn injury. The facts that *neuronal staining for activated ERK1/2 not only persisted,* but also that the *number of neurons expressing activated ERK1/2 increased* with the passage of

time are significant novel findings of this study which have important implications. After an acute noxious stimulus (provided by hind-paw injection of capsaicin), ERK1/2 activation reaches a peak within 2-5 min and then quickly subsides, such that it is barely detectable or absent by 2 hours after the stimulus (Ji et al., 1999). The findings of this study that activated ERK1/2 is: (i) still present in neurons at the 3 hour and 6 hour time-points post-burn injury; and (ii) that it is found in even more neurons at those later time-points than at the earlier time-points measured, strongly suggest that the ERK1/2 activation found at 3 hours and 6 hours post-injury, respectively, is not merely residual from the initial noxious heat stimulus, but rather that it has been caused by the ongoing inflammatory process itself. This is a key novel finding of this study. The data obtained contain evidence of a dissociation *within the physiological process of the burn injury* of these two distinct "noxious insults" comprising: first, the initial external heat stimulus of the burn injury, which causes direct damage to the tissue accompanied by heat-induced activation of heat-sensitive receptors which result in excitation of nociceptive primary afferents; and, second, the burn injury-induced massive inflammatory response whereby inflammatory mediators occasion an ongoing activation of their receptors on those nociceptors to maintain, and, indeed, increase, nociceptive signaling in those neurons. The further finding that, at 3 hours after scalding-type burn injury, in both TRPV1-null and wild-type mice, the contralateral side of the spinal cord now exhibited a substantial number of pERK1/2-immunopositive neurons remains intriguing, notwithstanding that the phenomenon of hyperalgesia developing in the hind-paw contralateral to a heat injury has long been noted (Coderre and Melzack, 1991; Polgar et al., 1998). One may speculate that this labelling is caused, or contributed to, by an action of descending fibres on these contralateral dorsal horn neurons since the concept of supraspinal pronociceptive modulation of pain is now well recognised (Lima et al., 2002; Tavares and Lima, 2007). In addition, or in the alternative, this labelling may result from cross-communication between opposite dorsal horn neurons (Coderre and Melzack, 1991; Shenker et al., 2003). In any event, the activation of ERK1/2 on both sides of the dorsal horn discloses a process in which spinal neurons are driving inflammatory

nociceptive activity after the excitatory effect of peripheral inflammagens has abated in primary sensory afferents.

3. Xenon provides effective analgesia in several pain states at sub-anaesthetic doses. The aim of this second study was to examine whether xenon may mediate its analgesic effect, in part, through reducing the activity of TRPV1, a receptor known to be involved in certain inflammatory pain conditions. The candidate studied the effect of xenon on capsaicin-evoked cobalt uptake in rat cultured primary sensory neurons and in human TRPV1 (hTRPV1)-expressing human embryonic kidney 293 (HEK293) cells. He also examined xenon's effect on the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in the rat spinal dorsal horn evoked by hind-paw injection of capsaicin. Xenon (75%) reduced the number of primary sensory neurons responding to the TRPV1 agonist, capsaicin (100 nM-1 μM) by ~25% to ~50%. Xenon reduced the number of heterologously-expressed hTRPV1 activated by 300 nM capsaicin by ~50%. Xenon (80%) reduced by ~40% the number of phosphorylated ERK1/2-expressing neurons in rat spinal dorsal horn resulting from hind-paw capsaicin injection. The key original finding is that xenon substantially reduces the activity of TRPV1 in response to noxious stimulation by the specific TRPV1 agonist, capsaicin, suggesting a possible role for xenon as an adjunct analgesic where hTRPV1 is an active contributor to the excitation of primary afferents which initiates the pain sensation. Next, to investigate whether the xenon-induced inhibition of TRPV1 in rat DRG neurons reduces nociceptive processing, the effect of xenon in reducing the release of calcitonin gene-related peptide (CGRP) from those neurons was examined. Exposure to xenon failed to effect a reduction of capsaicin-evoked CGRP release from cultured primary sensory neurons when stimulated by capsaicin. This latter novel finding suggests that xenon acts on several molecular targets on nociceptive primary sensory neurons, and that xenon’s action on one, or more, of those targets serves to offset the inhibitory, pro-analgesic, effect of xenon on TRPV1. It is concluded that xenon may not produce any analgesic effect *through peripheral nociceptors.*

4. Clinically relevant concentrations of isoflurane or sevoflurane sensitize TRPV1 to several of its activators, including capsaicin. It has, moreover, been suggested these volatile general anaesthetics may augment nociceptive signalling arising from surgical procedures and thereby contribute to post-operative pain. To investigate this suggestion, the candidate studied intraplantar capsaicin injection-induced phosphorylation of extracellular signal-regulated kinase 1/2 (pERK1/2) in spinal dorsal horn neurons (which is a recognised marker of spinal nociceptive processing) in rat during isoflurane or sevoflurane anaesthesia after 60 min under anaesthesia. Control animals were anaesthetised with pentobarbital (which of itself does not activate ERK1/2 in spinal dorsal horn neurons). Unilateral intraplantar capsaicin injection in control animals evoked pERK1/2 in a group of neurons in lamina I and lamina II of the ipsilateral spinal dorsal horn in a somatotopically appropriate area. In contrast, both anaesthetic gases (given for 60 min and without subsequent capsaicin injection) induced ERK1/2 activation in a different group of mainly lamina I neurons bilaterally. The total number of spinal dorsal horn neurons labelled on the ipsilateral side following capsaicin injection into the isoflurane-, or sevoflurane-, anaesthetised animals was significantly less than that produced by capsaicin alone. Further, capsaicin injection into isoflurane-, or sevoflurane-, anaesthetised animals reduced pERK1/2 induced by the gases alone on both sides. These key original findings are inconsistent with the suggestion that isoflurane-, or sevoflurane-, induced sensitisation of TRPV1 by capsaicin, or other agonist, is translated into induction of spinal nociceptive processing and consequential pain sensation.

PUBLICATIONS ARISING OUT THIS DISSERATION

A. Reasearch Articles:

White, J. P., Cibelli, M., Fidalgo, A. R, Paule, C. C., Anderson, P. J., Jenes, A., Rice, A. S., & Nagy, I. (2010). Sensitization of the transient receptor potential vanilloid type 1 ion channel by isoflurane or sevoflurane does not result in extracellular signal-regulated kinase ½ activation in rat spinal dorsal horn neurons. *Neuroscience 166,* 633-638.

White, J. P., Calcott, G., Jenes, A., Hossein, M., Paule, C. C., Santha, P., Davis, J. B., Ma, D., Rice, A. S., & Nagy, I. (2011). Xenon reduces activation of transient receptor potential vanilloid type 1 (TRPV1) in rat dorsal root ganglion cells and in human TRPV1-expressing HEK293 cells. *Life Sci 88,* 141-149.

White, J. P., Ko, C. W., Rei Fidalgo, A., Cibelli, M., Paule, C. C., Anderson, P. J., Cruz, C., Gomba, Sz., Matesz, K., Veress, G., Rice, S. C., Avelino, A., & Nagy, I. (2011). Severe burn injury induces a characteristic activation of extracellular signal-regulated kinase ½ in spinal dorsal horn neurons. *Eur J Pain* (in press).

B. Review Articles:

White, J. P., Cibelli, M., Rei Fidalgo, A., Paule, C. C., Noormohamed, F., Urban, L., Maze, M., & Nagy, I. (2010). Role of transient receptor potential and acid-sensing ion channels in peripheral inflammatory pain. *Anesthesiology 112,* 729-741.

White, J. P., Cibelli, M., Fidalgo, A. R., & Nagy, I. (2011) Extracellular signal-regulated kinases in pain of peripheral origin. *Eur J Pharmacol 650,* 8-17.

White, J. P., Urban, L., & Nagy, I. (2011). TRPV1 function in health and disease. *Curr Pharm Biotechnol 12*, 130-144.

Urban, L., White, J. P., & Nagy, I. (2011). Molecular structure of transient receptor potential vanilloid type 1 ion channel (TRPV1). *Curr Pharm Biotechnol 12,* 115-121.

C. Book Chapters:

Nagy, I., Paule, C. C., White, J. P., & Urban, L. (2008). Functional molecular biology of the transient receptor potential vanilloid type 1 (TRPV1) ion channel. In: Kofalvi, A., ed. *Cannabinoids and the Brain.* New York, Springer, pp. 101-130.

Nagy, I., White, J. P., Paule, C. C., & Kofalvi, A. (2008). An historical introduction to the endocannabinoid and endovanilloid systems. In: Kofalvi, A., ed. *Cannabinoids and the Brain.* New York, Springer, pp. 3-14.

Nagy, I., Paule, C. C. & White, J. P. (2009). Molecular mechanisms of TRPV1-mediated pain. In: Jancso, G., ed. *Neuroimmune Bioloy, Neurogenic Inflammation in Health and Disease, Vol 8.* Amsterdam, Elsevier B. V., pp. 75-99.

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TO THE MEMORY OF MY MOST SINCERE AND
WONDERFUL FRIEND, TOM HANNIGAN,
WHO PASSED TO GOD
ON 3RD JULY, 2007

PREFACE

This work has been prepared while reading for the Degree of Doctor of Philosophy of Imperial College London in the Department of Academic Anaesthetics, at Chelsea & Westminster Hospital. Its subject is one which is profoundly important. Pain is the almost inevitable accompaniment of serious injury and disease. It may also constitute an independent disease state. The reality of pain may be utterly destructive. Pain is that which terrifies every sane person. People can become reconciled even to terminal illness, but not to the pain which may frequently accompany it. People, otherwise well, can be tormented, quite beyond endurance, by such as back-pain, migraines, or thalamic pain. Unfortunately, although scientific progress in understanding pain in the last twenty years has been great, there is no reason to believe that a break-through, or a "wonder drug", will be found sooner rather than later. Therefore, for those in search of financial reward, patents, knighthoods, and peerages – which appear to be the modern currency of academic achievement in certain institutions – the study of pain is not recommended. However, one must struggle in vain to reconcile with any concept of a scientific or medical ethic the failure by Imperial College London to commit to the continuing study of pain as a priority.

During the past several years, I have been very fortunate to have enjoyed the company of fellow doctoral candidates in this Department. I must mention, in particular, Ms. Cleoper Paule, B.Sc., who is an exceptionally skilled and talented laboratory scientist, as well as Dr. Mario Cibelli, M.D., and Mr. Antonio Rei Fidalgo,

M.Sc., who always manage not to take themselves too seriously, which is an attribute which is seldom found in the post-modern era, but is much to be commended. I am very grateful to them indeed for their enduring friendship.

I wish to record a debt of gratitude to Professor Masao Takata, who has always shown a most kind and helpful interest in my progress and has made himself available to provide sound advice whenever required. I am very grateful to him.

A similar debt of gratitude is owed to Mr. Faruq Noormohamed, M.Sc., who brings his usual professionalism to bear in dealing with the myriad of minor and major problems which arise in the Department. An excellent research scientist, he is also always there to advise and offer kind assistance. I am very grateful to him.

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My very special thanks are reserved to Dr. Istan Nagy, M.D., Ph.D., who acted as my sole supervisor during my course of studies. Dr. Nagy possesses an unsurpassed knowledge of molecular pain, a willingness to engage in constructive argument without insisting on the necessity of winning, and very considerable laboratory skills. I am very fortunate indeed to have enjoyed the benefit of his tutelage. I am deeply indebted to him.

I am always sustained utterly by the memory of, and the assurance of my future re-union with, my most true and wonderful friend, Tom.

John P. M. White.

369, Fulham Road,
London.

21st August, 2009.

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All persons who made any contribution of any kind to the work involved in this dissertation are mentioned as co-authors in one, or more, of the published papers referred to above.

Experimental Studies:

All experimental studies were designed and executed by the candidate under the direction of Dr. Istvan Nagy, with assistance as hereinafter mentioned.

- **HEK293 cell studies.** These were undertaken exclusively by the candidate after training by Dr. Cleoper Paule. hTRPV1 cDNA was initially generously provided by Dr. J. B. Davis. Additional hTRPV1 cDNA was cloned in this laboratory by the candidate and Dr. Paule.

- **Animal studies.** Dr. Mario Cibelli, Consultant Anaesthetist, administered anaesthetics where required. Live animals were managed and/or sacrificed by Dr. Cibelli, Dr. Jenes, Dr. Hossein, Dr. Anderson, or Mr. Antonio Rei Fidalgo, as required. Almost all sectioning was performed by the candidate, but Mr. Rei Fidalgo assisted with much of the staining. Mr. Guy Calcott extracted and prepared DRG cultures. Almost all analysis of cobalt uptake and immunhistochemistry results and all interpretation of all analysed data was performed by the candidate. Dr. Jenes provided the diagrammatic illustration of the localisation of pERK1/2 neurons in the isoflurane/sevoflurane study. Burn injury was induced and spinal cords of burn-injured animals extracted after sacrifice by Dr. Antonio Avelino, University of Porto. The first three trials within the Burn Injury study were performed by Mr. C. W. Ko, an M. Res. student, under supervision. Pathology advice was provided by Dr. C. Cruz, Dr. W. Gomba, Dr. K. Matesz, and Dr. G. Veress.

- The xenon studies were designed, in part, from a pilot study by Dr. Peter Santha. Mr. F. Noormohamed provided technical advice on many matters. Dr. D. Ma provided technical advice in relation to methods of exposing cells to xenon.

Reviews:

All reviews were written exclusively by the candidate with advice from Dr. Nagy. Assistance in preparation for publication was provided by the persons named as co-authors.

Book Chapters:

The book chapters were written in joint authorship with Dr. Nagy.

CHAPTER 1

INTRODUCTION

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1.1. PERIPHERAL MECHANISMS OF INFLAMMATORY PAIN

The vast majority of persistent pathological pain conditions – including conditions as common as persistent back-pain and osteoarthritis – are instances of peripheral inflammatory pain. Peripheral inflammatory pain imposes a huge economic burden on society generally, in addition to the burden of misery and disability which it imposes on individual sufferers. Inflammatory agents, released as components of the body's response to peripheral tissue damage or disease, excite nociceptive primary sensory neurons to generate an electrical signal which will ultimately be interpreted by the brain as peripheral pain. These agents achieve this effect by activating their

own receptors on those neurons and, in addition, or in the alternative, by altering the sensitivity and expression, or by directly activating, one, or more, ionotropic receptors also found on those neurons. In the next chapter, I survey the identity, nature and mechanisms of action of these inflammatory agents on primary nociceptive afferents, with particular reference to ionotropic receptors, to examine the present state of research into peripheral inflammatory pain.

1.2. EXPERIMENTAL TECHNIQUES AND PROTOCOLS

During the course of my studies, I performed various techniques in molecular biology, extending over the areas of: cell culture; immunohistochemistry; agonist-activated cobalt uptake and imaging; and enzyme-linked immunosorbent assays (ELISAs). In chapter 3, the theoretical bases of these techniques are described, as are the protocols which were employed for their execution.

1.3. THE ROLE OF TRPV1 IN BURN INJURY

Pain resulting from severe burn injury is one of the most excruciating pain sensations that can be experienced and the absence of consistently effective analgesics for its treatment renders its clinical management unsatisfactory. Severe burn injury causes major tissue damage and the resulting attempts of the body to defend against infection and to effect repair of injured tissues generate a massive inflammatory response. This inflammatory response induces the formation and release of inflammatory mediators. The resulting excitation of a sub-population of nociceptive primary sensory neurons is responsible for initiating, and maintaining, the sensation of peripheral inflammatory pain.

The transient receptor potential vanilloid type 1 (TRPV1) is known to play an important role in several types of heat-induced hyperalgesia, although the more extensive claims made on its behalf have not been justified by the experimental results. I have investigated the role of this receptor in the context of a mouse model of scalding-type burn injury to the lower hind-paw and show that activation of TRPV1 is not always essential for the maintenance of inflammatory pain in mouse caused by at least certain types of severe burn injury.

1.4. WHETHER XENON MAY MEDIATE AN ANALGESIC ACTION THROUGH AN EFFECT ON TRPV1

Xenon is an inert gas which binds to a remarkable range of proteins, including ion channels, such as the ionotropic N-methyl-D-aspartate (NMDA) receptor for glutamate, as well as certain non-NMDA receptors. Research into the mechanism of action of xenon has focused, almost exclusively, on its role as a general anaesthetic. However, in addition to its anaesthetic effect, xenon, in sub-anaesthetic doses, provides analgesia. Such xenon-induced analgesia is believed to be mediated, at least in part, through inhibition of spinal nociceptive processing by way of reducing noxious stimulation-evoked spiking activity and long term potentiation in spinal cord neurons. If xenon indeed reduces the nociceptive input into the spinal dorsal horn by an action on receptors on nociceptive primary afferents, an inhibitory effect on TRPV1 activation may be one of the mechanisms through which it mediates that effect. Accordingly, this study was designed to determine the effect of xenon on TRPV1 activation in spinal dorsal horn neurons as well as in primary nociceptive afferents.

1.5. WHETHER ISOFLURANE AND SEVOFLURANE OCCASION PAIN-INDUCING SPINAL NOCICEPTIVE PROCESSING

Recent *in vitro* studies have shown that certain pungent gaseous anaesthetics, can activate, or increase the activation of, receptors on primary nociceptive afferents which have been implicated as mediators of hyperalgesia in inflammatory pain conditions. These receptors are the transient receptor potential ankyrin, type 1 (TRPA1) and TRPV1. The pungent general anaesthetics isoflurane and desflurane robustly activate TRPA1, with isoflurane producing inward currents in voltage-clamped TRPA1-expressing HEK293 cells and in cultured mouse dorsal root ganglion neurons. Moreover, clinically relevant concentrations of isoflurane, sevoflurane, enflurane and desflurane have also been shown to sensitise TRPV1 to capsaicin and protons and to reduce the threshold for heat activation of this ion channel. Hence, the suggestion that, in the clinical context, volatile general anaesthetics may augment nociceptive signalling arising from surgical insults. In particular, the finding that sevoflurane may exert a sensitizing effect on TRPV1 (albeit an effect which is substantially less than that elicited by isoflurane) raises an issue of clinical importance, given its preferred status as a non-pungent inhalational anaesthetic.

I have used activation of ERK1/2 in spinal dorsal horn neurons to elicit the extent to which exposure to isoflurane and sevoflurane, in clinically relevant concentrations results in nociceptive processing in the rat spinal cord. I have also examined the extent to which these inhalational anaesthetics are sensitizers of TRPV1 by exposing animals to clinically relevant concentrations of these gases followed by injection of the hind-paw with the archetypical TRPV1 agonist, capsaicin.

Contrary to the suggestions made on the basis of the *in vitro* data, in these experiments, isoflurane and sevoflurane are both shown to dramatically reduce, rather than increase, painful stimulus-evoked nociceptive processing in the spinal dorsal horn. Indeed, the data obtained suggest that volatile anaesthetics may mediate analgesia in surgery rather than provoke pain as previously suggested.

CHAPTER 2

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2.1. INTRODUCTION

2.1.2. Scope of Review

Peripheral inflammatory pain makes up the vast preponderance of persistent pain conditions, including, as it does, pain from osteo-, and rheumatoid, arthritis, back-pain from inflamed spinal disc injury, post-operative pain, and pain from tumours and burn injuries. Any attempt to understand the nature of inflammatory pain and, in doing so, to move forward and devise a remedy for it, must be prefaced by identification of the pharmacological issues involved and their essential parameters. Each of the individual topics addressed in this review is, in itself, worthy of an extended review. However, this paper is intended to serve a different purpose. It offers a tour of the relevant terrain as mapped by the literature, rather than a comprehensive "dig" at the site of any particular subject which is described. It is intended to contextualise the work of research scientists in many areas relating to the challenge of elucidating the peripheral mechanisms of inflammatory pain.

The inflammatory process gives rise to a multiplicity of chemical agents which are designed to protect against infection and to promote the repair of damaged tissue. However, the body's inflammatory response to injury, or disease, is not precisely regulated, and is often disproportionate, resulting in pain. Physiological pain – which abates with removal of the noxious stimulus – is a purely protective mechanism, warning the body of the threat which it has encountered. However, several pathological pain conditions may result from peripheral inflammation, namely: *hyperalgesia*, where an excessive sensation of pain is elicited by a mild noxious stimulus, such as heat (*thermal hyperalgesia*) or mechanical pressure (*mechanical hyperalgesia*); *allodynia* where pain is elicited by a harmlesss non-noxious stimulus;

and *spontaneous pain,* where pain is evoked without any precipitating stimulus. In cases of severe inflammation, as in the case of serious burn injury, these conditions can prevent necessary active treatment of the tissue damage. In other cases, the inflammation may not subside, or the pain may persist notwithstanding the fact that its initiating stimulus has abated, leading to a chronic pain condition.

2.1.2. The signal for peripheral inflammatory pain sensation

The inflammatory agents generated by the tissue damage or disease which is the cause of inflammatory pain include: bradykinin; eicosanoids; NGF; GDNF; serotonin; histamine; anandamide; ATP; cations, especially protons; and cytokines. Peripheral inflammatory pain is the result of an alteration of the excitability of peripheral sensory neurons resulting from the effect of these inflammatory agents.. Alteration of a neuron's firing pattern is reflected in its synaptic transmission of signal in the dorsal horn of the spinal cord, where first order primary afferent neurons synapse with secondary spinal neurons. The signal thus relayed to spinal secondary afferents then undergoes modulation by descending neuronal inputs, before being transmitted *via* these secondary spinal afferents to the thalamus. In the thalamus, these secondary afferents synapse with third order sensory neurons which relay the signal to the primary somatosensory cortex of the brain. In the brain, after further modulation by supraspinal (particularly thalamic and limbic) inputs, the signal falls to be interpreted as the sensation of pain. The main descending neuronal inputs are serotonergic and noradrenergic (Yoshimura & Furue, 2006; Braz & Basbaum, 2008; Geranton et al., 2008), Descending pain inhibitory, or facilitatory, pathways from the rostroventromedial medulla (RVM) are proposed to act ultimately in the spinal cord in

acute and chronic pain states through activation of 5-HT7 and 5-HT3 receptors, respectively (Dogrul et al., 2009).

A variety of agents released in inflammation contribute to alter the firing pattern of nociceptive primary sensory neurons. Many of these inflammatory agents act directly on their own receptors found on primary afferents to contribute to excitation of these neurons. Thus, bradykinin acts on B1 and B2 receptors expressed by primary sensory neurons. Certain of these inflammatory agents, in addition, or in the alternative, directly activate ion channels found on these neurons, or affect the sensitivity and expression of these ion channels. Present evidence suggests that TRPV1, TRPA1, TRPV4, and ASIC ion channels are of special relevance in the context of inflammation. These ion channels constitute an essential component of the mechanisms whereby inflammatory agents excite primary afferents to result in pain conditions. Yet other ion channels generally contribute – regardless of the special circumstances resulting from inflammation – to neuronal excitability. These include sodium, potassium, chloride, and calcium ion channels. Hence, the role of these ion channels -- so far as relevant to mediating the nociceptive consequences of the inflammatory response -- must be borne in mind as mediators of the effects of individual inflammagens.

2.1.3. Sodium, potassium and chloride ion channels

2.1.3.1. Na^+ channels

Altered function of certain Na^+ channels contributes to the increased hyper-excitability of primary afferent neurons in certain pathological pain states and the Nav1.9 sub-unit has been implicated in mediating inflammatory pain sensations

(Priest et al., 2005; Amaya et al., 2006). This channel is important in the development of inflammation-based bladder dysfunction (Ritter et al., 2009). Moreover, in a distinct population of L3-L5 knee joint afferent DRGs in a rat model of FCA-induced chronic inflammatory joint pain, Nav1.9, Nav1.8 and Nav1.7 show increased channel expression which is accompanied by unilateral joint hypersensitivity in the FCA-injected knee (Strickland et al., 2008). In at least certain instances of inflammation, individual inflammagens may, conjointly, produce an effect which they are individually incapable of mediating. In an important study, Delmas and colleagues (2008) have shown that bradykinin, ATP, histamine, PGE2, and noradrenaline, when applied separately at maximal concentrations, all fail to modulate Nav1.9 current. However, when applied conjointly as a soup of inflammatory mediators, they rapidly potentiate Nav1.9 channel activity, generating sub-threshold amplification and increased excitability (Maingret et al., 2008). These findings may have profound clinical implications because they suggest that, in certain pain states at least, an intervention which successfully prevents the contribution to neuronal excitation of certain only of the inflammagens may result in a significant analgesic effect. They also confirm the importance of the NaV1.9 channel as a mediator of inflammatory pain sensations. Again, Levine and colleagues (2008) have shown that the level of the pro-inflammatory cytokine, interleukin-6 (IL6), is increased following a partial-thickness burn injury in rat and that spinal intrathecal injection of oligodeoxynucleotides antisense for gp130, a receptor subunit shared by members of the IL-6 family of cytokines, attenuates both burn-induced, and intrademeral IL-6-induced, mechanical hyperalgesia, as does intradermal injection of anti-IL-6 function blocking antibodies (Summer et al., 2008).

PGE2-induced persistent hypernociception in rats results in up-regulation of Nav1.8 mRNA in DRG neurons. After a period of such recurring inflammatory stimuli, an intense and prolonged nociceptive response is elicited by a minimal inflammatory stimulus (Villarreal et al., 2009). A selective blocker of Nav1.8 reduces the nociceptive effects of CFA-induced inflammation in rat – as does a TRPV1 antagonist. Co-administration of both of these agents produces an additive effect in this rat pain model, suggesting that such a combination strategy to produce analgesia may be valuable in treating inflammatory pain (Joshi et al., 2009).

2.1.3.2. K^+ channels

In cultured rat trigeminal ganglion neurons, hypotonicity increases slow-inactivating K+ current but has no effect on fast-inactivating K^+ current. On the other hand, hypertonicity depresses both slow-inactivating, and fast-inactivating, K^+ currents (Chen et al., 2008).

2.1.3.3. Cl^- channels

In cultured rat DRG neurons, treatment with a soup comprising NGF, ATP and bradykinin for 1 to 3 hours raises intracellular Cl^- levels and the driving force for depolarizing Cl^- efflux (Funk et al., 2008).

2.1.4. TRPV1 mediates inflammatory thermal hyperalgesia and spontaneous pain sensations

2.1.4.1. TRPV1 as an ionotropic receptor

TRPV1 is a ligand-gated, non-selective, cationic channel with a high permeability for Ca^{2+} (Caterina et al., 1997; Marsh et al., 1987; Heyman and Rang, 1985). On activation, these cation-selective ion channels generate a net inward current which depolarises the neuronal membrane and increases the probability of action potential generation. TRPV1 is found in approximately 40 per cent of all primary afferents and is expressed in the perikarya, as well as in both the central and peripheral terminals, of primary sensory neurons; and it has a wide-spread distribution not only in peripheral tissues, but also in the central nervous system (Guo et al., 1999; Nagy, 2004; Mezey et al., 2000).

Multiple activators gate TRPV1. Ligands (such as capsaicin and related vanilloids), heat, protons, and depolarization induce TRPV1 opening directly (Caterina et al., 1997; Tominaga et al., 1998; Voets et al., 2004). It also appears that a reduction in the extracellular Na^+ concentration can directly gate and sensitise TRPV1 to such stimuli (Ohta et al., 2008). In addition, gating of this ion channel can also be evoked indirectly by various inflammatory mediators which activate their own target receptors, which are also expressed on nociceptive primary sensory neurons expressing TRPV1. These “own receptors,” in turn, induce activity in the neuron's intracellular second messenger system. That, in turn, results in post-translational modification of TRPV1 in a process called “sensitisation” which increases the responsiveness of TRPV1, as well as the extent of the expression of TRPV1.

TRPV1 is important in its ability to integrate the respective effects of many of the agents generated by the inflammatory response. In addition, "co-operation" occurs between various ligands of TRPV1 whereby one increases the effect of the other in activating TRPV1 in an apparently synergistic, rather than additive, manner (Caterina et al., 1997; Tominaga et al., 1998; Kress et al., 1996). The ability of certain ligands to potentiate each other's effect on TRPV1 suggests that the activation sites of different activators are in fact coupled in TRPV1. Given the polymodal nature of activation of TRPV1 and the potentiating effect of at least certain of the activating stimuli on the effect produced by another activator of TRPV1, this ion channel has been described as a "stimulus integrator" of exogenous stimuli (Tominaga et al., 1998). However, TRPV1 also acts similarly in relation to many endogenous agents, which makes it particularly relevant given the wide variety of inflammatory agents generated as components of the inflammatory response.

2.1.4.2. Thermal inflammatory pain

Heat hyperalgesia secondary to inflammatory tissue injury fails to develop in TRPV1 null mice as in wild type mice, although pathological thermal sensations after peripheral nerve injury remain the same in both wild type and TRPV1 null animals (Caterina et al., 2000; Davis et al., 2000), suggesting that TRPV1 is an essential component in the development of pathological thermal hyperalgesia at least in the contexts examined, but apparently limited to pain sensations of inflammatory origin. Thermal hyperalgesia following inflammation involves an increased level of TRPV1 expression (Amaya et al., 2003), as well as the sensitisation of existing TRPV1 channels. NGF and GDNF increase following inflammation and contribute to inflammatory thermal hyperalgesia *via* an increase in TRPV1 expression. These

increases in NGF and GDNF, respectively, each follow a different time course and they each act on distinct populations of DRG neurons (Amaya et al., 2004). Inflammatory mediators, such as ATP, bradykinin, and NGF, increase the temperature and proton sensitivity of TRPV1 and contribute to enhanced TRPV1 ion channel activity. Chuang and colleagues (2001) injected wild-type and TRPV1-deficient mice with bradykinin or NGF and measured paw withdrawal latencies from a radiant heat source before, and after, treatment. Each agent produced substantial sensitisation in wild-type animals but not in the knockout mice, demonstrating that TRPV1 is essential for the development of bradykinin-induced or NGF-induced thermal hypersensitivity *in vivo*. Increased activation of TRPVI channels expressed with BK2 receptors in HEK293 cells also results from bradykinin, while NGF produces increased responses in proton-evoked currents in oocytes expressing both TRPV1 and the NGF receptor, TrkA. Both bradykinin and NGF, therefore, mediate their effect by activation of TRPV1 and their own receptors (Chuang et al., 2001).

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2.1.4.3. Spontaneous pain sensations

That TRPV1 functions as a mediator of spontaneous pain sensations and is an important mediator of pain sensations resulting from inflammation is indicated by the fact that this ion channel is synergistically responsive to two major components of the inflammatory response, namely, local reductions in pH and local increases in temperature. The pH threshold for proton-evoked TRPV1 activation is ~6.5. At lower pH values, protons can themselves activate TRPV1. Lowering pH lowers the heat threshold for activation of the channel, and stabilises at least one of the open conformations of the channel (Ryu et al., 2003). Sensitivity to noxious heat is the most distinctive feature of TRPV1. Temperature affects the activation of TRPV1,

first, by reducing the threshold for the activation of TRPV1 by all other TRPV1 activators. Second, heat above ~43ºC independently activates TRPV1 (Tominaga et al., 1998). Below 43ºC, gating of TRPV1 occurs only infrequently and briefly. However, raising the ambient temperature rapidly increases the frequency of channel openings (Liu et al., 2003). The presence of either reducing, or oxidising, agents also appears to result in an increased response to heat by TRPV1 channels (Susankova et al., 2006). The point to emphasise is that, if the temperature activation threshold for TRPV1 is reduced to body temperature, i.e., 37 degrees C, whether by reduced pH, or otherwise, the result is spontaneous activation of TRPV1, leading to spontaneous pain sensations as a result of normal body temperature alone. Bradykinin has, importantly, also been identified as being capable of reducing the heat threshold for activation of TRPV1 well below body temperature (Sugiura et al., 2002; Liang et al., 2001). Moreover, it is well recognised that local body temperature is increased in inflammation. Hence, an effect of other inflammatory agents which reduces the temperature activation threshold to 37 degrees C may be unnecessary to result in the development and maintenance of spontaneous pain sensations. The central role of temperature in the activation of TRPV1 is demonstrated by the evidence that cooling inhibits capsaicin-induced currents in primary sensory neurons (Babes et al., 2002). This explains the efficacy of exposing burned, or itching, skin surfaces to cold water which causes TRPV1 activity to abate, reducing the noxious pain or itch sensations.

2.1.4.4. Animal models of inflammatory pain

Three principal animal models of inflammatory pain have gained currency in biomedical research, each involving intraplantar injection of an animal's hind-paw with either CFA, carrageenan, or formalin. TRPV1 activation is an essential feature

of the inflammation generated in both the CFA and carrigeenan models. Intraplantar injection of CFA in rat, results in thermal hyperalgesia extending, at least, over a four week period, and mechanical allodynia extending over a two week period, with peaks in each case at day 7. TRPV1 expression is increased in medium-sized myelinated A fibre (NF200 positive) neurons and in small non-peptidergic (IB4 positive) neurons from day 1 to day 14, and in small peptidergic (CGRP positive) neurons from day 1 to day 28. Moreover, intrathecal administration, at day 7, of a selective TRPV1 antagonist, significantly reduces thermal hyperalgeisa and mechanical allodynia (Yu et al., 2009). In carrageenan-induced hind-paw inflammation, thermal and mechanical hyperalgesia result from carrageenan hind-paw injection. TRPV1 null mice are unable to develop carrageenan-induced thermal hyperalgesia, demonstrating that TRPV1 activation is essential for the development of this form of inflammatory hyperalgesia (Davis et al., 2000). Hydrogen peroxide levels are also significantly raised in the carrageen-injected animals. At the same time, superoxide dismutase and catalase significantly reduce the mechanical and thermal hyperalgesia (Keeble et al., 2009). The third major model of inflammatory pain is that which is induced by formalin injection of the hind-paw, but this is exclusively mediated by TRPA1 (McNamara et al., 2007). Xylene-induced acute nocifensive behaviour in mice is mediated by activation of TRPV1 (Sandor et al., 2009). TRPV1 channels also appear to be involved in the development of acute mechanical hyperalgeisa during repetitive noxious colorectal distension in rats (Ravnefjord et al., 2009).

2.1.5. TRPAI contributes with TRPV1 in mediating inflammatory pain, including mechanical hyperalgesia and cold hyperalgesia

2.1.5.1. TRPA1 as an ionotropic receptor

TRPA1 is a noxious cold channel which is activated by cold at approximately 17 degrees C (Story et al., 2003; Bandell et al., 2004; Obata et al., 2005). Notwithstanding this, however, mice which lack the TRPA1 receptor display normal cold sensitivity (Bautista et al., 2006), suggesting that the association between TRPA1 activation and noxious cold is qualified. TRPA1 is a non-selective ligand-gated cation channel which is directly gated by Ca^{2+} (Doerner et al., 2007). This ion channel is found in a subset of nociceptive primary afferents of dorsal root and trigeminal ganglia that co-express TRPV1 (Story et al., 2003; Jordt et al., 2004; Kobayashi et al., 2005). TRPA1 is present, with TRPV1, in primary afferents innervating the masseter muscle in rats, and both of these ion channels are capable of functionally contributing to muscle nociception and hyperalgesia (Ro et al., 2009). The converse, however, is not true; and not all primary afferents which express TRPV1 also express TRPA1 (Kobayashi et al., 2005). Most compounds known to activate TRPA1 are capable of covalently binding cysteine residues. The covalent modification of reactive cysteines within TRPA1 can cause channel activation, rapidly signalling potential tissue damage through the pain pathway (Mcpherson et al., 2007).

2.1.5.2. TRPA1 and inflammatory pain

Mice which lack TRPA1 receptors have sensory neurons which are insensitive to formalin. Moreover, pharmacological blockade or genetic ablation of TRPA1 in mice produces marked attenuation of the characteristic flinching, licking, and lifting responses resulting from intraplantar injection of formalin (McNamara et al., 2007). . TRPA1 is activated by certain at least of the chemical agents generated by the inflammatory response. Hence, TRPA1 is responsible for the pain, inflammation and robust hypersensitivity to thermal and mechanical stimuli that results from the topical application of mustard oil (allyl isothiocyanate) to the skin (Jordt et al., 2004). TRPA1is the exclusive receptor through which mustard oil and garlic activate primary afferent nociceptors to produce inflammatory pain (Bautista et al., 2006). TRPA1 is activated by formalin which induces a robust calcium influx in cells expressing TRPA1. TRPA1 is activated indirectly by bradykinin (Bandell et al., 2004). Mice which lack the TRPA1 receptor suffer from obvious deficits in bradykinin-evoked nociceptor excitation and pain hypersensitivity (Bautista et al., 2006). Sensitization of TRPA1 by bradykinin is proposed to be mediated by PLC and PKA (Wang et al, 2008). TRPA1 channels are activated by 4-hydroxy-2-nonenal (HNE), an endogenous alpha, beta-unsaturated aldehyde that is produced when reactive oxygen species peroxidate membrane phospholipids in response to tissue injury, inflammation, and oxidative stress. Many agents produced during episodes of oxidative stress can activate TRPA1 expressed in sensory neurons (Andersson et al., 2008). Again, a PGD2 metabolite appears to be capable of directly activating TRPA1 (Taylor-Clark et al., 2007). 15d-PGJ2 induces acute nocicpetive responses when administered cuntaneously to mice by activating TRPA1 (Cruz-Orengo et al., 2008).

A functional interaction of PAR2 and TRPA1 in dorsal root ganglion neurons may contribute to the sensation of inflammatory pain. Frequent co-localisation of TRPA1 with PAR2 is found in rat dorsal root ganglion neurons. PAR2 my contribute to sensitising TRPA1 since activation of PAR2 increases the TRPA1 currents evoked by TRPA1 agonists in HEK293 cells transfected with TRPA1 and in dorsal root ganglion neurons (Dai et al., 2007).

TRPA1 appears to be important in airway inflammation and hyper-reactivity in asthma. Genetic ablation of this ion channel in mice inhibits allergen-induced leukocyte infiltration in the airways, reduces cytokine and mucus production, and almost completely abolishes airway hyper-reactivity to contractile stimuli (Caceres et al., 2009).

2.1.5.3. TRPA1 and mechanical hyperalgesia

Mechanical hyperalgesia resulting from inflammation is reduced when TRPA1 function is inhibited. Administration of a selective TRPA1 antagonist inhibits all formalin responses in rat C fibres and substantially diminishes mechanically-evoked action potential firing in C fibres. Importantly, it fails to affect TRPV1 function, as assessed by capsaicin responsiveness. Hence, the presence of TRPA1 in cutaneous sensory neurons is essential to identify at least certain noxious chemical stimuli and to respond to certain forms of mechanical stimuli (Kerstein et al., 2009). Sensitising TRPA1, *via* a PAR2-dependent mechanism, is important in mast cell activation-induced mechanical hyper-sensitivity of vagal nodose C-fibres in the guinea pig oesophagus (Yu et al., 2009). Experimentally induced colitis results in a significant increase in visceral sensitivity to colonic distension and chemical irritation

accompanied by up-regulation of TRPA1 in colonic afferent dorsal root ganglia. The effects are suppressed by ablation of the TRPA1 gene (Yang et al., 2008). Intrathecal and intraperitoneal injection of a TRPA1 inhibitor suppresses the response to noxious gastric distension (Kondon et al., 2009).

1.5.4 TRPA1 and cold hyperalgesia

Enhanced sensitivity to cold so that cold elicits an inappropriate sensation of pain -- described as "cold hyperalgesia" -- is a common symptom of both inflammatory pain and neuropathic pain conditions. Pharmacological blockade of TRPA1 in rat primary sensory neurons decreases the induction of TRPA1, while reversal of cold hyperalgesia caused by inflammation or nerve injury results from pharmacological blockade of TRPA1 (Obata et al., 2005). TRPA1 appears to make a major contribution to CFA-induced cold hyperalgesia in rats (Stucky et al., 2009).

2.1.6. TRPV4 mediates mechanical hyperalgesia in inflammation

2.1.6.1. TRPV4 as an ionotropic receptor

TRPV4 was previously known as vanilloid receptor-related osmotically activated channel (VR-OAC). TRPV4 is a non-selective, ligand-gated, cation channel that functions in the transduction of osmotic and mechanical stimuli. This receptor is necessary for the maintenance of systemic osmotic equilibrium, and for normal thresholds in response to noxious mechanical stimuli (Liedtke et al., 2005). In mice which lack the TRPV4 gene, the animals exhibit a marked reduction in the sensitivity of the tail to pressure and acidic nociception. Low pH, citrate, and inflation gate TRPV4 when expressed in vitro in Chinese hamster ovary cells; but neither heat nor capsaicin gate this ion channel in this model (Suzuki et al., 2003a). However, heat

above 25 degrees C activates currents and increases intracellular calcium concentration in HEK293 cells transfected with TRPV4 and in endogenous TRPV4 in mouse aorta endothelium (Watanabe et al., 2002). Anandamide and arachidonic acid employ epoxyeicosatrienoic acids to activate TRPV4 (Watanabe et al., 2003). Cell swelling utilises phospholipase A2-dependent formation of arachidonic acid, and its subsequent metabolism to 5',6'-epoxyeicosatrienoic acid, to activate TRPV4. Phorbol esters and heat activate TRPV4 by means of a different pathway which depends on an aromatic residue at the N terminus of the third transmembrane domain (Vriens et al., 2004). Src family tyrosine kinases mediate tyrosine phosphorylation of TRPV4 in different cell lines, with phosphorylation sites in the cytosolic N- and C-terminal tails of this ion channel. The N-terminal tyrosine residue is crucial in the activation of TRPV4 by heat, mechanical (shear) stress, hypotonic cell swelling, and phorbol 12-myristate 13-acetate. TRPV4 appears to be sensitised, rather than activated, by tyrosine phosphorylation. Moreover, reactive oxygen species strongly up-regulate TRPV4 in the presence of Src family tyrosine kinases (Wegierski et al., 2009).

TRPV4 is found in peripheral sensory neurons and, in addition, in the central nervous system. Hippocampal neurons express functional TRPV4 (Shibasaki, 2007). Both TRPV1 and TRPV4 are co-expressed in DRG neuronal cell bodies and in the central terminals of laminae I and II of the spinal dorsal horn. TRPV4 can be sensitised by PKC. TRPV1-mediated, and TRPV4-mediated, responses are observed in a population of the same DRG neurons (Cao et al., 2009). Both small (low threshold) and large (high threshold) dorsal root ganglia neurons express TRPV4 in mice. TRPV4 is found in free nerve endings, and is also specifically located at

cutaneous mechanosensory terminals co-localised with neurofilament 200, including Meissner, Merkel, penicillate and intraepidermal terminals (Suzuki et al., 2003b).

2.1.6.2. TRPV4 mediates hypotonic and hypertonic nociception

In vivo single-fibre recordings in rat show that hypotonic solution activates 54% of C-fibers, an effect enhanced by prostaglandin E2. This osmotransduction causes nociception; and the channel is required for hypotonic stimulus-induced nociception (Alessandri-Haber et al., 2003). TRPV4 also mediates pain resulting from hypertonicity in rat and the aggravation of that pain which results from the addition of an inflammatory mediator (Alessandri-Haber et al., 2005).

2.1.6.3. Inflammation-induced mechanical hyperalgesia

Mechanical hyperalgesia occasioned by agents produced in the inflammatory process is mediated by TRPV4. Thus, the nocifensive paw-withdrawal reflex elicited by hypotonic, or mechanical, stimuli in rat is enhanced by intradermal injection of either carrageenan, or of a soup of inflammatory mediators. When TRPV4 is knocked down by spinal administration of TRPV4 antisense oligodeoxynucleotide, the enhancement is blocked without altering baseline nociceptive threshold. Again, inflammatory soup fails to induce any significant mechanical or osmotic hyperalgesia in mice which lack the TRPV4 receptor (Alessandri-Haber et al., 2006). Moreover, on injection *in vivo* with PGE2 and serotonin of the mechanical receptive fields of C-fibres in wild-type and TRPV4 knock-out mice, the percentage of C-fibres responding to a hypotonic stimulus and the magnitude of the response is significantly greater in wild-type mice when compared to TRPV4 knock-out mice. TRPV4 is shown to be crucial in mediating mechanical hyperalgesia because only C-fibres from wild-type

mice, which possess the TRPV4 receptor, exhibit increased spontaneous activity and decreased mechanical threshold in response to PGE2 and serotonin (Chen et al., 2007). Mechanical hyperalgesia is reduced in TRPV4-deficient mice in various models of painful peripheral neuropathy which exhibit mechanical hyperalgesia (Allesandri-Haber et al., 2008). TRPV4 contributes to mechanically-evoked visceral pain (Brierley et al., 2008); and is necessary for PAR2-induced mechanical hyperalgesia and excitation of colonic afferent neurons in mouse (Sipe et al., 2008). TRPV4 appears to play a pivotal role in visceral nociception and hypersensitivity (Cenac et al., 2008).

FIGURE 1.

Table of Characteristics of TRPV1, TRPA1, TRPV4 and ASICs as Ionotropic Receptors

Principal Features

- ligand-gated ion channels
- have central aqueous pore
- channel-gated on ligand-binding
- gating results in ionic flux via pore
- for fast synaptic transmission
- Examples: TRPs, ASICs, $5HT_3$

Possible Inhibiting Mechanisms

- competitive antagonism
- conduction blocker of channel pore
- binding in channel pore to induce failure of "gating"

FIGURE 2.

Table of Known Activators of TRPV1, TRPA1, TRPV4 and ASICs in the Absence of Inflammation

Activator	TRPV1	TRPA1	TRPV4	ASICs
Heat	**Y**	**N**	**N**	**-**
Cold	**N**	**Y**	**-**	**-**
Depolarisation	**Y**	**-**	**-**	**-**
Hypotonicity	**N**	**N**	**Y**	**N**
Hypertonicity	**N**	**N**	**Y**	**N**
Capsaicin (& other vanilloids)	**Y**	**N**	**N**	**N**
Mustard oil	**N**	**Y**	**-**	**-**
Garlic	**N**	**Y**	**-**	**-**
Protons & Cations	**Y**	**-**	**Y**	**Y**
	(pH <6.5)			

Key: Y= yes; N= no; - = not known.

