ZOSTER-ASSOCIATED PAIN in RODENTS

Thesis presented for the degree of Doctor of Philosophy in the University of London by

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ABSTRACT

Persistent herpes zoster-associated pain is a significant clinical problem and an area of largely unmet therapeutic need. However, progress in elucidating the pathophysiology of zoster-associated pain has been hindered by the lack of an appropriate animal model. This thesis refines a recently described rat model of zoster-associated hypersensitivity and investigates behavioural, pharmacological, and gene correlates of neuropathic pain.

The influence of viral strain and inoculum concentration on neuropathic pain behaviour in rats was initially investigated. Reflex withdrawal responses were assessed to static punctuate and dynamic mechanical, noxious thermal and cooling stimuli. The model was further validated by examining the pharmacological profile to analgesics known to have a degree of efficacy in human neuropathic pain conditions (e.g. tricyclic antidepressants, opioids and gabapentin) as well as novel analgesic compounds (e.g. cannabinoids) and anti-virals (useful in determining the nature of the model). Behavioural paradigms were then taken beyond stimulus-evoked reflex limb withdrawal paradigms conventionally employed in pain models, to encompass more complex outcome measures of integrated pain behaviour reflecting neuropathic pain co-morbidities (e.g. anxiety-like behaviour in the open field paradigm). Pharmacological sensitivity testing in the open field paradigm was performed in parallel with two further models of traumatic peripheral neuropathy. Finally, a microarray approach was used to globally investigate changes in gene expression in dorsal root ganglia associated with a) Varicella Zoster virus infection and b) spinal nerve transection. In this way, common gene expression changes between models were examined.

This model will prove useful in elucidating the pathophysiology of zoster-associated pain and related co-morbidity behaviour and ultimately provide us with greater clinical validity and predictability.
Acknowledgements

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<tr>
<td>ACDP</td>
<td>Advisory Committee on Dangerous Pathogens</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
</tr>
<tr>
<td>AHZ</td>
<td>Acute Herpes Zoster</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
</tr>
<tr>
<td>ATF-3</td>
<td>Activating Transcription Factor-3</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3',5'-monophosphate</td>
</tr>
<tr>
<td>CB</td>
<td>Cannabinoid</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>c-fos</td>
<td>cellular human oncogene homologous to Fos</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>cpe</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>CPP</td>
<td>conditioned place preference</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary Ribonucleic Acid</td>
</tr>
<tr>
<td>DAMGO</td>
<td>D-Ala², MePhe³, Gly-ol enkephalin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal Root Ganglia</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalograph</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyograph</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated Plus Maze</td>
</tr>
<tr>
<td>FAAH</td>
<td>Fatty Acid Amide Hydrolase</td>
</tr>
<tr>
<td>FBJ</td>
<td>Finkel-Biskis-Jinkins</td>
</tr>
<tr>
<td>fdr</td>
<td>false discovery rate (Limma moderated F-statistic plus multiple testing correction)</td>
</tr>
<tr>
<td>Fos</td>
<td>FBJ murine osteosarcoma viral oncogene</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric Acid</td>
</tr>
<tr>
<td>GCOS</td>
<td>GeneChip® Operating Software</td>
</tr>
<tr>
<td>GIRK</td>
<td>G-protein-coupled Inwardly-rectifying K⁺ channel</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
</tr>
<tr>
<td>Hel</td>
<td>Human embryonic lung</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal Axis</td>
</tr>
<tr>
<td>HSV-I</td>
<td>Herpes Simplex Virus Type-1</td>
</tr>
<tr>
<td>IASP</td>
<td>International Association for the Study of Pain</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate-Early</td>
</tr>
<tr>
<td>IE62</td>
<td>Immediate-Early Gene Protein 62</td>
</tr>
<tr>
<td>IE63</td>
<td>Immediate-Early Gene Protein 63</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IENF</td>
<td>Intra-epidermal nerve fibres</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling</td>
</tr>
<tr>
<td>U.K.</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>U.S.A</td>
<td>United States of America</td>
</tr>
<tr>
<td>USV</td>
<td>Ultrasound Vocalization</td>
</tr>
<tr>
<td>VAS</td>
<td>Visual Analogue Scale</td>
</tr>
<tr>
<td>VDCCs</td>
<td>Voltage-dependent calcium channels</td>
</tr>
<tr>
<td>VGF</td>
<td>(Non-acronymic), a ubiquitous neuropeptide precursor encoded by the vgf gene (nerve growth factor inducible protein).</td>
</tr>
<tr>
<td>v1PAG</td>
<td>ventrolateral Periaqueductal Gray</td>
</tr>
<tr>
<td>vOka</td>
<td>vaccine Oka strain</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella Zoster Virus</td>
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Chapter 1

General Introduction
Hypothesis

Infection of rat dorsal root ganglia with varicella-zoster virus is associated with behavioural, pharmacological and gene correlates of neuropathic pain.

1.1 Persistent Pain

The complexity of pain is underlined by the definition adopted by the ‘International Association for the Study of Pain’ (IASP): “an unpleasant sensory and emotional experience associated with actual or potential tissue damage” (Merskey and Bogduk 2006). This definition suggests that pain is more than a sensory experience that discriminates the intensity, location, and duration of a stimulus; it is a highly subjective multidimensional experience characterised by affective-motivational and cognitive-evaluative aspects (Finnerup and Jensen 2006). However, whilst the capacity to experience pain has a protective role, eliciting coordinated reflex and behavioural responses that keep imminent or actual tissue damage to a minimum; persistent pain, i.e. pain that persists beyond injury and outlasts any potential for healing, is maladaptive and offers no biological advantage (Woolf and Mannion 1999). Persistent pain negatively impacts on the individual and may be associated with considerable psychological, physiological, and social dysfunction (Dworkin and Gitlin 1991; Siddall and Cousins 2004). Clinically, it may be defined as “pain that persists beyond the expected healing time” or as “pain that persists beyond three months” (Siddall and Cousins 2004). Inflammatory and/or neuropathic mechanisms may underlie persistent pain states resulting in hyperexcitability and frequently spontaneous pain.

1.2 Inflammatory Pain

Inflammatory pain may be defined as “the heightened pain sensitivity that occurs in response to tissue injury and inflammation” (Kehlet et al. 2006). Under these conditions, individual nociceptors (predominantly unmyelinated C-fibers but also thinly myelinated Aδ-fibers) become activated and sensitised to heat, mechanical, or chemical stimulation, resulting in an altered pain response. The electrophysiological correlates of this altered pain response include a lowered threshold for nociceptor activation, ongoing spontaneous activity, and an increased
frequency of firing after a suprathreshold stimulus (Levine and Taiwo 1994). Furthermore, specific C-fiber afferents, which in healthy tissue remain unresponsive to even severely noxious stimuli, are recruited in inflamed or chronically injured tissue (McMahon and Koltzenburg 1990). Activation of these originally ‘silent’ nociceptors enhances total nociceptive input, directly contributing to the production of a heightened pain response.

Altered pain sensation in the presence of inflammation is produced by the local release of an array of inflammatory mediators from circulating leucocytes, platelets, vascular endothelial cells, degranulating mast cells, and from the primary afferent neurone itself. These potent inflammatory mediators include acids, proteases, chemo-attractants, and a variety of prostanoids, cytokines, and chemokines, which act synergistically to facilitate the inflammatory process and sensitize the primary afferent nociceptor. Most inflammatory mediators modulate neuronal excitability, either directly, by altering the conductance of membrane channels and hence the transduction sensitivity of nociceptive afferents, or indirectly, by acting on neutrophils or the sympathetic post-ganglionic neurone (Levine and Taiwo 1994). This increase in responsiveness of nociceptors to natural stimuli is the phenomenon termed peripheral sensitisation and underlies the pathophysiological state that accompanies tissue damage and inflammation.

However, the persistence of pain after the resolution of inflamed or damaged tissue implies that a mechanism independent of peripheral sensitisation is acting. Repeated nociceptor sensitisation triggers secondary changes in the central nervous system (CNS) facilitating spinal processing of nociceptor activity i.e. C-fiber evoked ‘wind-up’ or secondary hyperexcitability (discussed below). This maintains the hypersensitivity that initially accompanies tissue damage and inflammation and is the phenomenon termed central sensitisation. This central component of inflammatory hypersensitivity reflects neural plasticity and involves a change in the functional state of sensory neurones innervating the inflamed area.

1.3 Neuropathic pain

Neuropathic pain may be defined as “pain initiated or caused by a primary lesion or dysfunction in the nervous system” (Merskey and Bogduk 2006). However, it is generally agreed that the above definition adopted by the IASP has shortcomings, attributable to an
incomplete understanding of the underlying pathophysiology. Specifically, the inclusion of the term 'dysfunction' is vague and may be a source of confusion among clinicians since it allows other types of pain, e.g. nociceptive and psychogenic conditions to be improperly diagnosed as neuropathic (Hansson et al. 2001; Rasmussen et al. 2004; Scadding 2006). For example, a neurobiological response to nerve injury, such as alteration of sodium channel expression or peripheral and central sensitisation, might be considered "dysfunction" in the nervous system. As there is evidence for sensitisation of primary afferents in post-herpetic neuralgia (PHN), but also in nociceptive pain states such as rheumatoid arthritis, this imprecision in definition clearly impacts on reliability in characterising neuropathic pain clinically. In addition, the current definition does not consider the multidimensional clinical features of neuropathic pain and related comorbidities. A more appropriate definition, proposed in part by a special interest group task force of the IASP currently working to redefine neuropathic pain, is 'pain arising as a direct consequence of a lesion affecting the somatosensory system' (Scadding 2006).

The spectrum of neuropathic pain covers a variety of disease states and symptomatology, and is reported to account for 25 – 50% of all pain clinic visits in the U.K. (Bridges et al. 2001b; Wallace 2005). However, the true incidence of neuropathic pain is unknown, and likely to be underdiagnosed (Hall et al. 2006). A recent study reported a prevalence of 8.2% for chronic pain of predominantly neuropathic origin in the U.K. (Torrance et al. 2006).

Classification of neuropathic pain is traditionally by aetiology of the insult to the nervous system or by anatomical location of the lesion; however this approach has shortcomings (Sindrup and Jensen 1999; Finnerup and Jensen 2006; Baron 2006). For example, pain in patients with different aetiologies may share similar pain mechanisms, whilst patients with the same aetiology may have different pain-generating mechanisms. Moreover, continuous, evoked and/or paroxysmal pain, which have different underlying mechanisms, can co-exist in the same patient. In addition, neuroplastic changes following nervous system injury may give rise to sensory and pain distributions that do not respect nerve, root or segmental territories (Baron 2006). As a result, there is now increasing favour for a symptom- or mechanism-based approach to the classification and clinical management of neuropathic pain (Woolf and Mannion 1999; Rasmussen et al. 2004; Finnerup and Jensen 2006; Baron 2006). The ultimate aim of such an alternative approach is to obtain better treatment outcomes.
1.3.1 Clinical features of neuropathic pain

Two broad types of neuropathic pain may be distinguished: *spontaneous* and *stimulusevoked* pain (Dworkin 2002). Spontaneous pain is present in the absence of any stimulation (i.e. stimulus-independent pain) and may be continuous (ongoing) or paroxysmal in presentation. Stimulus-evoked pain, also termed stimulus-dependent pain, is characterised by abnormal hypersensory phenomena such as hyperalgesia (an increased response to a stimulus that is normally painful), hyperpathia (an exaggerated or explosive response to a painful stimulus applied in an area of decreased sensitivity), alldynia (an abnormal pain response to a normally innocuous stimulus), and abnormal sensations such as dysesthesiae (unpleasant) or paraesthesiae (not unpleasant) (Dworkin 2002). Although these are characteristic signs found in some patients with neuropathic pain, others commonly experience pain in combination with hypo-sensory phenomena, such as hypoesthesia (reduced sensation to non-painful stimuli) and hypoalgesia (reduced sensation to painful stimuli), or a complete sensory loss. Importantly, these distinct clinical phenomena may coexist in various combinations in different neuropathic pain states.

Whilst neuropathic pain may be described as "burning", "electric", and/or "tingling" in quality (Boureau et al. 1990; Bridges et al. 2001b), such pain descriptors may not be reliable indicators. Rasmussen et al., (2004) found that single pain descriptors, specifically “burning”, “shooting” and “pricking” could not distinguish between patients with no, low, or high likelihood of having neuropathic pain. Interestingly, affective words (as described in the Short Form of McGill Pain Questionnaire) in contrast to sensory descriptors, were less frequently used in patients more likely to have neuropathic pain than in those likely to have neuropathic pain, a finding consistent with previous studies (Boureau et al. 1990). The study also found brush-evoked alldynia to be more frequent with increasing clinical suspicion of neuropathic pain. Whilst no symptoms and signs were found to be specific for neuropathic pain (Rasmussen et al., 2004), other studies in contrast have shown that the presence of a cluster of symptoms rather than a single symptom may allow separation of patients with neuropathic from non-neuropathic pain (Bennett 2001; Krause and Backonja 2003; Bouhassira et al. 2005).

Significant comorbidity between neuropathic pain and various neuropsychiatric disorders, including anxiety and depression, has also been reported (Dworkin and Gitlin 1991; Meyer-Rosberg et al. 2001; Nicholson and Verma 2004; Leo 2005). The high prevalence of these
disorders and associated clinical burden among patients with chronic neuropathic pain is well recognised. A recent study found depression- and anxiety-related symptoms of any severity in the majority of patients suffering from lesion of a peripheral nerve or nerve root (Meyer-Rosberg et al., 2001), with one study finding a prevalence rate for depression approaching 100% among chronic pain patients (Romano and Turner 1985). In turn there is evidence for the deleterious impact of affective illness on both the diagnosis and prognosis of neuropathic pain (Gallagher and Verma 1999; Verma and Gallagher 2002; Leo 2005). Additional pain-related co-morbidity symptoms have also been reported in neuropathic pain conditions, and include disturbances in sleep, concentration, appetite and social functioning (Meyer-Rosberg et al. 2001).

1.3.2 Hyperalgesia and allodynia

Hyperalgesia is a pathophysiological state that accompanies tissue damage and inflammation. It is defined as enhancement of the physiological response to normally painful stimuli in which threshold for pain is lowered and pain to suprathreshold stimuli is enhanced (Meyer et al. 1995). Two types of hyperalgesia with different neural mechanisms may be distinguished. Primary hyperalgesia involves increased sensitivity to noxious stimulation at the site of tissue injury; whilst secondary hyperalgesia involves increased sensitivity extending beyond the site of the injury to surrounding uninjured areas. Peripheral neural mechanisms, such as sensitisation of peripheral nociceptive terminals and neurogenic inflammation, are likely to account for at least some aspects of primary hyperalgesia; whilst the mechanism for secondary hyperalgesia lies within the CNS. Here central facilitation of transmission at the level of the dorsal horn and thalamus contributes significantly to the development of pathological pain (Gebhart, 2004; Suzuki and Dickenson, 2005). These dynamic changes in CNS function are a feature of neural plasticity. Additionally, hyperalgesia may be classified on the basis of modality i.e. mechanical, thermal, or chemical (Woolf and Mannion 1999).

In contrast to hyperalgesia where the nociceptive pathway has become hyper-excitable, in allodynia, pathways which normally signal innocuous sensations begin to encode painful stimuli. Importantly, allodynia may also involve a change in the quality of the sensation (i.e. there is a loss of specificity in the sensory modality) (Bennett 1995). In mechanical allodynia, two broad subtypes may present: static (punctate or blunt) and dynamic (brush-evoked) (Dworkin 2002). Although a peripheral sensitisation mechanism, involving an abnormal reduction of the
mechanical threshold in sensitised nociceptors, has been proposed, the main mechanism underlying allodynia is thought to be generated at the central level (i.e. central sensitisation) (Truini and Cruccu 2006). Activation of low threshold Aβ mechanoreceptors with abnormal coupling in the dorsal horn resulting in C-fibre activation is proposed to be involved (Coderre et al. 1993; Fields et al. 1998; Besson et al. 2005). Importantly, whilst allodynia and hyperalgesia are appropriate terms in humans since they are defined by pain, they cannot be accurately applied to animals where pain per se is not an outcome measure. In this context, hypersensitivity is a more appropriate term.

1.3.3 Pain in areas of sensory loss

The paradoxical combination of pain in areas of sensory deficit, which may involve all sensory modalities particularly loss of spinothalamic functions (cold, warmth, and pinprick), is characteristic of some patients with neuropathic pain and is frequently seen in a subset of PHN patients (Fields et al. 1998; Pappagallo et al. 2000; Jensen et al. 2001). Partial or complete loss of normal sensory input into the CNS as a result of injury-induced deafferentation of sensory afferents is responsible and initiates long-term changes in the CNS leading to pain and allodynia. Although aberrant connections and synaptic reorganisation are likely contributors to this paradoxical phenomenon (discussed below), alternative mechanisms that serve to maintain central sensitisation in the presence of analgesic skin include the generation of ectopic impulse activity in cell bodies of injured neurones, or in axons of neighbouring uninjured afferents (Fields et al. 1998). Ongoing C-fibre activity may also be generated by intracutaneous microneuromas (Fields et al. 1998).

1.4 Pathophysiology of Neuropathic Pain

Neuropathic pain may be peripheral or central in origin, depending on where the primary lesion lies, and with disease progression pain generating mechanisms may expand to involve both peripheral and central pathophysiology (Fields et al. 1998; Zimmermann 2001). However, in patients with neuropathic pain it is currently impossible to predict the mechanisms that are responsible on the basis of aetiology of the neuropathy or symptomatology, as no single pain mechanism is responsible for pain in a particular disease process, and one mechanism may be
responsible for many different symptoms. Furthermore, the same symptom in two patients may
be caused by different mechanisms, while more than one mechanism may operate in a single
patient. A further complication is that neuropathic mechanisms may evolve over the time course
of the disease (Woolf and Mannion 1999; Bridges et al. 2001b). Therefore, it is inappropriate to
attempt to generate a unifying hypothesis of pathophysiology for all neuropathic pain states.

Thus, the mechanisms involved in the generation of neuropathic pain are multiple and complex
and with current diagnostic tools impossible to accurately ascertain which mechanisms are
operating in a single patient. Both peripheral and central pathophysiological phenomena
contribute in varying degrees to the symptomatology (summarised in Table 1) (Woolf and
Mannion 1999; Bridges et al. 2001b). However, our knowledge of the underlying mechanisms
involved is derived from animal studies and therefore a consequence of peripheral nerve injury.
Any relevance to mechanisms of neuropathic pain is putative since we cannot measure ‘pain’ but
only hyper-reflexia in animals. Therefore, the relationship of the following consequences of
peripheral nerve lesions in rodents can only ever be related to pain by implication, and also only
related to the hyper-sensory sub-group of neuropathic pain patients as they tell us very little
about pain generators in those patients with a sensory loss sensory phenotype.

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<th>Peripheral Mechanisms</th>
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Table 1 Summary of the main peripheral and central phenomena contributing to the generation of neuropathic pain.
1.4.1 Peripheral mechanisms of neuropathic hypersensitivity

The peripheral nervous system (PNS) may contribute multiple mechanisms to the generation of neuropathic pain (discussed below). These peripheral changes drive central mechanisms, which serve to maintain neuropathic pain.