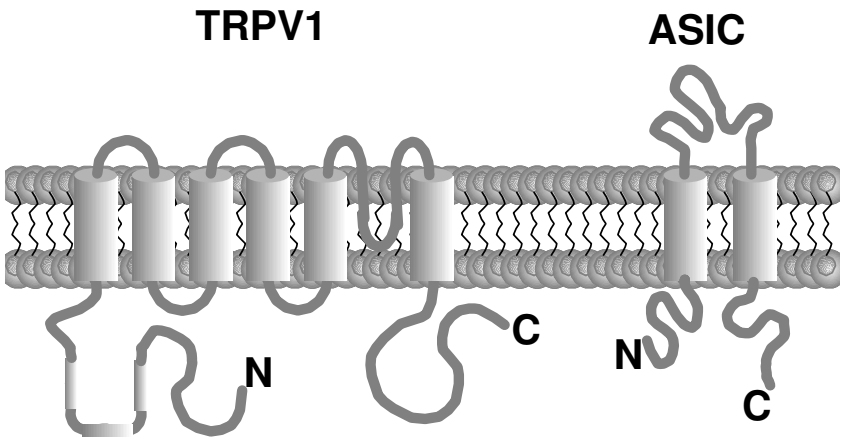
FIGURE 3.

Table of Inflammagens as Activators of Iontropic and Their Cognate (Own) Receptors in Peripheral Inflammation

Inflammatory Agent	TRPV1 (direct or indirect activation)	TRPA1	TRPV4	ASICs	Cognate (Own) Receptors (activation by ligand-binding)
Bradykinin	Y	Y	-	Y	B_{1-2}
PGE2	Y	-	Y	-	EP_{1-4}
PGI2	Y	-	-	-	IP
NGF	Y	-	-	Y	trkA
Artemin, neurturin, GDNF	Y	-	-	-	$GFRalpha_{1-4}$
Histamine	Y	-	-	--	H_{1-4}
Anandamide	Y	-	-	-	$CB_{1-2,}$ GPR55
Protons & Cations (pH <6.5)	Y	-	Y	Y	ASICs
LTB4	-	-	-	-	BLT_{1-2}
5-HT	-	-	-	Y	$5\text{-}HT_{1A\ \&\ 2A,}5\text{-}HT_3$
ATP	Y	-	-	-	$P2X_2$, $P2X_3$, $P2X_{2/3}$

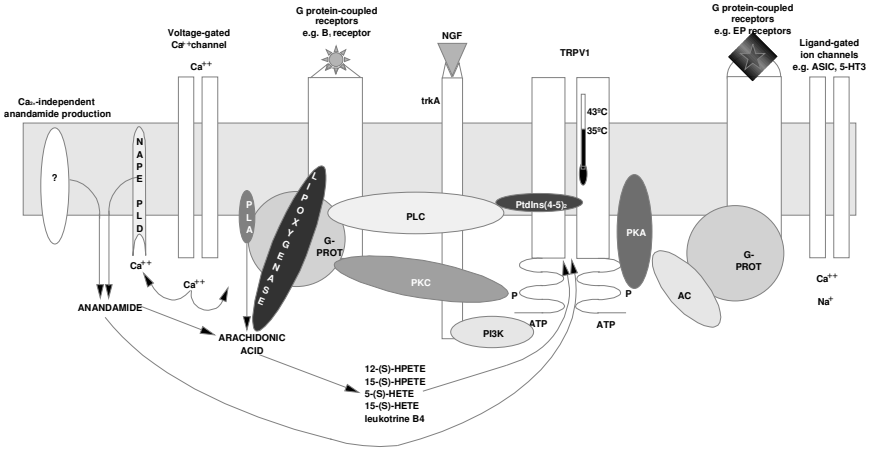
Key: Y = yes; - = not known.

FIGURE 4.



Predicted membrane structure of TRPV1 and ASIC ion channels. Like other members of the TRP family, TRPV1 has six transmembrane domains. Both the N- and C-termini are intracellular. The hydrophobic loop connecting transmembrane domains five and six is believed to be part of the channel. In contrast, ASIC ion channels have only two transmembrane domains. Both the N- and C-termini are again intracellular.

FIGURE 5.



Molecular mechanisms involved in the functioning of TRPV1 in inflammatory conditions. Reduction of the heat threshold of TRPV1 below body temperature may be induced by binding of endovanilloids, protons, anandamide, or lipoxygenase products. Post-translational modifications (removal of phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P2) and protein kinase C (PKC)-mediated phosphorylation) can also produce this effect. Protein kinase A (PKA)-mediated phosphorylation also sensitizes TRPV1. Phosphatidylinositol 3-kinase (PI3K) activity induced by nerve growth factor (NGF) and ATP-binding also potentiates responses evoked by endovanilloids. The molecular mechanisms involved in the functioning of TRPA1 and TRPV4 remain to be elucidated.

NAPE-PLD: N-acylphosphotidylethanolamine phospholipase D involved in anandamide production in primary sensory neurons. PLA: phospholipase A. PLC: phospholipase C. G prot: G protein. AC: adenylyl cyclase. Question mark refers to an unidentified enzyme producing anandamide in a Ca^{2+}-independent manner.

2.2. BRADYKININ AND OTHER KININS

2.2.1. Introduction

Bradykinin figures prominently in the context of inflammation and inflammatory pain, but it also serves important cardioprotective and other functions (Bhoola et al., 1992; Marceau et al., 1999; Vidal et al., 2005; Leeb-Lundberg et al., 2005; Araujo et al., 2006; Cayla et al., 2007; Wu and Pan, 2007). Bradykinin is one of a number of related peptides, derived from an action of the kallikrein enzyme, which are comprised in the so-called "kallikrein-kinin system" (Bhoola et al., 1992; Leeb-Lundberg et al., 2005; Marceau et al., 2005; Schmaier, 2008). Paradoxically, although the effect of bradykinin, when released in the periphery as part of the inflammatory response, is to induce the experience of pain sensations, yet, when bradykinin is released in certain areas of the brain, it can exert a powerful analgesic effect (Couto et al., 1998, 2006). Several reviews have recently addressed the role of bradykinin as a mediator of inflammatory pain sensations (Wang et al., 2006; Sharma and Al-Sherif, 2006; Moreau et al., 2005; Calixto et al., 2004).

2.2.2. Kalllikrein-kinin system

Bradykinin is a nonapeptide which is produced at sites of tissue injury. The proteolytic enzyme kallikrein acts on high-molecular-weight kininogen, a plasma alpha-globulin, to form either bradykinin or kallidin. Kallidin (which is also known as lysyl bradykinin (lys-BK) is a peptide which is almost identical with bradykinin, but comprises an additional amino acid residue. The kallikrein enzyme is derived from prekallikrein, which is an inactive precursor present in plasma. Prekallikrein can be converted to kallikrein in several ways, including by reaction with Hageman factor

(which is factor XII of the clotting sequence). Hageman factor is normally in an inactive form in the plasma and is activated by contact with surfaces having a negative charge, including collagen, basement membrane, and bacterial lipopolysaccharides. The increased vascular permeability which accompanies inflammation results in leakage of plasma, containing prekallikrein, Hageman factor, and kininogen, which leads to bradykinin formation. Kallikrein enzymes are also found in the pancreas, salivary glands, colon, and skin. In these tissues, they act on kinninogens to generate, for the most part, kallidin -- an action which is required for normal physiological function. (Bhoola et al., 1992; Leeb-Lundberg et al., 2005; Schmaier, 2008). Prekallikrein is also capable of digesting high-molecular-weight kininogen to release bradykinin, but this effect is inhibited by the presence of C1 inhibitor (Joseph et al, 2009). The "kallikrein-kinin system is exceptionally complex and has been well described as constituting an "endogenous metabolic cascade" whose complexity is derived from the importance of the several critical functions which it subserves (Moreau et al., 2005).

2.2.3. B1 and B2 kinin receptors

Bradykin receptors are denominated B1 and B2 kinin receptors, respectively (Moreau et al., 1998). Bradykinin mediates its effects through these receptors and also, as has been observed, through the actions of certain ionotropic receptors (*supra*). The relevance of B1 and B2 receptors in the context of inflammatory pain resides in their ability, on exposure to agonist generated by the inflammatory process, to set in motion an intracellular chain of events which results in excitation of the primary sensory neuron, or secondary (spinal cord) afferent, on which they are expressed. Both the extent of kinin receptor expression and the extent of the availability of

agonist are key factors in determining whether a nociceptive signal is generated and the strength of that signal.

Bradykinin receptors are found in primary sensory neurons, as well as in the spinal cord, and also in myocardial/coronary visceral afferent fibres in guinea pig (Steranka et al., 1998). The B1 and B2 kinin receptors are both G-protein coupled receptors. B2 receptors are found throughout the body and are constitutively expressed**.** B1 receptors are not expressed in most normal tissues, although there is a basal level of expression of these receptors in the spinal cord dorsal horn (Wotherspoon and Winter, 2000; Ma and Heavens, 2001). Generally, B1 receptors are induced within several hours under conditions of inflammation and tissue damage in the presence of pro-inflammatory cytokines, especially interleukin-1beta (Marceau et al., 1998; Phagoo et al., 1999; Phagoo et al., 2001; Prado et al., 2002). Interleukin-1beta is important in the events which result in the increased expression of B1 receptors. Bradykinin, acting at the B2 receptor, induces the synthesis of IL-1beta (Pan et al., 1996). In turn, this cytokine increases B1 receptor expression (Zhou et al., 1998). Up-regulation of the B1 receptor by IL-1beta and by agonists at the B1 receptor occurs through independent and synergistic intracellular signalling mechanisms in human lung fibroblasts (Phagoo et al., 2001). Thus, B2 receptors predominate in the acute phase of inflammatory pain while B1 receptors predominate in the chronic phase. The response of B2 receptors to bradykinin may vary with the cell upon which the receptors are expressed. Thus, in the lung fibroblast cell line IMR-90, binding of bradykinin to B2 receptors triggers down-regulation of receptor surface expression. However, the same treatment with bradykinin of cells from the human lung epithelial cell-line BEAS2B leads to up-regulation of B2 receptors in BEAS2B cells (Bengston

et al., 2008). HEK293 cells are widely employed in biological research as cells which are devoid of functional receptors and which, therefore, may be employed to explore in isolation the activation properties of receptors which are introduced into these naieve cells by transfection. However, these cells have now been shown to express endogenous functional B2 receptors. Exposure of HEK293 cells to bradykinin results in a concentration-dependent rise in intracellular Ca^{2+}, increases in ECAR and a rapid increase in ERK1/2 phosphorylation – all of which are blocked by a B2 receptor antagonist (Kramarenko et al., 2009).

B1 receptors and B2 receptors, when co-expressed by a cell, directly interact to enhance signalling (Kang et al., 2004). B2 receptors are stable plasma membrane components without agonist but in the presence of agonist, B2 receptors recruit beta-arrestin 2, internalise in a clathrin-dependent manner, and recycle rapidly. On the other hand, B1 receptors, when induced, are constitutively active, and constitutively internalise without agonist *via* a clathrin-dependent pathway. Agonist transiently stabilises these receptors by delaying B1 receptor endocytosis (Enquist et al., 2007). NGF, and to a lesser extent GDNF, up-regulate the expression of B2 receptor mRNA and B2 receptor protein in the surface membrane of mouse dorsal root ganglion neurones. NGF is also an important factor responsible for up-regulating B2 receptor expression after nerve crush injury *in vivo* (Lee et al., 2002). Functional B1 receptors are expressed in only a small percentage of freshly isolated dorsal root ganglion neurons, but their expression is up-regulated by exposure to the related neurotrophins GDNF and neurturin, while NGF has no effect in this regard. GDNF may be released from surrounding tissue following injury or inflammation and may act to up-regulate B1 receptor expression (Vellani et al., 2004). In rabbit aortic smooth muscle cells, interleukin-1beta and epidermal growth factor (EGF) each increase B1 kinin receptor expression at 4 hours (Kuombadinga et al., 2009). The metalloendopeptidase thimet

oligopeptidase (EC 3.4.24.15; EP24.15) serves as an intracellular peptidase in the degradation of bradykinin which has been specifically internalised via the B2 receptor, and also negatively regulates B2 responsiveness to bradykinin (Sanden et al., 2008).

Bradykinin (BK) and lysyl-bradykinin (Lys-BK) can each activate the B2 receptor. The ligands for the B_1 receptor are des-Arg^9-BK and Lys-des-Arg^9-BK, which are formed through the actions of carboxypeptidases on BK and Lys-BK, respectively. Both B1 and B2 receptors each act through $G\alpha_q$ to stimulate phospholipase Cβ followed by phosphoinositide hydrolysis and intracellular free Ca^{2+} mobilization and through $G\alpha_i$ to inhibit adenylate cyclase and stimulate mitogen-activated protein kinase pathways (Leeb-Lundberg, 2005). des-Arg9-bradykinin, des-Arg10-kallidin, and des-Arg10-kallidin-like-peptide are equipotent agonists at the B1 receptor. Bradykinin, kallidin and kallidin-like-peptide are equipotent agonists at the B2 receptor (Zubakova et al., 2008). There is also evidence from *in vitro* studies that kallikrein may activate B2 receptors in the absence of kininogen (Biyashev et al., 2006). A further important point is that B1 and B2 receptors are not exclusively activated by kinins. Thus, the endogenous opioid, dynorphin A, can activate bradykinin receptors. Activation of bradykinin receptors by dynorphin A induces calcium influx *via* voltage-sensitive calcium channels in sensory neurons (Lai et al., 2006; Lai et al., 2008). Chronic peripheral inflammation induces a significant up-regulation of the endogenous opioid dynorphin A, which, at pathological levels, may activate spinal bradykinin receptors to contribute to inflammatory hyperalgesia (Luo et al., 2008).

2.2.4. B1 kinin receptors evade desensitisation

The importance of kinins, and of the B1 receptor, in mediating long-term pain conditions is likely to be due in substantial measure to the B1 receptor's apparent immunity to desensitisation. In an important study, Proud and colleagues (1999) showed that the B2 receptor responds to agonist stimulation with a rapid loss of functional response, receptor sequestration, and ligand dissociation. On long term stimulation, this receptor becomes down-regulated. However, once B1 receptor expression is induced under pathological conditions, agonist stimulation of this receptor results in persistent signalling due to the absence of ligand dissociation and the failure of desensitisation and receptor sequestration to occur. Moreover, on long term stimulation, the B1 receptor actually exhibits increased expression (Faussner et al., 1999). Marceau and co-workers (2002) have described how inducible B1 receptors, when stimulated by des-Arg9-kinins, are neither phosphorylated nor internalised upon agonist stimulation but, instead, undergo a reversible re-distribution to caveolae-related rafts (Marceau et al., 2002). Calixto and colleagues (2004) observe that B1 receptors are apparently not subject to desensitisation and that ligand-binding at these receptors results in sustained elevation of Ca^{2+} concentration (Calixto et al., 2004).

3.5. Bradykinin involvement in mediating inflammatory pain sensation

Consistent with their role as inflammogens, kinins provoke the standard features of the inflammatory response. Thus, local vasodilation, exudation, and pain result from injection of BK into tissues (Marceau et al., 1999). Increases in the interstitial levels of bradykinin and kallidin in the trapezius muscle in patients with chronic work-related trapezius myalgia and in patients with whiplash associated pain, when

compared to healthy controls, suggest that these algesic kinins are involved in these forms of muscle pain (Gerdle et al., 2008). Treatment with B1 receptor agonist at a dose which has no effect on withdrawal latency to a noxious heat stimulus in the case of a naïve rat, may result in 50 per reduction of withdrawal latency following exposure of one hind-paw of the rat to strong ultraviolet radiation (Perkins and Kelly, 1993). Following an inflammatory insult, both B1 and B2 receptors are involved in mediating the resulting mechanical hyperalgesia (Davis and Perkins, 1994). Both B1 and B2 receptors are involved in mediating formalin-induced persistent pain in mouse (Correa and Calixto, 1993); and both these receptors are also involved in mediating bradykinin-induced rat paw oedema (Campos and Calixto, 1995). Mice lacking the B1 receptor are healthy, fertile and normotensive. However, under normal non-inflamed conditions, they are analgesic in behavioural tests of chemical and thermal nociception. Moreover, B1 receptor null nice exhibit a reduction in activity-dependent facilitation (wind-up) of nociceptive spinal reflexes (Pesquero et al., 2000). Substance P and capsaicin induce a mechanical hyperalgesia when injected into the rat knee joint. The development of both substance P-induced, and capsaicin-induced, hyperalgesia is blocked by injection of a B2 receptor antagonist. The same antagonist also reverses an established hyperalgesia similarly induced. B1 receptor antagonists (at least in the experimental doses employed) only block the development of substance P-induced hyperalgesia for a short interval after administration and fail to reverse an established substance P-induced hyperalgesia. However, the same B1 receptor antagonist blocks capsaicin-induced hyperalgesia and also reverses an established capsaicin-induced hyperalgesia (Davis and Perkins, 1996).

Kinins act at both B1 and B2 receptors at the spinal level to contribute to nociceptive processing and the development of inflammatory hyperalgesia. The intrathecal administration of B2 receptor agonists produces dose-related thermal hyperalgesia, demonstrated in the hot-plate test, which is abolished by the administration of a B2 receptor antagonist. The intrathecal injection of B1 receptor agonists also causes dose-related thermal hyperalgesia. However, this differs from that induced by B2 agonists in that B1 receptor agonists cause a weak, but prolonged, hyperalgesia. This effect is blocked by a B1 receptor antagonist (Ferreira et al., 2002). In the model of inflammatory hyperalgesia constituted by injection of CFA into one hind-paw of rat, there is a significant increase in B1 protein expression in both ipsilateral and contralateral dorsal root ganglion neurons measured 24 hours after injection, whereas axotomy results in reduction of B1 protein in the ipsilateral doral root ganglia. Intrathecal, or systemic, administration of the B1 agonist, desArg9BK, in the same model of inflammation, causes a marked mechanical hyperalgesia of the contralateral, un-inflamed, hind-paw (Fox et al., 2003). Calixto and colleagues (2001) demonstrated that the B1 receptor, but not the B2 receptor, is important in regulating the persistent inflammatory hyperalgesia induced by CFA in mouse. The role of the B2 receptor appears to be confined to a minor function in the amplification of the earlier stage of CFA-induced paw oedema formation. Thus, intradermal injection of CFA in mouse produces time-dependent and marked hyperalgesic responses in both ipsilateral and contralateral paws of wild-type mice. Ablation of the gene for the B1 receptor reduces the hyperalagesia in both ipsilateral and contralateral paws at 12 hours after injection. However, the absence of the B2 receptor has no such effect. Intradermal injection of CFA produces a time-related and long-lasting (up to 72 hours) increase in paw volume in wild-type mice. Loss of the B1 receptor does not

affect this swelling. However, the B2 receptor null mice exhibit a longer earlier stage of oedema development in the paw (Ferreira et al., 2001). Bradykinin, acting at the B1 receptor, mediates phorbol myristate acetate (PMA)-induced nociceptive behaviour in mouse as evidenced by the fact that injection of PMA into the mouse paw results in pain behaviour which is abolished by a selective B1 receptor antagonist or by B1 receptor gene deletion (Ferreira et al., 2008).

B2 kinin receptors, but not B1 receptors, are involved in the normal physiological functioning of the of the mouse colon (Hara et al., 2007). Studies of the B1 and B2 receptor activation in a mouse model of colitis show that both receptors are up-regulated in the context of inflammatory disease of the colon (Hara et al., 2007; Hara et al., 2008). During inflammatory states of inflammatory bowel disease, the endotoxin level is increased. Lipopolysaacharide promotes IL-1beta secretion from enteric glial cells, resulting in augmentation of the neural response to bradykinin through PGE(2) release via glial PLA(2) and COX-2 (Murakami et al., 2009).

Nociceptive inputs result in the production of bradykinin in the spinal cord, thereby providing local activators for spinal B2 receptors (Wang et al., 2005). Woolf and colleagues (2008) have found that B2 receptors are co-expressed in dorsal horn neurons with PKA and the delta isoform of PKC. Augmentation by bradykinin of AMPA and NMDA receptor-mediated currents in lamina II neurons requires co-activation of both PKC and PKA. The activation of PKA is downstream of COX1 (cyclooxygenase-1), while ERK1/2 activation is involved after the PKC and PKA co-activation. Moreover, while intrathecal administration of bradykinin induces thermal

hyperalgesia in rat *in vivo*, inhibition of ERK, PKA and PKC diminishes such hyperalgesia (Kohno et al., 2008). Aspirin is a widely used analgesic, anti-thrombotic, and anti-inflammatory drug which alters B2 receptor ligand binding properties and appears to function as an allosteric inhibitor of the B2 receptor. Aspirin reduces the apparent affinity of the receptor for [3H]-bradykinin by accelerating the dissociation rate of [3H]-bradykinin-receptor complexes. Aspirin also diminishes the ability of unlabelled bradykinin, or the B2 receptor antagonist, icatibant, to destabilise pre-formed [3H]-bradykinin-receptor complexes (Gardes et al., 2008).

Metabotropic glutamate receptors, mGluRs, more specifically of subtype-5, are involved in the nociceptive response induced by intrathecal injection of excitatory aminoacids, substance P, bradykinin, and cytokinesSP, BK and cytokines in mice (Jesse et al., 2008). Studies in human retinal pigment epithelial (ARPE) cells show that bradykinin stimulates glutamate uptake as well as the mRNA expression of excitatory amino acid transporter 4 (EAAT4) and excitatory amino acid carrier 1 (EAAC1), which is blocked by treatment with B1 and B2 receptor siRNA, suggesting a role of B1 and B2 receptors. The application of various additional antagonists shows that glutamate uptake is stimulated by bradykinin through a PKC-Akt-COX-2 signalling cascade in ARPE cells (Lim et al., 2008).

2.2.6. Bradykinin involvement in mediating neuropathic pain sensation

B1 receptor activation appears to be an important component in the development of neuropathic pain. Thus, partial sciatic nerve ligation induces an up-regulation of B1 receptor mRNA in the ipsilateral paw, sciatic nerve, and spinal cord of wild-type mice. While the paw-withdrawal responses to thermal, or mechanical, stimulation do

not differ between B1 receptor-null mice and wild-type mice in the absence of neuropathy, yet partial ligation of the sciatic nerve in the wild-type mouse produces a profound and long-lasting decrease in thermal and mechanical thresholds in the paw ipsilateral to the nerve lesion (Ferreira et al., 2005). B1 and B2 receptor-mediated mechanisms have been found to contribute significantly to the maintenance of hind-paw cold and mechanical allodynia and thermal hyperalgesia induced by L5/L6 spinal nerve ligation in rats (Werner et al., 2007). Treatment with a selective B1 receptor antagonist generally does not affect basal nociception in normal rats, whereas it induces a significant time-dependent and dose-dependent attenuation of hyperalgesia, or even restores nociceptive responses, in experimental diabetic neuropathy models. Again, diabetic hyperalgesia is absent in streptozotocin-induced type 1 diabetic knockout mice which lack the B1 receptor (Gabra et al., 2005a, 2005b, 2006). B1 and TRPV-1 receptor genes are over-expressed in the injured spinal cord of animals manifesting thermal hyperalgesia following experimental spinal cord injury compared with similarly injured animals without hyperalgesia (DomBourian et al., 2006). Thermal heat hyperalgesia resulting from experimental spinal cord injury is reduced by the administration of either a TRPV1 ion channel antagonist or a B1 receptor antagonist (Rajpal et al., 2007). The B1 receptor has also been shown to be important in mediating neuropathic pain-like behaviour in a mouse-model of brachial plexus avulsion (Quintao et al., 2008).

NGF, and to a lesser extent GDNF, up-regulates the expression of B2 receptors in mouse DRG neurones *in vitro*; and NGF is an important factor in up-regulating B2 receptor expression after nerve crush injury in mouse *in vivo* (Lee et al., 2002).

2.2.7. Mechanisms by which kinins mediate pain sensation

Bradykinin is a potent activator of primary afferents (Higashi et al., 1983; Oh and Weinreich, 2004). Bradykinin decreases K^+ and increases Ca^{2+}-activated Cl- conductances in guinea pig vagal afferent neurones (Oh et al., 2004). Bradykinin also inhibits the transient outward K^+ current in mouse Schwann cells by cAMP/PKA-dependent pathways *via* activation of B2 receptors (Zhang et al., 2009). Bradykinin and ATP facilitate the removal of action potential-induced Ca^{2+} loads by accelerating Ca^{2+} efflux from rat sensory neurons *via* protein kinase C and the plasma membrane Ca^{2+} pump isoform 4 (Usachev et al., 2002). In wild-type mice, intraplantar bradykinin injection produces immediate licking and flinching and a later decrease in mechanical threshold and hot-plate latency. However, in mice which lack the $Na_v1.9$ channel (which is present in TRPV1-expressing primary sensory neurons), the pain hypersensitivity elicited by bradykinin is reduced, while basal thermal and mechanical pain sensitivity remains unchanged (Amaya et al., 2006). Bradykinin also has important effects on TRPV1 and TRPA1 ion channels leading to nociceptive responses by primary sensory afferents. It seems unlikely, however, that bradykinin-evoked nociception is dependent only on its activation of TRPV1 and TRPA1, and it is likely that further ionic mechanisms are involved in mediating the excitatory effect of bradykinin on primary afferents (Katanosaka et al., 2008).

However, the intracellular messenger sequences initiated by B1 and B2 receptors remain to be comprehensively elucidated. Bradykinin stimulates the synthesis of inositol trisphosphate (IP3) and the breakdown of phosphatidylinositol bisphosphate, the synthesis of diacylglycerol, and the release of arachidonic acid from rat dorsal root ganglion neurones *in vitro*. Bradykinin also mobilises intracellular Ca2+ stores in rat

DRG cells from stores which are distributed equally between the cell soma and cell processes (Thayer et al., 1988). Bradykinin stimulates B2 receptors in rat dorsal root ganglion neurones to activate phospholipase C and occasion subsequent Ca^{2+} release which, in turn, triggers calcineurin-dependent NFAT (nuclear factor of activated T-cells) translocation to the nucleus and subsequent NFAT-dependent transcription. Activation of this cascade leads to increases in mRNA for cyclooxygenase-2 (Jackson et al., 2007).

Complex mechanism mediate the effect of bradykinin on the activation of TRPV1 ion channels. Bradykinin lowers the threshold temperature for heat activation of TRPV1 to below physiological body temperature (Sugiura et al., 2002). Bradykinin activates protein kinase C-epsilon (PKC-e) to enhance the activity of TRPV1 ion channels in primary sensory neurones. PKC-epsilon is translocated to the cell membrane by bradykinin. The TRPV1 ion channels become sensitised when constitutively active PKC-epsilon is incorporated into nociceptive neurons. Conversely, bradykinin-induced sensitisation is suppressed by a specific peptide inhibitor of PKC-epsilon (Cesare et al., 1999). In addition, B2 receptors are activated by bradykinin to, in turn, activate an intracellular second messenger pathway involving mobilisation of arachidonic acid by phospholipase A2, and generation of the 12-lypoxygenase product, 12-HPETE, which is capable of activating TRPV1. Bradykinin-induced thermal hyperalgesia is completely blocked by an inhibitor of 12-lipoxygenase (Shin et al., 2002). Again, bradykinin-mediated potentiation of thermal sensitivity *in vivo* requires expression of TRPV1 on primary sensory neurones. Diminution of plasma membrane phophatidylinositol-4,5-bisphosphate levels, through antibody-sequestration or PLC-mediated hydrolysis, mimics the potentiating effects

of bradykinin at the cellular level (Chuang et al., 2001). Moreover, bradykinin, through its B2 receptor, activates phospholipase C and cyclooxygenase-1. The B2 receptor facilitates non-selective Ca^{2+} channel activity, thereby increasing the intracellular Ca^{2+} concentration from the extracellular pool, leading to sensitisation of TRPV1 (Tang et al., 2004). In addition, a three hour treatment with bradykinin potentiates capsaicin-induced substance P release in cultured rat dorsal root ganglion neurones and also induces cyclooxygenase 2. This potentiation of substance P release by bradykinin can be reversed by administration of either a PKA inhibitor or a CB1 receptor agonist (Oshita et al., 2005). Bradykinin-evoked substance P release from cultured rat DRG neurones is attenuated by either a mitogen-activated protein kinase kinase (MEK) inhibitor or cycloheximide, which is an inhibitor of protein biosynthesis. However, while the long-term exposure of such neurones to bradykinin enhances substance P release induced by capsaicin, it attenuates substance P release induced by potassium (Tang et al., 2006).

The scaffolding protein AKAP79/150 is proposed to be essential in the formation of a signalling complex between several enzymes and TRPV1. The enzymes in question are the protein kinases, PKA and PKC, and the phosphatase, calcineurin. Modulation of the sensitivity of TRPV1 by PKA, PKC and calcineurin depends on the formation of this signalling complex between these enzymes, this scaffolding protein and TRPV1. A region in the C-terminal of TRPV1 is involved in the binding of this scaffolding protein. Failure of binding of this scaffolding protein, abolishes sensitization of TRPV1 by both bradykinin and PGE(2) (Zhang et al., 2008).

Bradykinin has recently been proposed to be also capable of potentiating the activation of the TRPA1 ion channel which is an important component of the transduction machinery through which environmental irritants and endogenous pro-algesic agents depolarise nociceptors to elicit inflammatory pain sensations (Wang et al., 2008). TRPA1 can, in fact, be activated by bradykinin. TRPA1 knockout mice show impaired responses to bradykinin and impairment of mechanical nociception. TRPA1 contributes to bradykin-induced visceral mechanical hypersensitivity (Yu and Ouyang, 2009).

2.2.8. Bradykinin in brain

An action of bradykinin on the activity of neurons in the brain may be relevant in when considering the effect in terms of pain sensations resulting from nociceptive inputs consequent on peripheral inflammation. This is because the electrical signal generated by the excitation of primary afferents by inflammatory mediators may be modulated by an effect of bradykinin in the brain. Thus, bradykinin, when introduced into certain brain areas, produces an anti-nociceptive effect. Thus, the microinjection of bradykinin into the lateral or fourth cerebral ventricles induces an antinociceptive effect, and to a lesser extent the third ventricle. The locus coeruleus, the principal nucleus, the oral part of the spinal sensorial trigeminal nucleus, and the sensory root of the trigeminal nerve, have been identified as brain sites where microinjection of bradykinin causes an anti-nociceptive effect. The antinociceptive effect is more intense when bradykinin is injected into the locus coeruleus (Couto et al., 1998, 2006). Thr^6-bradykinin (Thr^6-BK) is the active peptide in the venom of the social wasp, *Polybia occidendalis*. Injection of this peptide into the lateral ventricle of naive rats reduces noxious heat-evoked responses both in tail-flick and hot plate tests.

This Thr^6-BK-evoked anti-nociceptive effect is mediated through B2 receptors, and is superior to, or comparable with, the anti-nociceptive effects produced by intracerebroventricular injection of bradykinin, or morphine, respectively (Mortari et al., 2007). The powerful antinociceptive effect of Thr^6-BK, and, indeed, of bradykinin itself, when administered intraventricularly, appears to be profoundly paradoxical in the context of the established role of bradykinin in contributing to peripheral pain. The additional finding that Thr^6-BK and bradykinin may, on central administration, actually reduce noxious heat-evoked behavioural responses appears even more contradictory, because bradykinin contributes to the development of heat hyperalgesia in the periphery. However, the paradox is illusory. There is nothing in the kinin peptides which is specific to pain induction. On the contrary, everything depends on the localisation of their receptors and the functions subserved by the neruons which the bradykinin receptors serve to excite.

Bradykinin selectively transiently increases the permeability of the blood-brain barrier (BBB). This bradykinin-mediated increase in BBB permeability is due to accelerated formation of adenosine 5'-triphosphate-sensitive potassium channels (Zhang H et al., 2007). Bradykinin receptors are expressed in all major cell types in the brain, and migration of microglia to a site of injury in the brain is mediated by B1 receptors (Ifuku et al., 2007). Kinins mediate apparently contradictory effects in the brain. Administration of bradykinin into the right amygdala of adult male rats induces thermal hyperalgesia in the animals (Dalmolin et al., 2007). However, apart from this instance of kinins acting to increase nociception, in other cases they act in the brain to mediate an anti-inflammatory response, or even induce analgesia.

Bradykinin may exercise anti-inflammatory and neuroprotective effects in the central nervous system by modulating microglial function. In cultured mouse microglia, BK induces mobilisation of intracellular Ca^{2+}, microglial migration, and increases the release of NO and PGE2, in addition to attenuating lipopolysaacharide-activated TNF-alpha and Il-1beta release (Noda et al., 2006). The action of bradykinin in attenuating lipopolysaccharide-induced release of TNF-alpha and IL-1beta from microglial cells is anti-inflammatory in nature. However, bradykinin also enhances formation of PGE2 and expression of microsomal prostaglandin E synthase. Expression of bradykinin receptors and EP2/EP4 receptors are also enhanced (Noda et al., 2007). Kinins appear to influence prostaglandin synthesis in brain inflammation in an apparently regulated manner. Thus, bradykinin, a B2 receptor agonist, enhances both basal and lipopolysaccharide-induced PGE2 synthesis in rat neonatal glial cells in culture, whereas administration of a B1 receptor agonist attenuates both basal and lipopolysaacharide-induced production of PGE2 in glia (Levant et al., 2006).

2.3. EICOSANOIDS

2.3.1. Introduction

The term "ecosanoids" refers to the originating 20-carbon unsaturated fatty acid from which a family of lipid messengers are derived, “eikosi” meaning “twenty” in Greek. The principal such substrate is arachidonic acid. Hence, the eicosanoids are commonly referred to as “arachidonic acid-derived” lipid messengers. These bioactive lipid messengers are not constitutively expressed in tissues and must be generated *de novo* in response to appropriate stimuli. Free arachidonic acid is not available in cells but is found esterified in the cell’s membrane phospholipids. Many physiological and pathological stimuli, including general cell damage, can result in the release of arachidonic acid from membrane phospholipids either by an action of phospholipase A2 (PLA2) or an action of phospholipase C (PLC) followed by an action of diacylglycerol lipase. The biochemistry of eicosanoid production is complex, with several pathways being available for the synthesis of these mediators from arachidonic acid. The two pathways which have been most studied are those in which a cyclooxgenase enzyme, or a 5-lipoxygenase enzyme, initiates the process of synthesis. In both of these pathways, the initiating oxygenase catalyzes the synthesis of an unstable intermediate which is subsequently acted upon by other enzymes (Brock and Peters-Golden, 2007). Eicosanoids have important homeostatic functions in physiology which should not be overlooked (Harizi et al., 2008), especially in relation to neural function (Tassoni et al., 2008). Indeed, their association with inflammation and pain is essentially secondary to their physiological functions. The principal members of the eicosanoid family are: the prostanoids; the leukotrienes; and platelet activating factor.

2.3.2. Prostanoids: PGE2; PGI2; TxA2

The prostanoids include the prostaglandins (PGE2, PGF2alpha, PGD2, PGJ2), prostacyclin (PGI2), and thromboxane A2 (TxA2). Bioactive prostanoids derived from arachidonic acid are of two classes, designated "stable" and "labile," respectively. Stable prostanoids include the stable prostaglandins, such as PGE2, PGF2alpha, PGD2 and PGJ2. The labile prostanoids include the PG endoperoxides (PGG2, PGH2), TxA2, and prostacyclin (PGI2). TXA2 has a half-life of only 30 seconds at 37 degrees C. It produced mostly by blood platelets and possesses powerful vasoconstrictor, cytotoxic and thrombogenic properties (Gryglewski, 2008). However, unlike other prostanoids, it is not yet noted to be of importance in relation to inflammatory pain and will not receive further consideration here.

The prostanoids are a group of bioactive lipids which work as local mediators, exerting their actions on other cells near their cell of synthesis. Prostanoids are typically described as being the products of a "cyclooxygenase pathway," they are formed by the action of a cycloxygenase enzyme on arachidonic acid. The receptors which mediate the actions of prostanoids are G protein-coupled receptors with seven transmembrane domains. Eight types and subtypes of prostanoid receptors, which are encoded by different genes, have been identified. Together, these receptors constitute a sub-family in the super-family of the rhodopsin-type receptors (Narumiya et al., 1999).

There are two cyclooxygenase (COX) enzymes, namely, COX-1 and COX-2. Either of these enzymes can initiate the biosynthesis of both prostaglandins and

thromboxanes from free arachadonic acid within the cell. COX-1 is constitutively expressed in most tissues; and the prostanoids which it produces are most likely involved in normal homeostasis. Hence, the non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen, which inhibit both COX-1 and COX-2 have harmful side-effects with extensive use which are thought to be due to inhibition of COX-1. It was formerly assumed that COX-2 was an inducible enzyme only, with no house-keeping functions in normal human physiology and only producing harmful prostanoids in inflammatory conditions. Hence, drugs were developed which would target the COX-2 enzyme exclusively, thereby eliminating the effects of these harmful prostanoids believed to be emanating from the action of COX-2. Unfortunately, although specific inhibitors of COX-2 have been developed, it has transpired that COX-2 is also constitutively expressed in certain tissues, with the result that these inhibitors may affect the homeostatic functions served by these enzymes in the tissues where they are constitutively produced. In tissues where the COX-2 enzyme is not constitutively expressed, this enzyme is induced by an inflammatory stimulus to produce pathological prostanoids (Brock and Peters-Golden, 2007; Lin et al., 2006). Thus, COX-2 induction leads to a shift in arachidonic acid metabolism from the production of several prostanoids with diverse effects as mediated by COX-1 to the preferential synthesis of two prostanoids, prostacyclin and prostaglandin E2 (Brock et al., 1999). COX-1 and COX-2 are found both in the dorsal root ganglia and in the spinal cord (Inoue et al., 2006; Ebersberger et al., 1999; Beiche et al., 1998; Goppelt-Struebe and Beiche, 1992). Moreover, COX-2 is up-regulated in the dorsal root ganglia and in the spinal cord during peripheral inflammation (Goppelt-Struebe and Beiche, 1997; Beiche et al., 1998; Ebersberger et al., 1999; Inoue et al., 2006).

2.3.2.1. Prostaglandin-E2 (PGE2)

COX-1 and COX-2 catalyze the formation of prostaglandin H2 (PGH2) from arachidonic acid. Three different prostaglandin E synthases (PGE synthases) then act on PGH2 to transform it into PGE2. Two of these PGE synthases are membrane-bound (mPGES-1 and mPEGES-2), while the third is found in the cytosol (cPEGES). cPGES acts on PGH2 derived from the action of COX-1, while mPEGES-1 uses PGH2 endoperoxidase derived from the action of COX-2. m-PEGES-2 uses both these substrates. mPEGES 1 is mainly an induced enzyme, whereas the other two PGE synthases are constitutively expressed. mPEGES-1 is also glutathione-dependent. The availability of this PGE synthase is increased, as is that of COX-2, in response to various pro-inflammatory stimuli, but this increase is in both these enzymes is reversed by glucorticoids (Samuelsson et al., 2007). Glutathione is found to bind in a U-shaped conformation at the interface between sub-units in the protein trimer. The glutathione is exposed to a site facing the lipid bilayer which forms the specific environment for the oxidoreduction of PGH2 to PGE2 after displacement of the cytoplasmic half of the N-terminal transmembrane helix (Jegerschold et al., 2008).

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PGE2 may be induced in both dorsal root ganglia and in the spinal cord (Inoue et al., 2006; Ebersberger et al., 1999). PGE2 mediates its effects by binding to four G protein-coupled receptors, denominated EP1, EP2, EP3, and EP4, each of which shows selective ligand-binding specificity which distinguishes it from the others (Narumiya et al., 1999; Lin et al., 2006). In addition, at least four isoforms of the EP3 receptor exist. These EP3 isoforms, which differ only at their C-terminal tails and are produced by alternative splicing, couple to different G proteins to activate different second messenger systems (Namba et al., 1993).

2.3.2.1.1. PGE2 contributes to inflammatory pain

PGE2 generally exerts a pro-nociceptive effect, being capable of exciting primary sensory neurons. Infusion of PGE2 into healthy human subjects induces headaches in those subjects (Wienecke et al., 2009). The importance of PGE2 as a mediator of pain in humans is evidenced by the fact that NSAIDs, which inhibit the production of PGE2, are the most widely used analgesic drugs in clinical practice. An injection of PGE2 into the mouse paw results in oedema formation which is mediated by EP3 receptor activation, and also involves the stimulation of PLC, PKC, and MAPKs pathways and the participation of TRPV1 and NK1 receptors (Claudino et al., 2006). Moreover, PGE2 enhances the sensitivity of sensory neurons to various forms of noxious stimulation, in part, by the suppression of a delayed rectifier-like potassium current in these neurons (Jiang et al., 2003; Evans et al., 1999). PGE2 can also potentiate nociceptor activation by protons in human skin and muscle. Intradermal injections of PGE2 into the forearm skin, or intramuscular injections of PGE2 into the anterior tibial muscle, induces only very low levels of pain similar to injection of saline. Administration of low pH causes moderate pain within 5 seconds which declines rapidly over 15 to 20 seconds. However, co-injection of low pH with PGE2 leads to a biphasic pain response, with no increase in pain immediately after co-injection, but with significantly increased pain in the second phase, from 20 to 100 seconds, both in skin and muscle, with muscle pain being less than skin pain in the initial phase, but greater in the second phase (Rukwied et al., 2007).

Several receptor subtypes are involved in mediating the sensation of inflammatory pain. Thus, the EP4 receptor subtype is expressed by a subset of dorsal root ganglion

(DRG) neurons, and its levels, but not the levels of the other EP1-3 subtypes, increase in the DRG after CFA-induced peripheral inflammation in rat. Administration of both an EP4 antagonist and EP4 knock-down attenuates inflammation-induced thermal and mechanical behavioural hypersensitivity, without changing basal pain sensitivity. The EP4 antagonist also reduces the PGE(2)-mediated sensitisation of capsaicin-evoked currents in DRG neurons *in vitro* (Lin et al., 2006).

Spinal EP3 receptors are involved in hyperalgesia induced in mice by a low dose of PGE2, while spinal EP1 receptors are involved in allodynia in mice induced by PGE2 (Minami et al., 2001). Intraspinal release of PGE2 occurs during peripheral inflammation (Ebersberger et al., 1999). Spinal application of agonists at EP1, EP2, and EP4 receptors in nociceptive dorsal horn neurons, having their main input from the knee joint in rat, result in spinal hyperexcitability in the absence of inflammation which is similar to that resulting from PGE2 application. The effect of spinal EP receptor activation changes during joint inflammation. When the knee joint is inflamed for 7-11 hours, only activation of the EP1 receptor causes additional facilitation, whereas spinal application of EP2 and EP4 receptor agonists has no effect. However, an EP3-alpha receptor agonist reduces responses to mechanical stimulation. The EP3-alpha agonist also attenuates spinal hyperexcitability induced by spinal PGE2 (Bar et al., 2004).

PGE2 contributes to the pathogenesis of rheumatoid arthritis by its action on the EP4 receptor. Homozygous deletion of the EP1, EP2, or EP3 receptors does not affect the development of arthritis in a mouse model of the disease, whereas EP4 receptor-deficient mice show decreased incidence and severity of disease. These

animals also show reduced inflammation, as assessed by circulating IL-6 and serum amyloid A levels, reduced bone destruction, proteoglycan loss, and type II collagen breakdown in cartilage, when compared with EP4(+/+) mice (McCoy et al., 2002). PGE2 enhances dendritic cell-derived IL-6 production and induces a shift in the IL-23/IL-12 balance in favour of IL-23 in a mouse model of rheumatoid arthritis, resulting in increased IL-17 production and exacerbation of collagen-induced arthritis in mice (Sheibanie et al., 2007). PGE2, acting through EP receptors, controls human adult articular cartilage homeostasis and is linked to the pathophysiology of osteoarthritis (Li et al., 2009).

When dorsal root ganglia cultures are treated with the pro-inflammatory cytokines tumour necrosis factor alpha (TNF-alpha) or interleukin-1beta (IL-1beta) for 24 hours, COX-2 expression and PGE2 production is increased. The increase in PGE2 production is blocked by pre-treatment with a COX-2 inhibitor. At the same time, exposing these cultures to PGE2, or to either of these cytokines, does not alter EP receptor mRNA levels. Thus, these cytokines induce the functional expression of COX-2, but not EP receptors, in dorsal root ganglia cells in culture, which suggests that cytokine-induced sensitisation of sensory neurons is secondary to prostaglandin production and not to alterations in EP receptors (Fehrenbacher et al., 2005). The EP4 receptor appears to be mainly involved in the inhibition of cytokine release in C3H/HeN peritoneal macrophages, and gene expression of EP2 and EP4 appears to be differentially regulated during macrophage activation (Ikegami et al., 2001).

Prostaglandin D2 (PGD2) is known to be involved in inflammatory diseases like allergy and asthma. PGD2 acts through two seven-transmembrane receptors, DP

(DP1) and CRTH2 (DP2), which are also activated by several endogenous metabolites from the arachidonic acid cascade (Ulven & Kostenis, 2006). However, topical application of PGD2 to the spinal cord of the rat appears to attenuate spinal hyperexcitability under inflammatory conditions. Thus, in rats with inflamed knee joints, either PGD2 or a DP1 receptor agonist decreases the responses to mechanical stimulation of the inflamed knee and the non-inflamed ankle, thereby reducing established inflammation-evoked spinal hyperexcitability. Again, spinal co-application of PGD2 with PGE2 attenuates the PGE2-induced facilitation of responses to mechanical stimulation of the rat knee joint (Telleria-Diaz et al.).

2.3.2.2. Prostaglandin-I2 (PGI2; Prostacyclin)

Prostaglandin-I2 (PGI2) is also known as "prostacyclin." Prostacyclin has a half-life of 4 minutes at 37 degrees C. It is synthesised in the blood vessel wall (mainly by the endothelium). It has a range of important physiological actions, including acting as a physiological antagonist of TxA2. It exerts its effect through activation of adenylate cyclase, followed by an intracellular accumulation of cyclic-AMP in various types of cells, and is important in cell protection (Gryglewski, 2008). It is also an important inflammatory agent.

2.3.2.2.1. Prostacyclin contributes to inflammatory pain

Prostacyclin acts synergistically with other inflammatory mediators, such as histamine and bradykinin. It mediates its effects by binding to IP receptors. Infusion of prostacyclin into healthy human subjects induces headache associated with dilatation of the superficial temporal artery (Wienecke et al., 2008). Mice which lack the prostacyclin receptor are found to be viable, reproductive and normotensive.

However, their susceptibility to thrombosis is increased, and their inflammatory and pain responses are reduced to the levels observed in indomethacin-treated wild-type mice. Thus, prostacyclin is an antithrombotic agent and has a role as a mediator of inflammation and pain (Murata et al., 1997). PGI2 is also implicated as a mediator of chronic inflammation. In a rat model of osteoarthritis, using monoiodoacetate injection into the knee joint, an IP antagonist reduces pain with an efficacy approaching that of the NSAID diclofenac. In a chronic model of rheumatoid (i.e., inflammatory) arthritis -- namely, the collagen-antibody induced arthritis model in mice -- IP receptor-deficient mice display a 91% reduction in arthritis score. In fact, the development of inflammation in CIA, is not dependent on any single EP receptor type, although a partial suppression of CIA is achieved by the combined inhibition of EP2 and EP4. Thus, it appears that inhibition of PGI(2) synthesis by NSAIDs and COX-2 inhibitors, in addition to their inhibition of PGE(2) synthesis, contributes to their efficacy in treating the signs of arthritis (Pulichino et al., 2006; Honda et al., 2006). It should also be noted that both PGI2 and PGE2 are abundantly present in the synovial fluid of rheumatoid arthritis patients. Mice lacking the PGI2 receptor, when subjected to collagen-induced arthritis (CIA), also show significant reduction in the levels of pro-inflammatory cytokines, such as interleukin-6, in arthritic paws, while the addition of an IP agonist to cultured synovial fibroblasts significantly enhances IL-6 production and induces expression of other arthritis-related genes. Thus, both PGI2-IP and PGE2-EP2/EP4 signalling are important in the development of CIA, and inhibition of PGE2 synthesis alone may not be sufficient for suppression of symptoms of rheumatoid arthritis (Honda et al., 2006).

2.3.2.3. Excitation of dorsal root ganglion and spinal cord by PGE2 and PGI2

Prostaglandins released in the dorsal root ganglia excite those neurons by lowering the threshold for activation by heat of TRPV1 ion channels. PGE2 and PGI2 each, individually, increase, or sensitise, TRPV1 responses through their respective EP1 or IP receptors, predominantly in a PKC-dependent manner in both HEK293 cells expressing TRPV1 and in mouse DRG neurones. In the presence of PGE2 or PGI2, the temperature threshold for TRPV1 activation is reduced below 35 degrees C, so that body temperature itself is sufficient to activate TRPV1, resulting in the phenomenon of spontaneous pain sensations. A PKA-dependent pathway is also involved in the potentiation of TRPV1 through EP4 and IP receptors upon exposure to PGE2 and PGI2, respectively. In TRPV1 null mice and EP1 null mice, both PGE2-induced thermal hyperalgesia and inflammatory nociceptive responses are diminished. Moreover, PGI2-induced thermal hyperalgesia observed in wild type mice is almost completely absent in both TRPV1 null mice and IP null mice (Moriyama et al., 2005). ATP-sensitive potassium currents reduce the PGE2-mediated enhancement of excitability in adult rat sensory neurons. Thus, activation of K(ATP) can reverse the sensitisation produced by PGE(2) and may provide a mechanism of reducing the enhanced excitability that results from inflammation (Chi et al., 2007).

Synthesis of prostaglandins by spinal dorsal horn neurons is important for the induction and initial expression, but not for the maintenance, of spinal cord hyperexcitability. Spinal PGE2 application facilitates dorsal horn neuronal firing elicited by ionophoretic delivery of NMDA, suggesting that an interaction of prostaglandins and NMDA receptors may contribute to inflammation-induced central sensitisation. However, after development of inflammation, spinal indomethacin fails

to reduce responses to ionophoretic delivery of NMDA or AMPA, suggesting that such an interaction is not required for the maintenance of central sensitisation (Vasquez et al., 2001).

2.3.3. Platelet-activating factor (PAF)

Platelet-activating factor (PAF) describes a family of structurally-related lipid messengers which act on a wide variety of targets. Two major pathways for the production of these lipids have been described. One involves the production of PAF by an action of a PAF-phosphocholine transferase on CDP-choline and alkylacetylglycerol. The other involves an action of a lyso-PAF AT enzyme on lyso-PAF which results from an action of acetyl CoA and PLA2 (Teather et al., 2002a; Snyder, 1995). This latter pathway was believed to synthesise PAF under inflammatory conditions. However, it is now known that there are two types of lyso-PAF AT enzymes: one (lysoPAFAT/LPCAT2) is inducible and activated by inflammatory stimulation; and the other (LPCAT1) is constitutively expressed. Each lyso-PAF-AT synthesises inflammatory and physiological amounts of PAF, depending on the cell type (Harayama et al., 2008).

PAF has many different functions. It appears to be involved in polymorphonucler leuckcoyte recruitment to dermal inflammatory sites in rats (Belanger et al., 2008). It also functions in relation to bone metabolism and disease (Hikiji et al., 2008), and is an important mediator of anaphylaxis in animals such that interventions that block PAF can prevent fatal anaphylaxis (Vadas et al., 2008). The human PAF receptor (PAFR) is a G-protein-coupled receptor. After stimulation, this receptor is rapidly

desensitised. This refractory period depends on receptor phosphorylation, internalization and down-regulation (Dupre et al, 2007).

2.3.3.1. PAF contributes to inflammatory pain

PAF is an important mediator for inflammation. A receptor for PAF is found on the plasma membrane but, in addition, it has intracellular microsomal binding sites. The nociceptive responses in the late, but not the early, phase of formalin-induced nociception in rat is significantly reduced in animals receiving systemic PAF antagonists (Teather et al., 2002b). Morita and colleagues (2004) found that both thermal hyperalgesia and tactile allodynia are induced in mice by intrathecally administered platelet-activating factor (Morita et al., 2004). Inoue and co-workers found that PAF-receptor null mice display almost normal responses to thermal and mechanical stimuli. However, these mice exhibit attenuated persistent pain behaviours resulting from tissue injury by locally injecting formalin at the periphery, or resulting from capsaicin application, or from visceral inflammatory pain (Tsuda et al., 2007a). Inhibition of PAF receptors in hippocampal plasma membranes attenuates the inflammatory nociceptive response in rats, but intracellular PAF binding sites do not appear to be involved in processing inflammatory pain sensations (Teather et al., 2006). Injection into the rat paw of platelet-activating factor (1-30 nM per paw) results in a dose-related overt nociception, but only the dose of 10 nM per paw results in a significant and time-related mechanical hypersensitivity. Distinct mechanisms appear to be involved in the spontaneous nociception and the mechanical hypersensitivity caused by platelet-activating factor (Moretta et al., 2009).

2.3.4. Leukotrienes

Leukotrienes are metabolites of arachidonic acid which are produced by the lipoxygenase pathways. 5-Lipoxygenase acts on arachidonic acid to give 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is converted to leukotriene A4. This, in turn, can be converted to either leukotriene B4 (LTB4) or to a series of cysteinyl-leukotrienes which have amino acids incorporated in their structure (Murphy and Gigon, 2007). Leukotriene B4 acts through its receptors, BLT1 and BLT2. Both receptors are G protein-coupled seven transmembrane domain receptors. However, the two receptors differ in their affinity and specificity for LTB4 and also in their patterns of expression. Thus, BLT1 is a high-affinity receptor specific for LTB4, whereas BLT2 is a low-affinity receptor that also binds other eicosanoids. Again, the two receptors also differ in their pattern of expression. Thus, BLT1 is expressed primarily in leukocytes, whereas BLT2 is more widely expressed (Tager and Luster, 2003).

2.3.4.1. LTB4 contributes to inflammatory pain

LTB4 is known to be an important inflammatory mediator, but the nature and extent of its contribution to inflammatory pain is not settled. There is evidence of LTB4 involvement in rheumatoid arthritis in mice (Chen et al., 2006; Grespan et al., 2008), yet treatment of patients with active rheumatoid arthritis with a potent oral, long-acting, LTB4 receptor antagonist produces only modest improvements in disease activity, suggesting that LTB4 is not a major contributor to the inflammatory process in this disease (Diaz-Gonzalez et al., 2007). On the other hand, after intracutaneous infiltration in humans of LTB4 (10 uM) a significant decrease in the pain threshold on exposure to heat can be detected as compared with controls, with a maximum effect

between 6 and 24 hours after injection (Bisgaard et al., 1985). In the hyperalgesia induced by application of nucleus pulposus to the lumbar nerve root in the rat, an LTB4 antagonist injected into the epidural space decreases mechanical hyperalgesia at both three and seven days after epidural injection, but has no significant effect on sensitivity to noxious thermal stimuli (Kawakami et al., 2001). Joint injection of LTB4 induces a dose-dependent articular hypernociception (Guerrero et al., 2008). Importantly, several products of lipoxygenases, including LTB4, have been shown to directly activate TRPV1 in isolated membrane patches of rat sensory neurons (Hwang et al., 2000).

2.4. NERVE GROWTH FACTOR (NGF)

2.4.1. Introduction

Nerve growth factor (NGF) was initially identified as a molecule that promotes the survival and differentiation of sensory and sympathetic neurons. NGF has two known cell-surface receptors, TrkA and p75. TrkA is a receptor with tyrosine kinase activity that forms a high-affinity binding site for NGF. The ligand-binding domain of TrkA consists of two patches, one of which is specific for the interaction between NGF and TrkA (Wiesmann et al., 1999). Recently, the p75 receptor has also been found to be associated with inflammatory thermal hypersensitivity. Pro-NGF is the dominant ligand of the p75 receptor *in vivo;* and both pro-NGF and mature NGF play an important roles in inflammation-induced hyperalgesia mediated through the p75 receptor (Watanabe et al., 2008).

The functions of NGF in neural development have been characterised extensively, but recent studies indicate that its role in development is only one of several biological actions of NGF. In particular, NGF constitutes an important inflammatory mediator and is an important component in the process of sensitisation of TRPV1 ion channels in primary sensory neurons.

2.4.2. NGF production in inflammation

NGF is produced by a variety of cells in the context of inflammation. Monocytes, produce, store and release NGF, BDNF and NT-3 (Rost et al., 2005); as do eosinophils (Noga et al., 2003). Mast cells also synthesise, store and release NGF (Leon et al., 1994). Histamine -- itself an inflammatory mediator – powerfully

stimulates the production of NGF in both the central and peripheral nervous systems. Histamine is released by mast cells during inflammatory or allergic reactions. The main signalling pathway by which histamine stimulates the production of NGF involves the activation of the histamine H(1)-receptor, and stimulation of Ca(2+)-dependent protein kinase C and mitogen-activated protein kinase. Histamine and IL-1beta have an additive stimulatory effect on NGF secretion, but the interaction between histamine and IL-6 causes a long-term synergism (Lipnik-Stangelij, 2006). Brain macrophages also release NGF when stimulated by lipolysaacharide (Mallat et al., 1989). NGF is constitutively expressed in cells of the anulus fibrosus and nucleus pulposus in humans. Moreover, the pro-inflammatory cytokines, IL-1beta and TNF-alpha, stimulate the expression of nerve growth factor by these cells (Abe et al., 2007). It appears likely that other tissues constitutively express NGF and that pro-inflammatory cytokines have an important role in stimulating its production. Thus, for example, TNF-alpha markedly stimulates the synthesis and secretion of biologically active NGF in quiescent fibroblasts and also in glioblastoma cells (Hattori et al., 1993). Moreover, inflammation induced by intraplantar injection of Complete Freund's adjuvant in adult rats results in significant elevation in the levels of TNF alpha, IL-1beta and NGF in the inflamed paw (Woolf et al., 1997). The prostaglandins PGD(2) and PGE(2) potently induce the secretion of NGF from cultured mouse astrocytes, with PGE(2) or PGD(2) inducing an approximately 12-fold or 19-fold increase in NGF secretion after a 24-hour incubation, respectively. Even more strikingly, the sequential metabolites of PGD(2), PGJ(2), Delta(12)-PGJ(2), and 15-deoxy-Delta(12,14)-PGJ(2), induce NGF secretion to the culture medium 60 fold to 98-fold of the control after a 24-hour incubation. These prostaglandins also stimulate the secretion/synthesis of brain-derived neurotrophic

factor (BDNF) (Toyomoto et al., 2004). Activation of TNF-alpha receptors 1 and TNF-alpha receptors 2 by TNF-alpha in cultured astroycytes promotes expression of both NGF and GDNF. Both exogenous TNF-alpha and also TNF-alpha produced by astrocytes have this effect. Thus, TNF-alpha appears to be of considerable importance in contributing to the production of these neurotrophic factors in the context of inflammation (Kuno et al., 2006).

2.4.3. NGF and inflammatory pain

NGF acts on nociceptive afferent neurones, increasing their electrical excitability (Shu and Mendell, 2001). Acutely, NGF exerts profound effects on nociceptive transmission and produces pain and hyperalgesia. Thus, injection of NGF into human masseter muscle evokes long-lasting mechanical allodynia and hyperalgesia (Svensson et al., 2003). Injection of NGF into the tibialis anterior muscle of human beings results the development in an area of hyperalgesia at the injected site 3 hours after injection which expands both proximally and distally on day 1. This effect subsides by day 4 (Andersen et al., 2008). NGF is also up-regulated after muscle incision in rat and contributes to guarding pain behaviour (Wu et al., 2009). Again, increased NGF after rat plantar incision contributes to guarding behaviour and heat hyperalgesia (Banik et al., 2005). When nerve growth factor is injected into the temporomandibular joint of a rat which has been sensitised by a prior injection of carrageenan, significant nociceptive behaviour is elicited which is absent when the same injection is made into the same joint when not inflamed. Moreover, the nociceptive response appears to depend on the synergistic activity of NGF and sympathetic amines released during inflammation of that joint acting on beta2-adrenergic receptors (Pelegrinin-da-Silva et al., 2008). In CFA-induced hind-paw

inflammation, and in spinal nerve ligation and streptozotocin-induced neuropathic pain rodent models, a single intraperitoneal injection of a polyclonal anti-NGF antibody reverses established tactile allodynia from approximately day 3 to day 7 after treatment in rats and mice (Wild et al., 2007). There is also evidence that over-expression of NGF may induce endoneurial sprouting and trigger the development of thermal hyperalgesia in peripheral nuropathic pain states (Ruiz et al., 2004).

NGF plays a significant role in the maintenance of fracture pain, but not in the acute generation of such pain (Jiminez-Andrade et al., 2007). It has also been implicated in contributing to migraine pain, having been shown to make an important contribution to nociceptive signalling by trigeminal sensory neurons (D'Arco et al., 2007). It has also been suggested that NGF induces rapid, reversible, up-regulation of P2X3 receptor function *via* protein kinase C phosphorylation to contribute to migraine pain. These up-regulated receptors acquire the ability to repeatedly respond to extracellular ATP, thereby enhancing the signalling of painful stimuli (Giniatullin et al., 2008).

The p75 receptor has recently been associated with inflammatory thermal hypersensitivity. Thus, administration of neutralizing antibody to the p75 receptor blocks hyperalgesia which otherwise develops with CFA-induced inflammation or with an intraplantar injection of NGF. Blocking the p75 receptor also abolishes the up-regulation of CGRP levels in primary sensory neurons consequent on CFA injection. Pro-NGF is the dominant ligand of the p75 receptor *in vivo;* and both pro-NGF and mature NGF play an important roles in inflammation-induced hyperalgesia (Watanabe et al., 2008). In rat, most dorsal root ganglion neurons innervating

intervertebral discs, as well as the intervertebral discs themselves, express both the TrkA and p75 receptors (Sugiura et al., 2008).

NGF and its TrkA receptor are found in human degenerative lumbar facet joint specimens (Surace et al., 2009). NGF acts on nociceptive dorsal root ganglion neurons in rat to make them hyper-excitable so as to generate spontaneous action potentials and to generate spontaneous intracellular calcium fluctuations (Ozaki et al., 2009). In rats, bladder-derived NGF, acting through trk receptors, appears to at least contribute to peripheral sensitisation to mechanical stimuli associated with acute, and sub-acute, acrolein-induced cystitis (Guerios et al., 2008).

2.4.4. NGF involvement in sensitisation of TRPV1

Chronic exposure to high NGF increases the proportion of neurons that exhibit an inward current in response to anandamide (Evans et al., 2007). The importance of the role of NGF in the excitation of nociceptive primary afferents in the context of inflammation is strongly suggested by the evidence relating to its role in sensitising TRPV1 ion channels. NGF is known to be capable of sensitising TRPV1 ion channels (Shu and Mendell, 2001). By binding to, and activating, its trkA receptor, NGF sets in motion a biochemical chain of events which, if sustained, results in the sensitisation of TRPV1. As a cell membrane surface receptor, trkA relies on the further activation of intracellular messengers to mediate this effect. The issue relates to which one, or more, of three intracellular signalling pathways perform this function. These pathways are those denominated: PLC, PI3K, and MAPK, respectively.

Chuang and colleagues (2001) proposed that activation of trkA by NGF activates PLC which, turn, hydrolyzes PIP2 to sensitise TRPV1. PIP2 is proposed to tonically inhibit activation of TRPV1 and its hydrolysis by PLC results in releasing the ion channel from this inhibition (Chuang et al., 2001). PIP2 is proposed to inhibit TRPV1 by direct binding by PIP2 to a site within the C terminal domain of TRPV1. Deletion of this site precludes sensitisation of TRPV1 by NGF (Prescott and Julius, 2003). Woolf and co-workers (2002) proposed that p38 MAPK activation by NGF in primary sensory neurones after inflammation increases TRPV1 levels and contributes to thermal hyperalgesia (Ji et al., 2002). Bonnington and McNaughton (2003) found that specific inhibitors of ras/MEK (mitogen-activated protein kinase and extracellular signal-regulated kinase) pathway and of phospholipase C fail to abolish NGF-induced sensitisation of TRPV1, but a specific inhibitor of phosphatidylinositol-3-kinase (PI3K) totally abolishes the effect of NGF. In addition, pharmacological blockade of PKC or CaMKII prevents NGF-induced sensitisation, while blockade of PKA is without effect (Bonnington and McNaughton, 2003). Ji and co-workers (2004) proposed that NGF binds to TrkA which stimulates phosphatidylinositol-3-kinase to activate ERK in primary sensory neurones and thereby sensitises TRPV1 to generate inflammatory heat hyperalgesia (Zhuang et al., 2004).

According to McNaughton's group (2005), PI3K exercises the major role in sensitising TRPV1 consequent upon TrkA activation by NGF. They propose two pathways, major and minor, by which NGF activates trkA to induce sensitisation of TRPV1. Their major pathway involves the introduction of TRPV1 into the surface membrane and commences with autophosphorylation of the Y760 site of TrkA. Activation of PI3 kinase follows and Src is ultimately directly responsible for

phosphorylating TRPV1 at Y200. Their minor pathway involves activation of PKC-epsilon by trkA, leading to phosphorylation of TRPV1 at S502 and S801 (Zhang et al., 2005). Gordon and co-workers (2006) have also proposed that NGF acts through the PI3K pathway, and not through PLC, to facilitate trafficking of TRPV1 to the plasma membrane and hence increase TRPV1 function to contribute to hyperalgesia. In their model, TRPV1, PI3K and trkA physically interact in a signal transduction complex in which activation of trkA by NGF facilitates trafficking of TRV1 to the plasma membrane (Stein et al., 2006). Further evidence of the non-involvement of PLC in the trkA signalling sequence initiated by its being bound by NGF has been offered by Oxford and Zhu who propose that PI3K and MAPK pathways underlie the acute sensitisation of TRPV1 by NGF, but not the PLC pathway (Zhu and Oxford, 2007).

2.4.5. Surgical incisional injury and post-operative pain

Incisional injury in rat is commonly employed as a model for post-operative pain following surgical incision in humans. Most of the studies emanate from the Brennan laboratory. They developed a rat model of incisional pain involving a 1 cm longitudinal incision through skin, fascia, and muscle, of the plantar aspect of the hind-paw in halothane-anaesthetised rats. This results in a reliable and quantifiable mechanical hyperalgesia which persists for several days after surgery. Different areas around the wound manifest different withdrawal thresholds over time, while even remote sites, as much as 10 mm from the wound, show persistent mechanical hyperalgesia. Both the sural and tibial nerves are responsible for transmitting nociceptive input from the hyperalgesia-inducing incision (Brennan et al., 1996). Mechanical hyperalgesia, tactile allodynia and reduction in weight-bearing are

employed to characterise the development of post-incisional pain in rat (Whiteside et al., 2004).

NGF has been implicated as an important component of the inflammatory process mediating responses to thermal stimuli after plantar incision of rat paw, although it does not exercise any influence on responses to mechanical stimuli (Zahn et al., 2004). Increased NGF is found in rat skin over several days after plantar incision which contributes to heat hyperalgesia and some incision-induced pain behaviours (Banik et al., 2005). Not only is NGF increased in the area of the incision, but, in addition, a large-molecular-weight form of NGF protein is expressed in the region adjacent to the incision. Nerve bundles exhibit NGF immunoreactivity, with Schwann cells and axons being labelled (Wu et al., 2007). After muscle incision in the rat, NGF protein increases from 4 hours after incision and is sustained for several days. NGF is localised in many CGRP-positive axons, few N52-positive axons and is not found in isolectin B4-positive axons of incised muscle. The source of NGF includes keratinocytes in the epidermis and fibroblasts in deeper tissues (Wu et al., 2009). A distinct class of afferents outside the mechano-heat-sensitive afferent population is proposed to be a likely contributor to heat hypersensitivity after rat plantar incision. Knock-out of TRPV1 reduces spontaneous activity in these unclassified afferents in incised rat skin (Banik & Brennan, 2009).

Several other important studies on incisional pain are conveniently addressed at this juncture. Brain derived neurotrophic factor (BDNF) is increased in the ipsilateral lumbar DRG and spinal cord after hind-paw incision in rat. Intrathecal injection of BDNF antibody greatly inhibits the mechanical allodynia induced by incision (Li et

al., 2008). A decrease in pH occurs immediately after a plantar incision of the hind-paw, or incision of the gastrocnemius muscle, in rat, and is sustained for at least 4 days, during which time pain behaviours are evident in the animal. When tissue pH returns to normal, these pain behaviours diminish. The fall in pH is restricted to the incision site and does not extend to areas surrounding the incision (Woo et al., 2004). Antagonists of bradykinin's cognate B1 and B2 receptors, given either as a pre-treatment dose or in post-treatment doses, fail to reduce pain-related behaviour after plantar paw incision (Leonard et al., 2004). However, this does not preclude an effect of bradykinin after incisional injury that is mediated *via* another receptor, most obviously TRPV1. AMPA/kainate receptors are proposed to be implicated in mediating mechanical hyperalgesia inasmuch as antagonists for these receptors, if administered spinally, decrease the responses to mechanical stimuli after plantar incision (Lee et al., 2006; Zahn et al., 1998). However, spinal NMDA and mGluR antagonists do not have the same effect (Pogatzki et al., 2000).

2.5. BRAIN-DERIVED NEUROTROPHIC FACTOR (BDN)

2.5.1. Introduction

Brain-derived neurotrophic factor (BDNF) is misleadingly named inasmuch as this agent is neither confined to, nor originates exclusively within, the central nervous system, but is found extensively in the periphery. Like NGF, BDNF is one of the "neurotrophin" molecules and acts *via* a tyrosine kinase (trk) receptor, namely, the trkB receptor, which it shares with the neurotrophin NT-4/5 (Maness et al., 1994). In adult rat, BDNF is found mainly in the subpopulation of DRG cells that contain CGRP and express the trkA receptor for NGF. From 80 to 90 per cent of BDNF-expressing cells also express trkA. BDNF protein is anterogradely transported both peripherally and centrally so that BDNF is found in CGRP containing terminal arbors in the dorsal horn of the spinal cord. NGF causes an increase in BDNF within these neurons with the result that almost 90 per cent of trkA-expressing neurons contain BDNF mRNA after intrathecal NGF treatment BDNF is found in finely myelinated and unmyelinated axons and in axon terminals where it is most concentrated over dense-cored vesicles (Michael et al.,1997). BDNF is found in varicosities of sensory nerve terminals in laminae I and II of the dorsal horn. Most BDNF nerve terminals also contain CGRP or the low affinity neurotrophin receptor p75, but the converse is not the case (Luo et al., 2001).

The release of BDNF from the terminals of primary nociceptive afferents in the spinal cord is associated with increased nociceptive processing in the spinal cord. Activation of spinal ERK1/2 is a recognised marker of spinal nociceptive processing. Ji and colleagues (1999) showed that peripheral capsaicin injection into the left hind-

paw of rat activates ERK1/2 by phosphorylation (pERK1/2) in dorsal horn spinal cord neurons and induces pain-related behaviour in the animal (Ji et al., 1999). Noxious chemical, mechanical, or thermal, stimuli cause phosphorylation of the BDNF high-affinity receptor, trkB, in the spinal cord. Both *in vitro* and *in vivo* exogenous BDNF induces a rapid activation of ERK1/2. Moreover, sequestering BDNF *in vivo* with a TRkB-IgG fusion molecule significantly reduces the activation of ERK1/2 evoked by noxious stimulation (Pezet et al., 2002). BDNF in primary sensory neuron cell bodies and in central terminals potentiates nociceptive spinal reflex responses in an *in vitro* spinal cord preparation and induces c-fos expression in dorsal horn neurons. Moreover, exogenous BDNF enhances NMDA receptor-mediated responses (Kerr et al., 1999).

2.5.2. BDNF and inflammatory pain

Peripheral tissue inflammation produced by an intraplantar injection of CFA into rat hind-paw results in significant increases in the number of BDNF immunoreactive neurons in the L5 DRG and causes marked elevation of immuoreactive BDNF terminals in the spinal dorsal horn. This is consistent with peripheral inflammation causing an increase in BDNF synthesis in the DRG followed by an elevated anterograde transport of BDNF to the neuronal terminals in the dorsal horn of the spinal cord (Cho et al., 1997). Systemic NGF treatment – to mimic peripheral inflammatory states – increases BDNF levels in primary sensory neurons and increases nociceptive spinal reflex excitability. Behavioural nociceptive responses induced by intraplantar formalin and by intraplantar carageenan injections, respectively, are significantly attenuated by trkB-IgG, a BDNF antagonist (Kerr et al., 1999). In spinal cord slice preparations from rat with CFA-induced inflammation at

2 to 4 days after CFA injection, BDNF acts pre-synaptically to increase the frequency of excitatory miniature EPSCs in substantia gelatinosa neurons through activation of lidocaine-sensitive, TTX-resistant Na^+ channels. In addition, the number of monosynaptic A-beta afferent inputs to the substantia gelatinosa significantly increases 7 days after the onset of inflammation. This increase is significantly suppressed by treatment with a BDNF antiserum. Moreover, treatment with anti-BDNF antiserum significantly attenuates CFA-induced hyperalgesia and/or allodynia (Matayoshi et al., 2005). Intrathecal administration of BDNF in mouse produces an acute, dose-dependent, thermal hyperalgesic response. Selective inhibition of BDNF expression by intrathecal antisense oligodeoxynucleotide treatment results in antinociception in normal mice and attenuates carrageenan-induced hyperalgesia. Similar knock-out treatment directed against the full-length trkB receptor attenuates carrageenan-induced hyperalgesia (Groth & Aanonsen, 2002). Intrathecal co-administration of an NMDA receptor antagonist with BDNF dose-dependently inhibits BDNF-induced hyperalgesia, suggesting that BDNF mediates hyperalgesia in a process which is dependent on NMDA receptor activation (Groth & Aanonsen, 2002). BDNF contributes to pain hypersensitivity after surgical incision in rats. After hind-paw incision, BDNF is up-regulated in the ipsilateral lumbar DRG and spinal cord. Intrathecal injection of BDNF antibody greatly inhibits the mechanical allodynia resulting from the incision (Li et al., 2008).

2.6. ARTEMIN, NEURTURIN, AND GDNF

2.6.1. Introduction

Artemin, neurturin, and GDNF are members of the g*lial cell line-derived neurotrophic factor (GDNF)* family and are potent regulators of TRPV1 function and possess the ability to potentiate TRPV1 signalling and to induce behavioural hyperalgesia (Malin et al., 2006). Members of the GDNF family are produced in the form of a "prepro" precursor. The signal sequence is cleaved on secretion, and activation of the "pro" derivative is suggested to occur by proteolytic cleavage. GDNF family members appear to bind to heparin-sulphate side chains of extracellular-matrix proteoglygans, but little is known of the gene regulation and mechanisms of secretion of these proteins, or of the activation of their precursors (Airaksinen & Saarma, 2002). Within sensory terminals located in the sacral spinal cord, and in bladder-projecting sacral dorsal root ganglion neurons of adult rats, distinct distribution patterns in the spinal cord are found for nerve fibres immunolabelled for GFRalpha1 (GDNF receptor), GFRalpha2 (neurturin receptor), and GFRalpha3 (aretemin receptor), respectively (Forest & Keast, 2008).

2.6.2. Contribution to inflammatory pain

Artemin, neurturin, and GDNF, each individually potentiate capsaicin-evoked TRPV1 signalling in isolated mouse dorsal root ganglion neurons and cause thermal hyperalgesia when injected into mouse hindpaw *in vivo*. Artemin mRNA (but not neurturin or GDNF) is hugely up-regulated during cutaneous inflammation evoked by hind-paw injection of CFA, suggesting that artemin, in particular, enhances TRPV1 signalling in response to inflammatory injury. Hindpaw injection of artemin,

neurturin, GDNF, or NGF produces acute thermal hyperalgesia that lasts up to 4 hours. Moreover, a single combined injection of artemin and NGF produces hyperalgesia which persists for 6 days (Malin et al., 2006). Over-expression of artemin in the skin of mice enhances expression of TRPV1 and TRPA1 in cutaneous sensory neurons and results in increased behavioural sensitivity to heat and cold (Elitt et al., 2006). It also increases the total number of dorsal root ganglion neurons (Wang et al., 2008). In addition, over-expression of artemin in the tongue increases the expression of TRPV1 and TRPA1 in trigeminal afferents and causes oral sensitivity to capsaicin and mustard oil in mice (Elitt et al., 2008). Interestingly, artemin has recently also been found to have potent neurotrophic actions on injured C-fibres (Bennet et al., 2008). It also has a significant effect on the neurite growth of dorsal root ganglion neurons and activates axonal growth via Src family kinases and ERK-dependent signalling pathways in mature dorsal root ganglion neurons (Jeong et al., 2008). Exposure of isolated mouse sensory neurons in culture to GDNF, neurturin, and artemin enhances the capsaicin-stimulated release of immunoreactive calcitonin gene-related peptide (CGRP) two to threefold, but does not increase potassium-stimulated release of iCGRP. A similar profile of sensitisation is found in freshly dissociated spinal cord slices (Schmutzler et al., 2009). Acute administration of GDNF sensitises primary sensory neurons and produces mechanical hyperalgesia in the rat (Bogen et al., 2008).

2.7. SEROTONIN (5-HYDROXYTRYPTAMINE; 5-HT)

2.7.1. Introduction

Serotonin (5-Hydroxytryptamine; 5-HT) is formed from dietary tryptophan, which is converted by tryptophan hydroxylase to 5-hydroxytryptophan, and then to serotonin by a non-specific decarbosylase. It is transported into serotonin-containing cells by a specific transporter system. Degradation occurs mainly by monoamine oxidase forming 5-HIAA (5-hydroxyindoleacetic acid) which is excreted in the urine. Seven types of serotonin receptors have been identified, demoninated 5-HT1-7, with further subtypes (A-D) of 5-HT1 and 5-HT2. All are G-protein coupled receptors, except 5-HT3 which is a ligand-gated ion channel which is found in several locations, including on nociceptive primary sensory neurones. Serotonin is an important neurotransmitter in the central nervous system. It is also found throughout the body, outside the central nervous system, being present: in platelets in the blood; in mast cells; in enterochromaffin cells, which are found mainly in the stomach and small intestine; and in neurons of the myenteric plexus, where it functions as an excitatory neurotransmitter. About 95 per cent of peripheral serotonin is synthesised and stored in the enterochromaffin cells. Serotonin released into the circulation is taken up by platelets and stored in platelet dense granules (Jonnakuty and Cragnoli, 2008).

2.7.2. Peripheral serotonin, mast cells and inflammation

Serotonin is involved in inducing mast cell adhesion and chemotaxis (Kushnir-Sukhov et al., 2006). Moreover, human mast cells are capable, not only of storing serotonin for release, but, also, of synthesising serotonin (Kushnir-Sukhov et al., 2007). Mast cells are key cells of type I hypersensitivity reactions. They contribute to

host defence through the release of a myriad of pro-inflammatory and immunoregulatory molecules and can significantly influence multiple features of the inflammatory and immune responses (Metcalfe et al., 1997; Crivellato and Ribatti, 2005; Galli and Tsai, 2008). The 5-HT_{1A} receptor is the principal receptor mediating the effects of serotonin on mast cells. Serotonin induces the accumulation of mast cells in the dermis of mice which possess the 5-HT_{1A} receptor, but not in 5-HT_{1A} receptor null mice (Kushnir-Sukhov et al., 2006). Intracranial headaches, such as migraine, are putatively attributed to activation of sensory trigeminal nociceptive neurones that supply intracranial blood vessels and the meninges. Serotonin, prostacyclin (PGI(2)), and, to a lesser extent, histamine, can provoke a robust sensitisation and activation of meningeal nociceptive neurones and may be involved in the development of this type of headache (Zhang et al., 2007).

2.7.3. Peripheral serotonin contributes to pain of peripheral inflammation

Serotonin, when injected locally in the periphery causes pain, and wheal and flare responses (Jensen et al., 1990a; Jensen et al., 1990b). Serotonin released from mast cells contributes to the development of capsaicin-induced mouse ear oedema *via* 5-HT2 receptors in the ear skin to which capsaicin has been applied (Inoue et al., 1995). Intradermally injected serotonin produces a dose-dependent hyperalgesia in rat that is not attenuated by sympathectomy, polymorphonuclear leukocyte depletion or cyclooxygenase inhibition. The latency to onset of serotonin hyperalgesia is extremely short, with maximal hyperalgesia observed in less than one minute, and the effect is attenuated only by a 5-HT1A antagonist, suggesting that, in that model, the hyperalgesic effects of serotonin are exerted by direct action on primary afferent neurons *via* 5-HT1A receptors (Taiwo and Levine, 1992). Intradermal injection of the

5-HT1A receptor agonist, 8-OH-DPAT, produces a dose-dependent hyperalgesia which is attenuated by a cAMP kinase inhibitor, but prolonged by the inhibition of endogenous phosphodiesterase, supporting a role for the cAMP second messenger system in mediating serotonin-induced peripheral hyperalgesia (Taiwo et al., 1992). Mice which lack the serotonin transporter and which, therefore, have reduced levels of serotonin in their tissues, have attenuated hyperalgesia to heat, when compared to wild type mice, after hind-paw inflammation induced by intraplantar CFA injection (Palm et al., 2008).

The peripheral 5-HT2A receptor is involved in the development of thermal hyperalgesia in the carrageenan model of inflammation in rat. Intraplantar injection of carrageenan dramatically reduces paw withdrawal latency to noxious heat (47 degrees C) and causes paw swelling in the rat. Pre-treatment with ketanserin, a selective antagonist of the 5-HT2A receptor, in the hindpaw, results in dose-dependent inhibition of the hyperalgesia, up to, and including, full relief. This agent also moderately reduces carrageenan-induced paw swelling in a dose-dependent manner. Carrageenan administration induces conspicuous expression of c-fos-like immunoreactivity in the spinal dorsal horn of segments L4-5 which is markedly reduced by ketanserin (Wei et al., 2005). Serotonin, released in peripheral tissues after mild thermal injury to the rat hind-paw, acts *via* peripheral 5-HT2A receptors to contribute to the sensitisation of nociceptive primary afferents which results in allodynia and thermal hyperalgesia (Sasaki et al., 2006). The 5-HT2A receptor is mainly expressed in nociceptive sensory neurons in rat lumbar dorsal root ganglia (Van Steenwinckel et al., 2009). After formalin injection into the hind-paw of rat, serotonin is released into the formalin injection site in a formalin concentration-

dependent manner. Local pre-treatment with selective 5-HT2A and 5-HT2C receptor antagonists reduces the number of flinches in the early part of phase 2 of the formalin test in a dose-dependent manner. Moreover, peripheral pre-treatment with a mast cell membrane stabiliser completely suppresses serotonin release and inhibits phase 2 responses to the formalin test (Nakajima et al., 2009).

The 5-HT3 receptor is of varying significance in its contribution to the activation of primary nociceptive afferents. This receptor is not required for *acute* nociception in response to pain-inducing stimuli in several pain models in mouse, including the CFA intraplantar injection model of peripheral inflammation. However, it contributes to persistent nociception in the context of injury by an action on both myelinated and unmyelinated noceptive neurons in the same models, although it does not contribute to oedema. In addition, 5-HT3 receptors in the spinal cord facilitate the transmission of nociceptive impulses and have a pro-nociceptive effect (Zeitz et al., 2002). Serotonin markedly and specifically stimulates vagal mucosal chemosensitive afferents *via* 5-HT3 receptors (Grundy et al., 1995). Again, colonic serosal and mesenteric endings exhibit increased sensitivity to serotonin in inflammation, with both an increase in the proportion of responders and an increase in sensitivity, which is maintained after healing of the inflammation. This is associated with alterations in the roles of 5-HT3 receptors and mast cells (Coldwell et al., 2007). The 5-HT3 receptor also facilitates at-level mechanical allodynia following spinal cord injury (Oatway et al., 2004; Chen et al., 2009). The 5-HT(3A) receptor is found co-localised with GABA and enkephalins in the spinal dorsal horn and it has been suggested that serotonin may activate GABAergic and enkephalinergic neurons *via* 5-HT(3), thereby affecting the release of GABA and enkephalins (Huang et al., 2008)

Activation of the 5-HT7 receptor appears to be capable of exerting an anti-nociceptive effect in mice. Thus, mechanical hypersensitivity resulting from sensitisation to capsaicin in mice is reduced by systemic administration of 5-HT7 agonists and increased after administration of 5-HT7 antagonists (Brenchat et al., 2009)

2.7.4. Role of central serotonergic system

Central serotonergic neurons in the brain which project to the spinal cord – the so-called "descending serotonergic projections originating in the rostral ventromedial medulla – are believed to exercise vital functions in modulating nociceptive impulses in the spinal cord (Fields et al., 1991; Zhuo & Gebhart, 1991; Urban & Gehart, 1999; Millan, 2002; Suzuki et al., 2004; Zhao et al., 2007), in conjunction with central noradrenergic neurons projecting to the spinal cord (Fields and Basbaum, 1978; Basbaum and Fields, 1984; Millan, 2002. This hypothesis is supported by the success in the management of chronic pain enjoyed by tricyclic antidepressants which increase central serotonergic and noradrenergic neurotransmission (Lynch, 2001). In mice in which the central serotonergic system has been ablated, the animals survive to adulthood and exhibit normal locomotor activity (Zhao et al., 2006). These mice have normal acute thermal thresholds and visceral pain responses, but are generally less sensitive to mechanical stimuli. Thus, in general, the mechanical thresholds of the mutant mice are increased relative to the wild type mice, which suggests a facilitatory role of the central serotonergic system in modulating mechanical sensitivity in these circumstances. However, the contrary is found after hind-paw injection with capsaicin, and also in the carrageenan model of inflammatory pain,

where the mutants exhibit increased mechanical hypersensitivity in response to these noxious stimuli. Thus, in the capsaicin experiment, the mutants show significantly greater mechanical hypersensitivity when compared with wild type mice. Again, after injection of 5 per cent formalin into the hind-paw, flinching and licking behaviours of wild type and mutant mice were not significantly different during the first phase. In contrast, licking and flinching responses in the second phase were significantly enhanced in the mutants when compared with the wild type animals (Zhao et al., 2007). 5-HT2A receptors in the trigeminal sub-nucleus caudalis/upper cervical spinal cord junction region appear to mediate anti-nociceptive effects in craniofacial nociception (Okamoto et al. 2007).

2.8. HISTAMINE

2.8.1. Introduction.

Histamine is a basic amine and is an important neurotransmitter in both the central and peripheral nervous systems. Histamine mediates its physiological functions through binding to four known histamine receptors, namely: H1; H2; H3; and H4. These are all G protein coupled receptors and have been cloned (Yamashita et al., 1991; Gantz et al., 1991; Lovenberg et al., 1999; Nguyen et al., 2001). The H3 receptor appears to serve a general regulatory function. H3 receptor density is highest in the brain, but these receptors also exist in dorsal root ganglia, spinal cord and in certain peripheral tissues, including whisker pads and hairy and glabrous hind paw skin of rats and mice (Cannon et al., 2007a). "Constitutive activity" (i.e., spontaneous activity in the absence of agonist) of H3 receptors is found in rodent brain and controls histaminergic neuronal activity *in vivo*. Thus, a proportion of the H3 receptor population spontaneously undergoes an allosteric transition, leading to a conformation that can bind G proteins, independently of activation by any agonist (Morrisset et al., 2000; Arrang et al., 2007; Bongers et al., 2007).

In the periphery, histamine is found mainly in mast cells and basophils. Histamine is secreted when complement components C3a and C5a interact with specific membrane receptors, or when antigen interacts with cell-fixed IgE. Histamine has several well-known peripheral actions in humans including: stimulation of gastric secretion; contraction of most non-vascular smooth muscle; cardiac stimulation; vasodilation; and increased vascular permeability. It can also be responsible for type 1 hypersenstivity reactions, including urticaria and hay fever

(Jutel et al., 2005; Akdis et al., 2008; Ohtsu, 2008) Histamine also has an established effect of inducing the itch sensation when released in the periphery (Shim et al., 2007; Shim & Oh, 2008). It also has an important role as an agent which contributes to inflammatory pain sensations.

2.8.2. Histamine and inflammatory pain

Histamine contributes to pain sensation resulting from peripheral inflammation, although, paradoxically, histamine, acting through its H3 receptor, may apparently have an antinociceptive effect even in the context of inflammatory pain. More generally, H1 receptor null mice show significantly fewer nociceptive responses to the hot-plate, tail-flick, tail-presure, paw-withdrawal, capsaicin, abdominal constriction, as well as formalin, tests (Mobarakeh et al., 2000).

Injection of formalin into the paw of an animal results in a peripheral inflammatory response. Histamine is one of the agents generated by injection of formalin. The histamine thus released also contributes to the inflammatory response, with H1 receptors being relatively more important than H2 receptors in mediating formalin-induced nociceptive behaviour (Parada et al., 2001; Mobarakeh et al., 2000). Local administration of a H1 receptor antagonist prevents formalin-induced nociception in the temporomandibular joint (TMJ) in rats in a dose-dependent manner (Ting et al., 2007). Histamine H4 receptors participate in the early phase of acute inflammation induced by carrageean in rats, influencing both oedema formation and thermal hyperalgesia. Thus, highly selective H4 antagonists significantly reduce paw oedema and hyperalgesia in rats provoked by subplantar injection of carrageenan in the early (2 hour) phase of inflammation (Coruzzi et al., 2007).

However, histamine is apparently not unidirectional in its effects on nociception. Thus, the H3 agonist, pro-drug BP2-94, attenuates swelling and nociceptive behaviours in some mouse models of inflammatory pain (Rouleau et al., 1997; Rouleau et al., 2000). In rats subjected to the formalin test, activation of H3 receptors by administration of a H3 agonist reduces formalin-induced swelling and flinching (Cannon et al., 2007b).

2.8.3. Histamine and mechanical pain sensation

Histamine also appears to be involved in reducing at least certain forms of *mechanical pain* sensation. Activation of spinal H3 receptors attenuates nociceptive responses to low-intensity tail-pinch stimulation. Thus, intrathecal administration of a H3 receptor agonist produces an antinociceptive effect on the tail-pinch test in wild type mice, but not in knock-out mice lacking this receptor (Canon et al., 2003).

2.8.4. Activation of TRPV1 to mediate itch sensation

One effect of histamine on a subset of primary afferents is mediated by activation by histamine of phospholipase A2 and 12-lipoxygenase, which leads to the production of 12-HPETE and activation of TRPV1 ion channels. Activation of TRPV1 then leads to excitation of the primary sensory neurones on which they are expressed. Histamine-induced itching is proposed to be mediated, in part, by this pathway (Shim et al., 2007). Pain generally inhibits itch, which indicates an inter-communication between the (apparently) different neuronal pathways which mediate itch and pain (Shim & Oh, 2008).

2.9. ANANDAMIDE

2.9.1. Introduction

Anandamide (N-arachidonylethanolamine) is an endogenous ligand of cannabinoid receptors. It is a member of the group of bioactive lipids known as "long chain C18 N-acylethanolamines (NAEs)". In animal tissues, anandamide is principally formed, together with other NAEs, from glycerophospholipid by two successive enzymatic reactions: (i) N-acylation of phosphatidylethanolamine to generate N-acylphosphatidylethanolamine (NAPE) by Ca2+-dependent N-acyltransferase; and (ii) release of NAE from NAPE by a phosphodiesterase of the phospholipase D type (NAPE-PLD) (Okamoto et al., 2007; Simon & Cravatt, 2008). A major sub-population of capsaicin-sensitive primary sensory neurons expresses NAPE-PLD (Nagy et al., 2009). Fatty acid amide hydrolase (FAAH) is an integral membrane protein which has been identified as the primary enzyme responsible for NAE degradation in most tissues, including brain (Cravatt et al., 1996), and is found in neural tissue involved in peripheral nociceptive transmission in rat, such as dorsal root ganglia, sciatic nerve, and spinal cord (Lever et al., 2009). When the action of this enzyme is inhibited, there is a resulting increase in anandamide levels coupled with anti-inflammatory and analgesic effects mediated through cannabinoid receptors (Cravatt et al., 2001; Kathuria et al., 2003).