Abnormal nociceptor sensitivity

Pain sensations are normally elicited by activity in unmyelinated (C-) and thinly myelinated (Aδ-) primary afferent neurones. Following a peripheral nerve lesion these neurones become abnormally sensitive and develop spontaneous activity (Devor and Seltzer 2005). This hypersensitivity is thought to occur as a result of a series of molecular and cellular changes at the level of the peripheral nociceptor and dorsal root ganglia (DRG), which in turn leads to similar changes in the dorsal horn of the spinal cord. Following nerve injury, the expression of sodium channels on degenerating damaged C-fibers is triggered (Ji and Strichartz 2004). Products such as nerve growth factor (NGF) that are associated with Wallerian degeneration are also released in the vicinity of spared fibers, triggering channel and receptor expression (e.g. sodium channels, transient receptor potential TRPV1 receptors, and adrenoceptors) on uninjured fibers (Ji and Strichartz 2004). Intact fibers are exposed to an altered environment that contains inflammatory mediators and trophic factors that lead to sensitisation of primary afferents. Peripheral sensitisation is therefore an important mechanism in neuropathic pain.

Ectopic and spontaneous neuronal activity

In normal primary afferent nociceptive neurones, it is rare for firing threshold to be reached without the input of a stimulus. However, following nerve injury, many injured axons and associated cell bodies in the DRG undergo an increase in their intrinsic electrical excitability. Primary afferent fibres become sensitised to peripheral sensory stimulation, decreasing their threshold for activation and increasing their rate of firing to previously subthreshold stimuli. As a result, damaged peripheral nerves can exhibit increased evoked activity and acquire an abnormal ‘spontaneous’ (ectopic) discharge. Ectopic activity may be generated at abnormal locations along the axon of sensory neurones, or in areas of afferent sprouting and neuroma formation (Wall and Gutnick 1974; Wall and Devor 1983; Nordin et al. 1984; Devor and Dubner 1988). Abnormal ongoing or spontaneous discharge may also occur in DRG neurones, causing transmission not only to the CNS where it may trigger and maintain central sensitisation.
amplifying the signal from remaining afferents innervating partly denervated skin leading to
tactile allodynia; but also antidromically towards the peripheral terminal, which can lead to
further hyperexcitability in the peripheral axon (Xie et al. 1995; Kim et al. 1998; Devor and
Seltzer 2005). Development of ectopic activity may also be important in hyper-sensory
phenomena and ongoing stimulus-independent pain (Gracely et al. 1992; Bridges et al. 2001b).

Oscillations in resting membrane potential in primary sensory neurones are further thought to
contribute to their ectopic potential. A small number of A-fibres (10%) exhibit subthreshold
membrane oscillations in their resting state or under depolarisation conditions. An increase in
these oscillations is observed in sensory neurones from axotomised rats (Amir et al. 1999). Such
increases in oscillations lead to increased ectopic activity in these neurones which may underlie
paraesthesias, dysaesthesia as well as frank pain. Further, it is known that regenerating C-fibers
of damaged axons, as well as uninjured neighbouring afferent fibers develop abnormal spontaneous activity. However, the degree to which either damaged or intact sensory afferent populations are responsible for the initiation and/or maintenance of neuropathic pain is not known (Bridges et al. 2001b; Djouhri et al. 2006). Due to an increase in the excitability of the DRG, it has been proposed that chemically mediated cross-excitation or ‘ephaptic cross-talk’ may present an additional mechanism by which ectopic firing in neighbouring uninjured afferents may be evoked (Bridges et al. 2001b).

Abnormal ion channel expression

(a) Sodium channels. One prominent hypothesis underlying hyperexcitability and abnormal
ectopic firing in peripheral nerves following injury relates to the distribution of sodium (Na+) channels, which are critical to the physiology of excitable membranes (Fields et al. 1991; Bridges et al. 2001b). Reorganisation of, and alterations in the expression of various Na+ channels have been demonstrated in the cell bodies and terminal neuroma of peripheral nerves following injury e.g. upregulation of Na1.3 and a downregulation of Na1.8 and Na1.9 expression in DRG neurones following peripheral axotomy in humans and in animal models (Black et al. 2001; Gold et al. 2006). There is further support for the importance of Na+ channels from pharmacological studies e.g. agents that block voltage-sensitive Na+ channels were also found to block electrogenesis of abnormal neuroma firing activity (Matzner and Devor 1994). Similarly, Omana-Zapata et al., (Omana-Zapata et al. 1997) demonstrated a dose-dependent attenuation of ectopic discharges arising from the neuroma and DRG by intravenous lignocaine.
Several distinct voltage-gated Na\(^+\) channels are expressed in the nervous system. Whilst they share a common overall structure, they are functionally distinct (exhibiting different kinetics and voltage dependences) and pharmacologically distinguishable (e.g. with different sensitivities to tetrodotoxin) (Black et al. 2001). Three voltage-gated sodium channel (Na\(_v\)) subtypes, Na\(_v1.7\), Na\(_v1.8\) and Na\(_v1.9\), preferentially expressed in DRG and trigeminal neurones, have been proposed to be of particular relevance to neuropathic pain. A critical role of Na\(_v1.8\) in neuropathic pain is postulated on the observation that a “knock-down” of this channel subtype in DRG is sufficient to both prevent and reverse nerve injury-induced pain in rats after spinal nerve ligation injury (Lai et al. 2002). The major site of action of antisense to Na\(_v1.8\) was found to be the adjacent uninjured DRG neurones, thus it appears that Na\(_v1.8\) may not in fact contribute to neuropathic pain through a role in injured neurones, rather, nerve injury results in a redistribution of Na\(_v1.8\) to the axons of uninjured neurones (Gold et al. 2003). Redistribution of Na\(_v1.8\) along the injured sciatic nerve has also been observed in the chronic constriction injury model of neuropathic pain (Novakovic et al. 1998), and in patients with chronic neuropathic pain, Na\(_v1.8\) immunoreactivity has been demonstrated in peripheral nerve tissue (Coward et al. 2000). Similarly, heritable channel mutations, such as that of Na\(_v1.7\), have been shown to underlie the peripheral nerve hyperexcitability associated with painful erythromelalgia in humans (Gold et al. 2006). These channels are preferentially localised near the terminals of DRG neurones, where their slow closed-state inactivation may allow them to amplify sensory generator potentials (Black et al. 2001). Additionally, Na\(_v1.9\), which is expressed preferentially in small DRG neurones, contributes a depolarising influence to the cell’s resting potential (Black et al. 2001). In addition, the up-regulation of Na\(_v1.3\) (expressed at only low levels in the normal nervous system) in the injured peripheral nerve is thought to promote ectopic discharge and may be important in the generation of spontaneous pain. Thus, voltage-gated sodium channels are involved in the dynamic regulation of neuronal excitability: they are targets of modulation, and changes in their biophysical properties and expression over time and with injury have a profound impact on neuronal excitability and to the generation of neuropathic pain (Cantrell and Catterall 2001;Gold et al. 2003).

(b) Potassium channels. Potassium (K\(^+\)) channels play a major role in the control of all aspects of neuronal excitability and plasticity; however their significance in pain signalling is far from understood. Alterations in K\(^+\) ion channel expression may lead to an increase in firing susceptibility and frequency, and hence in the generation and maintenance of spontaneous
activity (Rasband et al. 2001; Passmore et al. 2003; Marker et al. 2004). Thus, K\(^+\) channels are prime molecular targets for suppressing hyperactive neurones, and might, therefore, prove useful in suppressing hypersensitivity. Four families of K\(^+\) channels with different structures, functional characteristics and pharmacological sensitivity, may be distinguished in neurones: voltage-gated (K\(\text{v}\)), calcium-activated (K\(\text{Ca}\)), inwardly-rectifying (K\(\text{ir}\)) and two-pore (K\(\text{2P}\)) K\(^+\) channels (Miller 2000; Wickenden 2002). The K\(\text{v}1\) subfamily is the most explored among subtypes of sensory neurones (Rasband et al. 2001). K\(\text{v}1.1\) and K\(\text{v}1.2\) are present in large-diameter sensory neurones whereas K\(\text{v}1.4\) is present in most small sensory neurones co-expressing Na\(\text{v}1.8\). These channels represent one potential mechanism that has so far not been exploited in the treatment of neuropathic pain (McCleskey and Gold 1999; Wickenden 2002; Wickenden et al. 2004).

(c) Calcium channels. Two types of voltage-dependent calcium (Ca\(^{2+}\)) channels (VDCCs) have been identified: the high-voltage activated Ca\(^{2+}\) channels of the N-and P/Q-type, and the low-voltage activated T-type Ca\(^{2+}\) channel. Activation of VDCCs, predominantly the spinal N-type channel isoform, is critical for neurotransmitter release from primary afferent terminals. Previous studies have demonstrated that neuropathy increases Ca\(^{2+}\) channel function inducing enhanced release of glutamate and substance P from primary afferent terminals, resulting in greater post-synaptic activation and hence altered central hyperexcitability (Yamamoto and Sakashita 1998; Matthews and Dickenson 2001; Suzuki and Dickenson 2002). In particular, N-type Ca\(^{2+}\) channel antagonists have been shown to attenuate mechanical and heat hyperalgesia in models of neuropathic pain (Xiao and Bennett 1995; White and Cousins 1998); whilst cannabinoid receptor agonists, known to have analgesic effect in nerve-injury models, have also been shown to attenuate Ca\(^{2+}\) flux at N-type channels (Pertwee 1997).

A Ca\(^{2+}\) channel subunit of particular relevance is the \(\alpha2\delta-1\) subunit. This subunit is upregulated in rat DRG neurones, on central afferents terminals and on neurones within the spinal dorsal horn following nerve injury (Newton et al. 2001; Luo et al. 2002), and is correlated with pain behaviour (Luo et al. 2001; Li et al. 2004) suggesting that \(\alpha2\delta-1\) may contribute to neuroplasticity in neuropathic pain. Furthermore, transgenic mice that constitutively over express \(\alpha2\delta-1\) in neuronal tissues demonstrate pain behaviour and exaggerated and prolonged dorsal horn neuronal responses to peripheral mechanical and thermal stimulation (Li et al. 2006). Gabapentin, which has proven analgesic efficacy in postherpetic neuralgia (PHN) (Hempenstall et al. 2005) and diabetic sensory neuropathy (Backonja et al. 1998) is reported to bind to the
α2δ-1 subunit of VDCCs through which it modulates neurotransmitter release from primary afferent terminals (Fehrenbacher et al. 2003). Through the inhibition of these channels, activation of post-synaptic N-methyl-D-aspartate (NMDA) receptors on spinal neurones can subsequently be reduced, and in this way, central sensitisation and spinal hyperexcitability may be targeted.

(d) Transient receptor potential (TRP) channels. The TRP family of ion channels comprises a group of thermosensing and chemosensing receptors that are expressed on the peripheral and central terminals of small-diameter nociceptive C fibres, as well as Aδ fibres where they contribute significantly to nociception (Jhaveri et al. 2005; Gereau 2006). At least six TRP channels are involved in the sensation of noxious cold (TRPA1, thermal activation threshold 17°C) (Story et al. 2003); cooling (TRPM8, 25 - 28°C) (Peier et al. 2002); warming (TRPV3 and TRPV4, 31 - 39°C); and noxious heat stimuli (TRPV1, 43°C and TRPV2, >50°C) (Jhaveri et al. 2005; Julius and McCleskey 2006). The prototypical member of this superfamily of ion channels is TRPV1 which is activated by noxious heat (>43°C), capsaicin and low pH (Caterina et al., 2000). In contrast, TRPA1 responds to noxious cold temperatures (<17°C) and to the irritant, mustard oil. Following nerve injury, the phenotype of cells expressing TRP channels, and the distribution of TRPV1 receptors in primary afferent neurones is fundamentally altered, such that TRPV1 and TRPA1 are also expressed by neurones of a non-nociceptive phenotype (Rashid et al. 2003a; Rashid et al. 2003b). Moreover, expression of TRPV1 has been shown to decrease in injured nociceptive neurones whilst increasing in neighbouring uninjured neurones (Hudson et al. 2001). This includes novel expression in large diameter, low threshold A-fibres which may indicate a phenotypic switch contributing to symptoms of neuropathic pain. Similarly, TRPA1 expression has been shown to increase in a subset of small diameter primary sensory neurones following nerve injury, likely inducing hypersensitivity to cold stimuli (Obata et al. 2003). Indeed, interfering with TRPA1 channel function using antisense knockdown technology may abolish cold hyperalgesia following spinal nerve ligation in the rat (Katsura et al. 2006).

Moreover, TRPV1 receptor antagonists have been shown to attenuate thermal hyperalgesia and mechanical allodynia in experimental models of neuropathic pain (Pomonis et al. 2003; Walker et al. 2003). Similarly, pharmacological blockade of TRPA1 in primary sensory neurones has been shown to reverse cold hyperalgesia caused by inflammation and nerve injury (Obata et al. 2005). Additional evidence for a role in neuropathic pain exists for other TRP channels. For
example, in a rat model of chemotherapy-induced painful peripheral neuropathy, the spinal administration of antisense oligodeoxynucleotides to TRPV4, which reduced expression of TRPV4 in sensory nerves, abolished Taxol-induced mechanical hyperalgesia (Alessandri-Haber et al. 2004). These findings lend support for the TRP channels as promising targets in neuropathic pain. Targeting specific TRP channels may prove useful analgesic strategies in the future.

**Role of peripheral inflammatory mediators**

Peripheral nerve injury as a result of trauma and/or infection evokes a cascade of cellular events including a neuroinflammatory response with the release of chemical mediators, including proinflammatory cytokines and chemokines such as interleukins IL-1β and IL-6, and tumour necrosis factor-α (TNFα) (Schafer et al. 2001; Sommer and Kress 2004; Ji and Strichartz 2004; Marchand et al. 2005). Moreover, IL-6 knockout mice fail to exhibit neuropathic pain after nerve injury (Ramer et al. 1998). Intact and injured DRG sensory neurones are known to express receptors which respond to TNFα, IL-1β, and IL-6. However, the direct mechanism of neuronal sensitisation remains to be fully determined. Indirect evidence suggests an action of TNFα on neuronal Na+ or Ca2+ channels, whereas IL-1β may be involved in a complex signalling cascade that leads to the production of pronociceptive compounds (such as nitric oxide, NGF and prostaglandins) from immune cells or Schwann cells (Ji and Strichartz 2004). These compounds may lead to changes in gene expression and neuronal excitability in intact nociceptors (McMahon et al. 2005).

**Sensory neurone apoptosis**

Nerve-injury induced cell loss in the dorsal horn may further contribute to neuropathic pain (Hokfelt et al. 2005). In support of this theory, Sugimoto and colleagues (1990) described neurones with signs of degeneration in the lumbar dorsal horn after unilateral constriction of the sciatic nerve, with a greater increase ipsilaterally (Sugimoto et al. 1990). Later, Azkue and colleagues (1998), using the terminal deoxynucleotidyl transferase nick end labelling (TUNEL) technique as a marker of apoptosis, demonstrated nuclear fragmentation in neurones in the superficial layers of the dorsal horn (Azkue et al. 1998). Apoptotic cell death was seen at 7 days, but not 3 or 14 days, after sciatic nerve transection, and could further be prevented with an NMDA receptor antagonist, suggesting the involvement of a glutamatergic mechanism. Scholz and colleagues (2005) recently provided evidence for glutamate-mediated apoptosis leading to
gamma-aminobutyric acid (GABA)ergic interneurone death, loss of postsynaptic inhibition of spinal neurones, and subsequent neuropathic pain (Scholz et al. 2005). Specifically, trans-synaptic apoptosis was induced in the superficial dorsal horn (laminas I - III) of the spinal cord by three distinct partial peripheral nerve lesions: spared nerve injury, chronic constriction, and spinal nerve ligation. Ongoing activity in primary afferents of the injured nerve and glutamatergic transmission caused a caspase-dependent degeneration of dorsal horn neurones that was slow in onset and persisted for several weeks (activated caspase-3 is an early mediator of apoptosis). The reported cumulative loss of dorsal horn neurones four weeks after spared nerve injury for example, was >20%, with GABAergic inhibitory interneurones being among those neurones lost (Scholz et al. 2005). A marked decrease in inhibitory postsynaptic currents of lamina II neurones coinciding with the induction of apoptosis was also demonstrated. Furthermore, the authors reported that blocking apoptosis with the caspase inhibitor zVAD, prevented loss of GABAergic interneurones and the reduction of inhibitory currents (Scholz et al. 2005). Moreover, Moore and colleagues (2002) have reported that partial nerve injury decreases dorsal horn levels of the GABA-synthesizing enzyme, glutamic acid decarboxylase, and induces neuronal apoptosis (Moore et al. 2002). This provides further support for GABAergic interneurones as prime candidates for the proposed cell loss following peripheral nerve injury.

1.4.2 Central mechanisms of neuropathic hypersensitivity

Sensory input from primary sensory neurones is transferred, via their central axons, to second-order neurones in the dorsal horn of the spinal cord. Synaptic contacts made between afferent central terminals and dorsal horn neurones are highly organised, both topographically and functionally to maintain accurate transfer of nociceptive information. Following peripheral nerve lesions, synaptic processing in the CNS can be subject to diverse forms of functional, chemical and structural plasticity that are highly involved in the maintenance of neuropathic hypersensitivity. Increased synaptic efficacy (central sensitisation), loss of inhibitory mechanisms, alterations in synaptic contacts and the activation of non-neuronal cells all play key roles in producing increased pain sensitivity in neuropathic pain, and these are outlined below.
Excitatory mechanisms

The sustained state of hyperexcitability of central dorsal horn neurones following the afferent barrage and spontaneous discharge associated with peripheral nerve injury induces functional changes in sensory processing mechanisms. At the spinal cord level, this is the phenomenon termed central sensitisation (Wall 1991; Coderre et al. 1993; Ji and Strichartz 2004). Central sensitisation may manifest in three ways: enlargement of the neurone’s peripheral receptive field; increased response to a subthreshold input (i.e. lowering of activation thresholds); and action potential discharge initiated by previously subthreshold inputs (Ji and Strichartz 2004; Saltar and Woolf 2005).

Glutamate is the major excitatory neurotransmitter released at the central terminals of primary afferent nociceptive neurones following noxious stimulation. Glutamate acts on a number of postsynaptic receptors, including metabotropic glutamate receptors and ionotropic alpha-amin-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate and N-methyl d-aspartate (NMDA) receptors. Whereas AMPA receptors are important for the rapid excitatory synaptic transmission of physiological nociception, NMDA receptors are intimately involved in CNS plasticity and play a critical role in the induction of neuropathic pain (Kim et al. 1997; Ji and Strichartz 2004; Yoshimura and Yonehara 2006). They may be activated through several mechanisms. First, glutamate transporter expression in the spinal cord is reduced after nerve injury, resulting in an elevation of synaptic glutamate concentration. Second, nerve injury produces a loss of GABAergic inhibition (i.e. disinhibition) onto dorsal horn neurones partially due to a loss of second-order afferent neurones. This facilitates A-fibre-mediated excitatory synaptic transmission in an NMDA-dependent manner. Third, nerve lesion reduces the expression of the potassium chloride co-exporter KCC2 in the spinal cord, leading to a change in chloride ion concentration and a reversal of GABA receptor conducted current, such that stimulation of GABA receptors produces depolarisation rather than hyperpolarisation (Ji and Strichartz 2004). Moreover, NMDA receptor antagonists can prevent and reverse neuropathic pain in experimental animal models (Mao et al. 1992; Kim et al. 1997; Sotgiu and Biella 2000; Suzuki et al. 2001; Yoshimura and Yonehara 2006).
Loss of inhibitory systems in the dorsal horn
The GABA pathway forms a major inhibitory neurotransmitter system in the CNS. Depression of spinal inhibitory mechanisms is thought to be important for sustained enhancement of excitatory transmission and central sensitisation (Sivilotti and Woolf 1994). In support of this, administration of GABA-mimetics reduces neuropathic hypersensitivity, whilst antagonism of the GABA receptors is associated with hypersensitivity (Malan et al. 2002). Moreover, peripheral nerve injury results in a substantial loss of GABA-mediated inhibitory currents, decreased extracellular concentrations of GABA (Stiller et al. 1996) as well as decreased concentrations of the GABA-synthesising enzyme, glutamic acid decarboxylase, in the dorsal horn (Moore et al. 2002), and decreased GABA receptor levels in the spinal cord (probably due to degeneration of the primary afferent neurone terminals on which the receptor is localised) (Castro-Lopes et al. 1995). Moreover, apoptosis in the dorsal horn following nerve injury may correlate to selective death of GABAergic inhibitory interneurones due to excessive glutamate release or a result of cell death-inducing signals within the spinal cord (Scholz et al. 2005). All of the above factors likely promote a functional loss of GABAergic transmission in the superficial dorsal horn.