2.9.2. Contribution to inflammatory pain

Anandamide is implicated in acute and inflammatory pain conditions by reason of its ability to activate TRPV1 ion channels. Anandamide is one of several endogenous agents which have been proposed as direct activators of the TRPV1 ion channel

(Zygmunt et al., 1999). Others of these endogenous agonists include N-arachidonoyl-dopamine (NADA) (Huang et al., 2002), N-oleoyldopamine (OLDA) (Szolcsanyi et al., 2004), lipoxygenase products, such as 12- or 15-(S)-HPETE (Hwang et al., 2000), and unsaturated C18 N-acylethanolamines (Movahed et al., 2005). All of these agonists have been shown to compete for the capsaicin binding site, leading to their characterisation as "endovanilloids" (i.e., endogenous vanilloids). Anandamide is the best characterised of the endogenous TRPV1 agonists The capacity of anandamide to activate TRPV1 in normal physiological conditions is very limited. This limitation is necessary in order to prevent unnecessary activity of TRPV1, thereby signalling pain, in the absence of a relevant pain-inducing stimulus. However, when TRPV1 is activated by other stimuli, such as inflammatory mediators, anandamide becomes a powerful activator of TRPV1 (Olah et al., 2001; Singh et al., 2005) and, hence, a contributor to the pain sensations mediated by inflammogens. Anandamide and other endogenous activators of TRPV1 have therefore been described as "conditional activators" of this ion channel. Consistent with a role for anandamide in mediating inflammatory pain is the finding that AM404, an anandamide re-uptake inhibitor, induces an anti-nociceptive effect when formalin is injected into the rat hind-paw. While Fos-positive neurons are increased in the dorsal superficial and deep laminae of the lumbar spinal cord in formalin-injected rats, AM404 significantly reduces such Fos induction (Borsani et al., 2007).

2.10. ATP (ADENOSINE 5'TRIPHOSPHATE)

2.10.1. Introduction

ATP (adenosine 5'triphosphate) is a metabolite which functions as a neurotransmitter in both the central and peripheral nervous systems. ATP, in its role as a metabolite, functions intracellularly. However, it mediates its action as a neurotranmitter extracellularly. ATP, in its role as a neurotranmitter in the peripheral nervous system, functions on its own as a mediator and, in addition, as a co-transmitter at noradrenergic nerve terminals. Notwithstanding that ATP is an endogenous agent, it is capable of inducing pain in humans in certain conditions, a phenomenon which was reported, some thirty years ago, when it was observed that it induces a sensation of pain when it is applied to a blister-base preparation (Bleehen and Keele, 1977). Since then, the role of ATP in nociception has been the subject of considerable research on the assumption that it may act as an *inflammagen* when released from cells which are lysed by tissue damage or otherwise (Chizh & Illes, 2000; North, 2003).

2.10.2. ATP and inflammatory pain

ATP is a ligand at type 2 purinergic receptors (P2), of which there are two subtypes, namely, P2X and P2Y. P2X receptors are ionotropic receptors, whereas P2Y receptors are G-protein coupled receptors. Several P2X receptor sub-types are known to be involved in mediating pain sensations. P2X2 and P2X3 receptor subunits can form homomultimeric P2X2 receptors, homomultimeric P2X3 receptors, or heteromultimeric P2X2/3 receptors (Lewis et al., 1995). P2X2 and P2X3 receptors account for virtually all ATP responses in sensory and autonomic ganglion neurons in

mice. In addition to P2X3 receptors, P2X2/3 receptors (and possibly P2X2 receptors) are important in sensory modulation in at least one model of persistent pain and in the regulation of urinary bladder reflexes in mice (Cockayne et al., 2005). P2X2 and P2X3 receptor expression occurs in a subset of dorsal root ganglion neurons in both mouse and rat (Chen et al., 1995; Lewis et al., 1995; Collo et al., 1996; Cockayne et al., 2005). After formalin injection to the hind-paw of rat, P2X3 immunoreactivity is detected mainly in the cytoplasm and plasma membrane of small and medium-size dorsal root ganglion neurons. P2X3 transcript expression in the dorsal root ganglia remains unchanged at 1 hour after formalin injection, but is increased after 12 hours. In the spinal cord, P2X3 staining is not obviously increased by 1 hour post-injection, but is increased by 24 hours (Pan et al., 2009). P2X2 and P2X3 receptor expression occurs predominantly on small to medium-sized trigeminal afferent neurons in rat. The majority of neurons innervating the dura express P2X2 and/or P2X3 receptors, suggesting that purines may be involved in nociceptive processing in pain conditions such as migraine headache (Staikopoulos et al., 2007). In cultured trigeminal neurons, CGRP and NGF each enhances P2X3 receptor trafficking to the neuronal membrane and activates the gene transcription of these receptors. These P2X3 receptors are then capable of responding repeatedly to extracellular ATP to induce pain sensation (Giniatullin et al., 2008).

P2X3 receptors are capable of undergoing rapid constitutive and cholesterol-dependent endocytosis which is accompanied by preferential targeting of the receptor to late endosomes/lysosomes, with subsequent degradation. At steady state the P2X3 receptor localises mainly in lamp1-positive intracellular structures, with a small number found at the plasma membrane. The amount of functional receptors

expressed on the cell surface is rapidly up-regulated in response to agonist stimulation, which also augments receptor endocytosis (Vacca et al., 2009). TRPV1 receptors are co-expressed with P2X3 receptors in many dorsal root ganglion neurons and interact with each other in an inhibitory manner, probably by a physical association established by a motif located at the C-terminal end of the P2X3 receptor distal to Glu362 (Stanhev et al, 2009). The C-terminal Src inhibitory kinase (Csk) directly phosphorylates the tyrosine 393 residue of the P2X3 receptor and potently inhibits receptor currents. In mouse trigeminal sensory neurons, the activity of Csk is tightly controlled by the extracellular amount of NGF. Silencing endogenous Csk potentiates P2X3 receptor responses thereby demonstrating constitutive Csk-mediated inhibition of receptor function (D'Arco et al., 2009). The activation of P2X3, 2/3 receptors by endogenous ATP is essential to the development of mechanical hyperalgesia induced by carrigeenan (Oliveira et al., 2009). Allodynia in mouse evoked by the P2X receptor agonist, –methylene ATP, is mediated by the prostaglandin F2 receptor, possibly *via* the functional coupling between the activation of P2X2/3 receptors on the central terminal of capsaicin-insensitive fibres and prostaglandin F2 receptors on spinal neurons (Kunori et al., 2009).

P2X4 receptor null mice exhibit reduced tactile allodynia and swelling of the injected hind-paw in CFA-hind-paw-induced inflammation (Tsuda et al., 2009). Blockade of P2X7 receptors *in vivo* in mouse results in significant anti-nociception in mouse models of inflammatory pain, an effect which is likely to be mediated by inhibiting the release of IL-1beta from macrophages and microglial cells which is otherwise released as a result of the action of ATP on these receptors (Honore et al., 2009). An inhibitory effect of P2X7 receptors on the expression of P2X3 receptors

has also been demonstrated. P2X7 receptors in glial satellite cells of dorsal root ganglia tonically inhibit the expression of P2X3 receptors in nociceptive neurons and thereby reduce pain through down-regulation of neuronal P2X3 (Chen et al., 2008).

The P2Y2 receptor is highly expressed in dorsal root ganglia in mice. Mice which lack this receptor exhibit impaired thermal nociception. Mutant mice fail to develop thermal hypersensitivity in response to CFA-induced inflammation. TRPV1 function is also reduced in P2Y2 null mice, as measured by the frequency and magnitude of capsaicin responses *in vitro* and behavioural responses to capsaicin administration *in vivo*. (Malin et al., 2008). Activation of P2X7 receptors on satellite cells of dorsal root ganglia evokes ATP release which, in turn, stimulates P2Y1 receptors in primary sensory afferents. P2Y1 receptor activation appears to be necessary sufficient for the inhibitory control of P2X3 receptor expression in dorsal root ganglia (Chen et al., 2008).

Caution is always required when considering the effect in humans of the results of animal experiments, but this is especially so in the case of ATP because substantial inter-species differences have been found in the expression of ATP receptors. Thus, only 30 per cent of dorsal root ganglia neurons in cat express P2X3 receptors compared with 90 per cent in rat and in mouse, and a substantial difference in P2Y1 receptor expression is also found in cat when compared to rat and mouse (Ruan et al., 2005). Nevertheless, there is evidence that extracellular ATP may contribute to certain forms of neuropathic pain and inflammatory pain by an action on its receptors located on primary sensory neurones. There is also evidence that ATP, acting on different of its receptors in the central nervous system, may be important in mediating

peripheral pain sensations. Thus, ATP, acting through the glial P2X7 receptor, exerts a wide-ranging influence on spinal neuronal activity following a chronic injury. There is evidence that antagonism of the P2X7 receptor may, in turn, modulate central sensitisation and produce anti-nociception in animal models of pathological pain (McGaraughty et al., 2007). Moreover, it has also been suggested that antagonism of spinal P2X3/P2X2/3 receptors results in an indirect activation of the opioid system to alleviate inflammatory hyperalgesia and chemogenic nociception (McGaraughty et al., 2005).

2.10.3. Extracellular ATP

In the periphery, ATP can be released as a result of tissue injury, visceral distension, or sympathetic activation (Chizh and Illes, 2000). Human plasma contains ATP, independent of platelet dense body release, which is subject to diurnal variation (Ryan et al., 1996), while ATP is also released as a neurotransmitter in non-pathological conditions. These sources of ATP obviously do not result in the experience of pain in humans, or of pain-like behaviour in mammals. Were it otherwise, individuals would, in normal conditions, be subjected to pain sensations in the absence of an appropriate noxious stimulus. It is apparent, therefore, that the extracellular ATP which acts as a noxious stimulant is sourced elsewhere, most obviously from cells damaged in tissue injury. The concentrations of ATP within the cytoplasm of lysed cells (>1 mM) are sufficient to excite P2X receptors (North, 2003). The release of ATP into the extracellular environment is inevitable on the occurrence of tissue damage and inflammation. ATP released from mechanically lysed cells results in a direct excitation of P2X receptors on sensory neurones *in vitro* (Cook and McCleskey, 2002). Of course, in the abnormal circumstances resulting

from tissue damage and inflammation, ATP discharged from neurones may conceivably combine with released cytosolic ATP to contribute to the experience of pain sensations.

2.10.4. ATP contributes to inflammatory pain

Even low concentrations of ATP evoke action potentials and large inward currents in pain-sensing and stretch-sensing neurons (Cook et al., 1997). Souslova and colleagues (2000) found that ablation of the P2X3 gene in mice results in the loss of rapidly desensitizing ATP-gated cation currents in dorsal root ganglion neurons, while the responses of nodose ganglion neurons to ATP show altered kinetics and pharmacology resulting from the loss of expression of P2X2/3 heteromultimers. Behavioural responses of these knock-out mice to noxious mechanical and thermal stimuli are also normal, except that formalin-induced pain behaviour is reduced. However, deletion of the P2X3 receptor causes enhanced thermal hyperalgesia in chronic inflammation. In addition, although dorsal horn neuronal responses to mechanical and noxious heat application are normal, P2X3-null mice are unable to code the intensity of non-noxious 'warming' stimuli (Souslova et al., 2000). More recently, Cockayne and co-workers confirmed that neurons from P2X2 null mice and P2X2/P2X3 mull mice exhibit reduced pain-related behaviours in response to intraplantar injection of formalin. They also showed that these knock-out mice have reduced urinary bladder reflexes and decreased pelvic afferent nerve activity in response to bladder distension (Cockayne et al., 2005).

Iontophoretic delivery of ATP to the forearm skin of human volunteers consistently produces a modest burning pain, within 20 seconds of starting

iontophoresis, which is maintained for several minutes. Persistent iontophoresis of ATP leads to desensitisation within 12 minutes but recovery from this is almost complete by 1 hour later. Pain sensation is dose-dependent and is greatly potentiated under conditions of hyperalgesia (Hamilton et al., 2000). In accord, inflammation also potentiates the effect of ATP in mediating pain-like sensations in the rat (Hamilton et al., 1999). Inflammation causes a two-fold to three-fold increase in both ATP-activated currents, alters the voltage dependence of P2X receptors, and enhances the expression of P2X2 and P2X3 receptors. The increase in ATP responses gives rise to large depolarisations that exceed the threshold of action potentials in inflamed DRG neurons (Xu and Huang, 2002). A selective non-nucleotide antagonist of P2X3 and P2X2/3 receptor-activation reduces thermal hyperalgesia in the CFA-induced model of chronic inflammatory pain in rat. However, it is ineffective in reducing nociception in animal models of acute pain, post-operative pain, and visceral pain (Jarvis et al., 2002).

ATP appears to be more generally implicated in mediating neuropathic pain. Acute systemic administration of a selective antagonist for the P2X3 and P2X2/3 receptors reverses nerve injury-induced tactile allodynia and thermal hyperalgesia (Jarvis et al., 2002). Moreover, the P2X3 receptor plays an important role in promoting heat hyperalgesia in a rat model of trigeminal neuropathic pain (Shinoda et al., 2007). Activation of cytosolic phospholipse A2 resulting from P2X3/P2X2/3 receptor-activation has been advanced as a key event in neuropathic pain (Tsuda et al., 2007b).

2.11. PROTONS AND OTHER CATIONS

2.11.1. Introduction

Protons (hydrogen ions) are potent activators of TRPV1 and they also affect neuronal excitability by activating acid-sensing ion channels. Cations also have the ability to activate TRPV1. The reduction in pH and local acidosis in inflamed tissues are potent contributors to pain and hyperalgesia.

2.11.2. High proton (low pH) concentrations result in pain

An increase in the local hydrogen ion concentration is a common accompaniment of inflammation consequent upon tissue damage or disease. It has long been known that low pH (down to 4.7) is commonly found in inflamed tissues and that acidic solutions are particularly painful when injected into the skin (Habler 1929; von Gaza and Brandi, 1926; Steen et al., 1996). Protons selectively induce lasting excitation of nociceptors in rat skin with sensitisation to mechanical stimulation *in vitro* (Steen et al., 1992). *In vivo*, a decrease in pH occurs immediately after plantar incision in rats and is sustained for at least 4 days. During the period of decreased tissue pH, pain behaviours are evident, with these diminishing when the tissue pH returns to normal. The fall in pH is localised at the incision site and does not extend to areas surrounding the incision. However, the magnitude of the pH change varies between tissues (Woo et al., 2004). Continuous administration of low pH buffered solutions into human skin causes dose-dependent sustained pain and hyperalgesia to mechanical stimulation (Steen and Reeh, 1993). Inflammatory mediators potentiate the pain induced by experimental tissue acidosis. Thus, continuous infiltration of human forearm skin with a phosphate buffered electrolyte solution of pH 5.2 results in constant ongoing

pain. Subsequent injections of an acidic combination of BK, 5-HT, HIS, PGE2 into the same (acidotic) forearm and injections of a non-acidic combination of the same inflammatory mediators in the other forearm produces a dose-dependent, transient burning pain in both arms which is markedly more intense and prolonged in the acidotic skin (Steen et al., 1996).

2.11.3. High cation concentrations result in pain

Acute pain-related behaviour may be evoked by elevated ionic strength. Intraperitoneal injection of $MgSO^4$ evokes writhing responses in mice, while salt (NaCl), when applied to injured tissue, evokes a burning pain sensation similar to that evoked by capsaicin, heat, or extracellular protons. Extracellular cations, therefore, can result in acute burning pain sensation (Ahern et al., 2005).

2.11.4. Proton activation of TRPV1

Protons are able to activate the TRPV1 ion channel at pH below 6.5. Hence, minor reductions in pH below (neutral) 7.4 do not, as such, activate this ion channel. Capsaicin-binding, the temperature threshold for activation of the ion channel, and channel-gating are all affected by pH. Lowering pH enhances the apparent binding affinity of capsaicin, and lowers the heat threshold for activation of the channel. It also promotes the occurrence of long openings and short closures, and stabilises at least one of the open conformations of the channel. Moreover, capsaicin-binding and protonation of the channel interact allosterically, where the effect of one can be offset by the effect of the other (Ryu et al., 2003). Jordt and colleagues thought that protons modulate TRPV1 activity by interacting with specific amino acid residues on the extracellular surface of the channel protein (Jordt et al., 2000). The response of

TRPV1 to protons may be the result of at least two different mechanisms, namely, first, activation of the channel and, second, potentiation of the currents generated by an already activated channel (Ryu et al., 2003). These mechanisms appear to be distinct and separate although originating at the site where protonation is initiated. Mutations at the extracellular E648 residue selectively abrogate proton-evoked channel activation without diminishing the channel's responses to other noxious stimuli. However, while mutation at this site blocks proton-evoked activation, it does not affect proton-evoked potentiation. Another extracellular residue, E600 in the region linking the fifth transmembrane domain with the putative pore-forming region of the channel, constitutes the key regulatory site involved in the dynamic potentiation of the TRPV1 response to capsaicin or heat. It has been suggested that this site could set the sensitivity to other noxious stimuli in response to changes in extracellular proton concentration (Jordt et al., 2000).

2.11.5. Cation activation of TRPV1

In addition to protons, excess positive charges carried by various other ions are also able to activate TRPV1. Ahern and colleagues showed that extracellular Na^{+}, Mg^{++}, and Ca^{2+} can directly gate the TPRV1 ion channel under extreme or pathophysiological concentrations. However, even discrete pathology may result in the generation of extracellular microenvironments which readily achieve concentrations of cations which are sufficient to directly gate TRPV1. For example, in bone, the $[Ca^{2+}]$ surrounding resorbing osteoclasts approaches 40 mM (Silver et al, 1998). A yet more significant effect of extracellular cations may occur under normal physiological conditions where millimolar increases in cation concentrations (1-5 mM) are sufficient to sensitise TRPV1 to various ligands, including capsaicin, AEA,

and NADA. Site E600 and, to a lesser degree, site E648, are proposed to mediate cation activation of TRPV1, whereas site E648 is proposed to selectively support cation sensitisation of agonist-evoked responses (Ahern et al., 2005). This is the converse of the response previously observed by Jordt and colleagues with protons, in which E600 mediates proton sensitisation of agonist-mediated currents (Jordt et al., 2000). It is also proposed that divalent cations are more potent agonists than protons, because they impart a greater net positive charge at their binding sites (Ahern et al., 2005).

2.11.6. ASICs mediate pain sensations from both minor and TRPV1-sensitive reductions in pH

Acid-sensing ion channels (ASICs) are activated by extracellular protons. In the periphery, they contribute to the excitation of primary sensory neurons when exposed to an acid solution, including that comprised in an acidic microenvironment. ASICs are H^+-gated Na^+ channels which belong to the degenerin/epithelial sodium (Deg/ENaC) superfamily of ion channels (Waldmann et al., 1997). Six different members of the ASIC subfamily have been cloned (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4), which are encoded by four genes. ASIC1b and ASIC2b are splice variants of ASIC1a and ASIC2a (Bassler et al., 2001). All ASICs -- with the exception of ASIC4 -- are expressed in sensory neurons of the dorsal root ganglion. Homomeric acid-sensing ion channel 1 (ASIC1) can be activated by extracellular H^+ in the physiological pH range. Extracellular, divalent cations like Ca^{2+} and Mg^{2+}, as well as the polyvalent cation spermine, shift the steady-state inactivation of ASIC1a and ASIC1b to more acidic values. This leads to a potentiation of the channel response and is due to a stabilization of the resting state. ASIC1b is an effective sensor of transient H^+ signals during slight acidosis and, in addition to

alternative splicing, interaction with divalent and polyvalent cations extends the dynamic range of ASIC H^+ sensors (Babini et al., 2002). ASIC2b (a splice variant of ASIC2a) is acid-insensitive (Smith et al., 2007b).

Studies of ASIC3 in rat suggest that protons open ASIC3 by speeding release of Ca^{2+} ions from a high-affinity binding site on the extracellular side of the pore. The bound Ca^{2+} ions block permeation and the channel conducts when multiple H^+ ions relieve this block (Immke and McCleskey, 2003). Consistent with this hypothesis, there is evidence that activation of ASIC1 (in fish) is mediated by displacement of Ca^{2+} from the extracellular domain either by reducing the concentration of extracellular Ca^{2+} or by competing with protons for the same binding sites (Zhang et al., 2006). ASIC4 – where it is expressed -- appears to perform a regulatory function in relation to acid-sensitive ASICs. Transfected ASIC4 is targeted to the plasma membrane in CHO-K1 cells, where it associates with ASIC1a and down-regulates exogenous ASIC1a expression. ASIC4 performs a similar function with ASIC3 in similar circumstances (Donier et al., 2008).

After activation in the presence of an extracellular acidic environment, ASICs become desensitised over time, although desensitisation slows as temperature decreases (Askwith et al., 2001). The rate of desensitisation is affected by mutations on, or near, the first transmembrane domain (Coric et al., 2003; Pfister et al., 2006). Cushman and colleagues have shown evidence of a specific conformation change in extracellular domain of ASIC3 associated with desensitisation (Cushman et al., 2007).

During conditions that normally induce steady-state desensitisation, exogenous and endogenous arginine-phenylalanine-amide-related peptides profoundly enhance

ASIC1a activity. The human ASIC1a requires more acidic pH to undergo steady-state desensitisation compared with mouse ASIC1a. Moreover, steady-state desensitisation of human ASIC1a is also affected by a greater number of peptides compared with mouse ASIC1a. Mutation of five amino acids in a region of the extracellular domain changes the characteristics of human ASIC1a to those of mouse ASIC1a (Sherwood & Askwith, 2008).

2.11.7. ASICs as mediators of inflammatory pain

ASIC expression is increased in inflammatory conditions. This increase is contributed to by the pro-inflammatory mediators NGF, serotonin, interleukin-1, and bradykinin. A mixture of these mediators increases ASIC-like current amplitude on sensory neurons as well as the number of ASIC-expressing neurons and leads to higher sensory neuron excitability (Mamet et al., 2002). Arachidonic acid potentiates the currents carried by ASIC1a and ASIC3 in rat dorsal root ganglion neurons (Smith et al., 2007b). Acidic microenvironments may be created by osteoclasts in bone disorders with increased osteoclastic bone resoprtion. The resulting hyperalgesia is mediated, in part at least, by up-regulation of ASICs' expression (Nagae et al., 2007). Three ASIC isoforms -- ASIC1a, ASIC2a, and ASIC2b -- are expressed at a high level in dorsal horn neurons of rat spinal cord. Peripheral CFA-induced inflammation results in increased expression of both ASIC1a and ASIC2a in dorsal horn neurons (Wu et al., 2004). Following formalin injection, ASIC1 null mice and ASIC2 null mice show enhanced pain behaviour, predominantly in the second phase of the test. However, this does not occur with ASIC3 null mice (Staniland & McMahon, 2009). Nitric oxide (NO) has a direct external effect on acid-sensitive ASIC ion channels and potentiates their activity (Cadiou et al., 2007). ASIC1a and ASIC2a are the most

abundant ASICs in mouse adult spinal cord and are co-expressed by most neurons throughout all the laminae (Baron et al., 2008). There is evidence that increased ASIC activity in spinal dorsal horn neurons promotes pain by central sensitisation (Duan et al., 2007).

Approximately 60 per cent of rat cutaneous sensory neurons express ASIC3-like currents. Moderate pH, either alone or in conjunction with hypertonicity and arachidonic acid, increases neuronal excitability and results in pain which is suppressed by a specific blocker of ASIC3. Blockade of ASIC3 and *in vivo* knockdown of this ion channel with a specific siRNA have potent analgesic effects against primary inflammation-induced hyperalgesia in rat (Deval et al., 2008). In mice, ASIC3 immunoreactivity is found in 31 per cent of knee joint afferents, but, after joint inflammation due to carrageenan-induced arthritis, ASIC3 immunoreactive neurons increase in number by about 50 per cent (Ikeuchi et al., 2009). ASIC3 is also associated with hyperalgesia in response to hind-paw injection with acetic acid of rats with the L5 SNL model of neuropathic pain (Omori et al., 2008).

The extent of the respective roles performed by ASICs and TRPV1 ion channels in mediating acid-induced pain varies between species. Thus, there are significant differences between rat and mouse in the contribution of TRPV1 and ASIC subunits to proton sensitivity of sensory neurons (Leffler et al., 2006). It may also be the case that the extent of the respective roles performed by ASICs and TRPV1 in mediating acid-induced pain varies between tissues and, indeed, within tissues (Sugiura et al., 2007). ASIC expression may also be affected by tissue damage. Thus, gastric ulcers alter the properties of acid-elicited currents by increasing pH sensitivity and current

density and changing current kinetics in gastric dorsal root ganglion neurons (Sugiura et al., 2005).

The TRPV1 ion channel is modulated by acid at lower pH values than ASICs (Tominaga et al., 1998; Jones et al., 2004). There is evidence that, in humans, TRPV1 plays a relatively minor role in signalling cutaneous acid induced pain of moderate intensity, with ASICs being the main mediators of pain in that context. However, it may well be that TRPV1 plays a more prominent role in more acidic conditions (Jones et al., 2004). In situ hybridization studies suggest that ASIC1a receptors are expressed in 20-25 per cent of (mostly small diameter) DRG neurons in rat; ASIC1b are expressed by 10 per cent, and ASIC3 by 30-35 per cent, of small-to-medium diameter DRG neurons. ASIC1a and ASIC1 are essentially found in distinct populations of DRG neurons. There is some overlap between ASIC1 and ASIC1b, respectively, with ASIC3 channels. ASIC1a is strongly expressed in 40-45 per cent of TRPV1 positive neurons. ASIC1b is very rarely found in neurons expressing TRPV1, but 30 per cent of TRPV1 positive neurons express ASIC3 receptors. Approximately 13 per cent of TRPV1 positive neurons also express both ASIC1a and ASIC3. (Ugawa et al., 2005).

2.11.8 Proton-sensing G-protein-coupled receptors (GPCRs)

Proton-sensing GPCRs have been identified in primary sensory afferents but the extent to which they contribute to neuronal excitation and peripheral nociception remains unclear. These GPCRs were initially identified as subserving other functions and it was only more recently discovered that, in addition, they are capable of being activated by certain extracellular proton concentrations (pH). OGR1 (ovarian cancer

G-protein-coupled receptor 1) and GPR4 (G-protein-coupled receptor 4) were first reported as proton-sensing GPCRs (Ludwig et al., 2003), followed by G2A (Murakami et al., 2004), and TDAG (T cell death-associated gene 8) (Wang et al., 2004; Ishii et al, 2005). The members of this GPCR group share 40 to 50 per cent homology with each other and sense extracellular protons (i.e., pH) through histidine residues of their receptors to stimulate a variety of intracellular signalling pathways through several types of hetero-trimeric G-proteins (Tomura et al., 2005). Acidification of the extracellular environment significantly increases the constitutive activities of OGR1, GPR4, and TDAG8, but not G2A, as measured by production of IP or cAMP (Radu et al., 2004). These proton-sensing GPCRs are all fully activated at pH 6.4-6.8 *in vitro* (Huang et al., 2007). GPCRs of the OGR1 family are widely expressed in neuronal and non-neuronal tissues in mouse. Their transcripts are expressed in mouse DRG and most (75-82 per cent) are present in small-diameter nociceptive neurons. Approximately 31 to 40 per cent of all DRG neurons express at least two proton-sensing GFCRs. Moreover, in ASIC3 null mice, gene expression of proton-sensing GPCRs is also altered (Huang et al., 2007).

The fact that proton-sensing GPCRs are found in DRG neurons indicates that these receptors are localised to structures which are relevant to the induction of peripheral nociception (Huang et al., 2007). The inflammatory agents capsaicin, carrageen, and CFA alter the expression of proton-sensing GPCRs in mouse DRG, each to a different extent. The expression of TDAG8 is increased 24 hours after CFA injection. Indeed, the number of DRG neurons that express both TDAG8 and TRPV1 is also increased. Moreover, TDAG8 activation sensitises the TRPV1 response to

capsaicin leading to the suggestion that TDAG8 may be involved in CFA-induced inflammatory pain through regulation of TRPV1 function. (Chen et al., 2009)

2.12. CYTOKINES

2.12.1. Introduction

"Cytokine" is a generic term which embraces many different kinds of cell signalling molecules. Thus, "lymphokines" and "monokines" are signalling molecules made by lymphocytes and monocytes, respectively. "Chemokines" are signalling molecules which possess chemotactic properties, while interleukins are cytokines made by one leukocyte which act on other leukocytes. Cytokines may exhibit autocrine, paracrine, or endocrine, actions where they act on same cells that secrete them, or on nearby cells, or on more remote cells, respectively. Some cytokines are described as "pro-inflammatory," while other cytokines are described as "anti-inflammatory." Maintaining the balance between both types may be important for reducing inflammatory pain sensation. Cytokines are literally legion, and of those identified, some are important in mediating the pain signal in inflammatory conditions. In carrageenan-induced inflammation, although the related dorsal root ganglion shows elevated expression of only Scya2 (chemokine C-C motif ligand 2), the inflamed tissues exhibit 42 known cytokine and endothelial-related genes with elevated expression (Yang et al., 2007). Carageenan injection in the rat tail results in both thermal and mechanical hyperalgesia which coincides with significant increases in the concentration in the tissues of TNF-alpha, IL-1beta, IL-6 and CINC-1 (cytokine-induced neutrophil chemoattractant), peaking at 6 hours after injection (Loram et al., 2007a). However, the pattern of cytokine release during carrageen-induced inflammation differs in muscle inflammation from that seen in cutaneous

inflammation (Loram et al., 2007b). Up-regulation of IL-6, IL-8 and CCL2 gene expression contributes to the development of acute inflammation and inflammatory pain in an oral surgery model of tissue injury and acute pain (Wang et al., 2009).

However, it is important to note that not only may the pattern of cytokine release vary from one injury type to another, but, also, that the fact that cytokines are released as a result of tissue damage and ensuing inflammation does not make their action a condition precedent to the development of inflammatory pain. In other words, one, or more, cytokines may not be essential, given other inflammagens, to produce the inflammatory pain sensation. Thus, surgery to the rat tail results, within two hours of surgery, in mechanical hyperalgesia which lasts for 6 days. However, the tissue concentration of IL-1beta, IL-6, and CINC-1 increases only within 24 hours of surgery, and TNF-alpha increases only within 48 hours of surgery. Thereafter, cytokine concentrations remain elevated for four days (IL-1beta and IL-6) to 8 days (CINC-1, TNF-alpha) after surgery. Hence, secretion of these inflammatory cytokines is not essential for the development of post-operative hyperalgesia in this model of surgery (Loram et al., 2007c).

Several cytokines which have attained particular prominence in the literature in the context of inflammatory pain are considered here.

2.12.2. Growth-related oncogene (GRO/KC: CXCL1)

Neuronal inflammation *per* se, in the absence of nerve injury, causes large increases in Na^+ channel density, increased K^+ currents, and overall enhanced neuronal excitability (Wang et al., 2007) Levels of the cytokine growth-related oncogene (GRO/KC: CXCL1) are rapidly elevated in dorsal root ganglion cells in inflammatory pain models. Thus, when CXCL1 is incubated overnight with acutely isolated small diameter rat (adult) sensory neurons *in vitro*, both TTX-resistant and TTX-sensitive currents increase from 2- to 4-fold, in both IB4-positive and IB4-negative sensory neurons, without altered voltage dependence or kinetic changes. Two of the three types of Na^+ channels expressed show marked increases in expression, while CXCL1 cause a marked increase in neuronal excitability and a striking ability of neurons to fire repetitively (Wang et al., 2008). In IB4-negative acutely cultured adult rat small diameter sensory neurons, voltage-activated potassium current densities of both transient and sustained components increase after overnight incubation with CXCL1, without marked changes in voltage dependence or kinetics (Yan et al., 2009).

CXCL1, CXCL5, and LTB4 act sequentially in antigen-induced arthritis (a model of rheumatoid arthritis) to induce neutrophil migration. Elevated levels of CXCL1 and CXCL5 are also found in the synovial compartment of rheumatoid arthritis patients suggesting that these chemokines are indeed implicated in the pathogenesis of rheumatoid arthritis (Grespan et al., 2008)

2.12.3. Interleukin-1alpha

Interleukin-1alpha (IL-1alpha) regulates substance P expression and release in adult sensory neurons. When cultured dorsal root ganglion neurons of adult rat are exposed to either IL-1alpha or IL-1beta, the neurons, in each case, secrete substantially more substance P on stimulation with capsaicin than do neurons of control cultures. However, the addition of IL-1beta reduces the amount of substance P secreted in the presence of IL-1alpha on its own. Both of these cytokines alter substance P expression via the IL-1 receptor (Skoff et al., 2009).

2.12.4. Interleukin-1beta

CFA-induced inflammation in rats results in signification elevation in Il-1B and NGF in the inflamed tissues and of the peptides, substance P and calcitonin gene related peptide in the L4 dorsal root ganglion 48 hours after CFA injection (Safieh-Garabedian, et al, 1995). IL-1beta when injected intrathecally in mice with CFA-induced inflammation results in a marked increase in spinal COX-2 mRNA and persistent thermal hyperalgesia in mice. Moreover, the hypersensitivity resulting from the CFA-induced inflammation is significantly reduced by repeated intrathecal. pre-injection with an agent which sequesters endogenous IL-1beta (Narita et al., 2008)

IL-1beta is believed to be important in the pathogenesis of osteoarthritis. Up-regulation of IL-1beta initiates a cascade of intracellular events that can result in activation of proteinases, creation of an articular milieu which is tissue destructive, inhibition of anabolic pathways, and reduced synthesis of cartilage extracellular

matrix (Daheshia & Yao, 2008). Hyperalgesia, synovitis, and multiple biomarkers of inflammation are suppressed by Il-1 inhibition in a mouse model of gout arthritis (Torres et al., 2009).

2.12.5. Tumour necrosis factor-alpha

Tumour necrosis factor-alpha (TNF-alpha) induces hyperalgesia and also up-regulates IL-1beta. CFA-induced inflammation in rat results in significant elevation in the levels of TNF-alpha, Il-1beta and NGF in the inflamed paw (Woolf et al., 1997). TNF-alpha when injected intrathecally in mice with CFA-induced inflammation results in a marked increase in spinal COX-2 mRNA and persistent thermal hyperalgesia in mice. Moreover, the hypersensitivity resulting from the CFA-induced inflammation is significantly reduced by repeated intrathecal. pre-injection with an agent which sequesters endogenous TNF-alpha (Narita et al., 2008).

Peripheral administration of TNF-alpha induces thermal hypersensitivity in wild-type mice but not in TRPV1 null mice. In contrast, TNF-alpha application results in similar mechanical hypersensitivity in TRPV1 null mice and in wild-type mice, suggesting a role for TRPV1 in TNF-alpha-induced thermal, but not mechanical, hypersensitivity (Jin & Gereau IV, 2006). Again, acute application of TNF-alpha rapidly enhances TTX-resistant Na^+ currents in isolated mouse dorsal root ganglion neurons. This increase in Na^+ currents is substantially reduced in dorsal root ganglion neurons taken from TNF receptor 1 mull mice and is blocked by a p38 mitogen-activated protein kinase inhibitor. Mechanical hypersensitivity induced by peripherally applied TNF-alpha is also significantly reduced by the same inhibitor (Jin & Gereau IV, 2006). In rats with chronic antigen-induced arthritis in the knee joint

neutralization of TNF-alpha results in pronounced anti-nociceptive effects (Boettger et al., 2008). Inhibition of TNF-alpha reduces nociception in the first and second phases of formalin-evoked nociception in rats. Thus, when formalin is injected into the rat right upper lip, there is a marked reduction of the first and second phases of formalin-evoked nociception as result of systemic pre-treatment with the preferential inhibitor of TNF-alpha synthesis, thalidomide (Seadi Pereira et al., 2009).

2.12.6. Endothelins

Endogenous endothelin (ET) peptides are involved in mediating inflammatory, and other, pain conditions (Khodorova et al., 2009; Hans et al., 2008). Local injection of endothelin-1 results in nociceptive behaviour and excitation of C-fibres and A-delta-fibres in rats. Subcutaneous hind-paw injection of ET-1 in rat results in hind-paw flinching within minutes, which peaks at 30 minutes, and lasts for 60 minutes. This response is strongly inhibited by an endothelin-A receptor antagonist (Gokin et al., 2001; Davar et al., 1998. However, local injection of a selective endothelin-B receptor agonist inhibits ET1-induced nociceptive behaviour and excitation of primary afferents in a naloxone-sensitive manner. Hence, ET(A) and ET(B) receptors provide for dual modulation of acute nociceptive signalling in cutaneous tissues (Khodorova et al., 2002). ET-1 potentiates TRPV1 by a PKC-dependent mechanism in rat dorsal root ganglion cells *in vitro* and in HEK-293 cells co-expressing TRPV1 and the ET(A) receptor (Plant et al., 2007). Sensitisation of TRPV1, through an ET(A)-PKC pathway appears to contribute to ET-1-induced thermal hyperalgesia. Thus, intraplantar ET-1 injection produces thermal hyperalgesia in a dose-dependent manner. This thermal hyperalgesia is reduced by inhibition of the ET(A) receptor and PKC. However, inhibition of the ET(B) receptor does not

have this effect. In addition, ET-1-induced thermal hyperalgesia is significantly reduced in TRPV1 null mice when compared with wild-type mice (Kawamata et al., 2008). The increased in capsaicin-induced nociception caused by ET-1 is abolished in rat by prior administration of an ET(A) receptor antagonist, but not by an ET(B) receptor antagonist. However, the increase of capsaicin-induced hyperalgesia in rat caused by ET-1 is attenuated by both of these antagonists (Mottta et al., 2009).

Endogenous endothelins contribute to knee-joint inflammation in zymosan-induced arthritis in mice. Acting through ET(A) and ET(B) receptors, these cytokines modulate oedema formation, neutrophil recruitment, and production of inflammatory mediators (Conte Fde, 2008).

2.13. NEUROGENIC INFLAMMATION

2.13.1. Introduction

Neurogenic inflammation is the term employed to describe that inflammation which originates from the release of inflammatory neuromediators from the peripheral terminals of primary afferent fibres (Jansco et al., 1967; Szolcsanyi, 1988, 1996). These neuromediators in turn act on target cells in the periphery, such as mast cells, immune cells, and vascular smooth muscle cells, to produce inflammation, which is characterised by redness and warmth, swelling, and pain (Durnett Richardson & Vasko, 2002). The neuromediators released include calcitonin gene-related peptide (CGRP) and substance P (SP), which are found in a population of primary sensory neurons (Gibbins et al., 1985; Alvarez et al., 1988; Aoki et al., 2005). Neurogenic inflammation arises in two contexts. First, there exists a "pure neurogenic form of inflammation" identified by Jansco and colleagues (1967), the existence of which is "supported by the finding that antidromic stimulation of the sensory nerve evokes an inflammatory response." A group of inflammation-inducing agents, such as capsaicin, exert their inflammatory action by this neurogenic route (Jansco et al., 1967). Thus, intradermal capsaicin injection is employed experimentally to induce neurogenic inflammation, manifested as CGRP and substance P release, with resulting arteriolar vasodilation, plasma extravasation and pain. Such inflammatory response as is evoked by the pin-prick of the injection, as such, bears no relation to the almost immediate "pure neurogenic" inflammatory response which develops in the area of the injection. This "pure neurgogenic" inflammatory response is to be distinguished from neurogenic inflammation which is secondary to major inflammation which initially results from tissue damage or disease. The inflammation resulting from this

tissue damage or disease initiates a barrage of activity of primary afferent fibres which, in turn, results in antidromic activation of those afferents and the release by them of inflammatory neuromediators which enhance the excitability of those afferents as well as contributing further to the primary inflammation. Many inflammatory diseases, including arthritis, inflammatory bowel diseases, and migraine, are believed to comprise a neurogenic element. The subject of neurogenic inflammation has been recently extensively reviewed (Jansco, 2009).

2.13.2. Cellular mechanisms of neurogenic inflammation

Antidromic activation of primary sensory afferents, driven by dorsal root reflexes (DRRs), are involved in mediating the release of inflammatory neuropeptides from the peripheral terminals of primary afferents (Jansco et al., 1967; Lin et al., 1999; Willis, 1999; Lin et al., 2000; Lin et al., 2007; Li et al., 2008). Excessive depolarisation of the central terminals of primary afferents in the dorsal horn of the spinal cord goes to generate DRRs. Willis and colleagues (2006) have proposed that DRRs are triggered by an excessive primary afferent depolarisation (PAD) which results from the opening of Cl^- channels and the efflux of Cl^- ions from the synaptic terminals of primary afferents. The high gradient of Cl^- ions in primary afferent neurons is maintained by the $Na^+K^{+2}2Cl^-$ co-transporter (NKCC). The increased activity of the NKCC co-transporter contributes to the generation of DRRs and subsequently to the development of neurogenic inflammation (Valencia-de Ita et al., 2006). Sustained PAD also results in the phenomenon of central sensitisation of nociceptor specific neurons in the dorsal horn of the spinal cord. A sustained discharge of peripheral primary sensory afferents develops a capacity on the part of nociceptor specific neurons in the dorsal horn of the spinal cord to acquire low-

threshold inputs and enhances their excitability. This sensitisation can be reduced, or even reversed, by $GABA_A$ receptor antagonists, thereby implicating $GABA_A$ receptors in the development of central sensitisation (Garcia-Nicas et al., 2006). DRRs are also dependent on $GABA_A$ receptor activation of nociceptive specific neurons of the spinal dorsal horn (Lin et al., 2007). Hence, $GABA_A$ receptor activation appears to be important in developing that enhanced excitability of peripheral, and spinal cord, nociceptive neurons which is characteristic of neurogenic inflammation, and central sensitisation, respectively.

Local intradermal paw injection in the rat of capsaicin, CGRP, or substance P results in cutaneous vasodilation and oedema. Removal of DRRs, either by spinal dorsal rhizotomy, or by intrathecal administration of the $GABA_A$ receptor antagonist, bicuculline, reduces the *capsaicin-induced* vasolidation and oedema dramatically. Hence, DRRs are essential for driving the release of inflammation-inducing neuropeptides, at least in the context of neurogenic inflammation induced by intradermal capsaicin injection in rat. On the other hand, inflammation induced by injection of CGRP or of substance P is not significantly affected after DRR removal. A TRPV1 antagonist, administered peripherally, can almost completely abolish the capsaicin-evoked inflammation, whereas pre-treatment at the periphery with antagonists at the receptors for CGRP and substance P, respectively, only reduce the inflammation (Lin et al., 2007). When primary afferent nociceptive fibres are sensitised by capsaicin (manifested by an increase in afferent responses and a lowering of the response threshold to mechanical stimuli), the capsaicin-evoked sensitisation is significantly reduced after DRRs are removed by dorsal rhizotomy. Moreover, peripheral after-treatment with CGRP dose-dependently restores

capsaicin–induced sensitisation under dorsal rhizotomised conditions. Hence, it appears that CGRP released by primary sensory afferents in conditions of neurogenic inflammation "feeds back" on those afferents to enhance their state of excitation (Li et al., 2008).

2.14. CONCLUSION

TRPV1 initially appeared to hold out the promise of explaining much of the mystery of peripheral inflammatory pain and, equally importantly, of providing a target for scientists attempting to devise a drug which would relieve that pain. That promise has not been fulfilled. TRPA1 has newly emerged as a potent contributor to primary afferent excitation in inflammation, and the importance of the role of voltage-gated sodium channels in this regard is also becoming apparent. In any event, there appears to be considerable redundancy at the periphery in terms of the availability of mechanisms which are capable of responding to inflammatory agents and contributing to neuronal excitation and nociceptive signalling. Hence, "combination therapy," in which two, or more, mediating mechanisms are blocked may be a logical development in proposed analgesic therapies. However, this ignores the fact that the ongoing elucidation of the extent of the distribution of TRPV1 throughout the body and its involvement in multiple physiological functions suggests that TRPV1 antagonists are likely to offer more of a lethal, than a "magic," bullet for analgesia. It may well prove more rewarding in terms of analgesic effect if researchers were to focus their attention on neutralising certain of the agents of inflammatory pain since it has been shown that the excitability of primary afferents may be reduced by eliminating certain of the complex of inflammatory mediators which would otherwise be active (Maingret et al., 2008; Summer et al., 2008). In any event, it is surely a

sobering consideration that billions of dollars, of euros, and of pounds, have yet to achieve the success in pain relief achieved by Friederick Serturner, at the age of 20, in 1805, when he obtained pure morphine from the poppy plant, and by Felix Hoffman who, in 1897, rediscovered acetylsalicylic acid, which was subsequently patented and marketed as “Aspirin.”

CHAPTER 3

EXPERIMENTAL TECHNIQUES AND PROTOCOLS

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3.1. INTRODUCTION

The experimental work required for this dissertation was based on various techniques in molecular biology, extending over the areas of: cell culture; immunohistochemistry; agonist-activated cobalt uptake and imaging; and enzyme-linked immunosorbent assays (ELISAs). Here, the theoretical bases of these techniques are described, as are the protocols which were employed for their execution.

I do not address the techniques involved in sacrificing animals, administering injections, administering scalding-type burn injury, inducing and maintaining anaesthesia in animals, or spinal cord extraction, as colleagues kindly performed on my behalf those aspects of the experiments which involved exposure to animal fur.

3.2. CELL CULTURE TECHNIQUES

3.2.1. Requirements for culturing cells

Most kinds of plant and animal cells are able to survive, proliferate, and even express differentiated properties in a tissue culture dish in appropriate conditions. These conditions are quite elaborate. Most obviously, they require a suitable environment and a medium which contains the necessary nutrients to sustain cell life, as well as various other factors, such as growth factors, which stimulate cell proliferation, and transferrin, which carries iron into cells. These nutrients and other factors were traditionally supplied by constituting a medium comprising specified quantities of small molecules such as salts, glucose, amino acids, and vitamins, together with a poorly defined mixture of macromolecules in the form of horse serum,

or foetal calf serum, or a crude extract made from chick embryos. Such media are still used today for routine cell culture. Various serum-free, chemically defined, media have been developed which, in addition to the usual small molecules, include one, or more, specific proteins that most cells require in order to survive and proliferate in culture, such as growth factors and transferrin (Alberts et al., 1994). It must also be emphasised that the growth of cells in culture requires a controlled environment, especially in terms of temperature, oxygen and carbon dioxide.