Nerve injury-induced degeneration of primary afferent fibres similarly results in loss of opioid receptors (the time courses of mu and delta opioid receptor decreases being comparable to the time course of primary afferent fibre degeneration) (Besse et al. 1992a; Besse et al. 1992b; Porreca et al. 1998; Rashid et al. 2004). For example, Besse and colleagues (1992a) demonstrated decreases in mu and delta opioid receptors in the superficial dorsal horn (in which primary afferent fibres project) of the rat spinal cord following unilateral dorsal rhizotomy. Specifically, the loss of mu opioid receptors (71 – 74%) was significantly more pronounced than the loss of delta opioid receptors (57 – 62%). In a similar study, Besse and colleagues (1992b) compared the loss of opioid binding sites in the superficial dorsal horn following loose ligation injury of the sciatic nerve with sciatic nerve section and lumbar dorsal rhizotomy, and proposed that the more deprived the dorsal horn was of primary afferent fibre input (i.e. the greater the relative severity of injury and hence degree of deafferentation), the more dramatic the loss of opioid sites ipsilateral to the lesion. Importantly however, opioid receptor regulation may occur independently of a direct alteration in primary afferent fibres i.e. altered nociceptive sensory input following nerve injury (loose ligation of the rat sciatic nerve) activates endogenous opioid systems resulting in a bilateral up-regulation in mu opioid binding sites (Besse et al. 1992b).
However, this up-regulation may be masked by the fibre degeneration. These mechanisms may in part account for the differing efficacy of opioids in neuropathic pain. Specifically, the reduced effectiveness of systemic morphine in neuropathic pain is proposed to be at least partly due to decreased mu opioid receptor expression in DRG and subsequent loss of peripheral morphine analgesia (Rashid et al. 2004).

Thus, there is evidence that a combination of increased activity in the excitatory and a concomitant decrease of activity in inhibitory (GABA and opioid) systems within the spinal cord both contribute to the phenomenon of central sensitisation following peripheral nerve injury.

**Anatomical reorganisation**

Structural changes (anatomical reorganisation) within the dorsal horn, brainstem and cortex, including cell death, degeneration or atrophy of axon terminals, sprouting of new axon terminals, and reorganisation of synaptic circuitry with loss of inhibitory mechanisms may further contribute to the persistence of neuropathic pain (Woolf et al. 1992; Koerber et al. 1994; Shortland et al. 1997; Bridges et al. 2001b). Although somewhat controversial (Bao et al. 2002), sprouting of central processes of surviving afferent axons may occur after injury (Woolf et al. 1992; Shortland et al. 1997), leading to the development of aberrant direct connections (i.e. synaptic reorganisation) in the spinal cord. Following peripheral nerve injury, small diameter (Aδ- and C-) primary afferent terminals degenerate in lamina II of dorsal horn of the spinal cord. As a consequence of the loss of synaptic contacts normally made onto pain-signalling interneurones, the central terminals of large diameter low-threshold Aβ-mechanoreceptive afferents, which normally terminate in deeper laminae (III and IV) sprout into lamina II and directly contact the deafferented cells (Woolf et al. 1992; Koerber et al. 1994; Shortland et al. 1997). This reorganisation of information results in low-threshold sensory information being interpreted as nociceptive and might be the neural correlate of the dynamic mechanical allodynia observed in a subset of PHN patients who suffer profound loss of small fibre sensory function as a result of C-fibre deafferentation. In these patients, thermal sensory loss and dynamic mechanical allodynia co-exist. The outgrowth of central Aβ-fibre terminals is prevented by exogenous NGF, presumably by provision of trophic support for damaged C-fibres, suggesting an important role for neurotrophins in the regulation of this manifestation of structural plasticity (Bennett et al. 1996). However, concerns about the specificity of bulk labelling techniques and the sampling of intracellular labelled intact and injured afferents have been raised (Tong et al.
1999; Hughes et al. 2003) such that damaged C-fibres have been proposed to abnormally take up the label. However, in favour of the sprouting theory, electrophysiological studies have demonstrated that stimulation of Aβ-fibres in injured nerves can produce activation of neurones in lamina II (Okamoto et al. 2001). Nevertheless, further work is required to resolve the basis for the differences in these studies, and to determine the extent to which sprouting of Aβ fibres actually contributes to tactile hypersensitivity after peripheral nerve injury.

Furthermore, in a very small subset of patients suffering from neuropathic pain, the pain can be somewhat dependent on activity in the sympathetic nervous system as a result of abnormal contact between the sympathetic and sensory nervous systems. This is often referred to as ‘sympathetically maintained pain’ (SMP) and may underlie the enhanced sensitivity to catecholamines in these individuals (Janig et al. 1996). SMP may involve a direct chemical coupling between the sympathetic and sensory nervous systems within peripheral effector sites or DRG; or an indirect coupling via peripheral sensitising mechanisms with release of inflammatory mediators from sympathetic terminals and the sensitisation of primary afferents (Bridges et al. 2001b; Janig and Levine 2005). (It is likely that onset of sympathetic sprouting involves release of neurotrophic factors and cytokines and is linked to Wallerian degeneration in the peripheral nerve. The terminals of sprouted neurones form functional synapse-like structures with the cell bodies and these may be involved in the formation and maintenance of abnormal excitation arising from the DRG (Bridges et al. 2001b)). In support of a sympathetically-mediated mechanism, Kress and colleagues (2001) demonstrated that infection with wild-type varicella zoster virus (VZV) isolates in vitro conferred norepinephrine sensitivity to rat nociceptive DRG i.e. infected neurones became de novo sensitive to adrenergic stimulation expressing functional α1- and/or β1-adrenergic receptors (Kress and Fickenscher 2001).

**Role of non-neuronal cells in pain plasticity**

Although pain hypersensitivity was originally thought to result exclusively from altered activity of neurones, there is accumulating evidence implicating a role for glial cells (specifically microglia and astrocytes) in the pathogenesis of pain (Watkins et al. 2001; DeLeo and Yezierski 2001; Watkins and Maier 2003; Hansson 2006). Until recently, glia which outnumber neurones in the CNS by about 10:1 (Salter and Woolf 2005), have been considered static constituents of the CNS serving primarily supportive functions. However, it is now thought that glia have key neuromodulatory, neurotrophic and neuroimmune functions in the CNS, and as such, they

Microglia are immune-derived cells constituting a resident population of macrophages within the brain and the spinal cord (5–10% of glia), which in response to neuronal damage become activated from their normal 'resting' state (i.e. undergo substantial morphological and phenotypic changes), and migrate to the relevant spinal segments, thus increasing the local microglial population (Watkins et al. 2001). Activated microglia express and release various cytotoxic and proinflammatory cytokines (such as IL-1β, IL-6, and TNF-α), proteases, chemokines, reactive oxygen intermediates, and excitatory amino acids, all of which are known to enhance pain transmission (Watkins et al. 2001). In this way, activated microglia dynamically modulate the function of neurones, contributing to central sensitisation and the propagation of neuropathic pain behaviour. Importantly, drugs that suppress glial activation and proliferation can prevent and reverse neuropathic pain (DeLeo and Yezierski 2001; Watkins and Maier 2003; Raghavendra et al. 2003). For example, minocycline-induced selective inhibition of microglial activation has been shown to attenuate development of behavioural hypersensitivity in a rat model of neuropathic pain (Raghavendra et al. 2003). However, no effect of treatment on existing nerve-injury induced hypersensitivity was observed, suggesting that microglia are more important in the initiation, rather than the maintenance of enhanced pain responses (Raghavendra et al. 2003). Furthermore, intrathecal administration of activated microglia in naïve rodents has been demonstrated to induce hypersensitivity phenomena (Tsuda et al. 2003; Narita et al. 2006b). In parallel with pharmacological studies, knockdown of the adenosine triphosphate (ATP) receptor P2X4 (induced in spinal cord microglia after nerve injury), with antisense oligodeoxynucleotides reduces neuropathic pain (Tsuda et al. 2003). Moreover, neuropathic pain behaviour in mice lacking the chemotactic cytokine receptor CCR2, which is similarly expressed on spinal cord microglia in response to neuronal injury, is diminished (Abbadie et al. 2003). The recruitment of microglia is commonly associated with the phosphorylation of p38 MAP kinase in the spinal cord, which is likely a key intracellular signal in the microglial response in neuropathic pain (Jin et al. 2003; Tsuda et al. 2004). The MAP kinase ERK (extracellular signal-regulated kinase) is also activated in microglia. Indeed, the sequential activation of ERK in neurones, then microglia, and finally astrocytes in a neuropathic pain model (Zhuang et al. 2005) suggests that microglial activation might be the first step in a
cascade of immune responses in the CNS. Thus, 'microgliosis' fundamentally contributes to the pathophysiology of neuropathic pain.

Astrocytes are intimate partners with neurones at synapses, and are required for normal synaptogenesis and synaptic stability (Salter and Woolf 2005). They are similarly activated in the spinal cord after peripheral nerve injury and release diffusible chemical mediators that regulate neuronal functioning and synaptic transmission e.g. glutamate which may act on NMDA receptors to excite neurones. Moreover, depression of astrocyte activation has been found to suppress the enhancement of nociceptive behaviours in models of inflammatory (Watkins et al. 1997) and neuropathic (Sweitzer et al. 2001) pain. Thus neuronal excitability, synaptic efficacy, and synaptic plasticity are all subject to control by astrocytes (Araque et al. 1999).

**Descending modulation**

In addition to the peripheral and spinal mechanisms discussed, supraspinal modulation of spinal cord excitability is thought to play an important role in neuropathic pain (Ossipov et al. 2000). Such descending modulation of spinal nociceptive processing can be either inhibitory or facilitatory (Gebhart 2004). Of central importance in controlling nociceptive transmission at the level of the spinal cord are the ventrolateral periaqueductal gray (vlPAG), and rostral ventromedial medulla (RVM) (which includes the nucleus raphe magnus and the adjacent reticular formation) (Fields et al. 1991). The vlPAG, which is closely associated with the brainstem including the rostral ventromedial medulla (RVM), receives ascending nociceptive input from the dorsal horn and is involved in the integration of inputs from areas such as the limbic forebrain, diencephalon, amygdala and hippocampus (Bandler and Keay 1996). The PAG is therefore associated with the affective and autonomic responses to pain. Descending inhibition from the vlPAG or RVM is mediated mainly by serotonergic and noradrenergic receptors in the spinal cord, though other neurotransmitters (e.g. GABA, glutamate, cholecystokinin, and substance P) descending from the brainstem also contribute to modulation of spinal nociceptive transmission. Likely via anatomically distinct pathways, the vlPAG and RVM can exert both facilitatory and inhibitory influences on the spinal cord (Zhuo and Gebhart 1997). The balance of these two supraspinal pathways and primary afferent input, ultimately determines the excitability of spinal neurones, so under pathological conditions enhancement of descending facilitatory controls to the spinal cord are likely to allow excitatory influences to predominate to
maintain spinal central sensitisation and hence secondary hyperalgesia. The enhanced pain afforded by activation of descending facilitatory influences is thus thought to prevent further damage and so contribute to protection of already injured tissue from further insult. However, in persistent pain states, there is prolonged input to the RVM that sustains facilitatory influences to the spinal cord (Gebhart 2004).

Facilitatory cells within the RVM are classed as ‘ON’ cells whereas cells that have inhibitory influences on the spinal cord are termed ‘OFF’ cells (Fields et al. 1995). Both cell types respond to manipulations of the PAG to produce behavioural analgesia (Fields et al. 1995). Following nerve injury, there is enhanced descending excitatory drive from the RVM (Burgess et al. 2002) that appears to be particularly important for those lamina I spinothalamic or spinoparabrachial projection neurones expressing the neurokinin-1 receptor (Suzuki et al. 2002). This may represent a central compensatory mechanism for the loss of normal sensory input following peripheral nerve damage (Suzuki and Dickenson 2005). The brainstem areas involved are also implicated in autonomic responses, emotions and, sleep. Therefore, these same pathways likely underpin the well established links between these states and pain and may provide a basis for an affective component of pain (Monassi et al. 2003). Intra-RVM cholecystokinin produces reversible thermal and tactile hypersensitivity in naïve rats (Kovelowski et al. 2000) and prevents both the activation of OFF-cells and the antinociception produced by systemic morphine (Heinricher et al. 2001). Additionally, although thought mainly to play an inhibitory role in supraspinal systems, supraspinal serotonergic inputs to the spinal cord originating in the RVM may play a role in facilitatory influences following peripheral nerve injury (Rahman et al. 2006). The serotonin receptor, localised to a novel group of small diameter afferents, and a larger number of presumed A-delta afferent fibres (Zeitz et al. 2002), has been implicated as the target receptor of this system, and is supported by pharmacological studies in which the serotonin antagonist, ondansetron, attenuates punctate mechanical responses after nerve injury (Suzuki et al. 2004). Moreover, a preliminary clinical study suggests that block of serotonin receptors has clinical utility in the treatment of pain (McClean et al. 2003). Finally, evidence suggests that cannabinoids produce their antinociceptive effect at least in part by recruiting the PAG-RVM modulatory system (Fields et al. 2005). Cannabinoid-1 (CB1) receptors are densely expressed in the PAG, and microinjection of CB1 agonists into the PAG or RVM produces antinociception. Furthermore, immunocytochemical studies characterising CB1 receptor expression in rat spinal cord have demonstrated receptor expression in the dorsolateral funiculus,
a major tract for descending control systems, in addition to the superficial dorsal horn (Farquhar-Smith et al. 2000).

1.5 Functional Genomics

The ultimate goal in pain genomics is the identification of which genes are relevant to pain, and which of these pain-relevant genes are thus responsible for individual differences in the perception of pain. The genes in question may affect variability in the susceptibility to developing pain syndromes, in the perceptual (sensory and affective) severity of pain, as well as in the behavioural responses to pain, and sensitivity to pharmacological agents. Identification of those genes relevant to pain will ultimately provide us with the opportunity to identify truly novel molecular targets for development of new pain therapies, and therefore allow us to individualise existing pain therapies in addition to using gene therapy to ultimately eliminate pain-related pathology (Mogil 2005).

1.5.1 Microarray technology

Advances in molecular genetic techniques, in particular microarray-based gene expression profiling has allowed significant progress to be made in the identification of potential genes relevant to pain (Costigan et al. 2002; Valder et al. 2003; Mogil 2005). Unlike other strategies, e.g. transgenic knock-outs, which consider genes largely in isolation, microarray is a powerful biological tool that offers efficient genome-wide screening with the capacity to analyse parallel changes in many thousands of genes. Importantly it is revolutionizing the rate at which information about gene expression can be collected (Reilly et al. 2004). The concept of microarray technology involves the hybridisation of mRNA transcripts from target tissues, typically from DRG or spinal cord, to a set of oligonucleotide probes. Using oligonucleotide microarrays, any gene whose expression differs significantly between experimental conditions can be identified and correlated. Much data now exists on hundreds of genes that have disregulated expression in rodent models of inflammatory and neuropathic pain conditions (Costigan et al. 2002; Reilly et al. 2004). Functionally related genes may be identified, e.g. ion channels or receptors and signal transduction related molecules (Reilly et al. 2004), specifically an up-regulation of genes that are expressed by immune and inflammatory cells, and a down-
regulation of genes that are involved in neurotransmission in DRG from sciatic nerve transected rats has been reported (Costigan et al. 2002). However, no studies to date have investigated gene expression changes in neuropathic pain models of different aetiologies, but which share mechanical hypersensitivity phenomena, in an attempt to identify commonly disregulated genes. This would ultimately have more relevance to neuropathic pain, rather than collating lists and lists of genes which may not be of direct relevance to neuropathic pain.

1.5.2 The importance of strain difference in mediating nociceptive sensitivity

Furthermore, there is an emerging rodent literature on the importance of strain differences in mediating nociceptive sensitivity which emphasises the issue of heritability of pain (Mogil et al. 1999a; Mogil et al. 1999b). Both rats and mice display clear and robust strain differences in nociceptive sensitivity, as inferred by their behaviour on a number of behavioural nociceptive assays (Mogil et al., 1999a; Valder et al., 2003). For example, nociceptive sensitivity among eleven inbred mouse strains across twelve different assays was found to vary by up to 54 fold (Mogil et al., 1999a). Further, this genetic variability in nociception was found to be associated with a median heritability of 46% (range 30% – 76%) across assays. Moreover, the effect of genotype is at least partially specific to the nociceptive assay being considered. For example, paw withdrawal latency to noxious heat was found to differ by approximately five fold in AKR/J and C57BL/6J mice strains; C-fibers from the behaviourally sensitive C57BL/6J strain, displayed significantly lower firing thresholds to heat and higher firing rates compared to those from AKR/J mice (Mogil et al., 1999a). In contrast, paw withdrawal threshold to punctate mechanical stimulation was found to be at least three times higher in the C57BL/6J mouse strain compared to that in AKR/J mice.

Significant correlations between certain nociceptive phenotypes and anxiety-like behaviour in rodents have also been demonstrated (Kim et al. 2002; Augustsson et al. 2005; Millstein and Holmes 2006). For example, in the open field and light-dark exploration tests, the most ‘anxious’ mouse strain was reported to be the 129S1 strain, which appears to be the most sensitive to punctate mechanical stimulation at baseline and develops the greatest magnitude of sensitivity following peripheral nerve injury (Millstein and Holmes 2006). In contrast, C57BL/6J mice have been reported to show markedly less anxiety-like behaviour as compared to 129S1 mice and likewise demonstrate less sensitivity to nociceptive tests at baseline and following
peripheral nerve injury (Millstein and Holmes 2006). This suggests a correlation between the level of anxiety associated with strain and the nociceptive response to mechanical stimulation. However, the strain difference between 129S1 and C57BL/6J mice, which is clear in the open field paradigm, is not apparent in the elevated plus maze paradigm (Millstein and Holmes 2006), therefore strain baseline anxiety-like behaviour is affected by behavioural paradigm.