Defined cell culture media generally consists of four basic chemical groups: amino acids; carbohydrates; inorganic salts; and vitamins. This balanced mixture provides the sources for protein synthesis, energy metabolism, cell membrane functions, and catalytic processes. Many of these basal media need to be supplemented with serum, or other appropriate proteins, for promoting cell growth. Historically, foetal calf serum, of 1 to 2 per cent volume of the media, has been used as a rich source of these complex protein requirements. However, the composition of serum varies from lot to lot, introducing a significant variability in products manufactured from this source.

Minimum Essential Media (MEM) is the most widely used media and was originally developed for primary cell cultures. It supports the growth of a broad spectrum of cells (including haematopoietic and bone marrow cells). It serves as the basis for other modified media, such as DMEM.

Dulbecco's Modified Eagle Media (DMEM) is a modification of MEM which has increased amino acids and vitamins and also contains non-essential amino acids, ferric

nitrate, and more glucose. It is suited for a broad range of mammalian cells and can be used as a basic component of speciality media.

Nutrient Mixture F-12 (Ham's Modification) has a higher level of amino acids, vitamins and trace elements. It was developed for cell lines but is suitable also for primary cell cultures.

3.2.2. Primary and secondary cell cultures

Primary cell cultures are cell cultures prepared directly from the tissues of an organism. Thus, we have used dorsal root ganglia neurons harvested from rat to investigate the effect, if any, of xenon in attenuating their response to capsaicin. Such cultures suffer from several drawbacks, most notably, the damage which the cells sustain while being harvested and the effect on their normal functioning of their being dissociated from the other tissues with which they profoundly interact *in vivo*.

Secondary cell cultures describe the cultures obtained from propagating cells derived from tissue, which introduces the next most fundamental aspect of cell culture technique, namely, the process of growing cells in a confluent monolayer by providing them with anchorage to pass "start" in their cell cycle. Dissociated cells plated on a dish in the presence of appropriate media adhere to the surface, spread out, and divide until a confluent monolayer is formed in which each cell is attached to the dish and contacts its neighbours on all sides. At this stage, the cells stop dividing, thereby exhibiting "density-dependent inhibition of cell division" unless they are cancerous ("transformed") cells which are not similarly restrained. Cells grown *in vitro* also generally exhibit "anchorage dependence of cell division" meaning that

they require to be able to adhere to the underlying plate surface in order to divide. A suitable surface to which propagating cells may adhere is constituted by the plastic surface of the culture-dish or T75 flask.

Many biochemical procedures require the isolation of a specific population, or populations, of cells. The required cells must be dissociated from their parent tissues and from other cell types. Disruption of the extracellular matrix and intercellular junctions that hold the cells together is typically achieved by the use of proteolytic enzymes, such as trypsin and collagenase, or with agents, such as EDTA, that bind, or chelate, the Ca^{2+} on which cell-to-cell adhesion depends. Large cells can be separated from small cells, and dense cells from light cells, by centrifugation. Yet another technique is based on the tendency of some cell types to adhere strongly to glass or plastic, which allows them to be separated from cells that adhere less strongly. A refinement of this latter technique relies upon the specific binding properties of antibodies. Antibodies that bind specifically to the surface of only one cell type in a tissue can be coupled to various matrices – such as collagenase, polysaccharide beads, or plastic – to form an "affinity surface" to which only cells recognised by the antibodies will adhere. The cells which are bound may then be recovered by gentle shaking, by treatment with trypsin to digest the proteins that mediate the adhesion, or, in the case of a digestible matrix (such as collagen), by degrading the matrix itself with enzymes, such as collagenase (Alberts et al., 1994).

3.2.3. Propagation techniques

Cell culture propagation techniques, in which mammalian cells are propagated *in vitro* for experimental use, are among the most remarkable techniques of modern

biological science. When cells from normal mammalian tissues are cultured in standard conditions, their ability to propagate is confined to a limited number of division cycles – about 50 for typical cells derived from humans. After this, they cease dividing in the process described as "cell senescence." Some cells are capable of avoiding cell senescence to divide indefinitely as "cell lines." These cells have undergone a genetic change that makes them effectively immortal. They will proliferate indefinitely in appropriate conditions and can be propagated as a cell line. Cell lines can also be developed from cancer cells which do not exhibit anchorage dependence and which proliferate to a very much higher density in a culture dish. Normal cells may similarly be "transformed" to constitute "transformed cell lines" by using a chemical or virus to alter their genetic make-up. The genetic uniformity of a cell line can be improved by cell cloning, in which a single cell is isolated and allowed to proliferate to form a large clone. A "clone" is any such collection of cells that are all descendants of a single ancestor. Such clones offer standardised cells which are ideal for comparative purposes over many experiments (Alberts et al., 1994).

The major advantages of these cloned cells reside in the efficiency with which they may be produced when compared with primary cultures and the consistency and reproducibility of the results which can be obtained from their use. The main disadvantage associated with the use of these cells is that these cells are atypical and their responses are not indicative of those of any other cell type. Moreover, after repeated passaging their characteristics may change and become quite different from those found in the starting population.

3.2.4. HEK293 technology

The HEK293 cell line is a clone of standardised cells which has proved to be particularly accommodating for reliably expressing DNA which has been artificially introduced into the cell in a process called "transfection." The HEK293 cell line is derived from human embryonic kidney (HEK) cells which were transformed by exposing human embryonic kidney cells to sheared fragments of adenovirus type 5 DNA (Graham et al., 1977); and the human-viral junctions of integrated adenovirus type 5 DNA in HEK293 cells have been cloned and sequenced (Louis et al., 1997). Remarkably, it has been found that these cells express significant amounts of protein and mRNA for three of the four major neurofilament (NF) proteins (NF-M, NF-L, and alpha-internexin), with, in addition, small amounts of NF-H, the fourth NF subunit. Subject to very few, apparently aberrant, exceptions, each of these NF proteins is found only in neurons. Hence, the presence of all of these proteins in HEK 293 cells is highly suggestive that these cell lines belong to the neuronal lineage. It has been suggested that the most likely explanation for this phenomenon is that Ad5 preferentially transforms rare neuronal lineage cells present in human and rodent kidney cultures. It has also been observed that the use of these cells as a non-neuronal control line in transfection studies is inappropriate given their many attributes of developing neurones and neuronal progenitor cells (Shaw et al., 2002).

HEK293 cells have limitations when employed as a model to examine the functioning of various cell types since they are essentially in a category of their own. However, they are highly efficient in expressing a reliable transient transfection. Moreover, they are relatively easy to manage and sustain in culture. They are,

therefore, useful where the behaviour of the cell itself is not the subject of study, but rather the protein which is expressed by the transfected DNA. Repeated passaging of these cells results in variations from their original characteristics. Hence, in this laboratory we do not use these cells after they have been passaged 20 times.

An important variant of the HEK293 cell line is the *HEK293T* cell line which provides longer temporal expression of the desired gene product.

3.2.5. Transfection

Transfection of animal cells typically involves opening transient pores in the cell plasma membrane to allow the uptake of material. Genetic material (such as supercoiled plasmid DNA or siRNA constructs), or even proteins, such as antibodies, may be transfected. Various proprietary transfection reagents, such as Lipofectamine, are widely used. Lipofectamine 2000 is a cationic liposome based reagent that provides high transfection efficiency and high levels of transgene expression in a range of mammalian cell types *in vitro* using a simple protocol. Optimum transfection efficiency and subsequent cell viability depend on a number of experimental variables, such as cell density, liposome and DNA concentrations, liposome-DNA complexing time, and the presence, or absence, of media components, such as antibiotics and serum (Dalby et al., 2004).

Tansfections may be transient or stable. Transient transfections do not require that the foreign DNA should be introduced into the nuclear genome of the host cell, with the result that the foreign DNA is lost at the later stage when the cells undergo mitosis. A stable transfection requires that the trasfected gene should remain in the

genome of the cell transfected and its daughter cells. To achieve a stable transfection, another gene must be co-transfected which gives the host cells some selection advantage, such as resistance to a certain toxin into their genome. Addition of the toxin, to which the transfected gene offers resistance, results in only those few cells being able to proliferate, while the others perish. Repeated application of this selection procedure results in only the cells with a stable transfection remaining for further cultivation. Geneticin, also known as G418, is a common toxin employed to produce stable transfection.

3.2.6. Cloning DNA of hTRPV1

DNA may be cloned by first obtaining a fragment of DNA which contains the gene of interest. In our experiments using HEK293 cells, this was hTRPV1. This DNA is then inserted into the purified DNA genome of a self-replicating genetic element – generally a virus or a plasmid. Plasmids are self-replicating extra-chromosomal circular DNA molecules, distinct from the normal bacterial genome. The terms "recombinant DNA technology," "DNA cloning," "molecular cloning," or "gene cloning" all refer to the same process: the transfer of a DNA fragment of interest from one organism to a self-replicating genetic element, such as a bacterial plasmid. The DNA of interest can then be propagated in a foreign host cell.

To "clone a gene," a DNA fragment containing the gene of interest is isolated from chromosomal DNA using restriction enzymes and then united with a plasmid that has been cut with the same restriction enzymes. When the fragment of chromosomal DNA is joined with its cloning vector in the laboratory, it is called a "recombinant DNA molecule." Following introduction into suitable host cells, the

recombinant DNA can then be reproduced along with the host cell DNA. Plasmids can carry up to 20,000 bp of foreign DNA. Besides bacterial plasmids, some other cloning vectors include viruses, bacteria artificial chromosomes *(BACs),* and yeast artificial chromosomes (*YACs*). *Cosmids* are artificially constructed cloning vectors that carry up to 45 kb of foreign DNA and can be packaged in lambda phage particles for infection into *E. coli* cells. BACs utilize the naturally occurring F-factor plasmid found in *E. coli* to carry 100 to 300 kb DNA inserts. A YAC is a functional chromosome derived from yeast that can carry up to 1 MB of foreign DNA. Bacteria are most often used as the host cells for recombinant DNA molecules, but yeast and mammalian cells also are used (Human Genome Project Information, 2008).

3.2.7. Protocols: generally

We employed HEK293T cells in the experiments which required cloned transformed cells. HEK293T cells are stored in liquid nitrogen until required for experiments. Hence, the first protocol describes the procedure for retrieving cells from liquid nitrogen for growth and propagation in a flask maintained in an incubator. Such cells multiply, and are then passaged so that certain of their number can be used for transfection, while the remainder are placed in storage in liquid nitrogen until required. Hence, the protocol for passaging cells is followed by that for transfection and that for preparing the cells for storage in liquid nitrogen.

Additional protocols describe the preparation of culture media and of poly-dl-ornithine-coated cover-slips and, finally, the preparation of a clone of hTRPV1.

3.2.7.1. Retrieving cells from liquid nitrogen

Generally, spray equipment with enthanol before introducing it into the fume cabinet, taking care, however, not to contaminate any cells or medium with such ethanol.

First Day

1. Place 35 mL of culture medium into a 50 mL Falcon tube.
2. Spray with ethanol and place the Falcon tube in an incubator at 37 degrees C.
3. Remove a vial of HEK293T cells from liquid nitrogen using a full face visor and glove protection.
4. Place the vial in 37 degrees C. water bath for 5–10 minutes, until completely thawed.
5. Then spray the vial with 70 per cent ethanol and wipe it down before placing it into the fume cupboard.
6. Remove the cell suspension from the vial and place it in the Falcon tube.
7. Then, add 10 mL culture media, drop by drop, for the first 1 mL volume. Slow addition of the culture media helps to reduce cell damage by slowing the environmental changes to which the cells must adjust.
8. Centrifuge at 1,100 rcp for 5 minutes at 26 degrees C.
9. Remove the supernatant, and gently tap the pellet left in the end of the tube.
10. Add 15 mL of culture medium to re-suspend the pellet.

11. Transfer the contents of this Falcon tube to a T75 flask, and label with date and passage number.
12. Place the flask in an incubator at 37 degrees C. in 5 per cent CO_2 overnight.

Second Day

1. Place 15 mL of culture medium in a 50 mL Falcon tube and then place the tube in a hot water bath to warm the medium to 37 degrees C.
2. Check the flask under a microscope to ascertain whether cells have attached to the flask surface.
3. Remove the existing medium in the flask and replace it with fresh 15 mL culture medium to remove DMSO present from the previous freezing process and to give the cells a further boost of nutrients to encourage growth.
4. Return the flask to the incubator for 3 days.

3.2.7.2 Passaging of cells

All steps in this technique must be performed in a fume-cabinet in aseptic conditions.

1. The fume-cabinet is cleaned.
2. Place 35 mL of culture media in a 50 mL Falcon tube and warm the same to 37 degrees C.
3. Defrost 1 x trypsin with EDTA (previously prepared in 5 mL aliquots).
4. Withdraw the flask with cells from incubation and remove the media therefrom.
5. Put 2.5 mL trypsin into the flask *gently*.
6. Wash the cells briefly (not more than 10 seconds) with this trypsin by rotating the flask 10 degrees either way.
7. Remove the "wash" medium.
8. Place 2.5 mL trypsin into the flask and incubate at 37 degrees C. until the cells appear to cease to adhere (which should be less than 5 minutes). Confirm under the microscope that the cells are no longer adherent. Tap the flask gently to dislodge the cells, if necessary.
9. In the fume-cabinet, place 2.5 mL media into the flask and tip gently to dislodge the cells.
10. Remove the whole contents of the flask and place the same in a a 50 mL Falcon tube.
11. Rinse out the flask with a further 2.5 mL of media and remove the contents of the flask into the Falcon tube.

12. Centrifuge for 5 minutes at 1,100 rcp (235 g.) at room temperature (26 degrees C.).
13. Remove the supernatant, leaving a pellet behind.
14. Tap the pellet and add 10 mL of fresh medium. Pippette up and down to disperse the cells in the fresh medium.
15. Take a haemocytometer and put 10 uL of cell suspension into each of its two chambers.
16. Count all viable cells in the centre grids under microscope. (Note: *viable cells* are those which possess a white halo.)
17. Add the number of cells found in the two centre grids areas and then divide by 2 to obtain the average number of cells per centre grids area.
18. Each centre grids area takes 0.1 uL of cell suspension. Therefore, one has obtained the average number of cells per 0.1 uL of cell suspension.
19. Then multiply by 10,000 to give the number of cells per 1 mL of cell suspension.
20. Each T75 flask should contain > 1.5 x 10(6) [1,500,000] cells and the amount in mL of cell suspension necessary to give this amount of cells must be calculated. This calculation is 1,500,000 divided by the number of cells per 1 mL of cell suspension.
21. Put 15 mL of culture medium in a fresh T75 flask (*less* if a large amount of cell suspension is to be added as the total volume in the Flask should not exceed approx. 20 mL).
22. Add the amount of cell suspension calculated to be necessary to comprise 1,500,000 cells.
23. Label the flask appropriately with cell type, passage number, and date.

3.2.7.3 Transfection and plating

The transfection procedure takes place over 2 days, as the cells require 24 hours to settle and begin to spread across the dish on their way to becoming a confluent monolayer in the dish.

Day 1

1. Take a 60 mL round dish.
2. Warm 5 mL of culture medium and put in the dish.
3. Add 1.5 million cells to the dish.
4. Incubate overnight at 37 degrees C.

Day 2

1. Check that the cells in the dish are between 50 per cent to 90 per cent confluent and return the dish to the incubator.
2. Prepare the following:

   On ice:

   a. DNA

   b. Lipofectamine

   c. Plus Reagent

   Heat: Warm 3 mL of DMEM.
3. Take 2 x 1.5 mL eppendorf tubes within the fume cup-board and label them (+) and (LIPO), respectively.
4. Add 290 uL DMEM to each of these tubes.
5. Then, to the (+) tube add:

a. 10.71 uL hTRPV1 (140 ng/uL)

b. 7.5 uL Plus Reagent (which is orange-labelled)

6. Incubate the (+) tube for 15 minutes at room temperature.

7. Add to the (LIPO) tube, 11.5 uL Lipofectamine (which is green-labelled).

8. After the (+) tube has been incubated for 15 minutes, add the contents of the (LIPO) tube to the (+) tube.

9. Incubate the (+) tube for a further 15 minutes at room temperature.

10. At the same time, remove the culture medium from the dish while leaving the cells therein.

11. Then, immediately, add 2 mL of DMEM to the dish *very gently*, drop by drop, and leave to incubate for 15 minutes at room temperature.

12. After the 15 minute incubation of the (+) tube, put the contents of this tube into the dish *very gently.*

13. Incubate for 3 hours in the incubator at 37 degrees C.

14. After the 3 hour incubation, add 5 mL of heated culture medium to the dish. *This stops the transfection which is now completed.*

15. The cells must now be plated.

16. Take 4 plates (16 wells) and "poly-dl-ornithine-coated" cover-slips which have been prepared in advance. (The plates should have their coverslips added before trypsin EDTA is added to the cells.)

17. Defrost 1 x trysin with EDTA (which have been previously prepared in 5 mL aliquots).

18. Remove the media from the dish.

19. Put 2.5 mL trypsin EDTA into the dish *gently.*

20. Wash the cells briefly (for not more than 10 seconds) with this trypsin EDTA by rotating the dish 10 degrees either way.
21. Remove the “wash” medium.
22. Place 2.5 mL trypsin EDTA into the dish and incubate for 5 minutes at 37 degrees C. Then confirm under the microscope that the cells are no longer adherent.
23. Again, in the fume-cabinet, place 2.5 mL culture media into the dish and tip gently to dislodge the cells.
24. Remove the 5 mL of solution in the dish and place the same in a Falcon tube.
25. Rinse out the dish with a further 2.5 mL of media and remove the contents of the flask into the Falcon tube.
26. Centrifuge for 5 minutes at 1,100 rcf (or 235 g.) at room temperatures (26 degrees C.)
27. Remove the supernatant, leaving a pellet behind.
28. Tap the pellet and add 5 mL of fresh media. Pippette up and down to disperse the cells from the pellet.
29. Take a haemocytometer and put 10 uL of cell suspension into each of its two chambers.
30. Count all viable cells in the centre grids under a microscope. (Note: *Viable cells* are cells which possess a white halo.)
31. Add the number of cells found in the two centre grids areas and then divide by 2 to obtain the average number of cells per centre grids area.
32. Each centre grids area takes 0.1 uL of cell suspension. Therefore, one has obtained the average number of cells per 0.1 uL of cell suspension.

33. Then, multiply by 10,000 to give the number of cells per 1 mL of cell suspension.

34. Each well must contain a minimum of 300 uL of cell suspension in culture medium.

35. Calculate on the basis of, say, 300 uL x 18 = 5,400 uL = 5.4 mL of culture medium in a Falcon tube. (Although cells are required only for 16 wells, allow sufficient suspension for 18 cells by way of precaution).

36. Each well must contain 25,000 cells in suspension. Therefore, calculate the cells required to provide 18 wells with 25,000 cells (namely, 450,000 cells).

37. Then, remove from the previously prepared 5.4 mL of culture medium sufficient of the same to allow for the addition of the volume of cell suspension which is required to provide the 18 wells with 25,000 cells (i.e., 45,000 cells). Next, add that volume of cell suspension to the remaining volume of culture media to give 5.4 mL cell suspension in culture media.

38. Pipette cell suspension in culture media into each of the 16 wells.

39. Label the wells appropriately with cell type, passage number, and date.

3.2.7.4. Preparing cells for liquid nitrogen

After cells have been passaged, there will generally be many more cells available than are the required for transfection. These cells can be preserved for future use by storing them in liquid nitrogen after having first prepared them appropriately for this purpose.

1. Obtain:
    a. 35 mL of culture medium (heated to 37 degrees C.)
    b. 5 mL of trypsin EDTA.
    c. 2 cryovials.
    d. Freezing medium
2. The freezing medium is comprised of:
    a. Culture medium (70 per cent by volume)
    b. DMSO (10 per cent by volume).
    c. Foetal calf serum (20 per cent by volume)
3. Thus, to make 1 mL of freezing medium add:
    a. 0.7 mL of culture medium
    b. 0.1 mL of DMS0
    c. 0.2 mL of foetal calf serum.
4. The amount of freezing medium required depends on the number of cryovials because each cryovial needs 1.5 mL of freezing medium.
5. Obtain the cell suspension intended for preservation from the flask by using trypsin EDTA to dislodge the cells as in the process of passaging those cells.

6. Defrost 1 x trypsin with EDTA (previously prepared in 5 mL aliquots).
7. Withdraw the flask with cells from incubation and remove the media therefrom.
8. Put 2.5 mL trypsin EDTA into the flask *very gently.*
9. Wash the cells briefly (for not more than 10 seconds) with this trypsin EDTA by rotating the flask 10 degrees either way.
10. Remove the "wash" medium
11. Place 2.5 mL trysin EDTA into the flask and incubate for 5 minutes at 37 degrees C. Then confirm under the microscope that the cells are no longer adherent. Tap gently to dislodge the cells, if necessary.
12. In the fume-cabinet, place 2.5 mL media into the flask and tip gently to dislodge the cells.
13. Remove the whole contents of the flask and place the same in a Falcon tube.
14. Rinse out the flask with a further 2.5 mL of media and remove the contents of the flask into the Falcon tube.
15. Centrifuge for 5 minutes at 1,100 rcf (or 235 g.) at room temperature (26 degrees C.)
16. Remove the supernatant, leaving a pellet behind.
17. Tap the pellet and add 10 mL of fresh media. Use the pipette repeatedly on the contents of the Falcon tube to disperse the cells within the culture medium.
18. Take a haemocytometer and put 10 uL of cell suspension into each of its two chambers.

19. Count all the viable cells in the centre grids under a microscope. (Note: *viable cells* are cells which possess a white halo).
20. Add the number of cells found in the two centre grids areas and then divide by 2 to obtain the average number of cells per centre grids area.
21. Each centre grids area takes 0.1 uL of cell suspension. Therefore, one has obtained the average number of cells per 0.1 uL of cell suspension.
22. Then multiply by 10,000 to obtain the number of cells per 1 mL of cell suspension.
23. Each cryovial must contain at least 4 x 10(6) and not more than 10 x 10(6) cells.
24. Therefore, the amount in mL of cell suspension necessary to give this amount of cells (4 x 10(6)) must be calculated.
25. Make a decision on the number of cryovials that it is intended to put into liquid nitrogen and the amount of cells that each cryovial should contain, having regard to the total amount available in the cell suspension.
26. Calculate the volume of cell suspension necessary to provide the required number of cells for the cryovials required and put that volume of cell suspension into a Falcon tube.
27. Centrifuge for 5 minutes at 1,100 rcf (or 235 g.) at room temperature (26 degrees C.).
28. Remove the supernatant, leaving a pellet behind.
29. Tap the pellet and now add the volume of freezing medium necessary to provide 1.5 mL of freezing medium per cryovial.
30. Use the pipette repeatedly on the contents of the Falcon tube to disperse the cells within the freezing medium.

31. Add 1.5 mL of the cell suspension in the Freezing medium to each cryovial.
32. Label each cryovial appropriately with cell type, passage number, date, and number of cells.
33. Put the cryovials at:
    a. 4 degreees C. for 20 minutes; and then
    b. – 20 degrees C. for 15 minutes.
34. Wrap each vial in cotton wool and place in – 80 degrees C. for two hours or overnight.
35. Then place the vials in the liquid nitrogen container, ensuring that the vials are in vapour phase. Note the basket and rack number and note details of the cells put down in the appropriate file.

3.2.7.5. Preparation of cell culture medium

In the fume-cabinet, in aseptic conditions, add to a 500 mL bottle of "Minimum Essential Medium" the following:

1. Penicillin streptomycin solution 10 mL.
2. L-Glutamine 5 mL
3. "MEM Non-Essential Amino Acids" 5 mL
4. Foetal bovine serum 50 mL.

3.2.7.6. Preparation poly-dl-ornithine-coated cover-slips

Polyamino acids facilitate the attachment of cells and proteins to solid surfaces in biological applications. In cell cultures normal attachment, growth, and development of many cell types are dependent on attachment factors and extracellular matrix components. While some cells are able to synthesize these components, others require an exogenous source, particularly when grown in serum-free culture. Sterile, cell culture-tested, polyamino acids facilitate attachment, cell spreading, growth, morphology, differentiation, and motility of cells. In histochemical applications both polymers of D- and L-lysine are used to coat slides to promote attachment of cells.

1. Obtain a batch of 100 cover-slips labelled “100 pcs Cover Glass 13 mm Borosillicate Glass.
2. Put these in a 50 mL Falcon tube and place sufficient ethanol in the Falcon tube to cover them (15 mL).
3. Rotate the Falcon tube in a rotating machine for 10 minutes.
4. Remove the ethanol and replace with 25 mL of pure distilled water.
5. Rotate the tube 10 times by hand.
6. Repeat the pure distilled water wash and hand rotation two more times.
7. Then place sufficient pure distilled water in a Falcon tube to reach the 15 mL level (including the volume occupied by the cover-slips).
8. Defrost a vial of poly-dl-ornithine.
9. Add 300 uL poly-dl-ornithine to the Falcon tube.
10. Rotate, using the rotating machine, for at least 1 hour.

3.3. IMMUNOHISTOCHEMISTRY

3.3.1. Introduction

Immunohistochemistry combines anatomical, immunological and biochemical phenomena to enable specific proteins to be identified by means of a specific antigen/antibody reaction tagged with a visible label. This technique enables the distribution and localisation of specific proteins within a tissue to be visualised. It involves staining the cells by means of antibodies against the target antigen. This first antibody can be labelled but it is more common (and improves the visualisation) to use a second antibody directed against the first (an antiIgG). This second antibody is conjugated either with fluorochromes or appropriate enzymes or colourimetric reactions or gold beads (for electron microscopy) or with the biotin-avidin system so that the location of the primary antibody and, thus, the target antigen, can be recognised. The term "immunohistochemistry" is frequently used interchangeably with "immunocytochemistry." Strictly, "immunochemistry" refers to the use of this technique in the context of tissues, rather than individual cells to which the term "immunocytochemistry" applies. Thus, *immunohistochemisty* is the demonstration of a target antigen in tissue, or in a cell, *in situ* by detecting specific antibody-antigen interactions where the antibody has been tagged with a visible label.

Immunocytochemistry has been described as a broad-based and powerful method for analyzing specific biochemical structures and sequences at the cellular level, allowing the study of multiple cellular compartments, including the cell body, axons and dendrites of neurons. Even minute levels of protein can be visualised in cellular compartments and membranes. At the same, there is a serious issue relating to the specificity of the process. It can be quite difficult to prove target antigen specificity,

particularly in view of the potential for cross-reactivity. Another disadvantage resides in the fact that, generally, immunocytochemistry is not quantitative. There are a number of reasons for this including multiple epitopes, different affinities for different sizes and sequences of various protein versions, variable fixation, and variable tissue penetration by antisera (Stanley et al., 1995).

3.3.2. Antibody staining

Antibodies are serum glycoproteins, known as immunoglobulins (Igs), which are secreted by terminally differentiated B cells (leukocytes). In the vertebrate immune system, antibodies are produced as a defence against foreign substances. Antibodies may be used as biochemical tools to identify and locate specific cell components. Of the five classes of antibodies, IgGs are the predominant class produced in the immune response. *Polyclonal antibodies* can be made against any antigen by injecting the antigen (isolated cell constituent or molecule) into a host animal (e.g. goat, rabbit or mouse), harvesting the plasma, and removing clotting factors to produce serum containing the antibodies. Animals produce a highly heterogeneous mixture of antibodies in response to challenge by a foreign antigen. These different antibodies come from different antibody producing cells. Polyclonal antibodies have an advantage over monoclonal antibodies in that they are more likely to identify multiple epitopes of the target protein. An alternative is to use "monoclonal antibodies." Monoclonal antibodies are produced by fusing spleen cells with myeloma cells and growing the resultant hybrids in tissue culture. The hybrids are grown in a selective medium containing hypoxanthine and aminopterin (HAT), the original myeloma cells being unable to survive in this medium. The hybrids are sub-cultured to produce clones derived from individual hybrids and each clone produces a

single class of antibody. Thus, monoclonal antibodies consist of a single molecular species whose cross-reactivity can be well characterised. In contrast, conventional antisera consists of a heterogeneous population of antibodies of different molecular species showing complex cross-reactivity. In addition, the characteristics of the antibody population in sera varies from bleed to bleed and from animal to animal and the supply of high-affinity specific antibodies is frequently exhausted after one, or two, years (Cuello et al., 1983).

Where a *secondary antibody* is used, it must be generated against the immunoglobulins of the primary antibody source, e.g., if the primary antibody is raised in rabbit, then the secondary antibody could goat anti-rabbit. The optimal titer of both the primary and secondary antibody should be determined for each batch. The proper working dilutions for every antibody must be optimised for the system in which it is being employed The same system does not always work for every antibody. The product data sheets may be used as a guide for dilution series starting points. The optimal antibody dilution will be that which gives the strongest specific antigen staining with the lowest non-specific background.

3.3.3. Fixatives to retain morphology of tissues and cells

For immunohistochemical analysis, it is essential that the morphology of the tissues and cells be retained and that the antigenic sites be accessible. Tissue blocks or tissue sections are typically immersed into a fixative solution. Fixation is necessary to prevent artifactual diffusion of soluble tissue components, to arrest enzymatic activity, to avoid decomposition of the structure, and to protect the tissue against the deleterious effects involved with the various stages of the immunohistochemical

process. Fixatives help tissue preservation by preventing autolysis caused by lysosomal enzymes and to prevent any bacterial or fungal growth. At the same time, fixatives will often cross-link the tissue so that it can become difficult for the immunoassay reagents to penetrate the tissue. Four variables must be considered in determining the optimal fixation conditions for immunohistochemical localisation, namely: (i) preservation of tissue morphology; (ii) immobilisation of the antigen; (iii) preservation of antigen immunoreactivity; and (iv) adequate penetrability of tissue to the immunochemical reagents. The optimal fixation conditions with respect to the first two variables usually involve rapid and complete fixation, whereas the optimal conditions for the second two variables are best achieved with the lowest possible degree of fixation. Therefore, the most appropriate fixation conditions necessitate a compromise between competing considerations. Well-defined localisation with a low intensity of staining reaction are normally the symptoms of excessive fixation, since fixation tends to lower immunoreactivity and penetrability of immunoreagents. On the other hand, less than optimal fixation is usually associated with an intense reaction that is diffuse and poorly localised (Berod et al., 1981).

Formaldehyde-based fixatives stabilise tissues by reacting primarily with basic amino acids to form methylene cross-links. The number of methylene bridges formed depends on the concentration of formaldehyde, temperature, pH and time of exposure. Formaldehyde fixatives are relatively mild. However, they are difficult to reverse (Puchtler & Meloan, 1984). They are well tolerated by tissues and allow for good penetration. The disadvantages of using this type of fixative include shrinkage and distortion of the tissue and, therefore, the antigenic sites. Examples of formaldehyde-based fixatives include Bouin's solution and 10% phosphate buffered neutral

formalin. Formaldehyde is the most commonly used fixative in immuno-histochemistry because of its high degree of tissue penetration and its good preservation on immunoreaction, compared to glutaraldehyde. Formaldehyde fixation is highly pH dependent (Berod et al., 1981).

Glutaraldehyde is a dialdehyde that reacts with amino groups, sulfhydryl groups and possibly with aromatic ring structures. Fixatives containing glutaraldehyde are stronger cross-linkers of protein than formaldehyde. They penetrate tissue more slowly, causing extraction of soluble antigens and modification of the structure. Glutaraldehyde inhibits enzyme activity more than does formaldehyde, the inhibition increasing with the time in the fixative (Hopwood, 1967). Tissues that have been fixed with a glutaraldehyde-based fixative must be treated before the immunoassay with inert amine-containing molecules because free, unsaturated aldehyde groups are available to covalently link amine-containing moieties such as antibodies. The most efficient aldehyde blockers are ethanolamine and lysine. Glutaraldehyde-based fixatives include 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer and 5% glutaraldehyde in 0.1 M cacodylate and 1% sodium metabisulphite, pH 7.5-7.8 (Pierce Protein Research Products, 2008).

Precipitating fixatives include ethanol, methanol and acetone. They precipitate large protein molecules and are good for cytological preservation. They result in poor penetration and possibly incomplete fixation. They are not good for electron microscope work because they cause tissue shrinkage.

3.3.4. Antigen retrieval

Fixation alters the tertiary structure of proteins (antigens) with the result that they may not be detected by specific antibodies. Antigen retrieval agents reverse at least some of these changes and are particularly necessary when tissues are fixed in cross-linking fixatives. Approximately 85 per cent of antigens fixed in formalin require some type of antigen retrieval to optimise the immunoreaction. However, polyclonal antibodies are more likely to detect antigens than monoclonal antibodies in the absence of antigen retrieval agents. The two most common antigen retrieval techniques are enzymatic and heat-based, respectively (Ramos-Vara, 2005).

3.3.5. Inhibition of endogenous tissue components

Tissues, being complex biological systems, can contain materials that react with the various reagents used to develop an immunohistochemical stain. Cellular components such as enzymes and the vitamin, biotin, can result in unwanted signals, falsely indicating the presence of the target antigen. The immunoperoxidase method of signal detection is commonly used for the immunostaining of cells and tissues. Final analysis of this staining method is complicated by the presence of endogenous peroxidase and “pseudoperoxidase” activity in the cells and tissues. Peroxidase reacts with hydrogen peroxide to reduce the diaminobenzidine (DAB) substrate or other peroxidase substrates, resulting in non-specific staining of the tissue or false positives. To determine if there is endogenous peroxidase activity, the fixed tissue should be reacted with a peroxidase substrate, such as DAB. Any coloured precipitate that forms in the tissue from this treatment indicates endogenous activity. If a tissue is rich in peroxidase activity, an alternative enzyme label, such as calf intestinal alkaline

phosphatase (AP), can be used. Several methods have been devised to inhibit or destroy endogenous peroxidase activity after tissue fixation. The most common methods are 3% H_2O_2, or H_2O_2 in methanol. The activity of an antibody is not affected by the addition of this inhibitor. To ensure that the peroxidase inhibitor does not affect immunoreactivity of the primary antibody, apply the inhibitor after the incubation with the primary antibody and before incubation with the peroxidase conjugate (Pierce Protein Research Products, 2008).

3.3.6. Blocking of non-specific sites

Blocking the reactive sites in tissues is essential for the development of an immunohistochemical reaction. Normal serum is the most popular blocking agent for immunohistochemical staining. The principle is that non-immune serum from the host species of the secondary antibody is applied to the tissue at the beginning of the procedure and will adhere to protein-binding sites either by non-specific adsorption or by binding of specific, but unwanted, serum antibodies to antigens in the tissue. If a direct technique is being performed, the blocking serum should be from the host species providing the primary antibody (Pierce Protein Research Products, 2008).

3.3.7. Avidin-biotin system

Avidin is a 68,000 molecular weight glycoprotein with an extraordinarily high affinity for the small molecular weight vitamin, biotin. This affinity is more than one million times higher than that of antibody for most antigens. Hence, the binding of avidin to biotin (unlike antibody-antigen interactions) is essentially irreversible. In addition to this high affinity, the "Avidin/Biotin System" can be effectively exploited because avidin has four binding sites for biotin and most proteins (including antibodies and enzymes) can be conjugated with several molecules of biotin. The first step in detecting a primary antibody is the addition of a biotinylated, affinity-purified secondary antibody. The biotinylated secondary antibody can then be detected using: (i) a preformed macromolecular complex between avidin and biotinylated enzyme which still retains biotin-binding sites (the Vectastain ABC system); (ii) avidin covalently conjugated to enzymes; or (iii) avidin acting as a bridge to biotinylated enzymes (Vector Laboratories, 2004).

The ABC system has several advantages. It increases the enzyme label at the tissue antigen site and provides increased detection efficiency. It requires less primary antibody and involves a short assay time. Against this, some tissues may require blocking of endogenous biotin to avoid non-specific staining. Moreover, the ABC complex is large, and may hinder tissue penetration in some applications. If the avidin-biotin complex becomes too large to penetrate the tissue, an alternative method can be used, called the "labelled avidin-biotin (LAB)" method. This employs an avidin-enzyme conjugate (or streptavidin-enzyme conjugate) to detect the bound

biotinylated-primary antibody on the tissue section. This smaller complex allows better tissue penetration (Pierce Protein Research Products, 2008).

3.3.8. Colour of antigen-antibody reaction

The antigen-antibody reaction is not visible under the microscope unless a label is used. Enzymes are the most commonly used labels, especially horseradish peroxidase and alkaline phosphatase. Each enzyme has specific substrates required to produce a coloured precipitate. Thus, for horseradish peroxidase, 3,3'diaminobenzidine tetrachloride (DAB) is the most commonly used chromogen, giving a brown colour. It is insoluble in organic solvents, but is inappropriate when endogenous peroxidase activity is very high. Several chromogens are used for alkaline phosphatase which is recommended for immunocytochemistry or cytological specimens. The choice of counter-stain will depend mainly on the colour of the immune reaction. The counter-stain must produce enough contrast to avoid confusion with the chromogen precipitate. It should lightly stain the tissues, without interfering with the chromogen precipitate. One of the most widely used is haemotoxylin which stains blue (Ramos-Vara, 2005).

3.3.9 Protocol – Immunohistochemistry (using DAB)

Preparation of tissue

1. After completion of the experimental protocol, the animals are perfused transcardially with 4 per cent paraformaldehyde.

2. The lumbar spinal cord is recovered from each animal and placed in a solution of 80 per cent sucrose dissolved in PBS until the tissue sinks.

3. The spinal cord is then cut into 20 um sections.

Day 1: Application of primary antibody

1. Place the sections in a bath and rinse three times (by bathing for 5 minutes for each rinse) with phosphate-buffered saline (PBS).
2. Prepare a "blocking solution" using PBS + Triton X (0.3%) to which normal donkey serum has been added in the ratio 90:10.
3. Apply the "blocking solution" and leave for 30 minutes.
4. Surround the sections with 2 x 2 parafilm to create a "well" to hold the primary antibody.
5. Prepare the primary antibody in PBS-Triton in the required concentration and then apply it to the sections.
6. Place a cover-slip over the parafilm.
7. Allow the sections to incubate in the primary antibody overnight at room temperature.

Day 2: Application of secondary antibody

1. After incubation overnight, rinse the sections in water to remove the cover-slips.
2. Then rinse the sections three times in PBS, by allowing them stand in fresh PBS for 5 minutes for each rinse.
3. Prepare the secondary antibody in 1:200 PBS-Triton
4. Apply the secondary antibody to the sections and allow them to incubate in it for 30 minutes at room temperature.
5. Then prepare the ABC solution immediately in order to allow it to stand for 30 minutes before use.
6. Rinse the incubated slides with PBS three times, using 5 minute rinses in PBS.
7. Then rinse with Tris (0.01 m or 0.05 m)
8. Prepare DAB and apply.
9. Wait for the change in colour.
10. Terminate the reaction by applying Tris.
11. Rinse with PBS once by allowing the slides to stand in the PBS for 5 minutes.
12. Then rinse with water.
13. The sections are then dehydrated with ethanol (50%; 75%; 90%; 96%; absolute).
14. Bathe in xylene for at least 1 hour, and keep in xylene until ready to mount.
15. Apply the mounting medium and then the cover-slips

3.4. AGONIST-ACTIVATED COBALT UPTAKE

3.4.1. Introduction

The agonist-activated cobalt uptake technique is a method of quantitatively analysing the extent of non-selective cation channel activation in neurons and other cells. The cells are placed in cobalt in solution with the result that their extracellular environment includes divalent cobalt cations. When the cation channels which are the subject of investigation are opened on being stimulated by an agonist, these extracellular cobalt ions are drawn by their concentration and electrical gradients within the cell where they may be caused to become precipitated by exposure to a solution of ammonium polysulphide. The extent of the resulting intracellular staining offers a measure of the extent to which the ion channels in question have been activated by the agonist, or putative agonist. The intracellular staining can be assessed visually, using a microscope, which is adequate where the technique is employed only to determine whether there has been agonist activation of the channels in question. However, such visual assessment is inadequate where one seeks to investigate the existence, or otherwise, of gradations in staining referable to alterations of the conditions in which the agonist is applied. Hence, the development of a computer programme which measures on an arbitrary scale the extent to which individual cells absorb light (i.e., are discoloured). While the scale is arbitrary, it is, nevertheless, relative which is all that is required since the enquiry relates to the extent of comparative, rather than absolute, staining by precipitated cobalt ions.

This technique was developed by Pruss and colleagues (1991) who showed that activation of kainate receptors causes Co^{2+} influx into neurons, type-2 astrocytes, and

O-2A progenitor cells. Based on the pattern of responses to kainate, glutamate, and quisqualte, it was possible to discriminate between three functionally different kainate-activated ion channels (Pruss et al., 1991). The technique has since been widely used to label neurons (Nagy et al., 1993; Lorusso et al., 1998; Singh Tahim et al., 2005).

The TRPV1 ion channel is potently activated by capsaicin which is typically employed as an agonist to activate this channel. Hence, the extent of capsaicin activation of these ion channels in the presence, and absence, of various substances can be assessed using this technique. The standard protocol for cobalt-labelling involves rinsing the cells twice for 2 minutes in a normal buffer followed by incubation for 5 minutes of the cells in cobalt buffer, to which there has previously been added, sufficient capsaicin to allow for activation by capsaicin (of the required concentration) of TRPV1 ion channels expressed by the cells. The result is that the capsaicin, at the required concentration, activates TRPV1 ion channels expressed in the cells in the presence of extracellular cobalt ions in solution. These cobalt ions thereby gain ingress to the cells, *via* the opened TRPV1 ion channel pores, where they discolour the cytoplasm when they are precipitated in the presence of ammonium polysulphide. The cells so discoloured are usually referred to as having been "labelled" as TRPV1-activated cells. The precipitation is achieved after a brief rinsing of the cells in standard buffer followed by a 2 minute incubation in 0.12% ammonium polysulphide (Sigma).

The extent to which TRPV1 ion channels are activated when challenged with different concentrations of capsaicin can be determined by examining the uptake of cobalt by individual cells as evidenced by the discolouration of the cells when visualised by a light microscope attached to a CCD camera (Leica) and a PC running the QWin software package (Leitz, Wetzlar, Germany). Following background subtraction, the area of the cells and their optical density, measured on an arbitrary scale between 0 and 255, are measured in 100-150 randomly systematically chosen neurons on each coverslip. With these settings, greater optical intensity values represent light, labelled cells, while smaller optical intensity values represent dark, non-labelled cells.

The main concerns with the use of the cobalt uptake technique arise from the fact that the extracellular solution is not a physiological solution. In particular, it does not comprise Ca^{2+}; and, moreover, the concentration of sucrose in the buffer creates an hypertonic extracellular solution. Ca^{2+} is excluded from the buffer to prevent these ions from competing with Co^{2+} for entry into the cell *via* TRPV1 ion channels, while the amount of sucrose employed has been found necessary to maintain the cells in the buffer in a healthy condition. Nevertheless, these concerns are misplaced in the context of the purpose for which this technique has been employed in the present experiments. First, the absence of Ca^{2+} in the extracellular solution would be important if the objective of the technique were to measure the effects of ingress of Ca^{2+} into the cell. However, the purpose of the technique is to assess the relative ingress of Co^{2+} ions as evidenced by staining of the cells as a relative measure of activation of TRPV1. Second, an apparently more substantial objection is that the composition of the extracellular solution is such as to result in its gaining entry into

the cell along its concentration gradient. While this occurs to some extent, it does not result in bursting of the cells because when the cells are ultimately visualised under the microscope, there is no evidence of cell lysis – a finding which has been consistently made in all of the experiments performed in this laboratory. Moreover, we have specifically compared the diameter of capsaicin-responding cells after cobalt uptake with the diameter of similar cells taken from DRG ganglia maintained in physiological solution and found no difference in the diameters of the respective cell populations. The final objection is that the nature of the extracellular buffer may be such as to activate mechanoreceptors resulting in ingress of Co^{2+} *via* these channels when thus activated. However, this objection cannot stand with the fact that the cobalt staining is abolished in the presence of a capsaicin antagonist and does not occur in TRPV1-null mice. In the result, this technique is cheap, efficient, and entirely suitable for the limited purpose for which it has been employed in our experiments.

3.4.2. Preparation of buffers

3.4.2.1. Standard (Normal) Buffer

Standard (normal) buffer is prepared by adding to 1 L of distilled water: NaCl: 3.358 g; KCL: 0.3725 g; HEPES 2.833 g; glucose: 2.16 g; $MgCl_2$: 0.4066; sucrose: 47.5797 g. The pH should be 7.4. Bring the buffer to pH 7.4 by the addition thereto of sufficient NaOH to raise it to 7.3 on the pH meter after which it will increase of its own accord to 7.4.

3.4.2.2. Cobalt buffer

The "cobalt buffer" is prepared by adding 1.2 g $CoCl_2$ to 1 L of the previously prepared standard (normal) buffer.

3.4.3. Agonist: capsaicin

Stock capsaicin (Tocris, Bristol, UK) is diluted in H_2O and DMSO (50:50) to provide several capsaicin concentrations, e.g.: 10 nM; 30nM; 100 nM; 300 nM; and 1 uM. Previous experiments in this laboratory have demonstrated that DMSO alone, at concentrations in excess of 1:500, is capable of activating TRPV1 ion channels. Hence, the importance of limiting the concentration of DMSO employed. The DMSO is necessary because capsaicin is highly hydrophilic and cannot be added to water alone. Thus, it is added to DMSO and water in which it dissolves.

To confirm that DMSO is not participating in activation of TRPV1 ion channels, but is merely acting as a vehicle for delivery of the capsaicin to those receptors, DMSO is added to the standard cobalt chloride buffer (the "vehicle control").

3.4.4. Agonist-activated cobalt uptake protocol

1. Pre-heat a precision incubator to 37 degrees C.
2. Pre-heat to 37 degrees C. Falcon tubes comprising standard buffer and cobalt buffer, respectively.
3. Obtain several small vials, the number depending on the number of different dilutions of capsaicin (or other variations) to be employed in the experiment. An additional vial will serve as the "without capsaicin" control.
4. Put 2 mL of cobalt buffer in each of the small vials.
5. Put 1 uL of each capsaicin dilution in all, except one, of these vials.
6. Place all the vials in the precision incubator at 37 degree C.
7. Label appropriately the wells assigned to the various concentrations of capsaicin employed, plus the control application of buffer.
8. Then place a cover-slip containing cells in each of these wells.
9. Rinse the cells on their cover-slip in each well twice with incubated standard buffer by putting 0.5 mL of incubated standard buffer into each well. Remove the buffer using a pipette, and rinse again using the

incubated standard buffer. Then, pipette out the added buffer to complete the second rinse.

10. Now, put more incubated buffer in each well and incubate the cells in this buffer for 5 minutes at 37 degrees C., after which remove this buffer.

11. Put 500 uL of each concentration of capsaicin in all of the wells except that for control to which should be added instead 500 uL standard buffer.

12. Incubate for 5 minutes at 37 degrees C.

13. In the fume-cabinet, add 60 uL of ammonium polysulphide to 5 mL of incubated buffer.

14. Terminate the incubation after 5 minutes and remove the solutions from the wells.

15. Wash each twice with standard buffer (which has not been incubated beforehand.)

16. After the second wash, put ammonium polysulphide on the cover-slips and incubate at room temperature for 2 minutes, before removing the ammonium polysulphide.

17. Apply 70 per cent ethanol to the coverslips to fix the tissue.

18. Use glycerol to mount the cover-slips on slides.

3.4.5. Image analysis protocol

1. Run Leica QWin V3 programme on computer.
2. Switch on camera control.
3. Adjust shutters on the microscope. Left to middle. Right to out.
4. Condenser to “H”, Filter to “1”
5. Use maximum objective on the microscope.
6. Click “Eye” for live image.
7. Click “Mono”.
8. Capture image of “empty field”, i.e., no slide.
9. Go to “Measure” and, then, “Measure Interactive”.
10. Then draw several areas and check grey level values.
11. Set optical intensity of background grey level to above 200 and less than 250..
12. To obtain this optical intensity, return to “live image”, then reduce/increase light from the micorscope, or alter “exposure time” on the camera control. Then, capture image and measure again.
13. When the requited optical intensity is obtained, press “Subtract (Sub)” button on Function Keys of the cameral control box.
14. Next, place a slide in the microscope and adjust the light to obtain the detial of the images.

15. Then, “capture image”, save as “---“, and return to “live image”.

16. Obtain from 150 to 250 images from each cover-slip.

17. After all the images of a given cover-slip have been collected, copy the same to the Excel file as follows:

    a. in the window entitled “Manual Results”, got to “Edit” and click “Copy”;

    b. Then go to “Desk Top,” open Excel and in any column click “Paste” and then “Save”.

18. Before taking images of the next cover-slip, click on “Reset” in the “Measure Interactive” window to eliminate previous data.

3.5. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

3.5.1. Introduction

Enzyme-linked immunosorbent assay (ELISA) is a plate-based assay employed to search for the presence, and quantify the amount, of peptides, proteins, antibodies or hormones. This technology involves the antigen being immobilised to a solid surface whereupon it is complexed with an antibody linked to an enzyme. The conjugated enzyme activity is measured by incubation with a substrate to produce a measurable product. ELISAs are typically carried out in a 96-well polystyrene plate which can passively bind antibodies and proteins. Horeseradish peroxidise (HRP) and alkaline phosphatase (AP) are the enzyme labels which are usually employed. When the ELISA is performed with an HRP or AP conjugate, there is a large selection of substrates available. The assay sensitivity required and the device available for signal detection (spectrophotometer, fluorometer or luminometer) will determine the choice of substrate (Thermo Scientific Proteomics, 2009).

3.5.2. Sandwich assay

Immobilization of the target antigen can be achieved by direct adsorption to the assay plated. Alternatively, the target antigen can be immobilised using a “capture antibody” that has been attached to the plate. The target antigen is then detected using a labelled primary antibody or using a labelled secondary antibody. The so-called “sandwich assay” is the most effective ELISA assay type. Here, the target antigen is bound between two primary antibodies – the capture antibody and the detection antibody to provide an assay which is both sensitive and robust. In a sandwich ELISA, it is essential that the secondary antibody should be specific for the detection

of the primary antibody only and not for the capture antibody. Generally, this is achieved by using capture and primary antibodies from different host species. (Thermo Scientific Proteomics, 2009).

3.5.3. ELISA procedure

3.5.3.1. Coating the well with capture antibody

An assay microplate is chosen which has a minimum protein-binding capacity of 400 ng/cm^2 and a coefficient of variation value of protein binding which is < 5 per cent to limit deviation in values that should be identical in the assay results between wells and plates (Thermo Scientific Proteomics, 2009). Clear polystyrene flat bottom plates are used for colorimetric signals, while black or white opaque plates are used for fluorescent and chemiluminescent signals. Plate-coating with the relevant antibody is achieved through passive adsorption of the protein to the plastic of the assay microplate as a result of hydrophobic interactions between the plastic and non-polar protein residues. The most common method of coating plates involves adding a 2 – 10 ug/ml solution of protein dissolved in an alkaline buffer such as phosphate-buffered saline (pH 7.4) or carbonate-bicarbonate buffer (pH 9.4). The plate is left to incubate for several hours to overnight at 4 – 37 degrees C. Then, after removing the coating solution, blocking buffer is added to ensure that all remaining available binding surfaces of the plastic well are covered. Some proteins, especially antibodies, are best coated on plates at a concentration lower than the maximum binding capacity in order to prevent non-specific binding in later steps by a phenomenon called "hooking." "Hooking" results from proteins getting trapped between the coating proteins which prevents effective washing and removal of non-bound proteins. When hooking non-specifically traps detection primary and secondary antibodies, high

background signal results in reducing the signal to noise ratio and thus the sensitivity of an assay (Thermo Scientific Proteomics, 2009).

Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in sandwich ELISA systems. Monoclonal antibodies have an inherent mono-specificity toward a single epitope which provides for precision in both detection and quantitation of small differences in antigen. However, these antibodies are very expensive. The design of a sandwich ELISA must take account of the fact that the capture and detection antibodies must recognise two different non-overlapping epitopes. When the antigen binds to the capture antibody, the epitope recognised by the detection antibody must not be obscured or altered. Capture and detection antibodies that do no interfere with one another and can bind simultaneously are called "matched pairs" and are suitable for developing a sandwich ELISA (Thermo Scientific Proteomics, 2009). The strength of the "matched pair" in capture and detection of the analyte is the most important single factor in the success of the ELISA (Millipore, 2009).

3.5.3.2. Blocking buffer

A "blocking buffer" is a solution of irrelevant protein, mixture of proteins, or other compound, that passively adsorbs to binding surfaces on the plate which are not already bound with capture antibody. The most important consideration when choosing a blocking buffer is the signal:noise ratio, which is measured as the signal obtained with a sample containing the target analyte as compared to that obtained with a sample without the target analyte. The objective is to determine the highest signal:noise ratio. Failure to use sufficient blocker will result in excessive

background and a reduced signal:noise ratio, while using too high a concentration of blocker may mask antibody-antigen interactions, or inhibit the enzyme, again causing a reduction in the signal:noise ratio. Serial testing is necessary to ascertain the most effective blocking buffer (Thermo Scientific Proteomics, 2009).

3.5.3.3. Washes

Washes must be performed between each step of the ELISA. These washing steps are necessary to remove non-bound reagents and to decrease background, thereby increasing the signal:noise ratio. Inadequate washing allows high background, while too much washing may result in decreased sensitivity caused by elution of the antibody and/or antigen from the well. Washing is done in a physiological buffer such as Tris-buffered saline (TBS) or phosphate-buffered saline (PBS) without any additives. A detergent such as 0.05 per cent Tween-20 may be added to the buffer to help to remove non-specifically bound material. (Thermo Scientific Proteomics, 2009).

3.5.3.4. Substrates for detection

The last stage in all ELISA procedures is the detection. Unless a radioactive or fluorescent tag is used, this requires the use of an enzyme substrate. The enzyme converts the substrate to a detectable product. In a properly constructed ELISA, the intensity of the signal produced when the substrate is added is directly proportional to the amount of antigen captured in the plate and bound by the detection reagents. Enzyme-conjugated antibodies (especially those involving horseradish peroxidase) offer the most flexibility in detection because of the variety of substrates available for chromogenic, chemifluorescent and chemiluminescent imaging (Thermo Scientific

Proteomics, 2009). Popular enzymes are those that convert a colourless substrate to a colour product, e.g., pnitrophenylphosphate (pNPP), which is converted to the yellow p-nitrophenol by alkaline phosphatase. Substrates used with peroxidase include 2,2'-azo-b9s(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), which yields a green colour (Millipore, 2009)

Apart from individually constructing an ELISA system from first principles, complete kits are available for the detection of specific cytokines and other targets, such as interferon gamma (IFN gamma) and interleukin 6 (IL-6).

3.5.4. Protocol for sandwich ELISA (Millipore, 2009)

1. Before the assay, both antibody preparations should be purified and one must be labelled.

2. For most applications, a polyvinylchloride (PVC) microtiter plate is best; however, consult manufacturer guidelines to determine the most appropriate type of plate for protein binding.

3. Bind the unlabeled antibody to the bottom of each well by adding approximately 50 µL of antibody solution to each well (20 µg/mL in PBS). PVC will bind approximately 100 ng/well (300 ng/cm^2). The amount of antibody used will depend on the individual assay, but if maximal binding is required, use at least 1 µg/well. This is well above the capacity of the well, but the binding will occur more rapidly, and the binding solution can be saved and used again.

4. Incubate the plate overnight at 4°C to allow complete binding.

5. Wash the wells twice with PBS. A 500 mL squirt bottle is convenient. The antibody solution washes can be removed by flicking the plate over a suitable container.

6. The remaining sites for protein binding on the microtiter plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hrs. to overnight in a humid atmosphere at room temperature. **Note:** *Sodium azide is an inhibitor of horseradish peroxidase. Do not include sodium azide in buffers or wash solutions if an HRP-labelled antibody will be used for detection.*

7. Wash wells twice with PBS.

8. Add 50 μL of the antigen solution to the wells (the antigen solution should be titrated). All dilutions should be done in the blocking buffer (3% BSA/PBS). Incubate for at least 2 hrs. at room temperature in a humid atmosphere.

9. Wash the plate four times with PBS.

10. Add the labelled second antibody. The amount to be added can be determined in preliminary experiments. For accurate quantitation, the second antibody should be used in excess. All dilutions should be done in the blocking buffer.

11. Incubate for 2 hrs. or more at room temperature in a humid atmosphere.

12. Wash with several changes of PBS.

13. Add substrate as indicated by manufacturer. After suggested incubation time has elapsed, optical densities at target wavelengths can be measured on an ELISA

plate reader. **Note:** *Some enzyme substrates are considered hazardous, due to potential carcinogenicity. Handle with care and refer to Material Safety Data Sheets for proper handling precautions.*

14. For quantitative results, compare signal of unknown samples against those of a standard curve. Standards must be run with each assay to ensure accuracy.

CHAPTER 4

A STUDY OF THE ROLE OF TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 1 RECEPTOR IN BURN INJURY

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NOTE: CERTAIN OF THE EXPERIMENTS DESCRIBED IN THIS CHAPTER WERE PERFORMED BY MR. CHIN WING KO, UNDER SUPERVISION, WHILE A STUDENT IN THIS DEPARTMENT FOR THE DEGREE OF MASTER OF RESEARCH (M.RES.).

4.1 INTRODUCTION

Pain resulting from severe burn injury is one of the most excruciating pain sensations that can be experienced and the absence of consistently effective analgesics for its treatment renders its clinical management unsatisfactory (Arturson, 1996; Patterson et al., 2004). Severe burn injury causes major tissue damage and the attempts of the body to defend against infection and to effect repair of injured tissues generate a massive inflammatory response. This inflammatory response induces the formation and release of inflammatory mediators (Arturson, 1996). The resulting excitation of a sub-population of nociceptive primary sensory neurons is responsible for initiating, and maintaining, the sensation of peripheral inflammatory pain. We hypothesised that severe burn injury results in sustained activation of ERK1/2 (pERK1/2) and that activation of the transient receptor potential vanilloid type 1 receptor (TRPV1) mediates, in part at least, this effect.

TRPV1 is a ligand-gated, non-selective, cationic channel (Caterina et al., 1999; Heyman & Rang, 1985; Marsh et al., 1987; Tominaga et al., 1998) which is known to play an important role in several types of heat-induced hyperalgesia. About 40 per cent of the total neuronal population of primary sensory neurons express TRPV1 and the vast majority of these are non-myelinated, small diameter, nociceptive fibres innervating many tissues (Avelino et al., 2002; Dinis et al., 2004; Guo et al., 1999; Sato et al., 2005). Heat hyperalgesia secondary to inflammatory tissue injury fails to develop in TRPV1 null mice as in wild type mice in several models of thermal hyperalgesia, including that induced by carrigeenan, mustard oil, and complete Freund's adjuvant (Caterina et al., 2000; Davis et al., 2000), suggesting that TRPV1 is an essential component in the development of pathological thermal hyperalgesia

resulting from peripheral inflammation (Pogatzki-Zahn et al., 2005). The development of thermal hyperalgesia following inflammation involves an increased level of TRPV1 expression (Amaya et al., 2004; Kasama et al., 2007; McNaughton, 2005; Stein et al., 2006). It also involves the sensitization of existing TRPV1 channels by inflammatory agents (Chuang 2001; Vellani et al., 2006; Zhang et al., 2008; Zhu and Oxford, 2007). There is evidence that TRPV1 may be implicated in severe burn injury-induced pain. Immersion of one hind-paw of wild type mouse into 51 degrees C water for 15 seconds under brief ether anaesthesia induces thermal and mechanical hyperalgesia, manifested as a fall in heat and mechanonociceptive thresholds, which is reduced in TRPV1 null mice (Bolcskei et al., 2005). TRPV1 also suggests itself as being peculiarly relevant in the context of the spontaneous on-going pain found in burn injury-induced inflammation because of its ability, when sensitized by various inflammatory agents, to be rendered spontaneously active by body temperature alone (refs: Nagy et al., 2008; Nagy et al., 2009).

To investigate the role of TRPV1 in mediating the pain sensations associated with severe burn injury, we have developed a rodent model of second degree, partial thickness, burn injury by inducing a scalding-type injury, occasioned by immersing one of the animals hind-limbs in water heated to 60 degrees C. for two minutes (under urethane anaesthesia). Activation by phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) in spinal dorsal horn neurons is a recognised marker of spinal nociceptive processing (Ji et al., 1999). Hence, to elicit the extent to which TRPV1 is implicated in burn injury-induced nociception, we compared phosphoylation of ERK1/2 (pERK1/2) in spinal dorsal horn neurons for this model of scalding-type burn injury in TRPV1 null mice and in wild-type mice.

4.2. MATERIALS AND METHODS

4.2.1 Animals

All experiments were performed in accordance with the requirements of the Animals (Scientific Procedures) Act, 1986 (U.K.) and adhered to the guidelines of the Committee for Research and Ethical Issues of IASP published in PAIN®, 16 (1983) 109-110. In addition, the use of the animals for these experiments was approved by the Animal Subjects Review Board of the University of Porto, Porto, Portugal. All efforts were made to minimise both the number of animals employed in these experiments and any possible suffering of these animals.

Male and female C57BL/6J wild type mice (Charles River Laboratories), and B6.129X1-Trpv1/J mice lacking the TRPV1-encoding gene, weighing from 15.0 g to 29.0 g, were used in these experiments. Animals were housed in the veterinary facility of São João Hospital (Porto, Portugal) and kept in a 12 hours day and night cycle with free access to food and water.

4.2.2. Experimental groups

Each experiment was performed using two groups of animals comprising four wild type (WT) and four TRPV1-null "knock out" (KO) mice, respectively. The animals in all groups were anaesthetised using an intraperitoneal injection of urethane (0.02mg/g, Sigma). Animals were constantly monitored to ensure that they remained deeply anaesthetised during the course of the experiment. In total, 28 wild type and 28 TRPV1 null mice were employed in these experiments.

A wild type group and a KO group were each subjected to one of seven experimental protocols as follows: (1) One hour survival after urethane anaesthesia and without any burn injury; (2) Urethane anaesthesia followed by insertion for two minutes of the right hind-limb below the knee into 37ºC water with survival for one hour; (3) Noxious thermal stimulus after urethane anaesthesia comprising insertion for one second of the right hind-limb below the knee into 60ºC water with survival for one hour; (4) Burn injury after urethane anaesthesia comprising insertion for two minutes of the right hind-limb below the knee into 60ºC water with survival for five minutes; (5) Burn injury after urethane anaesthesia comprising insertion for two minutes of the right hind-limb below the knee into 60ºC water with survival for one hour; (6) Burn injury after urethane anaesthesia comprising insertion for two minutes of the right hind-limb below the knee into 60ºC water with survival for three hours; and (7) Burn injury after urethane anaesthesia comprising insertion for two minutes of the right hind-limb below the knee into 60ºC water with survival for 6 hours. In all cases, the animals were constantly monitored throughout the experiment and were maintained unconscious under anaesthesia throughout the entire duration of the experiment by repeated i.p. injections of urethane, as required.

4.2.3 Tissue preparation

After the specified survival period, the animals were terminally anaesthetised with urethane and were then perfused through the aorta with calcium-free Tyrode solution followed by 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS, pH 7.5) for 15 minutes. Heparin (LEO Pharma A/S, Denmark) was injected into the left ventricle before the perfusion to prevent blood clotting. The spinal cord and the skin

from the right lower hind-limb of each animal were carefully dissected and immersed in 4% paraformaldehyde for 4 hours at 4ºC. These tissues were then placed in 30% sucrose in 0.1M PBS until saturated.

The L4-L5 lumbar segments of the spinal cord were in each case identified and dissected out. The ventral aspect of the contralateral side of each spinal cord block was identified and marked by a shallow incision. The tissue blocks were then embedded in OCT embedding medium (VWR, West Chester, PA) and 20µm transverse sections were cut on a cryostat. Sections were thaw-mounted on Superfrost Plus microscope slides.

4.2.4. Immunohistochemistry

The sections were first washed in PBS and incubated in PBS containing 0.3% Triton X (PBST) for 10 minutes. The endogenous peroxidase activity was quenched by incubating the section in 0.3% H_2O_2. Sections were then placed in 10% normal donkey serum (NDS) for 25 minutes. The primary antibody was anti-phospho extracellular signal-regulated protein kinase 1/2 (pERK1/2) raised in rabbit against the residues surrounding T202/Y204 of pERK1/2 (1:1000, Neuromics). The sections were incubated in the primary antibody overnight. Following washes in PBS, the sections were incubated with biotin-conjugated donkey anti-rabbit IgG (1:800; Jackson Immunoresearch) for 2 hours. The immunoreaction was visualised using the Vectastain Elite and IMPACT DAB kits (Vector). The slides were then dehydrated in ethanol, cleaned in xylene and covered by depex polystyrene. All the washes and dilutions were performed at room temperature in PBST, with the addition of 0.3% NDS for the dilution of the primary antibody.

4.2.5. Histopathological analysis

Skin tissue from the hind-paws exposed to hot water were embedded in paraffin, and sections were cut with a Reichert microtome at a thickness of 10 um. After deparaffination, the sections were stained with hematoxylin and eosin and coverslipped. Photographs were taken with a Nikon Eclipse 800 microscope with a Spot RT Slider digital camera (Diagnostic Instrument Inc., Sterling Heights, MI) and Spot 3.5.9.1. software (Diagnostic Instrument Inc.).

4.2.6. Data analysis and statistics

Four non-consecutive sections were randomly taken from the spinal cords of each experimental group for analysis. These slides were then analysed using a Leica microscope. Cells expressing pERK1/2 were identified at x 40 magnification in both the ipsilateral, and contralateral, sides of the spinal cord. The average number of pERK1/2-expressing cells over the four sections were then calculated for each side of the spinal cord. The ipsilateral and contralateral means (±SEM) for each group were calculated. Differences in the number of pERK1/2-expressing neurons between the different experimental groups were analysed using one way ANOVA. Whether differences found were significant was established using Tukey's *post-hoc* test. Differences were regarded significant at $p<0.05$.

4.3. RESULTS

4.3.1. Histopathology of skin

The hind-limb skin tissue burned by immersion in scalding water for 2 minutes at 60 degrees C showed major pathological signs not found in normal tissue from naive animals (Figure 1). The epidermis of this burn-injured tissue was no longer adherent to the dermis, having become detached or lost. The dermis itself remained adherent to the underlying sub-cutaneous connective tissue but was substantially disrupted. It was oedematous, with its blood-vessels dilated or destroyed (Figure 1b). These features were consistent with severe second degree, partial thickness deep burn injury.

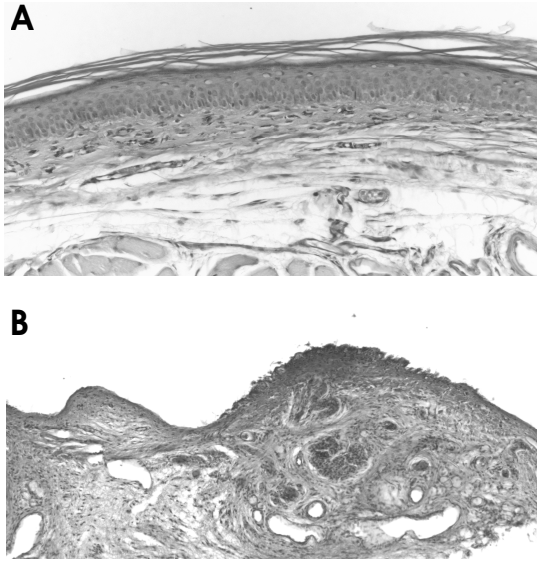


Figure 1.

Typical images of sections cut from control skin (A) and skin subjected to 120 seconds immersion into 60ºC water (B). Both sections are stained with hematoxylin eosin. Note that, while in the control section the structure of the skin is easily visible, that structure is severely damaged in the section cut from the burn-injured skin. The layers of the epithelium are no longer recognisable, and the vessels are severely diluted.

4.3.2. General features of pERK1/2-expression in the spinal cord

pERK1/2-immunopositivity, where found, could be observed in the cytoplasm and/or in the nucleus of neurons. In sections from animals of experimental groups (4), (5), (6) and (7), which were exposed to limb immersion in 60 degrees C. water for two minutes, the pERK1/2-immunopositive cells in the ipsilateral side of the spinal cord were predominantly found in the superficial laminae of the dorsal horn on that side, but were also seen in the deep dorsal horn. With increasing survival time after such burn injury, the distribution of labelled neurons altered in the ipsilateral spinal cord with an increasing number of labelled neurons being found in the deeper dorsal horn on that side, while an increasing number of labelled neurons were also observed in the dorsal horn of the contralateral side.

4.3.3. Experimental groups 1: no stimulus (naive animals)

Naive, anaesthetized, WT mice exhibited pERK1/2-expressing cells only extremely rarely in the dorsal horn on either side of the spinal cord (0.9±0.4 ipsilateral; 0.7±0.3 contralateral; n = 3). Similarly, only extremely few pERK1/2-immunopositive neurons were found in the dorsal horn on either side of the KO animals (0.7±0.7 ipsilateral; 0.5±0.5 contralateral; n =3). No significant difference was found between the combined number of pERK1/2-labelled neurons found on both sides of the spinal cord as between WT and KO mice. Furthermore, no significant difference was found when the number of labelled in the corresponding sides of the spinal cords of WT and KO animals were compared.

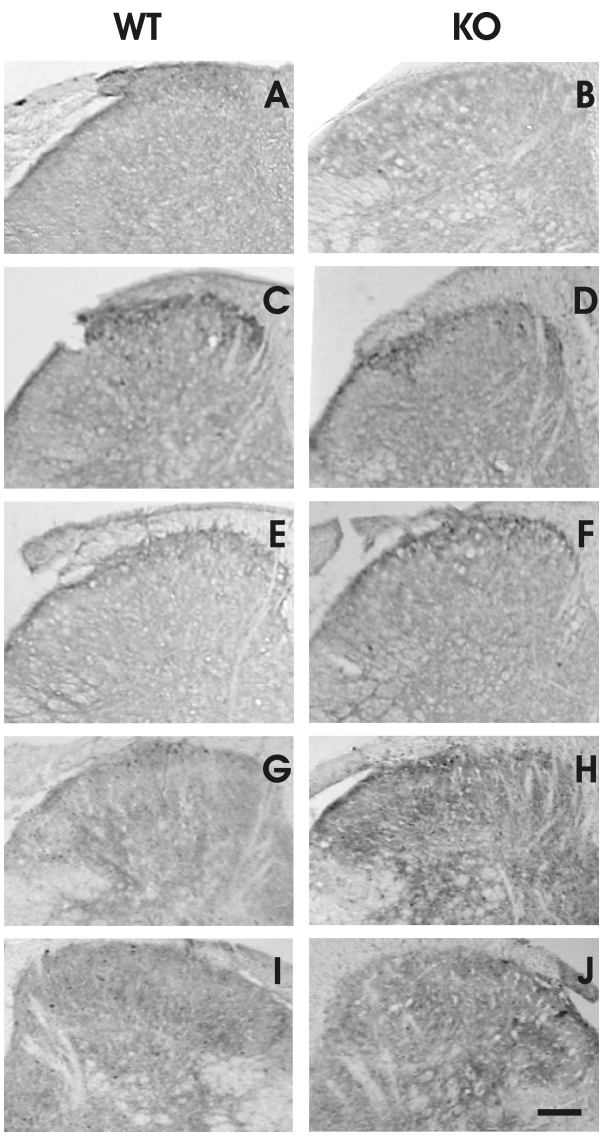


Figure 2.

Illustrative photomicrographs spinal dorsal horn of wild type (WT) and TRPV1 knock-out (KO) animals. Naive animals (A and B) and burn-injured animals with different survival times (C-J).

Only occasional isolated pERK1/2 immunopositive neurons are found in sections of either WT or KO naive animals (A and B), but sections of all burn-injured animals consistently express pERK1/2 immunopositive neurons in the spinal dorsal horn

.

(C and D) Severe burn injury (short survival time) in WT and KO mice. The ipsilateral dorsal horn sections shown here exhibit pERK1/2 immunolabelled neurons and neuropil in lamina I and outer lamina II. No significant difference is observed in pERK1/2 immunolabelling between the WT and KO animals.

(E and F) Severe burn injury (1 hour survival time) in WT and KO mice. The ipsilateral dorsal horn sections shown here exhibit substantially similar pERK1/2 immunoreactivity to that found at the short survival time. No difference is observed in pERK1/2 immunolabelling between the WT and KO animals.

(G to J) Severe burn injury (6 hours survival time) in WT and KO mice. At 3 hours and 6 hours survival time, the number of neurons labelled in the ipsilateral dorsal horn (G and H) exceeds that found at 1 hour survival time, although the number of such labelled neurons has diminished by 6 hours from the number observed at 3 hours, while their distribution has also altered. At 3 hours and 6 hours survival time, pERK1/2 labelling is found on the contralateral side of the dorsal horn (I and J), to an extent which was not present at 1 hour survival time. Interestingly, the distribution of the labelled neurons on the contralateral side differs markedly from that of the labelled neurons on the ipsilateral side and, moreover, the intensity of the staining is frequently less than that seen on labelled neurons on the ipsilateral side.

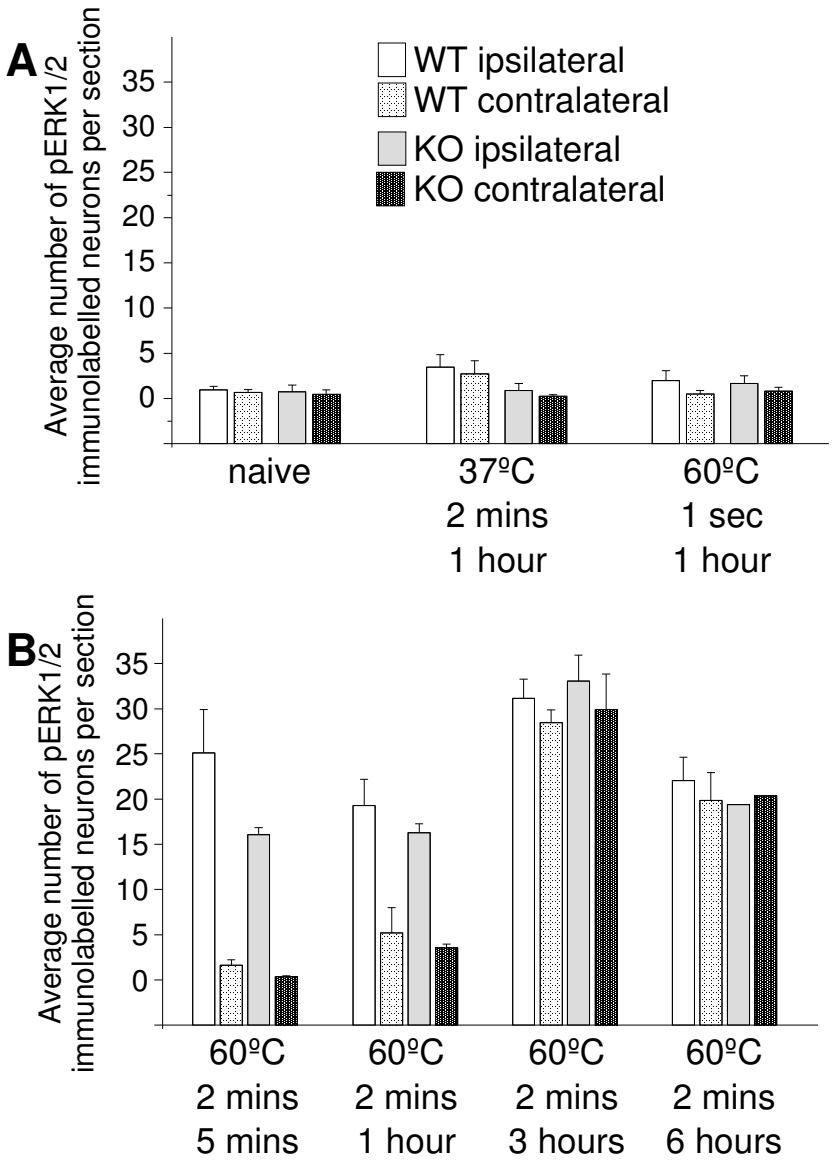


Figure 3.

Average number of pERK1/2 immunolabelled neurons per section in the ipsilateral and contralateral sides of the spinal dorsal horn of wild type (WT) and TRPV1 knock-out (KO) mice.

(A) The number of pERK1/2 immunopositive neurons in conditions not involving severe burn injury.

(B) The number of pERK1/2 immunopositive neurons in conditions involving severe burn injury.

Note that the number of immunopositive neurons is significantly increased on the ipsilateral side at 5 minutes to 6 hours survival times. Note also that at 3 hours to 6 hours survival times, pERK1/2 immunolabelled neurons are also expressed on the contralateral side of the spinal dorsal horn. By 6 hours, the number of labelled neurons is beginning to diminish in both ipsilateral and contralateral sides. Note that there is no significant difference between the extent of the immunolabelling between the ipsilateral and contralateral sides at both 3 hours and 6 hours survival times. Note also that there is no significant difference in the number of labelled neurons between the wild type and TRPV1 knock-out mice in any experimental group.

4.3.4. Experimental groups 2: non-noxious stimulus

Immersion of hind-limbs of WT and KO animals in 37 degrees C. water for 2 minutes resulted in few pERK1/2-immunopositive neurons being observed in any side of the dorsal horn of both WT and KO mice (3.5±1.4 ipsilateral, and 2.7±1.4 contralateral, in WT (n =3); 0.9±0.8 ipsilateral, 0.3±0.1 contralateral, in KO (n=4)). The number of pERK1/2-expressing cells was not significantly different as between the ipsilateral side and the contralateral side of either the WT or KO mice. No significant difference was found between the number of labelled cells found in the same sides of the WT and KO animals. Consistent with previous findings that innocuous stimuli do not evoke pERK expression in the dorsal horn of the spinal cord (Ji et al., 1999), no significant difference was found between the number of labelled neurons observed in the same sides of the spinal cords of naive, anaesthetized, animals and of animals which had had a limb immersed in 37ºC water.

4.3.5. Experimental groups 3: momentary noxious heat stimulus (1 hour survival time)

Immersion of the lower hind-paw into 60ºC water for 1 second with a survival time of 1 hour resulted in few pERK1/2-immunoreactive neurons being observed in either side of the dorsal horn of spinal cords of both WT (2±1.1 ipsilateral; 0.5±0.4 contralateral; n =4) and KO mice (1.7 ±0.9 ipsilateral; 0.8 ±0.4 contralateral; n =3). No significant difference was found in the number of labelled neurons as between the two sides of the spinal cord in either WT or KO mice. Furthermore, there was no significant difference found in the number of pERK1/2-expressing neurons in the same sides of the spinal cords of the WT and KO mice. Moreover, the number of labelled neurons in both WT and KO animals in any side of their spinal cord did not differ significantly from the number found in any side of the spinal cords of WT and KO animals which had received no stimulus at all (experimental groups (1)), or received an innocuous stimulus (experimental groups (2)).

4.3.6. Experimental groups 4: severe burn injury (short survival time)

Immersing the lower hind-limbs into 60ºC water for 2 minutes followed by a 5 minute survival time resulted in a major increase in pERK1/2-immunoreactivity in the dorsal horn of the ipsilateral side of the spinal cord of WT mice, but had little effect on the dorsal horn of the contralateral side. The average number of pERK1/2-immunolabelled cells in the ipsilateral side and contralateral side of WT mice was 25.1±4.8, (n=4) and 1.6±0.6 (n =4), respectively. In the ipsilateral side of the dorsal horn of WT animals, the great majority of the pERK1/2-immunopositive neurons were distributed in the medial part of the dorsal horn. The difference in the number of

pERK1/2-expressing cells as between the two sides of the dorsal horn in these animals was highly significant.

The number of pERK1/2-expressing neurons in the ipsilateral side of these WT animals was significantly higher than the number of such neurons found in the ipsilateral side of animals in the preceding experimental groups. On the other hand, there was no significant difference found between the number of pERK-expressing neurons on the contralateral side of WT animals in these and in the preceding experimental groups.

There was a major difference in pERK1/2-immunoreactivity as between the ipsilteral and contralateral sides of the spinal cords of the KO mice in these experiments. While the average number of pERK1/2-immunolabelled cells on the ipsilateral side of the dorsal horn of these animals was 16.1±0.8 (n=3), it was 0.3 ±0.1 in the contralateral side (n =3). The great majority of the pERK1/2-immunolabelled neurons were found in the medial part of the ipsilateral dorsal horn. The difference between the numbers of labelled neurons found in the ipsilateral and contralateral sides of the spinal cords was significant.

Comparison of the number of labelled neurons found in the ipsilateral side of the spinal cord in WT and KO animals showed an ~36% reduction in KO mice which difference was significant. The difference in the average number of labelled neurons in the contralateral side of the dorsal horn of the WT and KO mice, however, was not significant. The average number of labelled neurons in the ipsilateral spinal cord of KO mice was significantly higher than that in the ipsilateral side of the dorsal horn in

animals in the preceding experimental groups. However, the present experimental protocol did not evoke significant change in the number of neurons labelled in the contralateral side of these WT and KO animals, respectively, when compared to the contralateral sides of corresponding animals in the preceding experimental groups.

4.3.7. Experimental groups 5: severe burn injury (1 hour survival time)

Immersion of the lower hind-limb into 60ºC water for 2 minutes followed by 1 hour survival time also produced a substantial difference in pERK1/2-immunoreactivity between the ipsilateral and contralateral sides of the spinal cord in WT mice. While the average number of pERK1/2-immunolabelled neurons found on the ipsilateral dorsal horn was 19±1.3 (n=4), it was 2.9±1.7 (n=4) on the contralateral dorsal horn. The great majority of the labelled neurons in the ipsilateral dorsal horn were distributed in its medial part. The difference in the number of pERK1/2-immunoreactive cells between the two sides of the spinal cord in these animals was significant. Furthermore, increasing the duration of survival time following burn injury did not produce any significant change in the number of labelled neurons in the ipsilateral side of the dorsal horn of WT mice. The number of labelled neurons on the contralateral dorsal horn was not significantly different from the number found in the contralateral dorsal horn of WT animals in the preceding experimental groups.

In the KO animals in this experiment, pERK1/2-immunoreactivity was significantly greater in the ipsilateral side of the dorsal horn than in the contralateral dorsal horn. While the average number of labelled cells in the ipsilateral side of the spinal cord was 16.3±1 (n=4), it was 3.5±0.4 on the contralateral side (n=4).

The number of labelled neurons on the ipsilateral side of the dorsal horn in KO animals was significantly greater than the number found in the same side of the dorsal horn of KO animals in the first three experimental groups. However, the number of pERK1/2-immunopositive neurons on the ipsilateral side of the dorsal horn in KO animals did not differ significantly from that found either in WT animals which had received the same treatment or in the KO mice which had had their lower limbs immersed into 60ºC water followed by 5-minute survival (experimental groups no. 4). The number of labelled neurons found in the contralateral dorsal horn did not differ significantly from the number of such neurons found in the contralateral side of the spinal cord of animals of any other treatment groups.

4.3.8. Experimental groups 6: severe burn injury (3 hours survival time)

At 3 hours after severe burn injury, the number of neurons which were stained for ERK activation in both KO and WT mice had substantially increased when compared with the number of neurons stained for such activation at 1 hour after such injury. Remarkably, at this later time, in both TRPV1-null and wild-type mice, the contralateral side of the dorsal horn now exhibited a substantial number of p-ERK-immunopositive neurons, sometimes matching, or exceeding, the number of neurons stained for ERK activation on the ipsilateral side. Interestingly, the distribution of the labelled neurons on the contralateral side differed markedly from that of the labelled neurons on the ipsilateral side and, moreover, the intensity of the staining was frequently less than that seen on labelled neurons on the ipsilateral side.

Here, again, we found no significant difference between the number of neurons on either side of the spinal cord labelled for ERK1/2 activation in TRPV1 knock-out

mice when compared with the number so labelled in the corresponding sides in wild-type mice. Thus, the average number of p-ERK1/2 immunopositive neurons in the ipsilateral dorsal horn of WT mice was 31.2 +/- 2.1 (n = 3), while the average number of neurons stained on the contralteral dorsal horn was 28.5 +/- 1.4 (n = 3). In the case of KO mice, the average number of neurons stained on the ipsilateral dorsal horn of such mice was 33.1 +/- 2.9 (n = 3), while the average number of neurons stained on the contralateral dorsal horn was 29.9 +/- 3.9 (n = 3). No significant difference was found between the number of neurons stained for ERK1/2 activation when the same sides were compared in knock-out and wild-type mice, respectively.

4.3.9. Experimental groups 7: severe burn injury (6 hours survival time)

At 6 hours after severe burn injury, we found no alteration in the expression of p-ERK1/2 immunopositive neurons from that at three hours, as previously discussed, except that at this juncture it was apparent that there was a reduction in the extent of the labelling in both the ipsilateral and contralateral sides of the dorsal horn which was found in both TRPV1-null and wild-type mice.

We found no significant difference between the number of neurons on either side of the spinal cord labelled for ERK1/2 activation in TRPV1 KO mice when compared with the number so labelled on the corresponding sides in WT mice. Thus, the average number of p-ERK1/2 immunopositive neurons in the ipsilateral dorsal horn of WT mice was 22.1 +/- 2.6 (n = 3), while the average number of neurons stained on the contralateral dorsal horn was 19.9 +/- 3.1 (n = 3). In the case of KO mice, the average number of neurons stained on the ipsilateral dorsal horn of such mice was 19.4 (n = 2), while the average number of neurons stained on the contralateral dorsal

horn was 20.4 (n = 2). No significant difference was found between the number of neurons stained for ERK1/2 activation when the same sides were compared in KO and WT mice, respectively.

4.4. DISCUSSION

4.4.1. Experimental design

Histologically verifiable second degree burn injury was induced in mouse by exposing a lower hind-paw to hot water at 60 degrees C. for two minutes (under urethane anaesthesia). Using this model of scalding-type burn injury and DAB-labelling, we have compared the extent of the activation by such burn injury of ERK1/2 in the dorsal horn of the spinal cord of wild-type, and TRPV1 null, mice, respectively. At all of the post-injury time points beyond 5 minutes which we measured (1 hour; 3 hours; and 6 hours), no significant difference was found in the extent of ERK1/2 activation (pERK1/2) in the ispilateral dorsal horn between wild-type, and TRPV1 null, mice, respectively,

Ethical, and, indeed, in the United Kingdom, legal, considerations, dictate that animals must be deeply anaesthetised while subjected to this scalding-type burn injury. Hence, behavioural assessment of the effect of this type of injury is not possible and reliance must be placed instead on a surrogate marker of nociception. Activation of spinal ERK1/2 is a recognised marker of spinal nociceptive processing. Ji and colleagues (1999) showed that peripheral capsaicin injection into the left hind-paw of rat activates ERK1/2 by phosphorylation (pERK1/2) in dorsal horn spinal cord neurons and induces pain-related behaviour in the animal (Ji et al., 1999). There is also compelling evidence that activation of ERK1/2 is found in many inflammatory conditions in the somatosensory system (Cruz et al., 2005a; Cruz et al., 2005b; Karim et al., 2006; Seino et al., 2006; Tsuda et al., 2007). In accord, we found that both

non-noxious stimulation of the lower hind-paw and momentary noxious stimulation of the lower hind-paw each failed to evoke ERK1/2 activation. However, sustained noxious stimulation of the same part over 2 minutes, which resulted in severe tissue injury, resulted in substantial ERK1/2 activation within 5 minutes of the injury being sustained.

4.4.2. Involvement of TRPV2

The extent to which ERK1/2 was activated in the ipsilateral dorsal horn at five minutes after sustained noxious scalding-type injury was significantly greater in wild-type animals when compared to TRPV1 null animals. This difference in the level of ERK1/2 activation in wild-type mice, when compared with knock-out mice, can be attributed to the contribution to the excitation of primary sensory afferents made by TRPV1 present in the wild-type animals. However, there was also an increase in pERK in the ipsilateral dorsal horn of knock out animals, albeit that it was significantly less than the increase found in wild-type animals. One may speculate that this (lesser) increase in ERK1/2 activation in the knock-out animals was at least contributed to by activation of TRPV2 ion channels by the noxious heat stimulus. The transient receptor potential vanilloid receptor type 2 (TRPV2) is a non-selective cation channel, expressed primarily in myelinated afferents, which is activated in mouse and rat by temperatures in excess of ~ 52 degrees C (Ahluwahlia et al., 2002; Lewinter et al., 2004; Ma, 2001; Neeper et al., 2007; Patapoutian et al., 2003]. TRPV2 in rat dorsal root ganglion cells appears to contribute to peripheral sensitization during inflammation and to be important in mediating pain hypersensitivity to noxious high temperature stimuli (Shimosato et al., 2005). Given that there is increasing evidence of a role for TRPV2 in mediating high-threshold heat

responses in a sub-population of mammalian sensory neurons (Leffler et al., 2007; Qin et al., 2008), one may also speculate that TRPV2 may also be involved in mediating the pain occasioned by momentary exposure to scalding water (which fails to manifest itself in significant ERK1/2 activation). The pain sensation induced by such noxious stimulus is almost instantaneous, which is essential if the animal is to protect itself by promptly withdrawing its limb from the source of danger. This suggests the involvement in this phenomenon of myelinated nociceptive afferents which have the capacity for ultra-fast conduction of the pain signal, with the murine TRPV2 being the most obvious receptor capable of facilitating this response.

4.4.3. TRPV1 redundant except in period immediately after injury

While TRPV1 clearly had an effect in activating ERK1/2 in neurones of the ipsilateral dorsal horn at five minutes after burn injury, this effect of TRPV1 was not maintained and was absent when ERK1/2 activation was assessed at 1 hour, 3 hours, and 6 hours, after the scalding-type injury was sustained. We suggest that these findings can reasonably be interpreted as showing that the nociceptive response to this scalding-type burn injury in mouse is not dependent on activation of TRPV1 except in the period immediately after the injury is sustained and certainly ending within 1 hour after injury.

The conclusion that TRPV1 is not essential for the maintenance of the inflammatory nociceptive response occasioned by scalding-type burn injury raises an important question relating to whether the search for new analgesic agents ought to be focused upon attempts to neutralize the actions of various ion channels as opposed to eliminating, or at least reducing, the availability of inflammatory agents, as such. A

multiplicity of agents released in inflammation contribute to alter the firing pattern of nociceptive primary sensory neurons, including bradykinin, eicosanoids, nerve growth factor (NGF), artemin, glial cell line-derived neurotrophic factor (GDNF), serotonin, histamine, anandamide, adenosine 5'triphosphate (ATP), and cations, especially protons. These inflammatory agents act directly on their own receptors found on primary afferents (in respect of which they are specific ligands) to contribute to excitation of these neurones (Nagy and Rice, 2002). In addition, certain of these inflammatory agents also activate ion channels found on these neurons, or affect the sensitivity and expression of these ion channels. Present evidence suggests that the most important of these ionotropic receptors affected by these agents are TRPV1 (Ahern et al., 2005; Amaya et al., 2004; Avelino et al., 2002; Bolcskei et al., 2005; Caterina et al., 1999, 2000; Chuang et al., 2001; Davis et al., 2000; Dinis et al., 2004; Guo et al., 1999; Ji et al., 1999; Jordt et al., 2000; Kasama et al., 2007; McNaughton, 2005; Pogatzki-Zahn et al., 2005; Ryu et al., 2003; Sato et al., 2005; Stein et al., 2006; Sugiura et al., 2002, 2007; Tominaga et al., 1998; Vellani et al., 2006; Zhang et al., 2008; Zhu and Oxford, 2007), TRPA1 (Andersson et al., 2008; Bandell et al., 2004; Chao et al., 2008; Doerner et al., 2007; Jordt et al., 2004; Kobayashi et al., 2005; Taylor-Clark et al., 2008), TRPV4 (Alessandri-Haber et al., 2005, 2006, 2008; Liedtke, 2005; Suzuki et al., 2003a, 2003b), and ASIC (Babini et al., 2002; Bassler et al., 2001; Smith et al., 2007; Sugiura et al., 2005; Waldmann et al., 1997; Zhang et al., 2006) ion channels. Our findings suggest that, in the model of scalding-type burn injury which we employed, TRPV1 is an unnecessary "fifth-wheel" on the carriage mediating the effects of these *inflammagens,* except in the period immediately after the injury is sustained and certainly ending within 1 hour after injury.