1.6 Clinical Pathogenesis of Varicella Zoster Virus Infection

VZV is a highly virulent neurotropic alphaherpesvirus directly responsible for two distinct conditions in humans: varicella (chicken pox) and herpes zoster (shingles). Primary infection typically occurs during childhood, and is characterised by the inoculation of respiratory mucosal epithelium, viral transport to the skin by a cell-associated viraemia and development of cutaneous lesions. Following primary infection, the virus is retrogradely transported along axons of sensory neurones from the skin to establish a prolonged latent infection in the trigeminal, dorsal root, and autonomic nervous system ganglia in the PNS (Kennedy et al. 1998; Kennedy et al. 1999; Kinchington 1999; Kennedy 2002a; Mitchell et al. 2003; Gilden et al. 2003b). Reactivation of latent virus presents as acute herpes zoster (AHZ) and occurs most commonly in elderly and immunocompromised individuals, reflecting senescence of cellular immunity and VZV-specific host immunodeficiency (immunosenescence) (Kennedy et al. 1999; Abendroth and Arvin 2001; Kennedy 2002a; Mitchell et al. 2003; Gilden et al. 2003b). AHZ is typically characterised by a vesicular dermatomal rash and acute sensory disturbances, including pain. However, untreated, recovery may become complicated in 13 – 26% of patients (Scott et al. 2006). The most common complication being development of PHN (Hope-Simpson 1975; Hempenstall et al. 2005; Coen et al. 2006; Scott et al. 2006). PHN is a chronic neuropathic pain syndrome that persists even after the virus reverts to a latent state within sensory ganglia of affected dermatomes and long after the disappearance of herpetic skin lesions (Watson et al. 1991). Preherpetic neuralgias prior to vesicular eruption may also occur, and is thought to reflect inflammation and ganglionic replication prior to peripheral disease (Gilden et al. 1991; Kinchington 1999). The term, ‘zoster-associated pain’ describes the continuum of pain from AHZ to the development of PHN (Dubinsky et al. 2004).
1.6.1 Epidemiology and clinical aspects of AHZ

The incidence of AHZ and associated burden of disease increases with advancing age, and it is likely that this incidence will further increase over the coming decades, largely as a result of increasing longevity of the population (discussed in Chapter 2) (Oxman et al. 2005; Dworkin et al. 2006). In developed countries, the percentage of the population over 65 years of age is predicted to rise from 17.5% to 36.3% by 2050, and more than triple in the over-80 age group (Gibson 2006). This is important as the median age of patients with AHZ is approximately 64 years, and currently the reported prevalence of AHZ in those over 65 years of age is already high (3.9 – 11.8 per 1000 person years) (Dworkin et al. 2006). Moreover, the lifetime risk of AHZ is estimated to be 10 – 30% further increasing markedly with age, and affecting up to 50% of people who live to 85 years (Brisson et al. 2001; Thomas and Hall 2004).

Subclinical boosting of the immune response to VZV in mothers of children with varicella (Arvin et al. 1983), in addition to a decrease in incidence of AHZ in adults who have greater contacts with children in their daily lives (Brisson et al. 2002; Thomas et al. 2002), suggests that exposure to viral antigens may be important for maintaining cell-mediated immunity against varicella. However, with the recent introduction of childhood varicella vaccination programmes, epidemiological models suggest that a significant increase in AHZ may initially occur as a result of reduced opportunities for subclinical boosting (Dworkin et al. 2006). The incidence in disease is expected to peak approximately 20 years after childhood vaccination, returning to pre-vaccination levels after 40 years before a significant decline in disease is then seen (Dworkin et al. 2006; Ringkamp and Meyer 2006; Gold et al. 2006; Gereau 2006; Hammond 2006; Vierck 2006). However, this increase may be offset by adult vaccination which has recently been shown to reduce the incidence of AHZ and PHN by 51.3% and 66.5% respectively (Oxman et al. 2005).

In the presence of inadequate VZV-specific cell-mediated immunity, specifically age-related immunosenescence and iatrogenic immunosuppression (e.g. chemotherapy), reactivation of latent virus results in AHZ. However, frequent subclinical viral reactivation may also occur in the presence of adequate VZV-specific immune response. Active viral replication in the sensory ganglia is accompanied by an extensive inflammatory response (ganglionitis) in which destruction of neurones and supporting cells is observed (Gilden et al. 2000; Dworkin et al. 2006). This is thought to underlie the prodromal pain that often precedes the characteristic
dermatomal eruption of AHZ. The prodrome is typically of two to three days in duration. Pain may be constant or intermittent in nature and often has a distinctive quality for individual patients who commonly describe "burning", "shooting", "stabbing" and "throbbing" sensations, often evoked by touch and/or associated with pruritus (Dworkin et al., 2006). The interval from the onset of the prodrome to the appearance of herpetic rash represents the time required for the actively replicating virus to reach the dermal-epidermal junction and induce sufficient necrosis and inflammation in the skin. The rash of AHZ typically affects a single dermatome, and is accompanied by acute pain and sensory disturbances similar to that experienced in the prodrome. It is characterised by an initial macular and/or papular phase, rapidly followed by the appearance of vesicles, ulcerations, crusting of lesions and scarring, which may persist long after AHZ resolves. Moreover, dermatomal pain in the absence of cutaneous lesions, termed zoster sine herpete may occur and provides further support for direct involvement of the ganglia (Dworkin et al., 2006). Furthermore, in 15% of patients, severe herpetic rash may be complicated by involvement of cranial nerves, for example, zoster ophthalmicus and Ramsay-Hunt syndrome, while in less than 20% of patients, significant systemic symptoms may occur (Dworkin et al., 2006).

1.7 Postherpetic Neuralgia

PHN is a frequent and highly debilitating complication of AHZ with an increasing incidence that is directly related to advancing age (Nurmikko 1995). Thus, it is one of the most common neuropathic pain syndromes and one that is expected to increase as the aging population continues to grow. PHN is variably defined, but a widely used definition is 'persisting pain arising in areas affected by herpes zoster at least three months after healing of skin lesions' (Dworkin and Portenoy 1994). Retrospective population-based studies have reported that without treatment, the incidence of pain persisting three months after the development of herpetic rash is 8% to 15% (Bowsher 1999;Johnson and Patrick 2003). In comparison to other neuropathic pain conditions, PHN is reported to have a far greater incidence (40 per 100 000 person years) than trigeminal neuralgia (27 per 100 000 person years), or painful diabetic neuropathy (15 per 100 000 person years) (Hall et al. 2006).
PHN may be accompanied by varying degrees of sensory deficits including abnormal hyper-sensory phenomena, such as hyperalgesia and allodynia and/or hypo-sensory phenomena, such as partial or complete sensory loss (Nurmikko 1995; Fields et al. 1998; Pappagallo et al. 2000). In addition, it may be associated with significant pain-related co-morbidity symptoms, such as anxiety and depression (Meyer-Rosberg et al. 2001). For example, Dworkin et al., (Dworkin et al. 1992) found at an initial assessment during AHZ that those patients who later developed PHN had higher anxiety, greater depression and generally lower life satisfaction than patients who did not develop chronic pain.

Post-mortem studies in PHN patients have revealed characteristic pathophysiological findings (Watson et al. 1988; Watson et al. 1991; Rowbotham et al. 1996; Oaklander et al. 1998). These include atrophy of the spinal cord dorsal horn (with one study reporting atrophy at the affected level over four segments with loss of axons, myelin, and neuronal cell bodies despite involvement of only one ganglion) (Watson et al. 1988); destruction of infected DRG neurones, with fibrosis and scarring, and marked loss and fibrosis of myelinated nerve fibres of all types in the sensory nerve roots; as well as loss of peripheral epidermal innervation (discussed in Chapter 6) (Watson et al. 1991; Rowbotham et al. 1996; Oaklander et al. 1998). Oaklander and colleagues (1998) examined the density of epidermal neurites from subjects with PHN, and without PHN after unilateral AHZ. Skin biopsies were evaluated from the site of maximum pain or zoster involvement and from the homologous contralateral site. The authors found that those subjects with PHN had a significantly lower density of sensory neurites in herpes zoster-affected epidermis compared with subjects without pain. The authors also noted that those subjects with pain had additionally lost half of the neurites in the contralateral epidermis despite the lack of contralateral herpetic lesions or pain. In addition, some evidence for a more generalised subacute or chronic inflammatory process in PHN exists (Watson et al. 1991; Gilden et al. 2003a). Prominent collections of lymphocytes in association with extensive axonal and myelin loss in DRG have been described on post-mortem (Watson et al. 1991). The presence of similar lymphocytic aggregations have been reported in the region of the substantia gelatinosa, nerve roots, and peripheral nerves (Watson et al. 1991). Furthermore, the occurrence of inflammatory cells at multiple levels (and bilaterally in some patients) suggests that disease is more widespread than clinical features may initially suggest (Watson et al. 1991).
While advancing age and impaired cell-mediated immunity are well recognised risk factors for VZV reactivation and development of PHN (Nurmikko 1995; Kennedy 2002a), additional risk factors have recently been identified for patients with AHZ. Clinical studies have found PHN to be independently associated with, female gender (in addition to the increased life expectancy-associated prevalence of PHN), presence of prodromal pain preceding the herpetic rash, greater acute pain severity at onset and greater rash severity (Bowsher 1999; Johnson and Patrick 2003; Jung et al. 2004; Coen et al. 2006). Identification of those at greatest risk encourages targeted prescribing of early and effective treatment and reduces the incidence and duration of PHN.

1.7.1 Treatment of PHN

PHN has a well known aetiology allowing easy diagnosis, which means that it is now a widely adopted model for clinical trials of novel neuropathic agents. While there is a strong clinical evidence base supporting the efficacy of certain analgesic therapies in established PHN, most of these therapies do suffer from a narrow therapeutic index which limits their clinical effectiveness. Therefore, PHN remains an area of largely unmet therapeutic need (Hempenstall et al. 2005; Rice and Hill 2006). In a recent meta-analysis of the clinical trial literature, only 30% - 50% of patients were able to obtain more than 50% pain relief and this was often at the cost of side effects (Hempenstall et al. 2005). Orally administered treatments with the strongest evidence base include opioids (combined number needed to treat, NNT, for a 50% change in pain intensity is 2.67), tricyclic antidepressants (NNT 2.64) and gabapentin (NNT 4.39). In terms of adverse events, the numbers needed to harm (NNH) for all reported side effects was 3.57, 5.67 and 4.07 respectively. Thus it is evident that the clinical effectiveness of these therapies is limited by their narrow therapeutic index (Hempenstall et al. 2005; Rice and Hill 2006). In addition, no single treatment has been shown to be completely effective for all patients and combination therapy, which may further affect patient compliance, is often used. Indeed, some patients (60%) remain refractory to all measures (Kinloch and Cox 2005). Therefore, there is a need for new therapies that provide more predictable efficacy in all patients with improved tolerability. Furthermore, PHN is no longer viewed as a single disease entity but as more than one disorder with multiple mechanisms (Rowbotham 1999). Therefore it has been proposed that individual treatment paradigms should be mechanism-based (Fields et al. 1998; Rowbotham 1999). This may explain why response to any single intervention is so often inadequate,
presenting a need to develop novel analgesics directed at the mechanisms underlying PHN (Fields et al. 1998; Rowbotham 1999).

1.7.2 Mechanisms underlying PHN

Due to the wide variation in clinical presentation and individual response to therapeutic interventions, it is believed that PHN encompasses a spectrum of potential pain-generating mechanisms that may even coexist within the same patient. Existing evidence reveals significant contributions from both the peripheral and central nervous systems (Fields et al. 1998; Pappagallo et al. 2000), though the relative contributions to the pathophysiology of pain may vary among individual patients and over the time course of the disease. Based on quantitative sensory testing and response to topical therapies, three distinct subtypes of PHN have recently been proposed: (a) an ‘irritable nociceptor’ subtype with minimal deafferentation and thermal and mechanical allodynia; (b) a deafferentation subtype with sensory loss and no allodynia; and (c) a second deafferentation subtype with sensory loss and allodynia (Pappagallo et al. 2000). The proposed nerve-injury related mechanisms for these clinical groups are not mutually exclusive and it is possible that several mechanisms may coexist in a single patient, or that one subtype may evolve into another (Fields et al. 1998).

(a) Irritable' nociceptor group. These patients typically have minimal sensory loss and pronounced mechanical allodynia, as thermal sensory thresholds are often preserved. Pain in these patients is believed to result from intact, but abnormally hyperactive primary afferent nociceptors in the skin i.e. sensitised or ‘irritable’ cutaneous nociceptors (Fields et al. 1998). Ongoing activity in these sensitised nociceptors is thought to have a role in the initiation and maintenance of the central sensitisation of pain transmission neurones, and hence in the generation of mechanical allodynia. Inflammation and axonal damage following nerve injury may further lead to the generation of spontaneous activity at several sites along the primary afferent, and/or to the upregulation of excitatory adrenergic receptors on primary afferents, thus producing a hyperexcitable state in peripheral nociceptors. Further support for the irritable nociceptor concept comes from the observation that drugs that would be expected to block ectopic impulses and hence the abnormal spontaneous activity generated in damaged primary afferents, for example, antirrhythmics and anticonvulsants that block voltage dependent sodium channels, do have some efficacy in this subtype of patients (Field et al., 1998). In addition, an
Inverse correlation between pain severity and sensory loss has been demonstrated in some individuals i.e. pain severity appears to be associated with relative preservation, rather than loss of primary afferents. Therefore, abnormal activity generated in the cutaneous terminals of intact primary afferents appears to play a major role in the generation of PHN pain. Thus, patients with this subtype of PHN, which is thought to represent 25% of sufferers, usually experience pain relief with topical local anaesthetics, and suffer pain with topical capsaicin (Field et al., 1998; Hempenstall et al., 2005).

Persistent inflammation of peripheral nerve trunks may also produce an ‘irritable nociceptor’ syndrome. AHZ is accompanied by intense inflammation along the affected peripheral nerve that typically resolves in several weeks, and although ongoing inflammation in the skin of patients with PHN has not been reported, in a small subgroup of PHN patients, inflammatory infiltrates have been demonstrated throughout the affected peripheral nerve, DRG, and dorsal root (Watson et al., 1991; Fields et al., 1998). Moreover, Gilden et al., (Gilden 1994) described a small subpopulation of PHN patients with evidence of continuing low level viral expression whose pain responds to antiviral agents. Thus, continuing VZV expression could produce sensitisation and activity in primary afferents secondary to inflammation (Fields et al. 1998).

(b) Deafferented non-allodynic group. This subtype represents a distinct minority of PHN sufferers with less than 15% reporting no significant allodynia but experiencing severe spontaneous pain in a region of profound sensory loss to all modalities i.e. anaesthesia dolorosa (Fields et al., 1998; Papagallo et al., 2000). It is likely that a virtually complete cutaneous deafferentation of both the large and small fibres have occurred. Therefore, their pain must be the result of intrinsic CNS changes. The proposed mechanisms for severe spontaneous pain in these patients are deafferentation-induced hyperactivity of central pain transmission neurones, and/or disinhibition of CNS neurones due to a predominant loss of pain inhibitory interneurones (Fields et al., 1998).

(c) Deafferented alldynic group. This subtype represents those PHN patients who experience mechanical allodynia, thermal sensory deficits and spontaneous pain. A profound loss of small fibre sensory function, often accompanied by raised thermal thresholds, and marked allodynia coexist in areas of maximal pain. Mechanical allodynia in these patients is thought to be secondary to synaptic plasticity or aberrant connections and reorganisation within the dorsal
horn of the spinal cord resulting from partial deafferentation (Fields et al., 1998). Following peripheral nerve injury induced degeneration of nociceptive C-fibres, central terminals of intact Aβ-low-threshold mechanosensitive primary afferents are thought to form abnormal connections with deafferented central pain transmission neurones.

1.8 Biology of VZV

VZV is an exclusively human neurotropic alphaherpesvirus. It is the smallest of the alphaherpesvirus family, with a genome consisting of approximately 125 kbp. The VZV genome is packaged in an icosahedral capsid surrounded by the tegument, comprising viral proteins that initiate DNA replication when the virus enters the host cell; and a lipid membrane envelope containing the viral glycoproteins that are presumed to mediate cell entry (Jones and Arvin 2003). The entire double-stranded DNA genome has been sequenced, revealing 71 unique open reading frames (ORFs) that encode proteins expressed as putative immediate-early (IE) regulatory genes, early genes, and late genes (Kennedy 2002a; Gilden et al. 2003b; Zerboni et al. 2005). Although transcripts mapping to most of the ORFs have been identified in VZV-infected cells in culture, few have been analysed in detail (Kinchington 1999; Gilden et al. 2003b). VZV gene transcription during productive (lytic) infection is highly regulated and follows a complex cascade of events. IE genes are the first to be transcribed after virus infection, and encode proteins involved in viral gene regulation (Shiraki and Hyman 1987; Gilden et al. 2003b). Nuclear accumulation of IE proteins in infected cells thus regulates further viral gene expression. This is followed by the expression of ‘early’, and then ‘late’ genes, both of which are upregulated by IE genes. Early genes encode proteins necessary for DNA synthesis, while late genes are expressed after DNA replication and encode structural proteins.

1.9 VZV Latent Infection and Reactivation

Latent infection is characterised by the long-lasting presence of the viral genome, of selected viral gene transcripts, and by the absence of infectious virus (i.e. no ongoing virus replication) (Sadzot-Delvaux et al. 1995; Kennedy 2002a). However, the mechanisms involved in both latency and reactivation of the virus are not well understood. This is mainly because of the
poor growth of VZV in vitro, where the virus remains highly cell associated. Reactivation of latent virus usually occurs within a single ganglion where it appears the virus causes extensive destruction and inflammation of neurones and surrounding cells, including the permanent degeneration of central and peripheral axons (Fields et al., 1998). Importantly, whilst latency has traditionally been used to describe the presence of VZV in rodent experimental models, this is not ideal in the context of rodent models in which we observe behavioural changes clearly reflecting an active viral interaction with sensory neurones. Indeed, if the virus was truly latent or dormant, it may be argued that behavioural changes could not occur. Therefore, in this context, a more appropriate term might be to describe the virus as ‘resident’.

Although the exact mechanisms by which persistent pain follows reactivation of latent VZV are not well known, clinical virological correlations suggest that virus persistence in ganglia, or chronic ganglionitis, may be the cause (Gilden et al. 2003a; Gilden et al. 2003b). For example, VZV-specific DNA has been detected in peripheral blood mononuclear cells (MNCs) of PHN patients up to 8 years after AHZ, whereas in zoster patients who did not develop PHN, VZV DNA was present only up to 38 days, or not at all, after disappearance of pain (Devlin et al. 1992; Mahalingam et al. 1995). Further evidence that the persistent pain of PHN reflects a chronic ganglionitis comes from the detection of VZV DNA in both blood MNCs and cerebrospinal fluid of patients without herpetic rash (i.e. zoster sine herpete). Post-mortem examination in human PHN sufferers has additionally demonstrated neuronal loss and fibrosis in the DRG, in addition to atrophy of the dorsal horn and loss of axons and myelin in peripheral nerves (Watson et al. 1991). While the precise interaction with the host is not known, a decline in cell-mediated immunity and an interaction between the host immune response and several VZV-encoded transcripts and proteins (genes 4, 21, 29, 62 and 63) associated with latent viral infection in human and rat ganglia, are thought to be involved (Kennedy et al. 1999; Abendroth and Arvin 2001; Kennedy 2002b).

1.9.1 Viral gene expression during latent infection

There is considerable evidence from several studies employing different techniques (in situ hybridisation, polymerase chain reaction (PCR) and northern blot analysis) to show that the following viral genes are transcribed during latent VZV infection in human peripheral ganglia; genes 21, 29, 62 and 63, with variable evidence for genes 4 and 18 (Kennedy et al.
A possible mechanism by which these genes maintain latent infection may involve the restriction of typically nuclear proteins, e.g. IE63 and IE62 (encoded by VZV genes 63 and 62 respectively), to the cytoplasm of infected neurones (Gilden et al. 2003b). The most frequently detected VZV transcript is that mapping to gene 63, which has been proposed as a hallmark of VZV latency. In addition, expression of IE63 protein has been demonstrated in the skin of patients with early symptoms of herpes zoster (i.e. during VZV reactivation) (Debrus et al. 1995), therefore suggesting that IE63 protein plays an important role, both in the control of the infectious cycle, and in the maintenance of latent infection (Debrus et al. 1995) (Merville-Louis et al. 1989; Sadzot-Delvaux et al. 1990). Similarly, IE62 protein, a major component of the VZV virion (Kinchington et al. 1992), is a potent viral regulatory protein that is likely to be among the first viral genes expressed and translated during infection or reactivation. IE62 stimulates transcription of further viral genes thereby increasing the infectivity of VZV (Gilden et al. 2003b). This normally nuclear located protein localises to the cytoplasm of neurones during latency. Its presence in the nucleus is therefore indicative of active viral replication.

### 1.9.2 Cellular site of VZV during latent infection

The presence of VZV DNA in human sensory ganglia was first demonstrated in 1983 by Gilden et al. (Gilden et al. 1983). Although this finding has since been verified by numerous studies and is now well documented, a more controversial issue is the identification of the cell type within the sensory ganglia in which VZV establishes latent infection. Several studies support the detection of VZV exclusively in neurones of infected human ganglia (in the neuronal cytoplasm and/ or nucleus) (Gilden et al. 1987; Debrus et al. 1995; Dueland et al. 1995), while others demonstrate the presence of the virus in the cytoplasm of non-neuronal satellite cells (Croen et al. 1988; Meier et al. 1993), or in both (Lungu et al. 1995). For example, in a study by LaGuardia et al. (LaGuardia et al. 1999), the presence of VZV DNA in neuronal and non-neuronal cells of human trigeminal ganglia was investigated. Using quantitative PCR analysis of VZV DNA in the two cell-type populations, it was found that viral DNA was primarily present in neurones at a frequency of 2-5 copies per 100 neurones. In contrast, Croen et al., (Croen et al. 1988) reported an exclusively non-neuronal localisation of latent VZV. Using in situ hybridisation techniques, they identified the presence of latent VZV RNA in peri-neuronal satellite cells in latently infected trigeminal ganglia from 15 of 30 subjects. However, whilst the
The majority of evidence now supports a predominantly neuronal localisation for latent VZV, the exact location of the virus within neurones, that is whether it is present in the neuronal cytoplasm or nucleus, remains to be determined unequivocally. There are studies that lend support for both sites.

### 1.9.3 VZV burden in human ganglia during latent infection

Despite wide variation in ganglionic viral load during latency (Cohrs et al. 2000), which most likely reflects the severity of primary infection, ganglionic VZV burden during latent infection tends to be low (Mahalingam et al. 1993; LaGuardia et al. 1999; Cohrs et al. 2000; Levin et al. 2003). (Mahalingam et al. 1993; LaGuardia et al. 1999; Cohrs et al. 2000; Levin et al. 2003) For example, in a quantitative PCR study using primers specific for VZV latency genes 28 and 62, Mahalingam et al., (1993) detected 9 - 53 copies of VZV DNA per µg of ganglionic DNA in human trigeminal ganglia. This corresponds to 6-31 copies of the VZV genome per $10^5$ ganglionic cells. Even though the exact copy number may vary with technique used, there is further evidence to support the initial findings of Mahalingam et al., (1993). For example, LaGuardia et al., (1999) reported 2-5 copies of latent VZV DNA in human trigeminal ganglion neurones, per $10^4$ ganglionic cells.

### 1.10 Viral Genotype and Strain Variation

Even though VZV is a genetically stable alphaherpesvirus with limited variation in its sequence (i.e. has a low mutation rate) (Barrett-Muir et al. 2003), it has recently been shown that there are at least three distinct genotypes of the virus in humans (Breuer 2003) (discussed in Chapter 2). However, little is known about their biological differences and if there is an association with particular clinical phenotypes. For the most part, these three viral genotypes are found in separate geographical regions (Quinlivan et al. 2002). Genotype A is found in Africa, Asia and the Far East. By contrast, genotype B is mainly found in the United States and Europe, whilst genotype C is the most common variant found in the United Kingdom. However, there is growing evidence for change in the prevalence of strains circulating in the United Kingdom over recent years, with genotypes A and B also circulating in smaller numbers. This may relate to
patterns of immigration, and hence the introduction of new genotypes from continents such as Africa and Asia.

1.10.1 Oka vaccine strain

The live-attenuated Oka strain of varicella, derived from a Japanese strain and created by multiple passages of the wild-type parent strain in guinea pig embryo cells and human fibroblasts (Jones and Arvin 2003), was developed as a vaccine against VZV infection in the 1970s. Certainly, in Japan and the United States, vaccination against varicella with the Oka vaccine is routine and results in protective immunity in 90% of healthy adults (Gershon et al. 1992; Hawrami and Breuer 1997). In the U.K., varicella vaccination programmes have recently been introduced in children, with the Oka vaccine also being available to VZV-seronegative health workers who may be occupationally exposed to VZV infection, and to groups at risk of severe primary varicella (e.g. pregnant women) (Quinlivan et al. 2004). In addition, vaccination may be used to provide 'booster' immunity in elderly patients and so decrease risk of AHZ (Oxman et al. 2005; Dworkin et al. 2006). However, a small number of vaccinees do develop varicella, evidently as a result of either circulating wild-type virus or the vaccine strain. In addition, reactivation of the vaccine strain to cause zoster has been described in 6% of vaccinees (Gershon et al. 1992; Hawrami and Breuer 1997).

1.11 Animal Models of Neuropathic Pain

Current knowledge of the mechanisms contributing to the generation of neuropathic pain is based largely on animal models involving a discrete peripheral nerve injury (Zimmermann 2001; Bridges et al. 2001b), with a model of simple axotomy being the first widely used injury (Wall et al. 1979). More commonly, partial injury of the sciatic nerve is employed and includes the chronic constriction injury (Bennett and Xie 1988), partial sciatic nerve ligation (Seltzer et al. 1990), spinal nerve ligation (Kim and Chung 1992), and the spared nerve injury (Decosterd and Woolf 2000). Whilst these models do not necessarily mimic clinically relevant “dying-back” neuropathies in humans, such as diabetic or neurotoxic neuropathies, these models have allowed investigation of some of the different mechanisms that may play a role in clinically relevant
neuropathies, e.g. the relative role of injured and uninjured afferents in neuropathic pain states can be studied (Ringkamp and Meyer 2006).

However, these animal models have considerable short-comings in general which limits their use clinically. Firstly, whilst neuropathic pain is a devastating response to nerve injury, it is not the usual consequence, as most patients do not develop neuropathic pain following peripheral nerve injury, e.g. only approximately 20% of diabetics with peripheral neuropathy develop neuropathic pain (Bennett 2006). In contrast, development of neuropathic pain outcomes i.e. hypersensitivity phenomena is frequent and highly reproducible in animal models of peripheral nerve injury. Therefore they do not mirror the 'normal' human response to nerve injury. Further, current animal models which are generally limited to partial trauma of the sciatic nerve as discussed above do not reflect the diverse spectrum of human neuropathic pain aetiologies. Therefore, it is essential to develop animal models that reflect more common clinical disease and demonstrate good construct and face validity (Blackburn-Munro 2004). Also, for ethical reasons, most animal models of neuropathic pain study the animals for a period of weeks, rarely validating beyond 90 days, whereas the clinical duration of neuropathic pain is often for years (Bridges et al. 2001b). Finally, behavioural testing in animals is restricted to detection of hypersensitivity phenomena (i.e. correlates of human hyperalgesia and allodynia), rather than the more common neuropathic pain phenotype of sensory loss highlighting a mismatch between preclinical and clinical validation studies. Whilst mechanical hypersensitivity is of some clinical relevance, hypersensitivity to cold and heat which is reliably observed in many animal models of traumatic peripheral nerve injury, are less relevant in the clinical setting (Hansson et al. 2001). Further, alterations in cutaneous sensory thresholds to evoked stimuli are typically measured at a particular moment in time. However, such evoked spinal reflex withdrawal responses tell us little about integrated pain-related behaviours, nor about the nature of ongoing or spontaneous pain.

1.11.1 Animals and their response to pain

The ability to detect damaging or potentially damaging stimuli is present in many animal species (Flecknell 2000). However, possession of a peripheral detection system does not in itself mean that animals experience pain; as pain in humans is recognised as having both a sensory and an emotional component and it is the interpretation of nociceptive information centrally that
results in the highly subjective and individual experience of pain. While there are broad parallels in animal and human neuroanatomy (both possess nociceptors, and the types of nerve fibres that connect them to the CNS, are virtually identical), and in the processing of nociceptive information in the spinal cord and lower parts of the brain, it is difficult to know what is actually perceived by the animal and impossible to investigate the emotional experience of pain directly.

The absence of verbal communication in rats is undoubtedly an obstacle to the evaluation of pain. Therefore, we can only draw inferences from other indirect measures, such as the investigation of behavioural responses to noxious stimuli. Furthermore, we often have preconceptions that animals in pain behave in the same way as humans in pain, but we should also expect them to behave in a species-specific way, which may further vary with different clinical conditions. Some animal species may display very obvious pain-related behaviour, while in others, expressing such overt behaviour would simply alert predators (Flecknell 2000). In addition, animals may mask their behaviour when aware of being observed. They may also change their responses when in a familiar, secure environment, and express less pain-related behaviour when in an unfamiliar environment (Flecknell 2000).

Aside from immediate avoidance or defence reactions, such as struggling or biting when an injured limb is handled, animals display a range of more subtle behavioural responses to pain which may not be evident on stimulus-evoked reflex withdrawal testing. Indeed, animals may be conditioned to perform complex tasks to avoid brief painful stimuli (Cryan and Holmes 2005), while other studies have shown that animals can choose to self-administer analgesics when they develop chronic painful conditions (Flecknell 2000; Fattore et al. 2001; Colpaert et al. 2001). A re-interpretation of the IASP definition of pain so that it could be applied to animals may therefore be: “an aversive sensory experience caused by actual or potential injury that elicits progressive motor and vegetative reactions, results in learned avoidance behaviour, and may modify species-specific behaviour, including social behaviour” (Le Bars and Cadden 2005). Thus, more complex measures of integrated pain behaviours in animals are necessary to properly assess their experience of pain.
1.12 Integrated Pain Behaviours in Rodents

Current animal models of neuropathic pain have considerable limitations, thus one of the major challenges facing researchers in the pain field is how to make them more clinically relevant (Blackburn-Munro 2004; Mogil and Crager 2004). Inappropriate outcome measures (i.e. stimulus-evoked hind-limb withdrawal reflexes which assess hypersensitivity phenomena seen in only a small subset of patients with neuropathic pain) mean that current models do not have good clinical face validity in addition to having limited predictive validity for the identification of targets for the development of novel analgesic agents. Importantly, pain co-morbidity symptoms (e.g. anxiety and depression) in addition to ongoing stimulus-independent or spontaneous pain frequently seen in neuropathic pain are not reflected in classical mechanosensory tests. Therefore, paradigms reflecting more complex measures of integrated pain behaviours in animals are necessary. To date however, the investigation of pain co-morbidity behaviour in rodent models of neuropathic pain is somewhat limited (Kontinen et al. 1999; Narita et al. 2006a). This is partly because anxiety and depression are heterogeneous disorders with symptoms manifested at the psychological, behavioural and physiological level, making them difficult disorders to model in the laboratory (Cryan et al. 2005).

Similarly, the investigation of spontaneous pain in rodents is also limited as it is difficult to know what is actually perceived by the animal (Blackburn-Munro 2004; Mogil and Crager 2004; Djouhri et al. 2006; Pickering et al. 2006). Proposed dependent measures of spontaneous chronic pain include analgesic self-administration; attention deficits; autotomy; bite force; conditioned place preference; food intake (hypophagia, weight loss); disturbance in gait, weight bearing, grooming (including scratching, licking, or biting); paw lifting, guarding, flinching or shaking; locomotor activity; and ultrasonic vocalisation (discussed in Chapter 8) (Mogil and Crager 2004). However, there are challenges associated with these measures and indeed ethical considerations (specifically with regard to autotomy, which may reflect ongoing, ectopically generated pain or possibly paraesthesias, dysesthesias, or even numbness). For example, behavioural changes may not be specific to chronic pain and could be produced by other disease states. They may be labour-intensive or not easily detectable, and subject to observer bias (particularly if not automated). In the case of analgesic self-administration, outcome measures may be confounded by variable sensitivity to the analgesic itself. Behavioural measures may further be confounded by cognitive deficits, sedation, ataxia, fear or variable learning or memory...
in animals; and may ultimately be dependent on social or environmental factors (Mogil and Crager 2004).

1.12.1 Assessment of anxiety-like behaviour in rodents

The open field, elevated plus maze (EPM) and light-dark box are well characterised paradigms for the investigation of anxiety-like traits in animals, and importantly have proven sensitivity to clinically employed anxiolytics (Belzung and Dubreuil 1998; Prut and Belzung 2003; Cryan and Holmes 2005). These tests are based upon the fact that small rodents have an innate aversion to exposed, well-lit spaces, probably reflecting predator avoidance in the wild. However, as they are also naturally exploratory animals, this aversion conflicts with a drive to explore novel environments, especially when foraging (Cryan and Holmes 2005). The aversive area takes a different form in each of the tests; an exposed brightly lit central area in the open field, a similarly brightly illuminated chamber in the light-dark box, and open exposed arms in the EPM test. It is likely that the differing environments therefore induce different degrees of anxiety, with the EPM tending to be more anxiogenic (Carola et al. 2002). Outcome measures indicative of anxiety-like behaviour, such as thigmotaxis (i.e. “wall-hugging” pattern of ambulation), avoidance of the aversive areas, and scanning or ‘risk-assessment’ behaviour, demonstrate a certain amount of face validity given that anxiety disorders (including pain-induced anxiety) are typified by a pervasive avoidance and a heightened vigilance or apprehension of feared or potentially aversive situations (Cryan and Holmes 2005). Therefore, these paradigms lend themselves to the investigation of an ongoing pain state which may cause alterations in an animal’s natural pattern of behaviour thus proving useful in demonstrating more complex outcome measures of integrated pain behaviour.

As an alternative to exploration-based approach-avoidance conflict tests, anxiety-like behaviour in rodents may also be modeled by examining fear conditioning (i.e. increased flight from a predator or increased acoustic startle/freezing response), or by examining biological measures, such as stress-induced hyperthermia (Cryan and Holmes 2005). Specifically in mice, increased ultrasound vocalisation (>20 kHz) e.g. in pups separated from their mother may be observed (consistent with separation anxiety in humans). However, it has been found that ultrasound vocalisation behaviour in rats does not correlate with behavioural measures of persistent pain, and it is therefore not a useful integrated correlate of pain behaviour in rats (Wallace et al. 2005).
1.12.2 Assessment of depression-related behaviour in rodents

The tail suspension test (TST) is one of the most widely used paradigms for assessing depression-related behaviour in mice (Bai et al. 2001; Cryan et al. 2005), validated by its sensitivity to clinically effective antidepressants which cause mice to actively and persistently engage in escape-directed behaviours as compared to control (Cryan and Holmes, 2005). Further, the TST has been shown to be sensitive to various factors that influence or are altered by depression in humans, including genetic predisposition, previous exposure to stress, and drug-induced anhedonia (Cryan and Holmes 2005). The main outcome measure in the TST is passive immobility behaviour, which reflects a learned helplessness to a short-term inescapable stress. This is thought to correlate with similar observations in depressed patients (i.e. psychomotor impairments particularly in tasks requiring sustained effort).

However, while the TST may prove useful in investigating depression-related behaviour in neuropathic mice, it is not an appropriate test for rats since they are unable to support their own body weight when suspended. Suggested approaches suitable for modeling depression-related behaviour in rats may include evidence of social withdrawal and/or reduced activity in the home cage, abnormal loss in body weight after exposure to chronic stressors, abnormal sleep architecture, poor grooming or coat condition, and possibly deficits in specific memory tasks (Cryan and Holmes 2005).

1.12.3 Assessment of spontaneous pain in rodents

As previously discussed, conventional behavioural tests measure stimulus-evoked pain at a particular moment in time, whereas a significant proportion of patients with neuropathic pain suffer ongoing stimulus-independent or spontaneous pain. So arises the question of how we can reliably measure spontaneous pain in animals to better reflect the clinical scenario? While it may be possible to use integrated behavioural paradigms, it has been suggested that behaviours such as frequent licking, flinching and/or guarding of the affected hind paw are indicative of spontaneous nocifensive behaviours. Spontaneous foot lifting (SFL) has further been proposed as a measure of spontaneous pain behaviour in neuropathic and inflammatory pain models (Bennett and Xie 1988; Choi et al. 1994; Djouhri et al. 2006). For example, Djouhri et al., (2006), demonstrated significant duration and frequency of SFL in a modified L5 spinal nerve axotomy.
model (i.e. axotomy plus loose ligation of the L4 spinal nerve), and similarly in an inflammatory pain model (i.e. complete Freund’s adjuvant). However, SFL was not observed in a standard spinal nerve axotomy model, nor in a model of L4 loose ligation alone. Furthermore, while SFL was found not to be correlated with mechanical allodynia following nerve injury or inflammation, it was related to the rate of spontaneous firing in intact C-nociceptors.

However, nocifensive behaviours indicative of spontaneous pain may not be obvious and therefore appropriate measures, in all animal models. It may be necessary to investigate a number of more subtle behaviours to reflect this aspect of persistent pain, e.g. co-morbidity behaviours, such as suppression of appetite, disturbances in sleep, social interaction, or grooming behaviour could be investigated as indirect measures of ongoing or spontaneous pain.

1.13 Animal Models of VZV Latency and Persistent Infection

Whilst it is possible to examine human cadaveric DRG harbouring latent VZV, such tissue is not readily available nor amenable to manipulation for studying the events surrounding the establishment and maintenance of latent infection, viral reactivation, and persistent infection (Annunziato et al. 2001). Alternatives to studies in human DRG are therefore in vitro (Merville-Louis et al. 1989; Chen et al. 2003) and in vivo rodent models of VZV latent infection (Sadzot-Delvaux et al. 1990; Myers and Connelly 1992; Sadzot-Delvaux et al. 1995; Brunell et al. 1999; Fleetwood-Walker et al. 1999; Dalziel et al. 2004; Garry et al. 2005).

Successful demonstration of latent VZV infection in vivo was initially made by Sadzot-Delvaux et al., (Sadzot-Delvaux et al. 1990) who described the presence of viral proteins in DRG of infected rats following the subcutaneous (s.c.) injection of VZV-infected human malignant melanoma cells along the spine of healthy adult rats. Further studies in this rat model provided evidence of VZV DNA and its distribution primarily in neurones in the PNS (Sadzot-Delvaux et al. 1995; Annunziato et al. 1998; Brunell et al. 1999). This agrees with observations in human DRG containing latent virus, confirming that VZV DNA persists in the same sites in DRG of the two species (Annunziato et al. 1998). This was followed by the first pain-related associations (i.e. mechanical allodynia and thermal hyperalgesia) in rats following infection with VZV (Fleetwood-Walker et al. 1999). The authors reported a heightened sensitivity to punctate

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mechanical and noxious thermal stimulation for up to 33 days following the s.c. injection of virus-infected fibroblasts into the hind foot pad of healthy adult rats. In a more recent study further characterising the original model described by Fleetwood-Walker et al., (1999), Dalziel et al., (2004) extended the time course to show that rats infected with VZV develop a chronic mechanical allodynia, which is present for longer than 60 days post-infection and resolves by 90 days post-infection. Further support for these early findings has recently been provided by Garry et al., (2005) who demonstrated a dose-dependent hypersensitivity to both punctate mechanical and noxious thermal stimuli in rats following infection with high titre VZV. Immunohistochemical analysis of rat DRG further confirmed the presence of a viral immediate-early gene protein (IE62) in both myelinated A- and unmyelinated C-afferent sensory neurones (Garry et al. 2005).