It has yet to be shown that alteration of the functioning of any single ion channel type – whether TRPV1, TRPA1, TRPV4 or the ASICs – may effect the outcome in terms of neuronal excitation and resulting pain sensation in the context of inflammation. It has been shown that altered function of Na+ channels contributes to the increased hyper-excitability of primary afferent neurons in certain pathological pain states and the Nav1.9 sub-unit has been implicated in mediating inflammatory pain sensations (Amaya et al., 2006; Priest et al., 2005). It has also been shown that individual *inflammagens* may, conjointly, produce an effect which they are individually incapable of mediating. Thus, bradykinin, ATP, histamine, PGE2, and noradrenaline, when applied separately at maximal concentrations, all fail to modulate Nav1.9 current; but, when applied conjointly as a soup of inflammatory mediators, they rapidly potentiate Nav1.9 channel activity, generating sub-threshold amplification and increased excitability (Maingret et al., 2008). Again, Levine and colleagues (2008) have shown that the level of the pro-inflammatory cytokine, interleukin-6 (IL6), is increased following a partial-thickness burn injury in rat and that spinal intrathecal injection of oligodeoxynucleotides antisense for gp130, a receptor subunit shared by members of the IL-6 family of cytokines, attenuates both burn-induced, and intrademeral IL-6-induced, mechanical hyperalgesia, as does intradermal injection of anti-IL-6 function blocking antibodies (Summer et al., 2008). The findings in these studies may have profound clinical implications because they suggest that, in certain pain states at least, an intervention which successfully prevents the contribution to neuronal excitation of certain only of the *inflammagens* may result in a significant analgesic effect. At the same, our findings suggest that there is redundancy, at least as regards TRPV1, in the complex of receptors (ionotropic and

G-protein coupled) which mediate the effects of these *inflammagens* so that, except in the initial aftermath of such burn injury, the resulting sensation of pain is not dependent on activation of TRPV1. If this should also be the case in humans, then it would appear that, in the context of certain types of burn injuries at least, resources ought to directed toward attempting to eliminate, or reduce, the availability of inflammatory agents rather than attempting to modulate the activity of TRPV1 to induce analgesia.

4.4.4. Contralateral responses to unilateral inflammatory lesions

Our finding that, at 3 hours after severe scalding-type burn injury, the number of neurons which were stained for ERK1/2 activation in both knock-out, and wild-type, mice had substantially increased when compared with the number of neurons stained for such activation at 1 hour after such injury, is consistent with a delay being necessary before inflammatory agents may mediate their effects through certain of their receptors. Our further finding that at this later time, in both TRPV1-null and wild-type mice, the contralateral side of the spinal cord now exhibited a substantial number of p-ERK1/2-immunopositive neurons remains intriguing, notwithstanding that the phenomenon of hyperalgesia developing in the hindpaw contralateral to a heat injury has long been noted (Coderre and Melzack; Polgar et al., 1998). Interestingly, the distribution of the labelled neurons on the contralateral side differed markedly from that of the labelled neurons on the ipsilateral side and, moreover, the intensity of the staining was frequently less than that seen on labelled neurons on the ipsilateral side. Contralateral responses to unilateral inflammatory lesions are not unknown (Coderre and Melzack, 1991; Koltzenburg et al., 1999; Polgar et al., 1998; Shenker et al., 2003). One may speculate that this labelling is caused, or contributed to, by an

action of descending fibres on these contralateral dorsal horn neurons since the concept of supraspinal pronociceptive modulation of pain is now well recognised (Lima et al., 2002; Tavares and Lima, 2007). In addition, or in the alternative, this labelling may result from cross-communication between opposite dorsal horn neurons (Coderre and Melzack, 1991; Shenker et al., 2003). In any event, the activation of ERK1/2 on *both* sides of the dorsal horn discloses a process in which spinal neurons are driving inflammatory nociceptive activity after the excitatory effect of peripheral *inflammagens* has abated in primary sensory afferents.

CHAPTER 5

THE EFFECT OF XENON ON NOXIOUS STIMULUS-INDUCED ACTIVATION OF TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 1 RECEPTOR

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5.1. INTRODUCTION

In primary sensory neurons the membrane currents generated by neuronal ion channels, such as TRPV1, affect neuronal firing patterns to constitute, and initiate, the electrical transfer to the brain of, an integrated picture of pain-inducing stimuli. The transfer of signals which relay this information from primary sensory neurons to the spinal cord is mediated by the release of neurotransmitter substances by these neurons where they synapse with second-order afferent neurons in the spinal cord. Nociceptive fibres terminate in the superficial layers of the dorsal horn, forming synaptic connections with transmission neurons running to the thalamus and other nuclei in the brain and brain-stem. Primary sensory neurons release glutamate (fast transmitter) and various peptides, especially substance P and calcitonin gene-related peptide (CGRP), which act as slow transmitters, at their central terminals in the spinal cord to mediate nociceptive signals. In addition, these neurons may, in conditions of *neurogenic inflammation*, discharge CGRP and substance P at peripheral terminals which results in further increased neuronal excitation and pain.

Acute activation of TRPV1 in primary sensory neurons results in a short-lasting burning pain sensation. However, sustained TRPV1 activation in those neurones resulting from inflammation of peripheral tissues leads to the development of heat hyperalgesia and visceral hyper-reflexia (Caterina et al., 2000; Davis et al., 2000; Charrua et al., 2007). Activation of TRPV1 ion channels results in neurotransmitter release, including CGRP (Sauer et al., 1999). CGRP release by primary sensory neurons is commonly regarded as a marker of substance P release also (Gibbins et al., 1985). However, the difficulty in its use as a marker for TRPV1

activation resides in the fact that CRGRP release is ultimately the result of electrical activity in the cell which may be the result of the activation of receptors other than TRPV1. Moreover, in rat, CGRP is expressed by all types of primary afferents (MCarthy & Lawson, 1990; Lawson & McCarthy, 1996). In addition, CGRP expression may also be affected by nerve injury which is an inevitable consequence of DRG culture preparations (Honore et al., 2000).

Xenon is an inert gas which binds to a remarkable range of proteins, including ion channels, such as the ionotropic N-methyl-D-aspartate (NMDA) receptor for glutamate, as well as certain non-NMDA receptors (Franks et al., 1998; Gruss et al., 2004; Dinse et al, 2005; Dickinson et al, 2007; Salmi et al., 2008). Research into the mechanism of action of xenon has focused, almost exclusively, on its role as a general anaesthetic. However, in addition to its potent anaesthetic effect, xenon, in sub-anaesthetic doses, provides analgesia (Yagi et al., 1995; Petersen Felix et al, 1998). Such xenon-induced analgesia is believed to be mediated, at least in part, through inhibition of spinal nociceptive processing by way of reducing noxious stimulation-evoked spiking activity and long term potentiation in spinal cord neurons (Utsumi et al., 1997; Benrath et al., 2007; Watanabe et al., 2004). It appears likely that xenon-induced reduction of spinal nociceptive processing is not mediated solely through inhibition of NMDA receptor activity. All components of spinal cord ventral root potentials (VRPs) evoked by stimulation of primary afferents are reduced by xenon (Watanabe et al., 2004). Individual components of these VRPs are dependent on one, or other, of NMDA receptors, non-NMDA receptors, or tachykinin receptors (Nagy et al., 1994; Nagy & Woolf, 1996; Thompson et al., 1994). Hence, an inhibitory effect of xenon on VRPs may result from xenon's action on these receptors in the spinal

cord and, in addition, or in the alternative, from xenon's effect on one, or more, receptor types on primary nociceptive neurons resulting in reduced nociceptive input into the spinal dorsal horn.

Previous studies have shown that xenon reduces pain-related behaviour evoked by formalin injection, which induces an inflammatory reaction in tissues (Fukuda et al., 2002; Ma et al., 2004). Recently, it has been shown that the formalin injection-evoked pain-related behaviour is reduced by TRPV1 antagonists (Garcia-Martinez et al., 2006; Tang et al., 2007). These data suggest that, if xenon indeed reduces the nociceptive input into the spinal dorsal horn, that reduction could be mediated – at least in part – by an inhibitory effect on TRPV1. Accordingly, this work was designed to determine the effect of xenon on TRPV1 activity by studying TRPV1-mediated responses in primary sensory neurons, TRPV1-expressing human embryonic kidney 293 (HEK293) cells, and by capsaicin-injection-evoked activation of spinal dorsal horn neurons.

5.2. MATERIALS AND METHODS

5.2.1. Animals

All procedures were performed in accordance with the requirements of the UK Animals (Scientific Procedures) Act, 1986. All together, 23 female Sprague Dawley rats (each ~100 g in weight) were used in this study. The animals were kept at 12 hours light-dark cycles at controlled temperature and humidity, with food and water *ad libitum*.

5.2.2. Capsaicin injection-induced expression of phosphorylated extracellular signal regulated kinase 1/2

Sixteen female Sprague Dawley rats were randomly assigned to four groups. Xenon (80%) and oxygen (20%) was administered as a mixture at atmospheric pressure to the animals from two of these groups in a sealed Perspex chamber. The flow-rate of the gas mixture through the chamber was 2.1 l/minute, while the chamber itself had a volume of 3 l. After exposure to the xenon/oxygen mixture for 15 minutes, the animals in one of these groups received a subcutaneous injection of capsaicin (3mg/ml dissolved in 10% Tween 80; 25 µl) into the right hind-paw. The other xenon-treated group of animals were injected with the vehicle (10% Tween 80; 25 µl). The remaining two groups of animals were anaesthetised with sodium pentobarbital (60 mg/kg, i.p.), after which one group was injected with capsaicin, while the other group was injected with vehicle. Following 5 minute survival under anaesthesia, all animals were perfused transcardially with saline followed by 4% paraformaldehyde. The lumbar spinal cord was removed and placed into the same

fixative for an additional 4 hours. Tissues were then put into 30% sucrose until they sank. The ventral horn of the L4-L5 segments was labelled by a shallow longitudinal cut on the left side. Then, 20 µm transverse sections were cut on a crysostat and thaw-mounted on Superfrost Plus microscope slides (VWR, West Chester, PA). Sections were air dried overnight and stored at -20℃ until required.

The effect of xenon on subcutaneous capsaicin injection-evoked activation of spinal cord neurons was assessed by studying the number of cells expressing phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) in the dorsal horn of the spinal cord (Ji et al., 1999). Spinal cord sections were washed in 0.01M phosphate-buffered saline (PBS) and incubated in PBS containing 0.3% Triton X (PBST) for 10 minutes followed by incubation of the sections in 10% normal donkey serum (NDS, Jackson Labs, USA) in PBST for 20 minutes. Following brief washes in PBST containing 1% NDS, sections were incubated in a polyclonal primary antibody raised in rabbit against the residues surrounding T202/Y204 of pERK1/2 (1:700; Neuromics, USA) overnight. The immunoreaction was visualized by incubation with donkey anti-rabbit IgG conjugated to fluorescein isothiocyanate (1:500; Jackson Labs, USA) for 2 hours. Sections were covered by glass coverslips using Vectashield (Vector, UK). All the washes and incubations were done at room temperature in PBST.

Spinal cord sections were analysed in a Leica epifluorescent microscope. Immunopositive neurons with clearly visible perikarya were manually counted on both the ipsi- and contra-lateral dorsal horn in every second of 10 adjacent sections.

5.2.3. Preparation of cultured primary sensory neurons

Rats were terminally anaesthetised by Enflourane (Abbott, Maidenhead, UK) and their dorsal root ganglia (DRG) from the first cervical to the first sacral segments were dissected out and maintained in culture medium (Ham's nutrient F12 (Invitrogen, UK), supplemented with 1 ml L-glutamine (Invitrogen, UK); 50 IU/ml penicillin (Invitrogen, UK); 50 µg/ml streptomycin (Invitrogen, UK); and 4% Ultroser G (Pall BioPharmaceuticals, France)). Ganglia were incubated in collagenase (2000 U/ml, Sigma) for 3 hours at 37ºC in an atmosphere of 95% air and 5% CO_2. Following washes in the supplemented medium, the ganglia were triturated through a fire-polished Pasteur pipette, before being re-suspended and plated on poly-DL-ornithine-coated glass coverslips. The cells were then kept at 37°C in an atmosphere of 95% air and 5% CO_2 for 2 days in the presence of nerve growth factor (NGF, 50 ng/ml, Promega, UK). Each animal provided one culture. We then used the cobalt uptake technique, *infra*, to assess the effect of xenon on TRPV1 activity.

5.2.4. Preparation of HEK293 cells transiently transfected with human TRPV1

HEK293 cells were maintained and propagated using standard procedures. Briefly, cells were grown as monolayers in minimum essential medium (MEM, Invitrogen, UK) containing 10% foetal bovine serum (Invitrogen, UK), 2 mM L-glutamine (Invitrogen, UK), penicillin (5,000 IU/ml, Invitrogen, UK), streptomycin (5 mg/ml, Invitrogen, UK) and MEM non-essential amino acids (Invitrogen, UK) in an atmosphere of 95% air and 5% CO2 at 37ºC. Cells were harvested every 3-4 days, using trypsin-EDTA. Only cells with a passage number lower than 22 were used in this study. Sub-confluent HEK cells were transiently transfected with human TRPV1

(hTRPV1) using Plus Reagent and Lipofectamine (Invitrogen, UK). The hTRPV1 was cloned in a pcDNA3.1/V5-His-TOPO vector (Invitrogen, UK). Transfected cells were plated onto 13 mm coverslips coated with poly-DL-ornithine (Sigma, UK) in 25,000 cells/cm^2 density and cultured in the growth media for 1 to 4 days. We then used the cobalt uptake technique to assess the effect of xenon on TRPV1 activity.

5.2.5 Cobalt uptake

The cobalt uptake experiments were performed as described previously[28-29]. Briefly, cultured primary sensory neurones, or HEK293 cells attached to their coverslips, were washed in buffer A (in mM: NaCl, 57.5; KCl, 5; $MgCl_2$, 2; HEPES, 10; glucose, 12; sucrose, 139; at pH 7.4) twice for 2 minutes. Then, cells were pre-incubated in buffer A saturated with a mixture of 75% nitrogen and 25% oxygen (control) or 75% xenon and 25% oxygen.

The first experiment with primary sensory neurons was done at room temperature. The pre-incubation of cells in this experiment lasted for 15 minutes and was performed with buffer A that had been bubbled through with either of the gas mixtures for 15 minutes. The pre-incubation was done in four-well plates which were covered by parafilm in order to prevent the escape of the gases. Buffer A was changed for cobalt-uptake buffer (buffer A+5mM $CoCl_2$) that had been bubbled through the appropriate gas mixture for 15 minutes and contained or lacked capsaicin (100nM or 1μM; stock dissolved in DMSO; Tocris, UK). Incubation of the cells in the cobalt-uptake buffer lasted for 5 minutes in four well plates covered by parafilm.

In a subsequent experiment, in order to try to improve xenon delivery, the pre-incubation of primary sensory neurons was done in an air-tight chamber filled with either of the gas mixtures. Buffer A was saturated by exposing it to the gases in the chamber for 45 minutes before the pre-incubation. The flow-rate of the gas mixtures through the chamber was adjusted to 2.1 l/minute both during the buffer saturation and pre-incubation, while the chamber itself had a volume of 3 l. The pre-incubation time was 15, 30, or 60 minutes. The cobalt-uptake buffer that had been saturated also by exposing it to the gases for 45 minutes in the chamber and contained or lacked capsaicin (500nM) was put onto the neurons for 5 minutes. The temperature within the chamber throughout the experiment was maintained at 37ºC.

Having regard to the results obtained on primary sensory neurons, the pre-incubation of the HEK293 cells lasted for 45 minutes in the chamber with buffer A that had been previously saturated with the appropriate gas mixture in the chamber for 45 minutes. Incubation of the cells in the cobalt-uptake buffer that had also been saturated in the chamber by the appropriate gasses for 45 minutes either with, or without, capsaicin (10nM-1µM) lasted for 5 minutes.

Following the removal of the cobalt-uptake buffer both primary sensory neurons and HEK293 cells were washed briefly in buffer A at room temperature or 37ºC. Cells were then transferred into 0.4% mercaptoethanol in buffer A for 2 minutes, then into 70% ethanol for 30 minutes, and mounted on glass slides using glycerol. Analysis of the cobalt labelling was carried out as described previously[28-29]. Briefly, the optical intensities of more than 100 systematically randomly chosen cells were measured on each coverslip by a Leica light microscope attached to a CCD

camera and a PC running the QWin software package (Leica). The distributions of the optical intensities found on each cover-slip were then fitted by normal distributions. The cut of intensity distinguishing between responding and non-responding cells was established as the 95% confidence interval of the normal distribution fitting the intensities of the labelled cells (Figure 1).

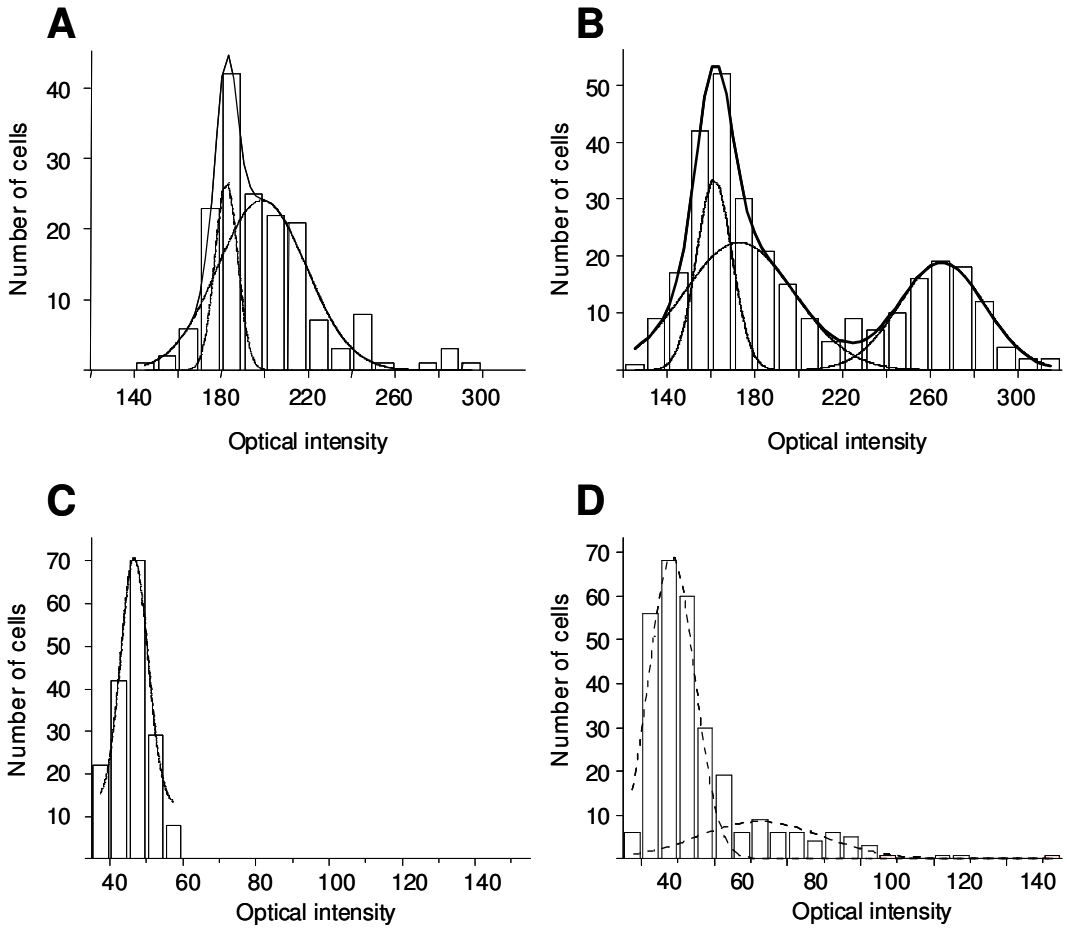


Figure 1.

Analysis of cobalt uptake experiments. Following the measurement of the optical intensity of at least 100 cells chosen randomly systematically, the distribution of the optical intensities were generated. The distributions were fitted with Guassian curves. (A) and (B) show typical distributions of optical intensities from two cover-slips containing cultured DRG neurons.

(A) shows the optical intensities of cells which were incubated in cobalt without capsaicin.

(B) shows the distribution of optical intensities which were incubated in cobalt in the presence of capsaicin.

Note that, while in the control conditions (A), the cells formed two groups regarding their optical intensities, capsaicin induced the formation of a third group of neurons with higher optical intensities. These latter cells represented the capsaicin-responding neurons. The cut-off intensity was determined as the lower 95% confidence interval of the curve fitted to the group of neurons with the highest optical intensities.

The reason for the presence of two groups in the control experiment is not known. Apparently, there is no correlation between the size of the neurons and their optical intensities (data not shown).

(C) and (D) show similar distributions of hTRPV1-transfected HEK293 cells. Note that, in control conditions (C), HEK293 cells formed only one group of cells regarding their optical intensities. Cobalt in these cells induced the formation of a second group of cells regarding their optical intensities.

5.2.6. Statistical analysis

The average number of pERK1/2-expressing neurons per section in each spinal cord was established. Then, the average number of pERK1/2-expressing cells of animals subjected to the same treatment was calculated.

The relative number of cobalt-labeled cells in each set of experiments in each culture was normalized to the highest relative number found in that set of experiments in order to make comparisons between primary sensory neurons and HEK cells easier. Normalized data obtained in the same condition were then averaged.

Differences between relevant groups were assessed by one way or multiple analyses of variance as appropriate. The statistical significance of the differences was assessed by the Dunnett's test. Differences were regarded as significant at $p<0.05$. Data are expressed as mean±SEM. n shows the number of cultures or animals which received the same treatment.

5.2.7. CGRP assay: ELISA

CGRP release was assessed using ELISA (SPI-BIO, France), employing the method previously described by Ahluwalia and colleagues (2003). All procedures were performed at room temperature. Briefly, cells were washed once with release buffer (in mM: NaCl, 136; KCL, 536; $MgCl_2$, 0.49; $CaCl_2$, 1.27; Glucose, 5.5.; $MgSO_4$, 0.4; KH_2PO_4, 0.44; NaH_2PO_4, 0.33; HEPES,10; pH 7.4). After washing, the cells were incubated in release buffer to which either 75%/25% N_2/O_2 or 75%/25% Xe/O_2 mixtures had previously been introduced by bubbling the gases through the

buffers for 15 minutes with the plates covered by parafilm in order to inhibit the escape of the gases. The cells were then placed in release buffer similarly treated, but which also contained the specified concentration of capsaicin (100 nM or 1 uM), or no capsaicin (as a control). The cells were incubated in these buffers for 2 minutes, again using parafilm to cover the plates. The buffers were removed, frozen immediately and then stored at -20 degrees C until the ELISA procedure was performed.

The CGRP content of the buffers was measured using the ELISA kit in accordance with the manufacturer's instructions. The absorption values of the acetylcholinesterase products were measured using a DYnex MRX-II 96 well plate reader (Dynex Technologies) using a 405 nm filter, controlled and analysed using the Revelation 4.22 software package running on a PC. Samples were measured in duplicate. CGRP data were normalised to the total protein content of the appropriate coverslips.

5.2.7.1 Protein content measurement

The protein content on each coverslip was measured in order to normalise the results of the CGRP release assay. After removal of the release buffer used for CGRP ELISA, the cells were removed from the coverslips in a lysis buffer (Cell Lysis Buffer 1x (Cell Signalling Technologies, Beverly, MA) with 1.7 mg/mL phenylmethylsulphonyl fluoride (Cell Signalling Technologies) and 3 mg/mL Dithiothreitol (Sigma) by scraping. The cell lysate was frozen immediately, stored at –20 degrees C, and used subsequently for measurement of the protein content.

Samples were first centrifuged at 4 degrees C, at 3000 rpm, for 10 minutes to separate the cells into a pellet. The supernatant was then removed. Protein standards were made using serial dilutions of Basic Standard Albumin into concentrations of (ug/mL) 1000, 500, 250, 125, 12.5, 1.25, fitted with a sigmoid curve. The protein measurement was done on 96-well plates in triplicate using the Bio-Rad protein assay kit (Bio-Rad Labs, GmbH). Absorption values were measured using the Dynex MRX-II (Dynex Technologies) plate reader with a 595 nm filter, using the Revelations 4.22 software package.

5.2.7.2 Statistical analysis for CGRP assay

Averages of appropriate data were calculated and the differences between relevant data were analysed by ANOVA. The significance of the differences was assessed using the Fisher *post-hoc* test. Data were regarded as significantly different at $p < 0.05$. Data are expressed as mean +/- standard error mean. "n" refers to the number of cultures tested in each condition.

5.3. RESULTS

5.3.1. Capsaicin-evoked phosphorylation of ERK1/2 in spinal dorsal horn neurons

The immunostaining was readily recognisable in the dorsal horn of the spinal cord (Figure 2A-B). Based on their size and shape, all immunopositive cells were identifiable as neurons. The great majority of the immunolabelled neurons were distributed in the superficial layers of the dorsal horn. Immunostained neurons could only very rarely be seen in the deep dorsal horn or around the central canal.

Injection of the vehicle into the hind-paw of rats resulted in negligible pERK1/2 expression in either the ipsilateral or the contralateral dorsal horn of the spinal cord, regardless of whether the animals had been treated with sodium pentobarbital or xenon (Figure 2C). Injection of capsaicin into the paws produced negligible pERK1/2 expression in the contralateral side of the spinal dorsal horn, too (Figure 2C). In contrast, injection of capsaicin into the paws resulted in a substantial number of pERK1/2-immunopositive cells in the ipsilateral spinal dorsal horn both in the sodium pentobarital-, and xenon-treated, rats (Figure 2C) In the sodium pentobarbital-anaesthetised rats, capsaicin injection resulted in pERK1/2 immunolabelling in 12.4±0.4 neurons/section (n=3), which was significantly different from that found in the ipsilateral side of the vehicle-injected animals (Figure 2C). Xenon anaesthesia significantly reduced the capsaicin injection-evoked pERK1/2 expression in the ipsilateral spinal dorsal horn (7.6±1.1 neurons/section; n=3; Figure 2C). The reduction in the number of pERK1/2 expression neurons was about 40%. Collectively, these results suggested that xenon, in the dose which we employed,

reduced capsaicin-evoked activation of rat spinal dorsal horn neurons. In order to ascertain whether inhibition of TRPV1 was involved in, or contributed to, the reduction in neuronal activation in the spinal cord, next, we studied the effect of xenon on capsaicin-induced TRPV1 responses in rat primary sensory neurons and in hTRPV1-expressing HEK293 cells.

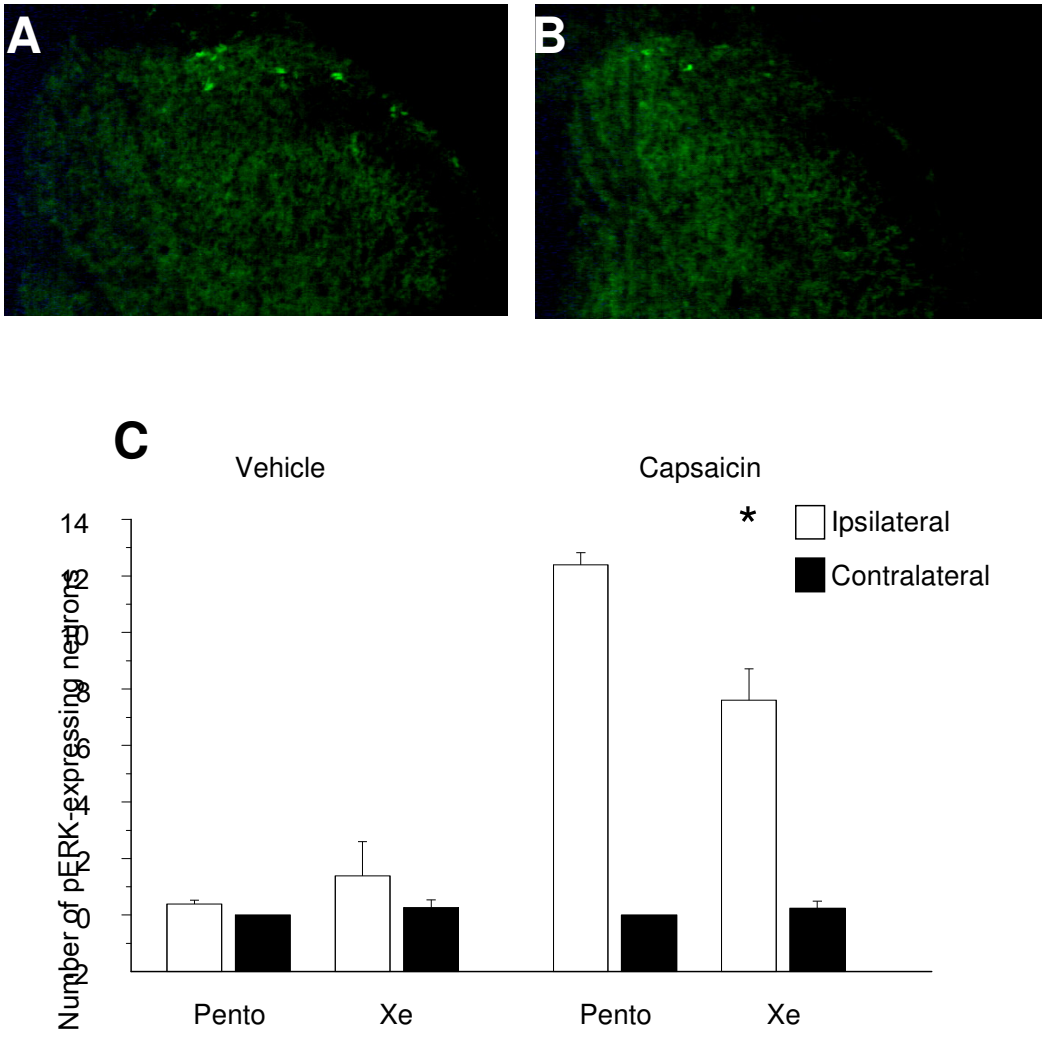


Figure 2.

The effect of xenon on the activation of spinal cord neurons evoked by subcutaneous capsaicin injection was assessed by studying the expression of phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) in the dorsal horn. Adult rats

were anaesthetised either by pentobarbital (Pento) or 75% xenon (Xe) and capsaicin was injected into the right hind paw. pERK1/2 expression was revealed by immunoflourescent staining.
(A) A typical image of the ipsilateral spinal dorsal horn from a pentobarbital-anaesthetised rat. Note that there are several pERK1/2-immunopositive neurons seen here in lamina I and outer lamina II.
(B) A typical image of the ipsilateral spinal dorsal horn from a xenon-anaesthetised rat. Note that the number of pERK1/2-immunoposive neurons is less than in the spinal cord of the pentobarbital-anaesthetised animals. Scale on (B) is 200μm.
(C) The number of pERK1/2-immunolabelled neurons was counted in every second section of 10 adjacent sections prepared from the L4-L5 spinal cords. Then, the number of immunopositive cells per section was calculated. Note that xenon application resulted in a significant decrease in the average number of pERK1/2-expressing neurons (n=3 in each data point).

5.3.2. Capsaicin-evoked activation of TRPV1 in primary sensory neurons

In the presence of the nitrogen/oxygen gas mixture, exposure of cultured dorsal root ganglion neurons to the capsaicin-free cobalt-uptake buffer resulted in labelling in a negligible proportion of neurons (Figure 3A, C). In contrast, exposure to the capsaicin-containing cobalt-uptake buffer in the same conditions resulted in a significant number of neurons showing cobalt-labelling (37.5±0.9% (n=3) of the total population at 1 μM capsaicin, Figure 3B, C). The proportion of labelled neurons by 100nM capsaicin was significantly less than by 1μM.

In the presence of the xenon/oxygen mixture, the cobalt-uptake buffer without capsaicin also produced only a negligible response (Figure 3C). While this value was lower than the proportion of labelled cells by the cobalt-uptake buffer alone in the presence of nitrogen/oxygen, the reduction was not significant. Capsaicin application resulted in a significant increase in the proportion of responding neurons (Figure 3C).

While that increase was less than that in the presence of nitrogen/oxygen at both concentrations, the reduction was significant only at 100nM capsaicin. The xenon-evoked reduction at 100nM capsaicin was about 30% (Figure 3C).

When primary sensory neurons were pre-incubated and incubated in the air-tight chamber at 37ºC, xenon reduced the proportion of primary sensory neurons labelled by capsaicin-induced cobalt-entry by ~15% (with 15 minutes pre-incubation; n = 2), ~50% (with 30 minutes pre-incubation; n = 2) and 54.7±8.7% (with an hour pre-incubation; n=3; data are not shown). These findings indicated that increasing the exposure time to xenon in an air-tight chamber can increase the efficacy of the xenon-evoked inhibitory effect on TRPV1.

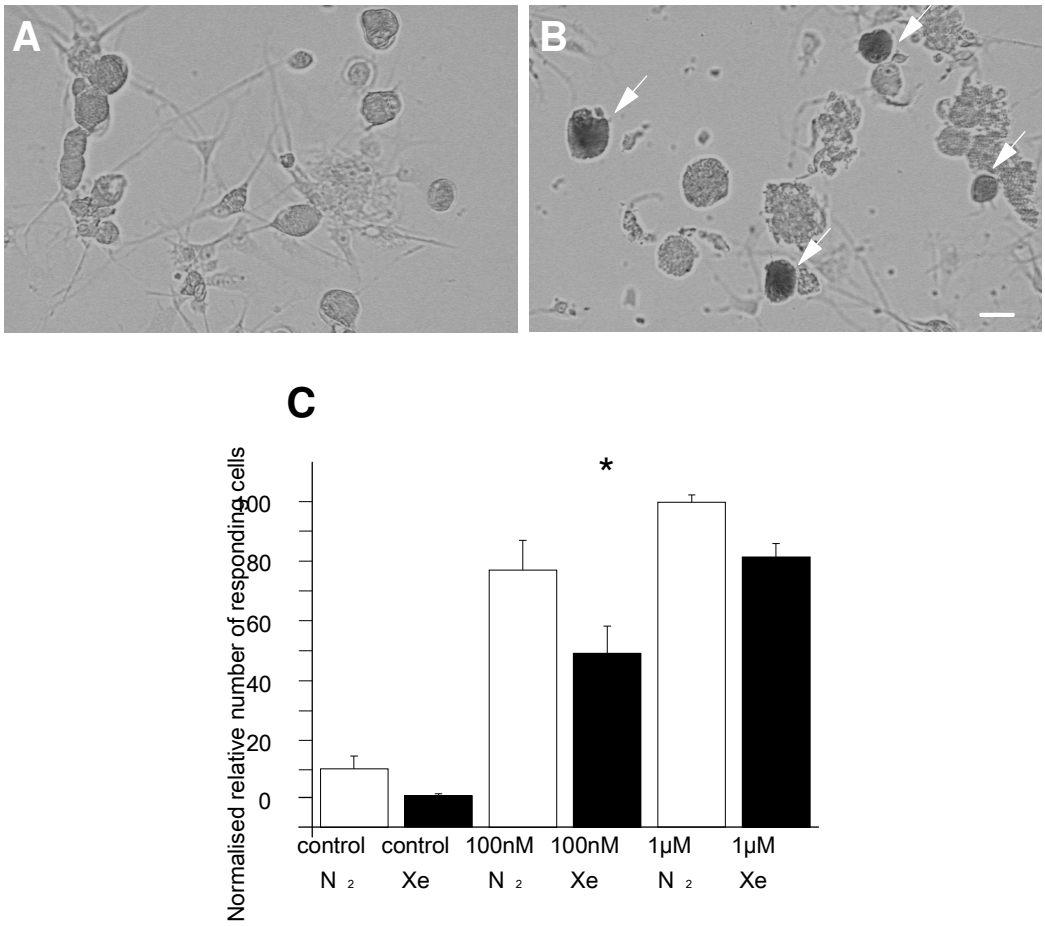


Figure 3.

Effect of xenon on capsaicin-evoked TRPV1 responses of rat cultured primary sensory neurons.

(A) Controls: a negligible number of neurons accumulated cobalt.

(B) Incubation with Co^{2+} and capsaicin for 5 minutes: a significant number of cells accumulated cobalt (dark cells indicated by the white arrows). Bar: 20μm.

(C) Averaged normalised relative number of cultured primary sensory neurons showing cobalt accumulation in 3-5 cultures. White columns: cultures incubated with 80% nitrogen and 20% oxygen. Black columns: cultures incubated with 80% xenon and 20% oxygen. Note that the proportion of capsaicin-responding neurons is reduced when the cells are pre-incubated in the buffer saturated with 80% xenon and 20% oxygen. This reduction was significant only at 100nM capsaicin.

** indicates significant difference ($p<0.05$).*

5.3.3. The effect of xenon on human TRPV1

Based on the data obtained on cultured primary sensory neurons these experiments were done in an air tight chamber with 45 minutes pre-incubation. To confirm that the 45 minute pre-incubation does not *per se* affect cobalt-labelling, first we compared the responses of the hTRPV1-expressing HEK293 cells with the usual twice 2 minutes wash and the wash followed by a 45 minute pre-incubation in buffer A saturated with nitrogen/oxygen. In both conditions capsaicin (10nM-1μM), evoked a concentration-dependent increase in the number of labelled cells between 10 nM and 300 nM (Figure 4). The relative number of cobalt-labelled cells at any capsaicin concentration was significantly higher than that produced by the cobalt-uptake buffer alone (Figure 4). We found the 300nM capsaicin application following only the usual wash produced labelling in about half of the cells in average (51.9±17.9%, n=3; Figure 4). The relative number of labelled cells at 1 μM capsaicin in both conditions was lower than at 300 nM, which was probably due to desensitisation of TRPV1 (Figure 4).

In the presence of xenon, capsaicin also produced a concentration-dependent increase in the number of cobalt-labelled cells between 10 nM and 300 nM (Figure 4). The 1 μM capsaicin-evoked labelling in this condition was also lower than that produced by 300 nM capsaicin. Comparison of the relative number of cells labelled by capsaicin-induced cobalt accumulation in the presence of nitrogen/oxygen and xenon/oxygen revealed that xenon reduced the capsaicin-evoked responses from 10 nM to 300 nM. However, a significant difference in the reduction was found only at 100 nM and 300 nM. On average, the xenon-induced reduction in the proportion of

cobalt-labelled cells was approximately 50% between 10nM and 300nM. The maximum reduction was found at 100 nM capsaicin, where the labelling was reduced by about 55%. Collectively, these results confirmed that the xenon-evoked reduction in the capsaicin injection-induced pERK1/2 expression in the spinal dorsal horn was contributed by xenon-evoked inhibition of TRPV1 activity on primary sensory neurons.

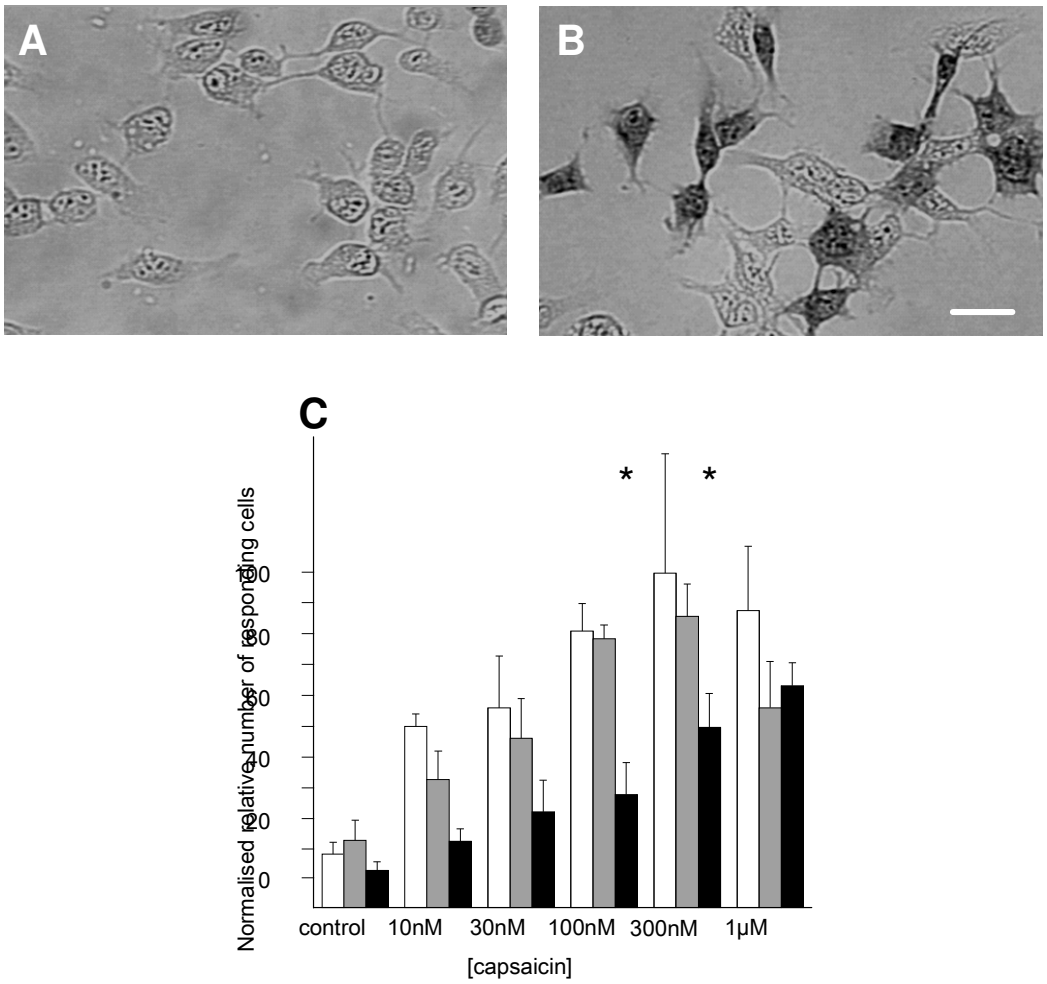


Figure 4.

Effect of xenon on capsaicin-evoked responses of hTRPV1 expressed by HEK293 cells after transient transfection.
(A) Controls: a negligible number of cells accumulated cobalt.
(B) Incubation with Co^{2+} and capsaicin for 5 minutes, a significant number of cells accumulated cobalt (dark cells). Bar: 20µm.
(C) Averaged normalised relative number of hTRPV1-expressing HEK293 cells showing cobalt accumulation in 3 cultures at each concentration. Empty bars: incubation with Co^{2+} and capsaicin for 5 minutes. Gray bars: incubation for 45 min in 80% nitrogen-20% oxygen. Black bars: incubation for 45 min with 80% xenon-20% oxygen. Note that incubation of cells in the presence of xenon (black bars) resulted in reduction of the number of capsaicin-responding neurons. This reduction was significant only at 100nM and 300nM.
** indicates significant difference ($p<0.05$).*

5.3.4. Results: capsaicin-evoked release of CGRP from DRG neurons *in vitro*

5.3.4.1. CGRP release from cells in buffer exposed to N_2 /O_2 mixture

Basal (non-stimulated) release of CGRP was 1.09 +/-0.20 pg/ug protein. In the presence of 100 nM capsaicin, CGRP release was increased to 1.96 +/-0.50 pg/ug (n=3). In the presence of 1 uM capsaicin, CGRP release was 1.32 +/- 0.38 (n=3), which was not significantly different from the control or from 100 nM capsaicin application.

5.3.4.2. CGRP release from cells in buffer exposed to Xe/O_2 mixture

In the presence of xenon, basal CGRP release was increased but this increase was not significant (1.34 +/- 0.09 pg/ug) (n=3). The presence of xenon failed to produce any significant changes in the extent of the CGRP release evoked by capsaicin when compared to the release evoked in the presence of the N_2 /O_2 mixture. At the lower concentration of capsaicin in the presence of xenon, CGRP release was 1.92 +/-0.44 pg/ug. At the higher concentration of capsaicin, CGRP release was 1.46 +/- 0.35 pg/ug in the presence of xenon.

As expected, capsaicin application resulted in the release of CGRP in these preparations at both of the concentrations at which it was applied. However, neither response was concentration dependent and, in fact, the release of CGRP evoked by 1 uM concentration of capsaicin was less than that which resulted from exposure of the cells to 100 nM concentration of capsaicin, although this difference was not statistically significant.

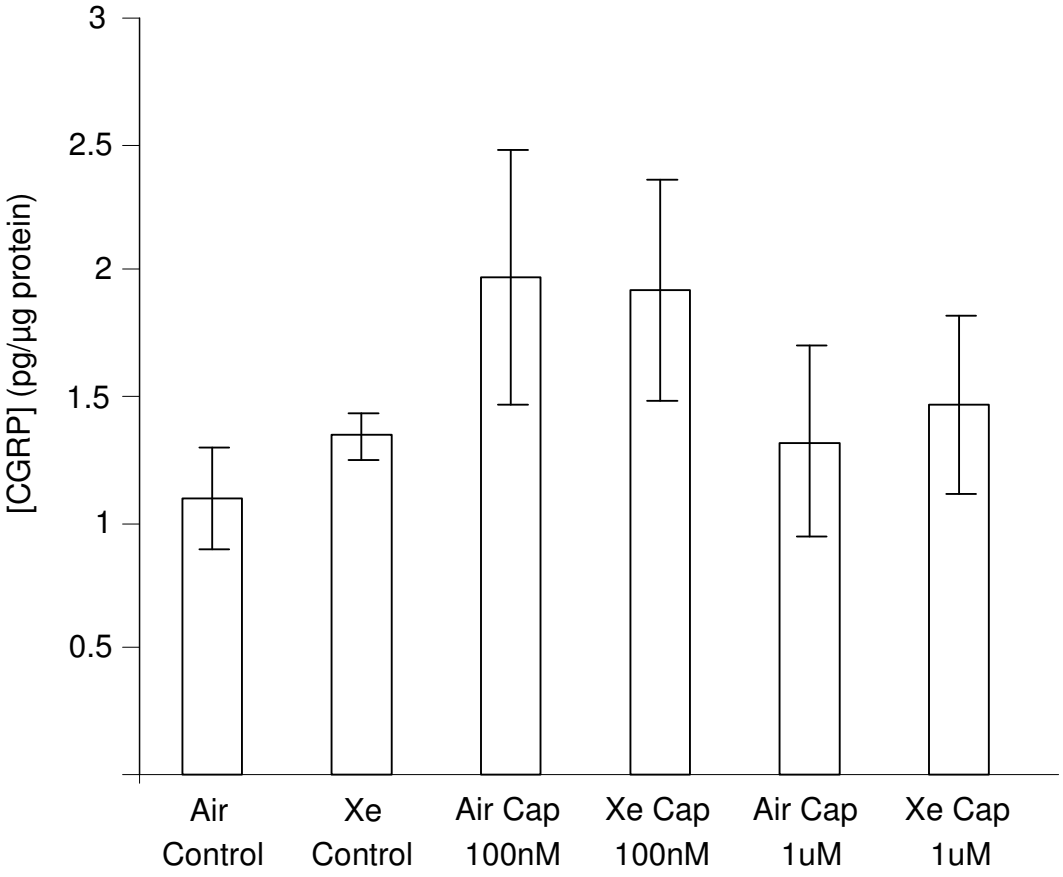


Figure 5.