However, none of the animal models described to date reproduce VZV pathophysiology completely (Sadzot-Delvaux et al. 1990; Myers and Connelly 1992; Fleetwood-Walker et al. 1999; Dalziel et al. 2004; Garry et al. 2005) and there is still the need to refine current animal models to show improved construct, face, and predictive validity. Lack of an appropriate animal model in which VZV may be experimentally reactivated from a latent state in sensory ganglia (i.e. showing appropriate construct validity), has therefore hindered progress in elucidating the pathophysiology of persistent zoster-associated pain. Indeed, reactivation of virus has only been demonstrated ex vivo after repeated stresses (Sadzot-Delvaux et al. 1995). The main challenges in establishing a reliable animal model are due to the fact that VZV is a species-specific human herpesvirus and therefore not a pathogen in rodents. Also, the virus remains highly cell-associated, restricted to only a few fibroblast cell lines. Therefore, it is difficult to obtain stable cell-free virus stocks or the high titres of virus required to produce behavioural changes in animals (Kinchington 1999). Indeed, early attempts to produce disease by experimental inoculation of animals led only to seroconversion (i.e. viraemia) without clinical signs (Myers et al. 1980; Myers et al. 1985).

Remarkably, rodent models of herpes virus infection have been described using the herpes simplex type-1 virus (HSV-1), another member of the alphaherpes family of viruses that similarly establishes lifelong latent infection in human peripheral sensory ganglia and is reported to induce behavioural changes following infection in rodents (Takasaki et al. 2002; Dalziel et al. 2004; Kuraishi et al. 2004). For example, Dalziel et al., (2004) reported the development of
hypersensitivity to punctate mechanical stimulation following HSV-1 infection in the rat, including a number of notable differences when compared to VZV-infected animals. Specifically, following HSV-1, but not VZV infection, cutaneous lesions and hind limb paralysis developed. Also, the onset of behavioural change was earlier (measurable by day 1 - 2 post-infection), of shorter duration (resolution of mechanical hypersensitivity by day 7 post-infection), and of less magnitude following HSV-1 infection. The development of characteristic herpes zoster-like skin lesions following HSV-1 infection (Dalziel et al. 2004; Kuraishi et al. 2004), and the persistence of pain-related responses after healing of these lesions in some animals (Takasaki et al. 2002) in addition to similarities in the biological properties of HSV-1 and VZV (Dalziel et al. 2004), have led some authors to claim that infection with HSV-1, rather than VZV (which is further complicated by species specificity), represents a more appropriate animal model for herpes zoster infection and postherpetic pain. However, it is VZV, not HSV-1 that is responsible for the pathogenesis of persistent zoster-associated pain and this must be reflected when demonstrating construct validity in an animal model. Therefore, it is more likely that these models actually reflect the acute pain observed following development of a vesicular lesion during herpes simplex eruption and is likely due to active viral replication (Dalziel et al. 2004).

As an alternative to the viral infection models, it has recently been proposed that resiniferatoxin-induced depletion of capsaicin-sensitive C-fibers may offer a reliable model for PHN in animals (Chen and Pan 2005). Systemic administration of resiniferatoxin, a highly potent analog of capsaicin that binds to the transient receptor potential ion channel (TRPV1) expressed on primary sensory neurones, results in paradoxical changes in thermal and mechanical sensitivities in the rat (i.e. thermal insensitivity and mechanical hypersensitivity) (Pan et al. 2003; Chen and Pan 2005). Such paradoxical changes are similarly present in patients with small-fiber neuropathies, such as PHN (Rowbotham and Fields 1996). However, the neurotoxin model is not specific to PHN; neither does it demonstrate construct validity with respect to VZV pathophysiology, therefore it cannot be regarded a model of PHN.

This thesis therefore aims to refine current animal models of herpes zoster-associated pain, ultimately providing greater clinical validity and predictability by identifying behavioural, including complex co-morbidity behaviour, and gene correlates of neuropathic pain associated with VZV infection in rats. This will further our understanding of the mechanisms underlying
persistent herpes zoster-associated pain. Accordingly, I have listed below the various broad experimental approaches employed in this thesis:

**Hypothesis**

Infection of rat dorsal root ganglia with varicella-zoster virus is associated with behavioural, pharmacological and gene correlates of neuropathic pain.

- **Assessment of simple reflex withdrawal behaviour:** Further characterisation, specifically investigation of the response to dynamic mechanical, and cold stimuli; and the influence of viral strain, and viral inoculum concentration (dose-response relationship) (Chapter 2).

- **Pharmacological sensitivity testing:** Investigation of the pharmacological profile of the model to analgesics known to have a degree of efficacy in human neuropathic pain conditions (e.g. tricyclic antidepressants, opioids and gabapentin) as well as novel analgesic compounds (e.g. cannabinoids) and anti-virals (useful in determining the nature of the model) (Chapter 3).

- **Assessment of complex integrated pain behaviours:** Behavioural paradigms will be taken beyond the simple reflex withdrawal paradigms conventionally employed in pain models, to encompass measures of integrated pain co-morbidity behaviour, specifically anxiety-like and depression-related behaviours. This will be assessed in parallel with traumatic models of peripheral nerve injury (Chapter 4); and in mice (Chapter 5).

- **Immunohistochemistry:** Detection of viral proteins in sensory ganglia and investigation of cutaneous innervation densities in VZV-infected animals (Chapter 6).

- **Differential gene expression in DRG:** A microarray approach will be used to globally investigate changes in gene expression associated with VZV infection. This will be performed in parallel with a traumatic model of peripheral nerve injury (spinal nerve transection) in order to identify gene expression changes common to both neuropathic pain models (Chapter 7).
Chapter 2

Characterisation of Nociceptive Reflex Behaviour in a Rat Model of Zoster-Associated Pain
2.1 Introduction

Persistent herpes zoster-associated pain is a significant clinical problem of which the underlying pathophysiology is not fully understood. The need for the investigation of the mechanisms involved in the establishment and maintenance of persistent herpes zoster-associated pain has resulted in the exploration of animal models of this condition. Following the introduction of an in vivo rodent model of VZV persistent infection (Sadzot-Delvaux et al. 1990), Fleetwood-Walker and colleagues (1999) described the first pain-related associations of rodent VZV infection, specifically reporting mechanical allodynia and thermal hyperalgesia. Support for these initial findings have been provided in more recent studies by Dalziel and colleagues (2004), in which VZV-induced mechanical allodynia was reported to have a chronic resolving nature; and by Garry and colleagues (2005) who described a dose-dependent mechanical and thermal hypersensitivity following VZV infection in the rat. Additionally, Garry and colleagues (2005) demonstrated the presence of a viral immediate-early gene protein (IE62) in both myelinated A- and unmyelinated C-fibre primary sensory neurons in rat DRG. This is consistent with previous studies examining human sensory ganglia from VZV-infected individuals in which viral transcripts have been detected using polymerase chain reaction (PCR) and in situ hybridisation on autopsy (Croen et al. 1988; Lungu et al. 1995; Kennedy et al. 1998) (discussed in Chapter 6). However, none of the animal models described to date (Myers et al. 1985; Sadzot-Delvaux et al. 1990; Myers and Connelly 1992; Fleetwood-Walker et al. 1999; Dalziel et al. 2004; Garry et al. 2005) reproduce VZV pathophysiology completely and there is still the need to refine current models to better reflect the clinical scenario.

Although Varicella Zoster is a genetically stable alphaherpes virus with limited variation in its sequence (i.e. has a low mutation rate) (Barrett-Muir et al. 2003), at least three distinct genotypes of the virus, based on single nucleotide polymorphisms, have been demonstrated in humans (Breuer 2003). However, little is known about their biological differences and indeed their association with particular clinical phenotypes. For the most part, these three viral genotypes are found in separate geographical regions: Genotype A is found in Africa, Asia and the Far East; genotype B is mainly found in the United States (U.S.), South America, and Europe; whereas genotype C is the most common variant found in the United Kingdom (U.K) (Quinlivan et al. 2002). However, there is growing evidence for change in the prevalence of strains circulating as varicella in the U.K. over recent years, with genotypes A and B also
circulating in smaller numbers (Barrett-Muir et al. 2003). This may relate to patterns of immigration, and hence the introduction of new genotypes from continents such as Africa and Asia, and the Far East (Barrett-Muir et al. 2003). Currently, information regarding the phylogenetic and epidemiological relationships between VZV strains globally is limited (Quinlivan et al., 2002). Such information would be useful for understanding patterns of spread of VZV infection, examining the evolution and recombination of the virus, and identifying strains that may be associated with particular clinical phenotypes (Barrett-Muir et al. 2003). For example, it has been proposed that different ethnic groups may be more susceptible to infection with particular viral strains, or that, within a given geographical area, one strain may be more virulent than another (Quinlivan et al., 2002). Although not yet known to be a critical factor in development of zoster-associated pain (Jung et al. 2004), I have examined the influence of viral strain on hind-limb reflex withdrawal responses in a rat model of VZV infection, and hypothesised that infection with different viral strains results in different patterns of reflex withdrawal behaviour, specifically, infection with a viral isolate from a zoster patient known to be associated with development of PHN following AHZ infection results in prominent reflex withdrawal behaviour, compared to a reduced effect following infection with a viral isolate known not to be associated with development of PHN.

In Japan and the U.S., vaccination against VZV with the live-attenuated Oka strain of varicella (vOka), derived from parental Oka (pOka), a wild-type Japanese strain, is routine and results in protective immunity in 90% of healthy adults (Gershon et al. 1992; Hawrami and Breuer 1997). In the U.K., varicella vaccination programmes have recently been introduced in children, with the Oka vaccine also being available to VZV-seronegative health workers who may be occupationally exposed to VZV infection, and to groups at risk of severe primary varicella (e.g. pregnant women) (Quinlivan et al. 2004). Although vaccination prevents >97% of severe varicella infections in children, vaccine-associated problems can occur (Quinlivan et al. 2004). Importantly, a small number of vaccine recipients have been found to develop varicella, evidently as a result of either circulating wild-type virus or the vaccine strain (Hawrami et al., 1997). In addition, reactivation of the vaccine strain to cause zoster has been described in up to 6% of vaccinees (Gershon et al. 1992) and may even occur without any obvious breakthrough varicella after vaccination (Quinlivan et al. 2002). Therefore, there clearly exists the potential for developing PHN in these individuals. Importantly, vaccination with live-attenuated vOka has also been found to markedly decrease the morbidity associated with AHZ and the incidence of
PHN among immunocompetent adults aged sixty years or older (Oxman et al. 2005). Given that PHN has an increasing incidence that is directly related to advancing age (Nurmikko 1995), and that life expectancy of the population is progressively increasing (Gibson 2006); the impact of mass vaccination has the potential to ultimately eradicate VZV-associated disease globally. In a recent randomised, double-blind, placebo-controlled trial, Oxman and colleagues (2005) specifically demonstrated 61.1% reduction in the “burden of illness” due to herpes zoster, 51.3% reduction in the incidence of herpes zoster, and 66.5% reduction in the incidence of PHN. Significant efficacy with respect to the incidence of PHN was demonstrated regardless of how PHN was defined (i.e. whether pain was present for more than 30, 60, 120, or 182 days after the onset of herpetic rash), with a trend toward greater efficacy for PHN of longer duration (Oxman et al., 2005). Consistent with the observation that the Oka vaccine significantly reduced the pain and discomfort among subjects in whom herpes zoster developed, and the incidence of both AHZ and PHN (Oxman et al., 2005); I have additionally investigated the influence of infection with vOka on stimulus-evoked paw withdrawal responses in the rat. I have hypothesised that infection with the vOka does not result in hypersensitivity phenomena.

In addition to examining the importance of viral strain in the development of persistent zoster-associated hypersensitivity assessed in response to punctate mechanical, noxious thermal and cold stimuli; the relationship between viral inoculum concentration (‘dose’) and mechanical hypersensitivity (‘response’) was also examined. This is important in determining whether the concentration of virus in the inoculum is a critical factor in development of VZV-induced behaviour. Therefore, I hypothesised that VZV-induced mechanical hypersensitivity is related to the viral inoculum concentration. Although it is beyond the scope of this chapter, it should be noted that viral inoculum concentration may not necessarily be the critical factor in VZV-induced hypersensitivity; rather it may be that it is the concentration of infectious virus present in the neurones of sensory ganglia that determines the degree of VZV-induced hypersensitivity, and this would be a subject for future studies (discussed in Chapter 8).

Whilst dynamic mechanical allodynia is a prominent feature in neuropathic pain, this is poorly reflected in current animal models, which focus on reflex withdrawal responses to static punctate mechanical stimuli. From clinical observations in PHN patients, hypersensitivity to dynamic mechanical stimuli (‘brush-evoked’ allodynia), rather than to static punctate mechanical stimuli, is particularly experienced in a large subset of PHN patients (Nurmikko and Bowsher, 1990;
Nurmikko, 1995; Rowbotham and Fields, 1996; Fields et al., 1998; Pappagallo et al., 2000; Chung et al., 2004) and yet this has not been adequately investigated in animal models. For example, Pappagallo and colleagues (2000) reported that 60.3% of individuals (median age 74 years) with pain for longer than 3 months after resolution of cutaneous herpes lesions (median duration of PHN 19 months) had dynamic mechanical allodynia evoked by brush stimulus, compared to only 36.5% of individuals who reported pain after punctate mechanical stimulation (11mN von Frey filament). Moreover, the intensity of ongoing pain was correlated with intensity of allodynia induced by dynamic stimuli in those with PHN of less than 1 year duration, but not in those with PHN of more than 1 year duration. This is consistent with previous findings (Rowbotham and Fields, 1996). Similarly, Nurmikko and Bowsher (1990) reported mechanical allodynia to gentle brushing of the skin in 87% of PHN patients. Moreover, sensory deficits in two or more modalities in the area of PHN were present in 93% of patients (Nurmikko and Bowsher 1990). Since hypersensitivity to dynamic mechanical stimuli has yet to be documented and is poorly reflected in animal models of VZV-associated pain, I have additionally investigated this phenomenon in VZV-infected animals, in parallel with a model of traumatic peripheral nerve injury.

This chapter further characterises the recently developed rat model of zoster-associated hypersensitivity (Fleetwood-Walker et al. 1999) with the aim of providing improved face validity. Ultimately, this will allow greater clinical predictability of efficacy in human randomised controlled trials of PHN.
2.2 Methods

2.2.1 Animal maintenance

Experiments were performed on adult male Wistar rats with a mean weight of 300g (range 240 - 350g) (Harlan, Bicester, U.K.) in accordance with the U.K. Animals (Scientific Procedures) Act 1986 and associated British Home Office regulations (under personal licence number PIL 70/18246 and project licence number PPL 70/5214). Animals were housed in groups of 3 or 4 in individually ventilated colony cages and maintained on a 14:10 hour light/dark cycle with free access to food and water.

2.2.2 Microbiology safety

VZV is classified as a category II pathogen (according to the ‘Advisory Committee on Dangerous Pathogens’ (ACDP) categorisation of biological agents) and as such, all procedures were carried out in a microbiological safety cabinet in accordance with local safety guidelines. Surfaces were disinfected with 1% Virkon and any residue rinsed off with 70% ethanol. A sterile technique was routinely maintained for all procedures.

2.2.3 Viral strains

VZV strains Dumas and Ellen were kindly provided by Prof. R. G. Dalziel, University of Edinburgh, U.K. Dumas is a clinical isolate for which the VZV sequence is known (Davison and Scott 1986). The viral strain was originally isolated from a chicken pox patient in The Netherlands in the late 1970s by A. M. Dumas (Dumas et al. 1981). Since it was the first viral strain to be sequenced, Dumas is considered to be the prototype for European and North American strains of VZV (Davison and Scott 1986; Grose et al. 2004). In comparison, Ellen is a highly passaged standard laboratory strain (passaged more than 100 times since its isolation in 1964) (Moffat et al. 1998). Two further low tissue culture passage viral strains were harvested from an East London cohort of patients participating in a separate study on zoster-associated pain (local ethics committee permission for this study was obtained) (Zarnegar et al. 2005). Viral strains recovered after low passage are indistinguishable from the parent strain, as are viruses transmitted directly from one person to another (Quinlivan et al. 2002). Low passage strains are
therefore thought to be more virulent than highly passaged ones (Quinlivan et al. 2002; Gomi et al. 2002). Both patients were in their late 60s with similar demographics and had suffered from AHZ infection following a brief prodromal illness. However, PHN was known to develop in only one patient (defined as the persistence of pain for more than 3 months after resolution of cutaneous herpetic rash). Patient demographic information and symptomatology is presented in Table 2. The live-attenuated Oka vaccine strain was purchased from GlaxoSmithKline (\(10^{3.3}\) pfu/0.5 ml, batch number A70CA092A). Oka was originally isolated from a child with varicella in Japan in the 1970s and attenuated by multiple passage in cultured cells by M. Takahashi (Gomi et al. 2002). Therefore, although the Oka vaccine strain is an avirulent virus, the parental virus is thought to be virulent in vivo (Gomi et al. 2002). The difference in virulence between the two strains is thought to be due to several base substitutions representing at least eight critical amino acid differences in gene 62, suggesting that IE62 might play an important role in the VZV replicative cycle and in the attenuation of VZV (Gomi et al. 2002).
<table>
<thead>
<tr>
<th></th>
<th>Viral Strain Associated with Development of PHN</th>
<th>Viral Strain Not Associated With Development of PHN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>69 years</td>
<td>67 years</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td>Caucasian</td>
<td>Caucasian</td>
</tr>
<tr>
<td><strong>Prodromal Illness</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Duration:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Symptoms:</strong></td>
<td>24 Hours Pain and abnormal sensations (skin sensitivity and tingling), fever and fatigue</td>
<td>7 Days Pain, fever and fatigue. No abnormal sensations</td>
</tr>
<tr>
<td><strong>Herpes Zoster</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rash site:</strong></td>
<td>Cervical/thoracic distribution C3-T1 (right)</td>
<td>Thoracic distribution T9/10 (right)</td>
</tr>
<tr>
<td><strong>Symptoms:</strong></td>
<td>Constant pain, mechanical allodynia (Visual analogue score (VAS) 5, progressively worsening), numbness, dysesthesiae, ‘burning’ and itch. Complicated by Ramsay Hunt Syndrome</td>
<td>Pain only, specifically ‘sensitivity to touch’ (VAS 2, improving), no abnormal sensations. Some sleep and disturbance in carrying out normal activities of daily living.</td>
</tr>
<tr>
<td><strong>Anti-Viral Treatment</strong></td>
<td>7 Day course of valacyclovir commenced 2 days after onset of rash</td>
<td>7 Day course of acyclovir commenced 2 days after onset of rash</td>
</tr>
<tr>
<td><strong>Analgesic Treatment</strong></td>
<td>Co-proxamol, gabapentin 900mg, Lofepramine 70mg</td>
<td>Co-proxamol</td>
</tr>
<tr>
<td><strong>Progress at 6 Weeks (after onset of herpes zoster)</strong></td>
<td>Rash scarred in patches, constant pain and mechanical allodynia, worsening, ‘burning’ (VAS 5), paraesthesiae, itch, sleep disturbance and unable to carry out normal activities of daily living.</td>
<td>Resolution of symptoms 2 weeks after onset (no rash, pain or abnormal sensations). No complications, therefore no further follow up.</td>
</tr>
<tr>
<td><strong>Progress at 3 Months</strong></td>
<td>Pain (VAS 5) and paraesthesiae, mechanical allodynia, ‘burning’, ‘numbness’ and itch; partial relief with gabapentin 900mg, able to sleep and carry out some activities of daily living</td>
<td></td>
</tr>
<tr>
<td><strong>Progress at 6 Months</strong></td>
<td>Mechanical allodynia (VAS 5), no numbness/ burning, ‘dull’, partial relief with analgesics</td>
<td></td>
</tr>
<tr>
<td><strong>Progress at 12 Months</strong></td>
<td>No pain (VAS 0) but persisting numbness &amp; tingling precipitated by touch. Comfortable on gabapentin 300mg and lophepramine 70mg</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Patient demographic information and symptomatology from whom the low tissue culture passage viral strains were obtained. Symptoms and signs were determined on questionnaire (VAS – visual analogue score) at intervals up to 12 months after onset of herpetic rash.
2.2.4 Preparation and injection of viral inoculum

VZV is a fragile and highly cell-associated virus that must be propagated on fibroblast cell lines. Primary human embryonic lung (Hel) cells (kindly supplied by Prof. J. Breuer, Royal London Hospital, U.K.) were inoculated with VZV and propagated for six to eight passages in cultured Hel cells maintained in Dulbecco's modified minimum essential medium with Earle's salts, supplemented with 10% fetal bovine serum and 1% L-glutamine (all reagents supplied by Gibco Invitrogen Ltd., Paisley, U.K.). VZV-infected fibroblasts were harvested when ~ 80% of cells exhibited cytopathic effects (cpe) on microscopy (equivalent to $10^4$ - $10^5$ plaque forming units). Cpe refers to viral lytic infection and is characterised by the destruction of normal fibroblast cell architecture and by the presence of vacuoles and granules (Fig. 2.1).

Virus-infected cells were gently scraped from the flask surface onto which they had formed a monolayer culture and the cell suspension centrifuged at 1500 rpm, 4°C for 15 minutes. The resulting pellet from each 75cm$^2$ flask was resuspended in 150 µl sterile phosphate buffer solution (Invitrogen). Animals were anaesthetised (pentobarbitone, 40mg/kg i.p. Animalcare Ltd., York, U.K.) and subcutaneously injected with 50 µl viral inoculum into the left (ipsilateral) hind footpad using a 25 gauge needle. Control animals received similar injection of uninfected fibroblast cells (mean count 6-8 x 10$^6$ cells/75cm$^2$ flask).
Figure 2.1 VZV-infected fibroblast cells in culture. A) Early appearance of VZV (strain Dumas)-infected human embryonic lung (Hel) cells 24 hours after inoculation; (i) x40 magnification, (ii) x250 magnification. Fibroblast cell morphology is maintained early on (up to 3–4 days after viral inoculation). Arrow indicates the characteristic linear and uniform appearance of the fibroblasts. B) VZV (strain Dumas)-infected Hel cells at 80% cytopathic effect (cpe); (i) x250 magnification, (ii) x400 magnification. Arrow indicates areas of loss of normal Hel cell morphology with presence of vacuoles and granules (lytic infection).
2.2.5 Reflex withdrawal testing

Prior to assessment, animals were habituated to the testing environment for one hour on two separate days, and similarly acclimatised for at least 15 minutes before each testing session. Environmental stress factors in the laboratory such as strong odours, bright light, noise including ultrasound-emitting devices, and activity by humans that may influence and modify behavioural responses were kept to a minimum (Sales et al. 1999; Chesler et al. 2002a; Chesler et al. 2002b; Khasar et al. 2005). Exposure to stress may strongly influence nociceptive behaviour in animals with the potential to modulate pain sensitivity in either direction (Jorum 1988). Whilst a variety of environmental and/or stressful stimuli may result in 'stress-induced analgesia' (Jorum 1988; Vendruscolo et al. 2004); acute and chronic stresses have also been shown to produce exaggerated responses (i.e. hyperalgesia) in various behavioural tests (Jorum 1988; Imbe et al. 2006). Therefore, animals that are well handled and adapted to the environment in the test situation are more likely to exhibit consistent and reliable patterns of behaviour.

Ambient temperature, air humidity and light levels were routinely recorded at the start of each testing session and efforts were made to maintain these at constant levels (20 - 24°C, 30 - 40%, and 275 - 315 lux respectively) throughout testing. The importance of controlling ambient temperature and relative humidity, particularly with regard to mechanical von Frey filament testing is discussed at the end of this chapter (Andrews 1993; Allmann-Iselin 2000). Efforts were also made to score reflex withdrawal responses at the same time of day (i.e. in the morning), as late-day testing has been found to be associated with increased nociceptive sensitivity (Chesler et al. 2002a; Chesler et al. 2002b). Finally, care was taken to ensure that the testing environment was thoroughly cleaned with 1% trigene solution between animals.

Animals were placed in individual plexiglass observation chambers (dimensions 23 x 18 x 14 cm) with a 0.8 cm diameter plastic mesh floor for testing the response to mechanical and cooling stimuli. A thermo-conductive glass floor was used for testing to noxious thermal stimulation. On a given test day, the sequence of behavioural tests was reflex withdrawal to cooling stimulus, dynamic mechanical, punctate mechanical, and lastly, thermal stimulus. The rationale for this sequence was to prevent the theoretical risk of inducing thermal injury. In addition, animals were allowed to rest for 15 - 20 minutes between testing to different modalities. Hind-limb reflex withdrawal testing was performed by a single experimenter. Three sets of baseline recordings were taken and a mean threshold response was then
calculated. Care was also taken to ensure that the testing environment was thoroughly cleaned with 1% trigene solution between animals.

(a) Static punctate mechanical stimulation. Assessment of static punctate mechanical hypersensitivity was made by determining paw withdrawal response thresholds to mechanical stimuli provided in two ways: 1) A calibrated force transducer with a 0.5mm² diameter tip (electronic “von Frey device” Somedic, type 735) was applied, at a rate of 8 – 15 grams/sec, to the mid-plantar glabrous surface of each hind paw in turn until an active limb withdrawal response was observed. The mean paw withdrawal threshold (PWT) was calculated from a set of five applications, 3 minutes apart (Bridges et al. 2001a). 2) Nylon “von Frey” monofilaments (Alan Ainsworth, London) were applied in ascending order of nominal bending force to the mid-plantar glabrous surface of each hind paw in turn using a method modified from Wallace and colleagues (Wallace et al. 2003). Five applications at a rate of one per second were made for each filament and 3 minutes was allowed before testing the same paw. The PWT was defined as the force required to elicit an active paw withdrawal response from at least three out of five applications and a mean of three readings calculated. Both methods were employed to assess mechanical hypersensitivity because the electronic von Frey device is less established than conventional von Frey filaments. However, the electronic von Frey device is superior in many respects (see discussion).

(b) Dynamic “brush-evoked” mechanical stimulation. Assessment of hypersensitivity to dynamic mechanical stimulus was made by determining the paw withdrawal latency (PWL) to light brushing of the plantar surface of the hind paw as described by Field and colleagues (1999a and 1999b). Care was taken to perform this procedure in fully habituated rats. Briefly, the stimulus (cotton bud) was applied three times to each paw in turn, for up to 15 seconds or until an active paw withdrawal response was observed, often accompanied by flinching or licking of the paw. For baseline PWL, a mean of five readings was calculated. If no reaction was exhibited within 15 seconds, the procedure was terminated and animals were assigned this withdrawal time. Thus, 15 seconds effectively represents no withdrawal. Hypersensitivity to dynamic stimulus was considered to be present for paw withdrawal <8 seconds (Field et al. 1999a;Field et al. 1999b).
(c) **Noxious thermal stimulation.** Response thresholds to a noxious thermal stimulus were assessed using a radiant heat source (Basile plantar test, Ugo Basile, Comerio, Italy) directed onto the mid-plantar glabrous surface of the hind paw, as described by Hargreaves and colleagues (Hargreaves et al. 1988). The latency to withdrawal of the paw from a focused beam of radiant heat at a constant temperature (46 - 49°C) was biologically calibrated to evoke a response latency of 10 seconds in a naive rat. A maximal cut-off latency of 21.4 seconds was used to prevent tissue damage. Sampling was repeated three times to each paw with three minutes between testing. Mean withdrawal latency was then calculated.

(d) **Cool stimulation.** Hypersensitivity to an innocuous cool stimulus was assessed using the acetone drop application technique modified from Carlton and colleagues (Carlton et al. 1994) in which a volume of 0.1 ml acetone was applied to the mid-plantar glabrous surface of each hind paw. A response was taken as positive if the acetone evoked an active paw withdrawal. Each paw was tested 5 times with 3 minutes allowed between each test. A value of percentage positive withdrawal was then calculated.

2.2.6 **Viral strain comparison**

Animals received injection with one of the following strains: Dumas (n = 9); Ellen (n = 6); Oka vaccine (n = 5); viral isolate known to be associated with development of PHN following AHZ infection (n = 6); viral isolate from a zoster patient not associated with development of PHN (n = 6). All animals received subcutaneous (s.c) injection of 50 μl viral inoculum (harvested at approximately 80% cpe) into the left glabrous hind footpad. Control animals received injection of uninfected fibroblast cells (n = 9). Behavioural testing to punctate mechanical, noxious thermal and cooling stimuli was performed at intervals post-infection.

2.2.7 **Duration of VZV-induced mechanical hypersensitivity**

Following infection with VZV (strain Dumas, 80% cpe) as described above, behavioural testing was extended until sensory thresholds had returned to baseline levels. In this way, the nature and duration of VZV-induced hypersensitivity to static punctuate and dynamic mechanical stimuli were determined.
2.2.8 Viral dose-response

VZV (strain Dumas) was harvested at three different cpes (or 'doses'). In order to more accurately determine the quantity of infectious virus at each given dose, plaque titration was performed using a method modified by Grose and Brunel (Grose and Brunel 1978). Briefly, serial dilutions of viral inoculum were propagated onto Vero cells (derived from an African green monkey kidney cell line) and allowed to form plaques. The number of plaque forming units (pfu), characterised by the formation of syncitia with sharp boundaries and glassy appearance, was then determined one week later (Fig. 2.2). This allowed a retrospective estimate of the amount of virus present in the original inoculum (in pfu/ml) to be calculated. Vero cells were used for plaque titration in preference to HeI cells as resulting viral plaques are more clearly demarcated (Ilobi and Martin 1989). Animals were then randomly allocated (n = 6 per group) to receive s.c injection of 50 μl viral inoculum at 15% cpe (3 x 10^2 pfu/ml), 35% cpe (8.6 x 10^3 pfu/ml) or 100% cpe (6 x 10^5 pfu/ml). Response was defined as 'the mean percentage decrease from baseline in ipsilateral PWTs to punctuate mechanical stimulation on day 14 post-infection (time of maximum behavioural change)'. In this way, a dose-response relationship was constructed. Separate animals were used for each dose examined.

2.2.9 Statistical analysis

Power calculation to determine the sample size for each experiment and subsequent statistical analysis was performed using Sigmasat (Jandel Scientific Software version 2.0). The expected difference between means (i.e. control versus experimental) and the expected standard deviations were required in order to determine the power for each experiment. All experiments were of suitable power (0.8 – 0.91). For all intra- and inter-group comparisons, the Kruskal-Wallis one way analysis of variance (ANOVA) on ranks was performed followed by the Tukey test (Dunn's test where sample sizes are unequal) or Dunnett's test where appropriate. The Tukey (or Dunn’s) test was used for pairwise comparisons between ipsilateral and contralateral paws at each time point while Dunnett’s test was used to compare the ipsilateral (or contralateral) response thresholds at each time point back to respective baseline values. Significance level was taken at p<0.05. Variance is expressed as standard error of the mean (sem).
Figure 2.2 Viral plaque assay A) VZV was propagated on Vero cells and titrated to endpoint. Arrow indicates a typical plaque forming unit (x300 magnification); B) Vero control cells (x120 magnification). Cells were stained with 0.1% crystal violet solution for permanence.
2.3 Results

In all virus-infected and control injected animals, the initial erythema and swelling at the site of injection completely resolved by 24 hours post-infection. Furthermore, on general observation, animals did not appear to exhibit obvious signs of non-weight bearing, nor indeed obvious motor impairment ipsilateral to the site of infection throughout the experimental period. Importantly, cutaneous lesions did not develop and phenotypic changes were only evident on formal sensory testing.

Mechanical hypersensitivity is not influenced by viral strain

For laboratory-adapted Dumas and Ellen strains, hypersensitivity to punctate mechanical stimulation developed in all animals in the limb ipsilateral to VZV infection by day 4 post-infection (Fig. 2.3A). Statistical significance was obtained at all time points for PWT comparisons between the ipsilateral paw and mean baseline and between the ipsilateral and contralateral paws within groups. A maximum effect was generally observed from day 14 post-infection (mean percentage decrease from baseline in PWTs was 22.1% ± 1.02 for Dumas-infected rats and 18.2% ± 1.14 for Ellen-infected rats) (Fig.2.3B). Development of mechanical hypersensitivity was similar following infection with both strains, however Dumas was observed to produce a greater degree of behavioural change on days 11 and 21 post-infection. Neither strain demonstrated evidence of hypersensitivity to thermal or cool stimuli (Fig. 2.3C & D). Animals infected with the viral isolate known to be associated with PHN after AHZ developed markedly lowered thresholds in the ipsilateral paw (when compared to either baseline values or the contralateral paw) in response to punctate mechanical stimulation but displayed no change in response to a noxious thermal or cool stimulus (Fig. 2.4A & B). The heightened mechanical sensitivity was apparent at all time points from day 4 post-infection and became maximal from day 14 post-infection: mean ± sem percentage decrease in PWTs was 31.7% ± 1.49 (compared to 22.1% ± 1.02 for Dumas-infected animals) (Fig. 2.4A & B). Following infection with the viral isolate with no known PHN association, mechanical hypersensitivity similarly developed in the paw ipsilateral to VZV infection. The mean percentage decrease from baseline in PWT on day 14 post-infection was 19.1% ± 1.69 (Fig. 2.4A & B). There was no change in response to noxious thermal or cool stimulus (Fig. 2.4C & D). However, animals infected with the live-attenuated Oka vaccine strain did not demonstrate statistically significant hypersensitivity in any sensory modality (Fig. 2.5). Overall, with the exception of the Oka vaccine strain, there appear to be no consistent differences in mechanical hypersensitivity between viral strains.
Figure 2.3 Comparison of hind-limb withdrawal responses in the ipsilateral paw following infection with VZV (strains Dumas (n = 9) -■- and Ellen (n = 6) -▲-) and uninfected fibroblast-injected control (n = 9) -○- animals: A) Paw withdrawal thresholds (PWT) in response to punctate mechanical stimulation (electronic von Frey device); B) Ipsilateral PWT responses expressed as the mean percentage decrease from baseline (*p<0.05 statistical difference between viral strains, one way ANOVA followed by Tukey test); C) Paw withdrawal latency (PWL) to noxious thermal stimulation; D) Paw withdrawal rates to cool stimulation. (For figures A, C and D, +p<0.05 statistical difference between virus-infected and control animals, one way ANOVA followed by Tukey test; *p<0.05 significant difference between response threshold at this time point compared to average pre-infection baseline threshold, one way ANOVA followed by Dunnett's test). Time at which VZV infection was performed (†).
Figure 2.4 Comparison of hind-limb withdrawal responses in the ipsilateral paw following infection with a viral isolate known to be associated with development of PHN following herpes zoster infection — (n = 6), a viral isolate not associated with the development of PHN following herpes zoster infection — (n = 6) and the Dumas strain - - (n = 9): A) Paw withdrawal thresholds (PWT) in response to punctate mechanical stimulation (assessed using the electronic von Frey device) (+p<0.05 significant difference between viral strains and uninfected fibroblast-injected controls; #p<0.05 significant difference between viral isolate associated with, and not associated with PHN development; *p<0.05 significant difference between Dumas and viral strain associated with PHN development (Kruskal-Wallis One Way ANOVA on Ranks, followed where appropriate by Dunn’s test or Student-Newman-Keul’s test); B) Ipsilateral PWT responses expressed as the mean percentage decrease from baseline (*p<0.05 statistical difference between PHN-associated and not associated strains; +p<0.05 statistical difference between PHN-associated strain and Dumas strain; #p<0.05 statistical difference between viral strain not associated with development of PHN and Dumas strain) (one way ANOVA followed by Tukey test); C) Paw withdrawal latency (PWL) to noxious thermal stimulation; D) Paw withdrawal rates to cool stimulation. Time at which VZV infection was performed (1).
Figure 2.5 Time course of the effect of injection of the live-attenuated Oka vaccine strain. Fibroblast cells were inoculated with $10^{3.3}$ pfu/0.5 ml Oka and harvested at 80% cytopathic effect (cpe). Reflex withdrawal thresholds are illustrated in response to A) punctate mechanical stimulation (assessed using the electronic von Frey device); B) noxious thermal stimulation and C) cool stimulation. Hypersensitivity in any sensory modality was not observed. Time at which VZV infection was performed (↑).
Chronic resolving nature of VZV-induced mechanical hypersensitivity

In all animals, significant hypersensitivity to punctuate mechanical stimulation was observed (when compared to mean baseline and contralateral limb) at all time points up to day 49 post-infection, after which time sensory thresholds returned to baseline values. Behavioural change developed progressively from injection until day 35 post-infection when the decrease was maximal (53.9% ± 4.9 from mean baseline). From this time behavioural change, though significant, was reduced until complete resolution of hypersensitivity was observed on day 53 post-infection (Fig. 2.6A).

In contrast, hypersensitivity to dynamic mechanical stimulus followed a slower course of development with change evident from day 14 post-infection (becoming significantly different to the contralateral limb on day 28 post-infection). Resolution of hypersensitivity to dynamic mechanical stimulus (defined as PWL >8 seconds) occurred on day 42 post-infection, although it was not until day 49 post-infection that PWL had returned to baseline values. Hypersensitivity to dynamic mechanical stimulus was also observed in the contralateral limb on day 14 post-infection (Fig. 2.6B).
Figure 2.6 Nature and duration of VZV-induced mechanical hypersensitivity as assessed using A) punctuate mechanical stimulus (electronic Von Frey device) and B) dynamic mechanical stimulus (cotton bud). Animals (n = 3; power calculation 0.81) were infected with VZV (Dumas strain) on day 0 and behavioural testing performed at intervals until sensory thresholds returned to baseline values. The dotted line in B) represents the threshold for hypersensitivity i.e. PWL < 8 seconds is consistent with dynamic mechanical hypersensitivity as described in the method by Field et al., (1999a and 1999b). +p<0.05 statistical difference in threshold response between ipsilateral and contralateral hind paws (one way ANOVA followed by Tukey test); *p<0.05 significant difference between response threshold at this time point compared to average pre-infection baseline threshold (one way ANOVA followed by Dunnett's test).
Mechanical hypersensitivity is influenced by concentration of viral inoculum

The mean percentage decreases from baseline in ipsilateral PWTs on day 14 post-infection, as measured using a) the electronic von Frey device, were 1.2% (± 1.1) at 15% cpe; 17.5% (± 1.6) at 35% cpe and 30.3% (± 1.6) at 100% cpe; and b) graded von Frey filaments, were 5% (± 1.7) at 15% cpe; 42.4% (± 1.9) at 35% cpe and 64.6% (± 3.6) at 100% cpe. Dose-response relationship approximated to a hyperbola (Fig. 2.7) while log-dose response was linear within this range. Thus, VZV infection of rats is associated with a mechanical hypersensitivity that is sensitive to the concentration of virus injected.