CGRP release by cultured DRG neurons when challenged with 100nM and 1µM capsaicin, respectively, in the presence, and absence, of xenon. Note that xenon increased, though non-significantly, the basal release of CGRP in control conditions. Note also that only 100nM capsaicin induced a significant increase in CGRP release. Finally note also that xenon, when present, does not significantly affect CGRP release.

5.4. DISCUSSION

5.4.1. Summary of results

The present studies show that xenon, at the concentrations employed, significantly reduces capsaicin-induced activation of spinal cord neurons, and that this effect is mediated, at least in part, by reduced activation of TRPV1.

Unlike volatile anaesthetics, xenon and nitrous oxide, at sub-anaesthetic concentrations, can reduce heat-induced pain (Yagi et al., 1995; Petersen-Felix et al., 1998). In rats, xenon suppresses both the pain-related behavioural, and the spinal expression of the nociceptive marker, cFos, induced by formalin injection (Fukuda et al., 2002; Ma et al.). Our results demonstrate a significant inhibitory effect of xenon on the activity of TRPV1 in peripheral sensory afferents. We also demonstrate a similar inhibitory effect of xenon on the activation of spinal dorsal horn neurons evoked by peripheral capsaicin injection and consequential TRPV1 activation in nociceptive primary afferents. At both capsaicin concentrations (100 nM and 1 uM), however, no effect of xenon on CGRP release was observed. Surprisingly, the presence of xenon does not reduce CGRP release in this preparation. Overall, however, these findings may well have important clinical implications for the control of severe inflammatory-type pain to which TRPV1 is a recognised contributor (Caterina et al., 2000; Davis et al., 2000; Charrua et al., 2007; Garcia-Martinez et al., 2006; Tang et al., 2007).

5.4.2 Properties of xenon

The element xenon is one of the "noble gasses" and exists as single atoms in the atmosphere. Xenon does not readily form covalent bonds with other atoms under normal biological conditions because its outer electron shell is completely filled with electrons. However, although traditionally characterised as an "inert" gas, it is now recognised that xenon can form certain compounds. Importantly, it is also capable of forming non-covalent bonds with atoms and molecules in many instances. Under gas pressures ranging from 8 to 20 bar, xenon is able to bind to discrete sites in hydrophobic cavities, ligand-binding and substrate-binding pockets and into the pore of channel-like structures (Prange et al., 1998). Even at moderate pressures, xenon binds to pre-existing atomic-sized cavities in the interior of certain globular protein molecules, such a sperm whale myoglobin and horse haemoglobin crystals, through weak van der Waals forces (Li.de la Sierra et al., 1997; Schoenborn et al., 1965). Since the interaction of xenon with proteins is the result these non-covalent, weak-energy forces, the process of xenon binding is completely reversible (Tilton & Kuntz, 1982; Tilton et al., 1984; Tilton et al, 1986; Tilton et al., 1988). The binding of xenon to proteins is characterised by the large diversity of its binding sites. Xenon binds to intra-molecular, as well as to inter-molecular, sites, to closed inaccessible cavities, as well as to exposed pockets and channel-pores. Xenon may bind into void sites, or replace existing water molecules. Thus, in contrast to the small, closed, hydrophobic cavities observed in the majority of proteins, the xenon binding sites in serine hydrolases are large solvent-accessible pockets that, in the case of elastase, collagenase and cutinase contain ordered water molecules which are displaced upon xenon binding (Prange et al., 1998).

Xenon is known to be capable of exercising an inhibitory effect on ligand-gated ion channels, similar to TRPV1, including glutamatergic, serotonergic (Suzuki et al., 2002) and nicotinic acetylcholine channels (Suzuki et al., 2003). Glutamate receptor ion channels are of three types, described by their preferred ligands, namely: NMDA, AMPA, and kainate glutamate channels. Franks and colleagues showed that xenon can inhibit NMDA ion channels in cultured hippocampal neurons in a non-competitive manner at 80 per cent xenon which is sufficient to maintain surgical anaesthesia (Franks et al., 1998). All three types of glutamate channels are inhibited by xenon in cortical neurones (Dinse et al., 2005). Xenon inhibits the human (alpha7)5 nACh receptor in a concentration-dependent manner, with this inhibition being non-competitive and independent of membrane potential (Suzuki et al., 2003). In contrast, xenon inhibits the 5-hydroxytryptamine 3 receptor, again independently of membrane potential, but the inhibition is competitive (Suzuki et al., 2002). Finally, in this connection, it may be noted that xenon can activate, rather than inhibit, certain ion channels and is capable of activating two-pore-domain potassium channels (Gruss et al., 2004).

Notwithstanding the demonstration of a substantial effect of xenon in reducing TRPV1 ion channel activation in HEK 293 cells upon challenge with the potent agonist, capsaicin, we continued to entertain considerable doubt as to the extent to which xenon was delivered effectively to the HEK cells. Therefore, in our subsequent experiments using DRG neurones, we considered it appropriate to attempt to improve the likelihood of effective delivery of the gas to the cells by bubbling it through the buffer in which the cells were maintained prior to their exposure to capsaicin. Having

regard to the results, in terms of TRPV1 activation, after this attempt at improving xenon delivery, our process of "bubbling" manifestly did not offer the answer to the problem which we perceived of improving delivery of xenon to the cells *in vitro*.

5.4.3 CGRP assay

Xenon elicited a minor increase in the basal release of CGRP in the rat DRG cells preparation which was clearly occasioned by the action of xenon on unidentified receptors. The extent of this increase did not reach statistical significance in our experiments, but we believe that the phenomenon is real in the context of rat DRG neuronal preparations exposed to xenon. However, we do not believe that it is of physiological significance in mediating a pain sensation. Spontaneous release of neurotransmitter from neurones at rest is a recognised phenomenon which is not known to have a physiological effect in the transmission of information relating to pain stimuli. Moreover, the transmission of information relating to pain stimuli manifestly requires a form of neurotransmitter release which is both organised and specific. In any event, this effect of xenon was small in terms of the increase of neurotransmitter release elicited, and was not elicited when the neurones were subsequently challenged with capsaicin. Therefore, it appears unlikely that it could constitute a significant adverse effect of the administration of xenon *in vivo.* Moreover, if xenon is indeed capable of mediating a pain sensation by activating receptors leading to neurostransmitter release, one would expect to find evidence of significant pain sensations attributable to xenon on the part of patients, who receive an anaesthetic dose of the gas, as they recover consciousness.

More surprising is our finding that xenon does not reduce the extent of CGRP release evoked by capsaicin. Several reasons may be suggested for this finding. First, it may be that there is a disconnection between TRPV1 activity and CGRP release in rat DRG preparations. In addition, or in the alternative, it may be that the maximal effect of xenon of reducing TRPV1 activity by approximately only 30 per cent in rat DRG neurones *in vitro,* using the gas-delivery method which we used, is insufficient to reduce CGRP release on challenge with capsaicin.

5.4.4. Limitation of experimental design

It is considered that there must always remain considerable doubt as to the extent to which effective *in vitro* exposure of cells in solution to xenon is achieved. This is because xenon atoms are uncharged with the result that there is no ionic flux which might attract these atoms through the channel pore. The result is that delivery of xenon to, and through, the channel pore is dependent upon the concentration of atoms in the external solution and the resulting concentration gradient from without to within the cell which will tend to draw xenon atoms inward. To this must be added the consideration, discussed above, that xenon can make binding sites for itself under pressure. Since that pressure was not available in this case, and since even the presence of xenon atoms at TRPV1 ion channels was due to little more than mere accidental propinquity, the conclusion must be that it is highly doubtful that xenon exhibited a maximal, or near maximal, effect of its potential to inhibit the activation of TRPV1. In addition to this consideration, TRPV1 expression in the DRG neurones may have differed substantially from that in the transfected HEK cells and, moreover, one cannot know how the auxiliary proteins which interact with TRPV1 ion channels in the natural state, and which are obviously absent in the transfected cells, affected

the respective results. Notwithstanding these reservations, our results demonstrate a significant effect of xenon on TRPV1 activity which may well have clinical implications for the control of severe inflammatory-type pain, such as that which is found most obviously in severe burn injuries, which is recognised as being mediated by TRPV1 (Davis et al., 2000).

5.4.5 Mechanism of xenon's actions

The further issue relates to the mechanism by which xenon may mediate its effect on TRPV1 and, here, there is evidence that xenon may achieve its effect by several mechanisms, rather than by a single mechanism.

First, xenon may act as a competitive antagonist at one, or more, of the binding sites for ligands of TRPV1. Since TRPV1 possesses different binding sites for certain of its activators, xenon, given its known promiscuity in the matter of binding to other atoms or molecules, may well bind to one, or more, of these sites. There is some evidence that xenon indeed acts as a competitive antagonist of the 5-hydroxytryptamine 3 receptor, which is also a ligand-binding ion channel (Suzuki et al., 2002).

Second, there is substantial evidence that, in any event, xenon in fact acts as a "conduction blocker" of the TRPV1 channel pore. This mechanism of action for xenon would be entirely consistent with the manner in which xenon mediates its effect on NMDA channels (Franks et al., 1998). Thus, xenon atoms may occupy the TRPV1 channel pore thereby blocking, or reducing, the ingress of cobalt through the channel pore, notwithstanding ligand-binding by capsaicin which would otherwise

gate the channel to allow the cobalt ions into the cell. In like manner, xenon may be expected to block, or reduce, the generation of membrane currents through that channel. This hypothesis is supported by a recent study by Oseguera and colleagues of the action of tetrabutylammonium (TBA) on HEK 293 cells transfected with rat TRPV1. TBA is a member of a family of classic potassium channel blockers widely used for determining the localisation of the activation gate and the properties of the pore of several ion channels. TBA was found to act as an ion channel conduction blocker in TRPV1 channels expressed in HEK 293 cells transfected with rat TRPV1. Pore-blocking by TBA of the TRPV1 ion channel is voltage-dependent and the properties of block are consistent with an open-state blocker, with the TBA molecule binding to multiple open states, each with different blocker affinities. The kinetics of TRPV1 channel closure and burst-length analysis in the presence of blocker are consistent with a state-dependent blocking mechanism, with TBA interfering with closing of an activation gate (Oseguera et al., 2007). Garcia-Martinez and co-workers (2006) investigated the effect of a synthetic compound, described as H-Arg-15-15C, in blocking TRPV1 activation at sub-micromolar concentrations. H-Arg-15-15C acts as a non-competitive capsaicin antagonist with modest voltage-dependent blockade activity. When assessed in rats, this compound is reported by these authors to: prevent the irritant activity of local administration of capsaicin and formalin; reverse the thermal hyperalgesia evoked by injection of complete Freund's adjuvant; and to attenuate carrageenan-induced paw inflammation -- without affecting normal nociception, and with little cross-reactivity with other receptors (Garcia Martinez et al., 2006). However, this mechanism of xenon’s action at NMDA receptors is now believed to be less likely (Weight et al., 2008).

The third possible mechanism by which xenon may inhibit the activity of TRPV1 ion channels involves xenon binding to a pocket in the channel pore, or, indeed, to another binding site for xenon and thereby inducing a conformational change in the protein which results in a failure of "gating" in the ion channel. The active site of enzymes can provide a specific binding cavity for a single xenon atom without inducing major changes in protein structure. Thus, the catalytic site of serine proteinases provides a specific binding cavity for xenon (Schiltz et al., 1995; Prange et al., 1998). Again, xenon occupies the haeme cavity of cytochrome P-450 monooxygenases and competitively inhibits its catalytic activity (La Bella et al., 1999). Colloc'h and colleagues (2007) recently employed x-ray crystallography techniques to study the binding characteristics of nitrous oxide and xenon, using urate oxidase and annexin V as structural models. Urate oxidase provides a model representative of a variety of intracellular globular proteins that possess large hydrophobic cavities, while annexin V is a protein that binds to biological membranes in a reversible calcium-dependent manner and possesses a hdyrophilic pore, believed to be the calcium conduction pathway, that lies at the interface of four homologous and structurally-related domains (Colloch et al., 1997; Gerke et al., 2002). Annexin V possesses structural characteristics and functional properties of ion selectivity and voltage-gating which allow it to be regarded as a prototype for the NMDA receptor (Berendes et al., 1992; Demange et al., 1993). They identified the primary binding sites for xenon within these two soluble proteins as flexible gas cavities with no visible water (Colloc'h et al., 2007). They postulate that occupation and expansion of large hydrophobic flexible gas cavities within intracellular or membrane proteins may lead to conformational rigidity, thereby disrupting protein and cell functions. Thus, they propose that the presence of a gas bound to a hydrophobic cavity within

the transmembrane domain of the NMDA receptor channel may be capable of altering the flexibility of the pore, thereby limiting calcium exchange and disrupting NMDA receptor function. This proposition would appear to be of no less validity in the context of the TRPV1 ion channel.

CHAPTER 6

A STUDY OF WHETHER ISOFLURANE AND SEVOFLURANE OCCASION PAIN-INDUCING SPINAL NOCICEPTIVE PROCESSING

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6.4. Discussion

Figure 1 Microphotographs illustrating pERK1/2 labelling of neurons in the spinal dorsal horn

Figure 2 Bar chart illustrating the average number of pERK1/2-labelled neurons in the ipsilateral and the contralateral spinal dorsal horn after various treatments

6.1. INTRODUCTION

Recent *in vitro* studies have shown that certain pungent gaseous anaesthetics, can activate, or increase the activation of, receptors on primary nociceptive afferents which have been implicated as mediators of hyperalgesia in inflammatory pain conditions. These receptors are the transient receptor potential ankyrin, type 1 (TRPA1) and the transient receptor potential vanilloid, type 1 (TRPV1). TRPV1 is a ligand-gated, non-selective, cationic channel (Caterina et al., 1999; Heyman & Rang, 1985; Marsh et al., 1987; Tominaga et al., 1998) which is known to play an important role in several types of heat-induced hyperalgesia. Heat hyperalgesia secondary to inflammatory tissue injury fails to develop in TRPV1 null mice as in wild type mice in several models of thermal hyperalgesia, including that induced by carrigeenan, mustard oil, and complete Freund's adjuvant (Caterina et al., 2000; Davis et al., 2000), suggesting that TRPV1 is an essential component in the development of pathological thermal hyperalgesia in these models of peripheral inflammation (Pogatzki-Zahn et al., 2005). The development of thermal hyperalgesia following inflammation involves an increased level of TRPV1 expression (Amaya et al., 2004; Kasama et al., 2007; McNaughton, 2005; Stein et al., 2006). It also involves the sensitization of existing TRPV1 channels by inflammatory agents (Chuang et al., 2001;Vellani et al., 2006; Zhang et al., 2008; Zhu & Oxford, 2007; Nagy et al., 2008; Nagy et al., 2009). TRPA1 is a non-selective ligand-gated cation channel which is directly gated by Ca^{2+} (Doerner et al., 2007), and is found in a subset of nociceptive sensory neurons of dorsal root and trigeminal ganglia that co-express TRPV1 (Story et al., 2003; Jordt et al., 2004; Kobayashi et al., 2005). Many of the exogenous activators of TRPA1 produce an inflammatory response when applied to the body;

but, more importantly, TRPA1 is activated by certain at least of the chemical agents generated by the inflammatory response (Jordt et al., 2004; Bautista et al., 2006; McNamara et al., 2007).

The pungent general anaesthetics isoflurane and desflurane robustly activate TRPA1, with isoflurane producing inward currents in voltage-clamped TRPA1-expressing HEK293 cells and in cultured mouse dorsal root ganglion neurons (Matta et al. 2008). Moreover, clinically relevant concentrations of isoflurane, sevoflurane, enflurane and desflurane have also been shown to sensitise TRPV1 to capsaicin and protons and to reduce the threshold for heat activation of this ion channel (Cornett et al., 2008). Hence, the suggestion that, in the clinical context, volatile general anaesthetics may augment nociceptive signalling arising from surgical insults (Cornett et al., 2008). In particular, the finding that sevoflurane may exert a sensitizing effect on TRPV1 (albeit an effect which is substantially less than that elicited by isoflurane) raises an issue of clinical importance, given its preferred status as a non-pungent inhalational anaesthetic.

Extracellular signal-regulated protein kinase 1/2 (ERK1/2) when activated by phosphorylation (pERK1/2) in spinal dorsal horn neurons is a recognised marker of spinal nociceptive processing (Ji et al, 1999). We have used activation of ERK1/2 in dorsal horn neurons of the spinal cord in rat to elicit the extent to which exposure of these animals to isoflurane and sevoflurane, in clinically relevant concentrations, results in nociceptive processing in the rat spinal cord. We have also examined the extent to which these inhalational anaesthetics are sensitizers of TRPV1 by exposing

animals to clinically relevant concentrations of these gases and then injecting a hind-paw with the archetypical TRPV1 agonist, capsaicin.

6.2. METHODS

6.2.1. Animals

All procedures were performed in accordance with the requirements of the UK Animals (Scientific Procedures) Act, 1986. Altogether, 21 male Sprague Dawley rats (each ~ 100 gms in weight) were used in this study. The animals were kept at 12 hours light-dark cycles at controlled temperature and humidity, with food and water *ad libitum*.

6.2.2. Treatment groups

Animals were divided into five treatment groups. Animals were randomly assigned to the several groups so each group had 4 animals except for the isoflurane group which contained 5 animals. In all cases, the animals were sacrificed by administration of a terminal dose of pentobarbital. In previous experiments in this laboratory, we have confirmed that pentobarbital administered at such doses in those circumstances does not evoke any significant activation of ERK1/2 in spinal dorsal horn neurons.

Group 1 animals: capsaicin (< 5 minutes). These animals were exposed to pentobarbital at a dose sufficient to render the animals unconscious (60 mg per kg, i.p.). They were then immediately given a subcutaneous right hind-paw injection of capsaicin (1 mg/ml dissolved in 10% Tween 80; 25 uL). At 3.5 to 4.5 minutes after capsaicin injection, the animals were sacrificed. Activation of ERK1/2 in the contralateral spinal cord of these animals served as a control for the effect of capsaicin

on ERK1/2 activation in this group and for the effect of isoflurane and sevoflurane on ERK1/2 activation in the subsequent groups.

Group 2 animals: isoflurane (1 hour). These animals were exposed to isoflurane at a dose of 1.5 Mac which was sufficient to render the animals unconscious and were then maintained in that condition for a period of 1 hour, after which they were sacrificed.

Group 3 animals: isoflurane (1hour) + capsaicin (< 5minutes). These animals were exposed to isoflurane at a dose of 1.5 Mac which was sufficient to render the animals unconscious and were then maintained in that condition for a period of 1 hour. Then, these animals, still under isoflurane anaesthesia, were then given a subcutaneous right hind-paw injection of capsaicin (1 mg/ml dissolved in 10% Tween 80; 25 ul). At 3.5 to 4.5 minutes after capsaicin injection, the animals were sacrificed.

Group 4 animals: sevoflurane only. These animals were exposed to sevoflurane at a dose of 1.5 Mac which was sufficient to render the animals unconscious and were then maintained in that condition for a period of 1 hour, after which they were sacrificed.

Group 5 animals: sevoflurane (1 hour) + capsaicin (< 5 minutes). These animals were exposed to sevoflurane at a dose 1.5 Mac which was sufficient to render the animals unconscious and were then maintained in that condition for a period of 1 hour. Then, these animals, still under sevoflurane anaesthesia, were then given a

subcutaneous right hind-paw injection of capsaicin (1 mg/ml dissolved in 10% Tween 80; 25 ul). At 3.5 to 4.5 minutes after capsaicin injection, the animals were sacrificed.

6.2.3. Perfusion

All animals were perfused transcardially with saline followed by 4% paraformaldehyde. The lumbar spinal cord was removed and placed into the same fixative and allowed to stand over-night. Tissues were then put into 15% sucrose until they sank. Then, the tissues were put into 30% sucrose until they sank. The ventral horn of the L4-L5 segments were labelled by a shallow longitudinal cut on the left side. Cords were then mounted into OCT embedding medium (VWR, West Chester, Pa) and 20µm transverse sections were cut on a crysostat and thaw-mounted on Superfrost Plus microscope slides (VWR, West Chester, Pa). Sections were air dried overnight and stored at -20℃ until required.

6.2.4. Immunohistochemistry

The sections were first washed in PBS and incubated in PBS containing 0.3% Triton X (PBST) for 10 minutes. The endogenous peroxidase activity was quenched by incubating the sections in 0.3% H_2O_2. Sections were then placed in 10% normal donkey serum (NDS) for 25 minutes. The primary antibody was anti-phospho extracellular signal-regulated protein kinase 1/2 (pERK1/2) raised in rabbit against the residues surrounding T202/Y204 of pERK1/2 (1:2000, Neuromics). The sections were incubated in the primary antibody overnight. Following washes in PBS, the sections were incubated with biotin-conjugated donkey anti-rabbit IgG (1:800; Jackson Immunoresearch) for 2 hours. The immunoreaction was visualised using the Vectastain Elite and IMPACT DAB kits (Vector). The slides were then dehydrated in

ethanol, cleaned in xylene and covered by depex polystyrene. All the washes and dilutions were performed at room temperature in PBST, with the addition of 0.3% NDS for the dilution of the primary antibody.

6.2.5. Analysis of pERK1/2 immunolabelling

Spinal cord sections were analysed in a Leica light microscope using magnification of 400X (40X objective lens; 10X projector lens). Immunopositive neurons with clearly visible perikarya were manually counted on both the ipsi- and contra-lateral dorsal horn in every second of 10 adjacent sections.

6.2.6. Statistical analysis

The average number of pERK1/2-expressing cells found in the dorsal horn on both sides in every section was calculated. Differences in the number of pERK1/2-expressing neurons between the different experimental groups were analysed using one way ANOVA. Whether differences found were significant was established using Fisher's least significant difference test assuming that the data were normally distributed. Differences were regarded as significant at $p<0.05$. "n" = number of animals from which sections were taken in each group.

6.3. RESULTS

6.3.1. Group 1 animals: capsaicin (< 5 minutes)

Animals given a right hind-paw injection of capsacin (under pentobarbital anaesthesia) with survival for 3.5 to 4.5 minutes after capsaicin injection exhibited ERK1/2 activation at the L4/L5 level as originally described by Ji and colleagues (Ji et al., 1999). Labelling of neurons for pERK (18.7 +/- 6.2; n= 3) was found in an area on the lateral aspect of the medial superficial dorsal horn, with labelled neurons being found predominantly in laminae I and IIo of the superficial dorsal horn, while fewer labelled neurons were found in more ventral laminae. No pERK immunoreactivity was evoked in the dorsal horn contralateral to the capsaicin injection (Figures 1, 2 & 3).

6.3.2. Group 2 animals: isoflurane (1 hour)

Exposure to isoflurane for 1 hour potently activated ERK1/2 in the dorsal horn of *both* sides of the spinal cord at the L4/L5 level. There was no significant difference between the number of neurons labelled on the respective sides of the spinal cord (12.5 +/- 3.2; 9.3 +/- 0.7; n = 3). However, the location of the pERK1/2-labelled neurons induced by isoflurane differed from that found with the capsaicin-induced labelling, with the isoflurane-induced labelling being found on the more medial aspect of the medial dorsal horn on both sides of the spinal cord. This difference in location was pronounced and it was patent that labelling induced by the gas involved neurons in a different part of the dorsal horn from the part where capsaicin induces pERK1/2 labelling of neurons. In addition, the vast majority of labelled neurons induced by administration of isoflurane were found in lamina 1 (Figures 1, 2 & 3).

6.3.3. Group 3 animals: isoflurane (1 hour) + capsaicin (< 5 minutes)

Injection of capsaicin into the hind-paw of animals which had been maintained under isoflurane anaesthesia for 1 hour, completely abolished labelling of neurons on the contralateral side of the spinal cord. A comparison of the number of neurons labelled on the ipsilateral side of the spinal cord by capsaicin alone (18.7 +/- 6.2; n= 3) with the number of neurons labelled by capsaicin after treatment with isoflurane (9.8 +/- 1.8; n = 3) shows that prior treatment with this gas reduces the capacity of capsaicin injection to label neurons. Moreover, the neurons found to be labelled after gas followed by capsaicin administration were distributed over two locations, namely, that part of the medial dorsal horn previously identified with capsaicin only–induced labelling, and that part of the same area previously identified with gas only-induced labelling (Figures 1, 2 & 3).

6.3.4. Group 4 animals: sevoflurane only

Exposure to sevoflurane for 1 hour potently activated ERK1/2 in the dorsal horn of *both* sides of the spinal cord at the L4/L5 level. There was no significant difference between the number of neurons labelled on the respective sides of the spinal cord (7.6 +/- 2.5; 4.8 +/- 1.5; n = 3). As with isoflurane, the location of the pERK1/2-labelled neurons induced by sevoflurane differed from that found with the capsaicin-induced labelling, with the sevoflurane-induced labelling being found on the more medial aspect of the medial dorsal horn on both sides of the spinal cord. Likewise, the vast majority of labelled neurons induced by administration of sevoflurane were found in lamina 1.

Sevoflurane demonstrated the same pattern of p-ERK1/2 labelling on both the ipsilateral side and contralateral side of the spinal cord as did isoflurane. However, on each side, fewer cells were labelled by sevoflurane than by isoflurane, although this difference was not statistically significant (Figures 1 and 2).

6.3.5. Group 5 animals: sevoflurane (1 hour) + capsaicin (< 5 minutes)

Injection of capsaicin into the hind-paw of animals which had been maintained under sevoflurane anaesthesia for 1 hour, completely abolished labelling of neurons on the contralateral side of the spinal cord. A comparison of the number of neurons labelled on the ipsilateral side of the spinal cord by capsaicin alone (18.7 +/- 6.2; n= 3) with the number of neurons labelled by capsaicin after treatment with sevoflurane (6.8 +/- 1.1; n = 4) shows that prior treatment with this gas reduced the capacity of capsaicin injection to label neurons. As was also the case with isoflurane, the neurons found to be labelled after sevoflurane followed by capsaicin administration were distributed over two locations, namely, that part of the medial dorsal horn previously identified with capsaicin only–induced labelling, and that part of the same area previously identified with gas only-induced labelling (Figures 1 and 2).

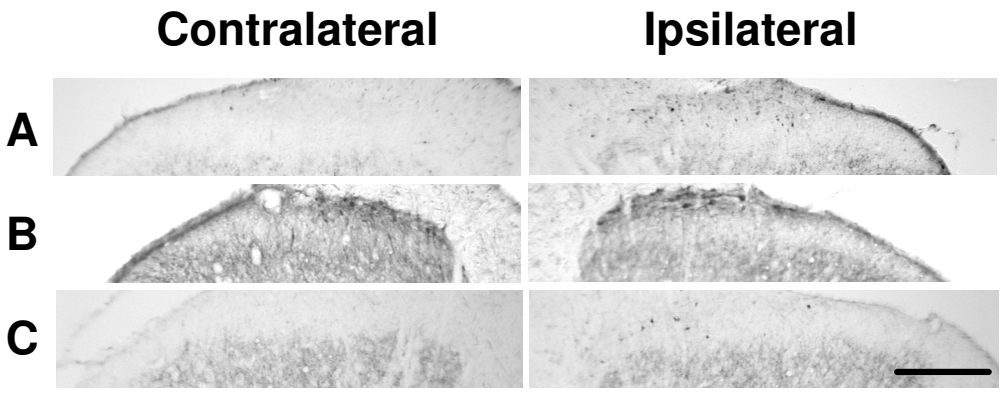


Figure 1

Microphotographs illustrating pERK1/2 labelling of neurons in the spinal dorsal horn. A: after capsaicin injection; B: after isoflurane anaesthesia for one hour; C: after isoflurane anaesthsia for one hour followed by capsaicin injection. Note the capsaicin injection-induced pERK1/2 expression in laminae 1 and IIo of the ipsilateral side of the spinal cord, and the absence of such expression on the contralateral side. Isoflurane anaesthesia results in pERK1/2-labelling on both sides, but in different area of the superficial medial dorsal horn. Note how capsaicin injection, after isoflurane anaesthesia, abolishes pERK1/2 expression on the contralateral side and how the capacity of capsaicin to induce pERK1/2 expression on the ipsilateral side is reduced. Bar indicates 100µm.

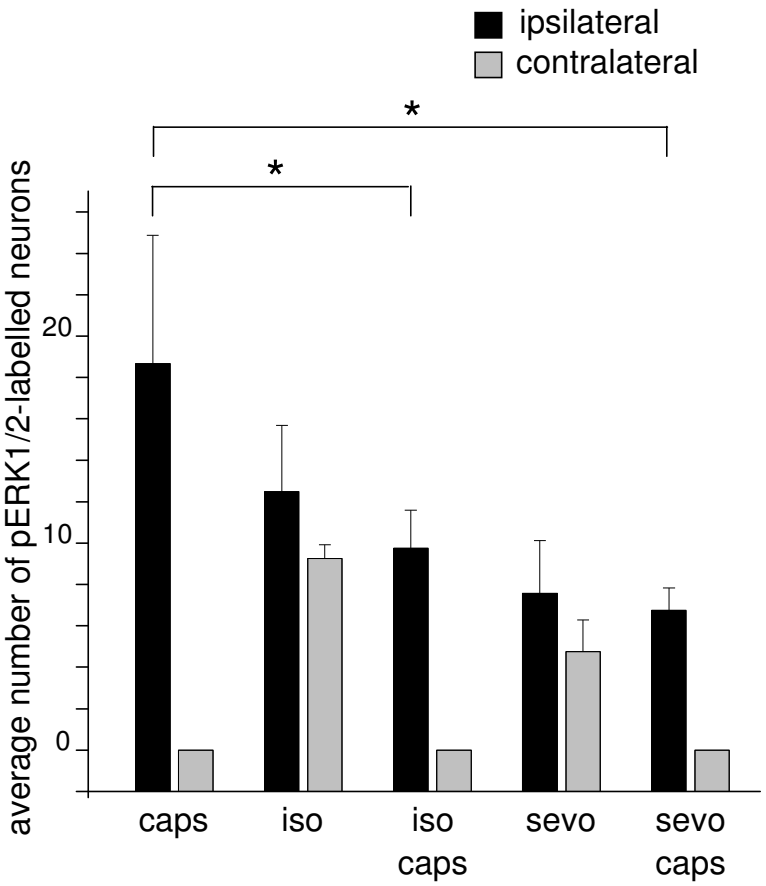


Figure 2.

Bar chart illustrating the average number of pERK1/2-labelled neurons in the ipsilateral and the contralateral spinal dorsal horn after: capsaicin injection (caps); isoflurane anaesthesia for one hour (iso); isoflurane anaesthesia for one hour followed by capsaicin injection (iso caps); sevoflurane anaesthesia for one hour (sevo); and sevoflurane anaesthesia for hour followed by capsaicin injection (sevo caps). Asterisks indicate significant differences.

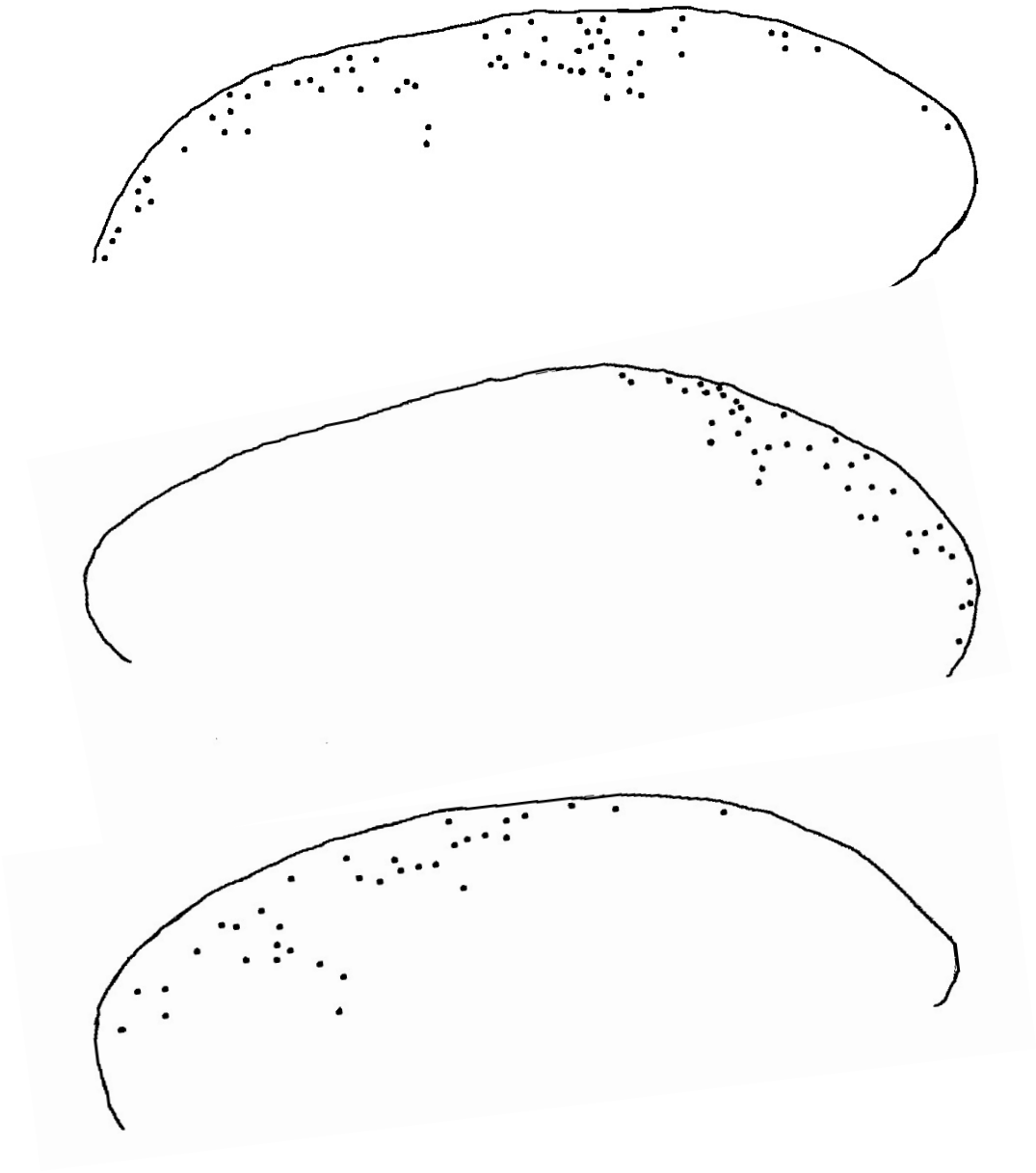


Figure 3.

Diagram illustrating the combined distribution (over three sections) of pERK1/2-immunopositive neurons in the spinal dorsal horn. The images generated reflect the position of pERK1/2-immunopositive neurons when combined from the three sections, (A) after capsaicin injection under pentobarbital anaesthesia; (B) after isoflurane anaesthesia alone; (C) capsaicin injection under isoflurane anaesthesia. The solid line indicates the border between lamina I and the white matter.

6.4. Discussion

Activation of spinal ERK1/2 is a recognised marker of spinal nociceptive processing. Ji and colleagues (1999) showed that peripheral capsaicin injection into a hind-paw of rat activates ERK1/2 by phosphorylation (pERK1/2) in dorsal horn spinal cord neurons and induces pain-related behaviour in the animal.[26] We have shown that isoflurane or sevoflurane, when administered to rats at 1.5 MAC, each result in potent activation of ERK1/2 in the dorsal horn of both sides of the spinal cord at the L4/L5 level. However, the location of the pERK1/2-labelled neurons induced by each of these gasses differs from that found with capsaicin-induced labelling, with the gas-induced labelling being found on the more medial aspect of the medial dorsal horn on both sides of the spinal cord. This difference in location is pronounced, allowing for no doubt that labelling induced by the gas involved neurons in a different part of the dorsal horn from that part where capsaicin induces pERK1/2 labelling of neurons, which is of proven association with nociceptive processing in the dorsal horn. Therefore, it is not possible to assert that the ERK1/2 activation induced by isoflurane and sevoflurane, respectively, is a marker of spinal nociceptive processing. Moreover, TRPA1 is expressed in a sub-population of neurons expressing TRPV1 [19,21]. Hence, if administration of either of these gases were to result in activation of primary afferents *via* activation of TRPAI or TRPV1, then one would necessarily expect to find the resulting neuronal labelling within the area of the medial dorsal horn occupied by those cells within which ERK1/2 is activated by administration of capsaicin alone.

We have also shown that the effect of capsaicin injection after 1 hour exposure to isoflurane or sevoflurane is to abolish neuronal labelling (attributable to gas

administration) on the contralateral side of the spinal cord. At the same time, the number of neurons attributable to capsaicin-induced labelling is significantly reduced from that found where capsaicin is administered on its own. A comparison of the number of neurons labelled on the ipsilateral side of the spinal cord by capsaicin alone with the number of neurons labelled by capsaicin after treatment with either gas shows that prior treatment with either of these gases reduces the capacity of capsaicin injection to result in pERK1/2 labelling of neurons. This effect of the gasses in reducing labelling for capsaicin administration is even more pronounced when one has regard only to the number of pERK1/2 labelled neurons found in the area of the dorsal horn identified with capsaicin only–induced labelling.

There is no evidence that ERK1/2 activation of spinal dorsal horn neurons by isoflurane or sevoflurane is in any way attributable to the excitation of primary afferent neurons resulting from ion channel activation by these gases. Neither TRPV1 nor TRPA1 activation of primary afferents can be involved since neither gas induces pERK1/2 labelling in that area of the medial dorsal horn of the spinal cord where capsaicin induces cell labelling [19,21]. On the other hand, the fact that these gases are systemically active allows for the possibility that descending inhibitory inputs, additional to those which normally obtain, are brought to bear by higher order brain areas which potently reduce nociceptive processing in the spinal cord. These same inputs may potently reduce nociceptive processing in the spinal dorsal horn during surgery, thereby reducing the pain which would otherwise be induced by surgery. Certainly, our results suggest that, under general anaesthesia with either isoflurane, or sevoflurane, patients are unlikely to be subjected to ongoing excitatory inputs from primary afferents which result in nociceptive processing in the spinal cord during the

course of surgery. On the contrary, surgical patients appear likely to benefit from an analgesic effect induced by these gases during the course of surgery.

CHAPTER 7

SUMMARY OF RESULTS AND CONCLUSIONS

Contents

7.1. The role of TRPV1 in burn injury

7.2. Whether xenon may mediate an analgesic action through an effect on TRPV1

7.3. Whether isoflurane and sevoflurane occasion pain-inducing spinal nociceptive processing

7.1. THE ROLE OF TRPV1 IN BURN INJURY

Activation of ERK1/2 in the spinal dorsal horn is a recognised marker for spinal nociceptive processing. At 5 minutes after severe scalding injury to WT mouse hind-paw, a substantial number of phosphorylated ERK1/2 (pERK1/2) immunopositive neurons were found in the ipsilateral dorsal horn. At 1 hour post-injury, the number of pERK1/2-labelled neurons remained substantially the same. However, at 3 hours post-injury, a further increase in the number of labelled neurons was found on the ipsilateral side, while a remarkable increase in the number of labelled neurons on the contralateral side resulted in there being no significant difference between the extent of the labelling on both sides. By 6 hours post-injury, the number of labelled neurons was reduced on both sides without there being significant difference between the two sides. A similar pattern of severe scalding injury-induced activation of ERK1/2 in spinal dorsal horn neurons over the same time-course was found in TRPV1 KO mice,

except that the extent to which ERK1/2 was activated in the ipsilateral dorsal horn at 5 minutes post-injury was significantly greater in WT animals when compared to TRPV1 null animals. This difference in activation of ERK1/2 in spinal dorsal horn neurons was abolished within 1 hour after injury, demonstrating for the first time that TRPV1 is not essential for the maintenance of ongoing spinal nociceptive processing in inflammatory pain conditions in mouse resulting from at least certain types of severe burn injury. The facts that *neuronal staining for activated ERK1/2 not only persisted,* but also that the *number of neurons expressing activated ERK1/2 increased* with the passage of time are significant novel findings of this study which have important implications. After an acute noxious stimulus (provided by hind-paw injection of capsaicin), ERK1/2 activation reaches a peak within 2-5 min and then quickly subsides, such that it is barely detectable or absent by 2 hours after the stimulus (Ji et al., 1999). The findings of this study that activated ERK1/2 is: (i) still present in neurons at the 3 hour and 6 hour time-points post-burn injury; and (ii) that it is found in even more neurons at those later time-points than at the earlier time-points measured, strongly suggest that the ERK1/2 activation found at 3 hours and 6 hours post-injury, respectively, is not merely residual from the initial noxious heat stimulus, but rather that it has been caused by the ongoing inflammatory process itself. This is a key novel finding of this study. The data obtained contain evidence of a dissociation *within the physiological process of the burn injury* of these two distinct "noxious insults" comprising: first, the initial external heat stimulus of the burn injury, which causes direct damage to the tissue accompanied by heat-induced activation of heat-sensitive receptors which result in excitation of nociceptive primary afferents; and, second, the burn injury-induced massive inflammatory response whereby inflammatory mediators occasion an ongoing activation of their receptors on

those nociceptors to maintain, and, indeed, increase, nociceptive signaling in those neurons. The further finding that, at 3 hours after scalding-type burn injury, in both TRPV1-null and wild-type mice, the contralateral side of the spinal cord now exhibited a substantial number of pERK1/2-immunopositive neurons remains intriguing, notwithstanding that the phenomenon of hyperalgesia developing in the hind-paw contralateral to a heat injury has long been noted (Coderre and Melzack, 1991; Polgar et al., 1998). One may speculate that this labelling is caused, or contributed to, by an action of descending fibres on these contralateral dorsal horn neurons since the concept of supraspinal pronociceptive modulation of pain is now well recognised (Lima et al., 2002; Tavares and Lima, 2007). In addition, or in the alternative, this labelling may result from cross-communication between opposite dorsal horn neurons (Coderre and Melzack, 1991; Shenker et al., 2003). In any event, the activation of ERK1/2 on both sides of the dorsal horn discloses a process in which spinal neurons are driving inflammatory nociceptive activity after the excitatory effect of peripheral inflammagens has abated in primary sensory afferents.

7.2. WHETHER XENON MAY MEDIATE AN ANALGESIC ACTION THROUGH AN EFFECT ON TRPV1

Xenon (75%) reduced the number of primary sensory neurons responding to the TRPV1 agonist, capsaicin (100 nM-1 μM) by ~25% to ~50%. Xenon reduced the number of heterologously-expressed hTRPV1 activated by 300 nM capsaicin by ~50%. Xenon (80%) reduced by ~40% the number of phosphorylated ERK1/2-expressing neurons in rat spinal dorsal horn resulting from hind-paw capsaicin injection. The key original finding is that xenon substantially reduces the activity of TRPV1 in response to noxious stimulation by the specific TRPV1 agonist, capsaicin,

suggesting a possible role for xenon as an adjunct analgesic where hTRPV1 is an active contributor to the excitation of primary afferents which initiates the pain sensation. Next, to investigate whether the xenon-induced inhibition of TRPV1 in rat DRG neurons reduces nociceptive processing, the effect of xenon in reducing the release of calcitonin gene-related peptide (CGRP) from those neurons was examined. Exposure to xenon failed to effect a reduction of capsaicin-evoked CGRP release from cultured primary sensory neurons when stimulated by capsaicin. This latter novel finding suggests that xenon acts on several molecular targets on nociceptive primary sensory neurons, and that xenon's action on one, or more, of those targets serves to offset the inhibitory, pro-analgesic, effect of xenon on TRPV1. It is concluded that xenon may not produce any analgesic effect *through peripheral nociceptors.*

7.3. WHETHER ISOFLURANE AND SEVOFLURANE OCCASION PAIN-INDUCING NOCICEPTIVE SPINAL PROCESSING

To investigate this suggestion, intraplantar capsaicin injection-induced phosphorylation of extracellular signal-regulated kinase 1/2 (pERK1/2) in spinal dorsal horn neurons (which is a recognised marker of spinal nociceptive processing) in rat was studied during isoflurane or sevoflurane anaesthesia after 60 min under anaesthesia. Control animals were anaesthetised with pentobarbital (which of itself does not activate ERK1/2 in spinal dorsal horn neurons). Unilateral intraplantar capsaicin injection in control animals evoked pERK1/2 in a group of neurons in lamina I and lamina II of the ipsilateral spinal dorsal horn in a somatotopically appropriate area. In contrast, both anaesthetic gases (given for 60 min and without

subsequent capsaicin injection) induced ERK1/2 activation in a different group of mainly lamina I neurons bilaterally. The total number of spinal dorsal horn neurons labelled on the ipsilateral side following capsaicin injection into the isoflurane-, or sevoflurane-, anaesthetised animals was significantly less than that produced by capsaicin alone. Further, capsaicin injection into isoflurane-, or sevoflurane-, anaesthetised animals reduced pERK1/2 induced by the gases alone on both sides. These key original findings are inconsistent with the suggestion that isoflurane-, or sevoflurane-, induced sensitisation of TRPV1 by capsaicin, or other agonist, is translated into induction of spinal nociceptive processing and consequential pain sensation.

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