Figure 2.7 Viral dose-response relationship. Dose was defined as percentage cytopathic effect (cpe). Virus-infected fibroblasts were harvested at either 15%, 35% or 100% cpe. Response was defined as ‘the mean (± sem) percentage decrease (from baseline) in mechanical ipsilateral PWTs on day 14 post-infection’ and was assessed using both the electronic von Frey device (— ■ —) and standard von Frey filaments (— ■ —).
Discussion

This study refines a previously described rodent model of VZV infection (Fleetwood-Walker et al., 1999) and specifically investigates behavioural response to different viral strains, relationship between viral inoculum concentration and neuropathic pain behaviour and the nature and duration of VZV-induced hypersensitivity to static punctate and dynamic mechanical stimuli.

In this study, hypersensitivity to punctate mechanical stimulation (suggestive of static mechanical allodynia) developed in a dose-related fashion ipsilateral to VZV infection; and (with the exception of the vOka), was not significantly influenced by viral strain. Any difference between wildtype clinical isolates may relate to differences in host immunity, rather than to virus specific factors. Overall, this provides evidence that viral strain is not an important factor in development of persistent zoster-associated pain. However, since it has been proposed that different ethnic groups may be more susceptible to infection with particular viral strains (Quinlivan et al., 2002), it would be of interest to examine the influence of animal strain on VZV infection in future studies. In animals that demonstrated statistically significant (p<0.05) mechanical hypersensitivity, (e.g. 84.3% of all animals infected in the open field pharmacological sensitivity study, Chapter 4, Fig. 4.8), a 32.9% reduction, from baseline ipsilateral PWTs was observed on day 14 post-infection (Chapter 4, Fig. 4.7). The magnitude of effect at this time is not dissimilar to that seen in other pain models. Using the same behavioural testing protocol, I demonstrated a 41.7% reduction in ipsilateral PWT in a partial peripheral nerve-ligation model (PSNL) (Fig. 4.2) and a 51.5% reduction in a model of more severe traumatic peripheral neuropathy L5 spinal nerve transection (SNT) (Fig. 4.6). However, in contrast to these models, in which hypersensitivity to noxious thermal and cold stimuli were evident (data not shown), I observed no hypersensitivity in response to noxious thermal stimulation, and report for the first time an absence of hypersensitivity to cold stimulation following infection with any of the viral strains examined. Indeed in some animals, it appears that there may be a trend towards longer PWL over time, possibly reflecting a thermal sensory loss. Overall, the nature of the model, specifically with regard to the latency to onset, peak effect and decline of VZV-induced mechanical hypersensitivity, and the magnitude of the effect, is in general concordant with previous studies (Fleetwood-Walker et al. 1999;Dalziel et al. 2004). However, my findings are in contrast to reports of thermal hyperalgesia following infection with VZV (Fleetwood-Walker et al. 1999;Garry et al. 2005). This dissimilarity is likely to be
due to experimental differences, particularly in viral inoculum concentration. For example, in the original study by Fleetwood-Walker and colleagues (1999), the thermal hyperalgesia effect was very small especially when compared to the magnitude of mechanical hypersensitivity, and particularly when the y-axis scale is considered. In the study by Garry and colleagues (2005), a significant thermal hyperalgesia was only seen at very high viral concentrations in excess of those used in our study. Again, the effect size is small when compared to the magnitude of mechanical hypersensitivity. Importantly, my findings of a lack of thermal and cold hypersensitivity appear to be consistent with clinical observations in PHN patients. For example, (Pappagallo and colleagues (2000) reported significant deficits in the detection of warmth and cool sensation (hypoesthesia) and pain induced by noxious heat and cold stimuli (hypoalgesia) in a cohort of 63 PHN patients as a whole (median duration PHN 19 months; median age 74 years) rather than hyperalgesia. Indeed, in only a small subset of these patients (28.5%), heat hyperalgesia (defined by a greater than 1°C decrease in mean threshold between the affected and unaffected sides) to heat stimulation was reported. Similarly, only 20.6% of patients reported hyperalgesia to cold stimulation; and only 9.5% exhibited decreased thresholds for both heat and cold pain (i.e. heat and cold hyperalgesia) (Pappagallo et al. 2000). In a similar study, 65.7% of PHN patients (defined as pain persisting for greater than 1 month after healing of cutaneous herpetic lesions; mean duration 48 months; mean age 75 years) reported heat hypoalgesia (Rowbotham and Fields, 1996). In addition, the magnitude of the heat pain sensory deficit observed in these patients as a group was inversely correlated with both pain intensity and severity of allodynia (Rowbotham and Fields, 1996).

In established PHN, the clinical phenotype may vary between individual patients and over the time course of the disease. A spectrum of abnormal sensory phenomena have been documented, with hyper-sensory phenomena, such as allodynia and hyperalgesia, at one extreme and a predominantly hyposensory picture with pain in the context of partial or complete sensory loss at the other (Fields et al., 1998; Pappagallo et al., 2000). The findings of VZV-induced dynamic mechanical hypersensitivity in combination with thermal insensitivity in this study further support clinical observations in the field and provide the model with a degree of face validity (Nurmikko, 1995; Rowbotham and Fields, 1996; Fields et al., 1998; Pappagallo et al., 2000). For example, Pappagallo and colleagues (2000) reported that in 52% of PHN patients, mechanical allodynia was associated with normal or elevated thresholds to thermal stimuli, compared to only 22% of patients who reported allodynia to cutaneous mechanical stimuli and thermal hyperalgesia. However, whilst
dynamic mechanical allodynia is a prominent feature in neuropathic pain, it is poorly reflected in current animal models, which focus on response to static punctate mechanical stimulation. In parallel with the zoster-associated pain model, I have provided additional evidence using a well described model of traumatic peripheral neuropathy in which hypersensitivity to dynamic mechanical stimulus (cotton bud) was observed in the ipsilateral paw of L5 spinal nerve transected animals 14 days post-surgery (Fig. 2.8).

Similarly, Field and colleagues (1999a and 1999b) demonstrated a time course of both dynamic and static components of mechanical allodynia in rat models of traumatic peripheral nerve injury (sciatic nerve chronic constriction injury and spinal nerve ligation), and streptozocin-induced neuropathy. Consistent with observations in the VZV model (Fig. 2.6), dynamic alldynia was reported to follow a slower course of development compared to static alldynia (Field et al., 1999a and 1999b). The contralateral changes in threshold observed in my study may reflect an underlying viraemia. Although somewhat controversial, it has been proposed that following peripheral nerve injury, axotomised A-fibre afferents sprout into previously uninnervated areas of the spinal cord leading to a reorganisation in the CNS (Woolf et al., 1992; Shortland et al., 1997; Bao et al., 2002). The difference in the onset of dynamic, compared to static mechanical hypersensitivity in the VZV model may reflect differences in sprouting of Aδ- and Aβ- fibres in the spinal cord, and would be an interesting aspect for future study. The authors further demonstrated that morphine (1 - 3 mg/kg, s.c.) dose-dependently blocked static, but not dynamic mechanical sensitisation (Field et al., 1999a). Since it has been shown that morphine can block small (C- and Aδ-) but not large diameter (Aβ-) fibre evoked responses into the spinal cord dorsal horn (Dickenson and Sullivan 1986), Field and colleagues (1999a) suggest that nerve-injury induced static allodynia is signalled by high threshold nociceptive afferents, and that dynamic allodynia is mediated by Aβ-fibres. Further support for this is provided by clinical studies showing that selective blockade of Aβ-fibres, induced by compression-ischemia, totally abolishes the dynamic, but not static allodynia in both neuropathic pain patients (Ochoa and Yarnitsky 1993) and following experimental tissue damage (Koltzenburg et al. 1992).
Figure 2.8 Dynamic mechanical hypersensitivity 14 days after A) spinal nerve transection (SNT); and B) VZV infection. Hypersensitivity to dynamic mechanical stimulus was assessed by determining the paw withdrawal latency (PWL) to light brushing of the plantar surface of the hind paw. Briefly, a cotton bud stimulus was applied for up to 15 seconds or until an active paw withdrawal response was observed. Hypersensitivity to dynamic stimulus was considered to be present for paw withdrawal <8 seconds (Field et al., 1999a and 1999b).

A) In SNT-injured animals (n = 12), there was a significant decrease in ipsilateral PWL (5.1 ± 1.0 seconds) when compared to baseline values (11.6 ± 0.8 seconds) (*p<0.05, paired t test) and sham-operated animals (n = 12) (13.2 ± 0.5 seconds) (*p<0.05, t test). There is no evidence for a contralateral change in PWL. B) In VZV (Dumas)-infected animals (n = 12), there was a significant decrease in ipsilateral PWL (6.4 ± 0.6 seconds) when compared to baseline values (13.1 ± 0.4 seconds) (*p<0.05, paired t test). Although a significant contralateral decrease in PWL was observed (8.9 ± 0.7 seconds) compared to baseline, this is greater than the 8 second threshold for dynamic allodynia described by Field and colleagues (1999a and 1999b).
Vaccination with the live-attenuated Oka strain of varicella has been found to markedly decrease the morbidity associated with AHZ and the incidence of PHN among immunocompetent adults aged sixty years or older (Oxman et al. 2005). In a recent randomised, double-blind, placebo-controlled trial, Oxman and colleagues (2005) specifically demonstrated 61.1% reduction in the burden of illness due to herpes zoster, 51.3% reduction in the incidence of herpes zoster, and 66.5% reduction in the incidence of PHN. Significant efficacy with respect to the incidence of PHN was demonstrated regardless of how PHN was defined (i.e. whether pain was present for more than 30, 60, 120, or 182 days after the onset of herpetic rash), with a trend toward greater efficacy for PHN of longer duration (Oxman et al., 2005). The vaccine also significantly reduced the pain and discomfort among subjects in whom herpes zoster developed, consistent with the lack of reflex withdrawal behaviour in animals following infection with the Oka viral strain in this study (Fig. 2.5). Importantly however, with the introduction of varicella vaccination programmes in children, the currently aging population will no longer be exposed to subclinical viral infection with consequent boosting of VZV-specific cell-mediated immunity, thought to protect against herpes zoster and hence PHN (Oxman et al. 2005; Dworkin et al. 2006). This is consistent with the observation that adults who have greater contacts with children in their daily lives have a decreased incidence of AHZ (Arvin et al. 1983; Brisson et al. 2002; Thomas et al. 2002). Subclinical boosting of the immune response suggests that exposure to viral antigens may be important for maintaining cell-mediated immunity against VZV and with the introduction of childhood varicella vaccination programmes, epidemiological models suggest that a significant increase in AHZ will initially occur before the anticipated decline is eventually seen as a result of reduced opportunities for subclinical boosting (Dworkin et al., 2006). The incidence in disease is expected to peak approximately 20 years after childhood vaccination, returning to pre-vaccination levels after 40 years before a significant decline in disease is then seen (Dworkin et al., 2006). However, this increase may be offset by adult vaccination, which has recently been shown to reduce the incidence of AHZ and PHN by 51.3% and 66.5% respectively (Oxman et al. 2005). Furthermore, it appears that U.K. strains of VZV are more similar to U.S. than to Japanese strains, (Hawrami and Breuer 1997; Quinlivan et al. 2002) and since vOka is derived from a wild-type Japanese strain (notably, the vaccine preparation used is actually a mixture of related strains which are mutated at several loci compared with pOka from which it derived), this could have implications on protective immunity from other more prevalent U.K. strains of VZV.
2.4.1 Study limitations and suggestions for improvement

**Rodent VZV infection**

A major challenge in establishing a reliable rat model of herpes zoster-associated pain is that VZV is a highly species-specific human herpesvirus and therefore not a pathogen in rodents. Infected animals do not develop varicella, nor do they develop herpetic lesions, thus the model fundamentally does not share the same disease pathogenesis as in humans, and therefore, cannot be claimed to be a model of PHN *per se* (discussed in Chapter 8). An important limitation is that the virus also remains highly cell-associated, restricted to only a few fibroblast cell lines (Kennedy 2002a). Therefore, it is difficult to obtain high titres (>10^5 pfu/ml) of virus comparable to those obtained by Garry and colleagues (2005) in culture (Kinchington 1999). Additionally, this approach is time-consuming and inefficient. A further limitation in this animal model is a relative lack of standardisation in the precise measurement of the concentration of virus injected into each animal.Whilst cpe is a standard and widely used microbiological technique that quantifies the destruction of normal fibroblast cell architecture caused by viral lytic infection, and was the method employed in the original study by Fleetwood-Walker et al., (1999), it has an element of subjectivity and provides only an indirect indication of the concentration of infectious virus in the inoculum. To refine this, viral plaque assay was additionally performed in some experiments. Although this method provides a more objective measure, it is extremely time-consuming and importantly provides only a retrospective estimate of viral concentration, as the actual number of infectious viral particles per plaque is unknown. Ideally, the exact concentration of infectious virus at the time of injection should be known, and should be consistent among animals; although a more important measure may actually be the number of virions in each DRG neurone at specific time points post-infection. In this respect, the study by Garry and colleagues (2005) is superior i.e. viral titre and percentage infectious cells was determined prior to animal inoculation: 500 μl of VZV-infected fibroblast cells were thawed from vapour phase liquid nitrogen and diluted in saline 1:10 before being centrifuged for 5 minutes at 1000×g. The virus-cell pellets were then resuspended in saline to display titres of 3.2×10^7 plaque-forming cells/ml (64% infectious cells).

Following viral infection, formal assessment to confirm resolution of initial erythema and swelling at the site of injection; and to determine the presence of any motor deficits were not performed. Measurement of dorsal-plantar paw thickness using a vernier caliper could be used as an index of oedema. Similarly, laser Doppler could be used to formally assess paw
erythema. Any deficit in motor function could be assessed on Rotarod performance tests (Holmes et al. 2001) or ‘catwalk’ gait analysis (Vrinten and Hamers 2003)

**Limitation of assessment of hypersensitivity to static punctate mechanical stimulation using the electronic von Frey device and conventional mechanical von Frey filaments**

Whilst mechanical von Frey filaments are conventionally used to assess static punctate mechanical hypersensitivity in experimental animals and in humans (Bove 2006), several important factors suggest that the electronic (Somedic, type 735) von Frey device, although less well established in the literature, may be superior in determining sensory thresholds. A typical series of von Frey filaments comprises twenty flexible nylon monofilaments of standard length arranged in ascending order of exponentially increasing stiffness, nominal bending force, and diameter (filaments are made according to a logarithmic scale of the weight in grams required for the filament to bend when it is applied perpendicularly onto a surface). Filaments are applied sequentially to the mid-plantar glabrous surface of the hind-paw and the nominal bending force to elicit active limb withdrawal is determined. However, this approach has several disadvantages. Firstly, the variability of filament bending force with environmental conditions, specifically changes in temperature and relative humidity are known to affect calibration of von Frey filaments, and hence reliability of testing. The force required to bend a filament is reduced by high temperature and humidity (i.e. increased nociceptive sensitivity) (Andrews 1993; Chesler et al. 2002a; Chesler et al. 2002b). Despite efforts to maintain the ambient temperature and relative humidity at constant levels in the laboratory, there was still significant variation during testing (20 - 24°C and 30 - 40% respectively), which may have influenced accuracy of the data. In contrast, the electronic von Frey device, by nature of the highly sensitive non-flexible metal transducer tip, is not influenced by such changes in environmental conditions, thereby allowing more consistent data. Additionally, the electronic von Frey device is required to be routinely calibrated at the start of each testing session (a two point calibration at 0g and 20g weights was performed) and is accurate to 0.01 mg. However, calibration of the von Frey series (accurate to 1 mg using an analytical balance) was performed less often and ideally should have been performed whenever changes in the operating environment were noted (Andrews 1993).

Second, force delivery with von Frey filaments is incremental and requires multiple applications as opposed to a single continuous application with the electronic von Frey device. Interestingly, it has been shown that the force required to bend a filament varies between successive depressions (maximum coefficient of variation, 16%) (Andrews 1993) which highlights the crudeness of conventional nylon filaments and may have implications
on testing protocols, particularly those that employ multiple applications e.g. Wallace and colleagues (2003) in which each von Frey filament is applied ten times at a rate of one per second; or Carlton and colleagues (1994) in which each trial of five applications <2 seconds apart is repeated five times at approximately 15 second intervals. Multiple applications and repeated testing may also lead to habituation (Greenspan and McGillis 1994), or alternatively, “wind-up” phenomenon, with the possibility of falsely lowered paw withdrawal thresholds (PWTs). (“Wind-up” is characterised by a progressive increase in the response to repeated low-frequency stimuli (Mendell 1966) and may contribute to hypersensitivity in humans. However, the exact relationship of the relatively short-lived phenomenon of wind-up and the persistent state of central sensitisation remains to be fully elucidated (Woolf 1996)). To minimise the risk of this occurring, 3 minutes was allowed before testing the same hind-paw, and 15 – 20 minutes was allowed before testing to different modalities.

Third, a typical series of von Frey monofilaments is arranged in ascending order of diameter (0.08 – 1 mm) of the nylon filament. The graded tip diameter, and hence cross-sectional area means that a variable stimulus area is presented (Greenspan and McGillis 1994; Bove 2006). In addition, tip geometry may lead to unpredictable force generation, particularly if the tip has a square-cut end (Bove, 2006). When the filament bends, the square tip tilts, resulting in the presentation of an edge, the shape and area of which varies with degree of bending (Bove, 2006). Thus, not only does tip geometry change during stimulus application, but so does the applied pressure. Furthermore, compliance of the tissue being stimulated may dictate the tip area (Bove, 2006). These factors ultimately decrease sensitivity of testing. An advantage of the electronic force transducer is that it has a stable probe of fixed diameter, which is important because for a given applied force, the threshold is lower for probes of smaller diameter (Greenspan and McGillis 1994). Its use furthermore allows standardisation of measurement of PWTs, recording of experiments with real time data collection using the computer software program, AcqKnowledge (v3.02), and subsequent analysis.

A further important disadvantage to conventional von Frey filaments is the potential for observer fatigue and/or animal habituation to the stimulus and a protracted period of testing compared to the electronic device. Further, there appears to be no accepted standard protocol for application of von Frey filaments despite being widely used (Dixon 1980; Chaplan et al. 1994; Fleetwood-Walker et al. 1999; Suzuki et al. 2001; Wallace et al. 2003; Garry et al. 2005; Suzuki et al. 2006). Inconsistency in the number of stimuli for each filament; the pattern of application; and the means of analysis, whether 50% threshold interpolation,
percentage withdrawal to 1 or 2 filaments, or other derived method (discussed below) may vary considerably between studies. In addition, the units of expression may vary (i.e. grams, Newtons, or Pascals). These differences in protocol design mean that it is not possible to directly compare data between different studies. For example, Chaplan and colleagues (1994) employed a series of eight von Frey filaments (Stoelting) with logarithmically increasing stiffness from 0.41 – 15.1 g (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g). For each rat the percentage response at each stimulus intensity was first characterised i.e. von Frey filaments were applied in ascending order of strength to the plantar surface of the hind paw with enough force to cause slight buckling and held for 6 – 8 seconds. (Although the force exerted by the tip of a bent filament is reported to be maintained throughout a relatively long bending excursion (Bove 2006), differences in duration of stimulus may imply testing of different sensory nuances). Each filament was presented ten times at intervals of several seconds, and the number of positive (i.e. active limb withdrawals) responses multiplied by ten. This was repeated either until the maximum stimulus was reached (15.1 g) or until a filament strength was reached that caused 100% response). The 50% withdrawal threshold was then determined using the ‘up down’ method of Dixon (1980). Starting with the filament in the middle of the series (2.0 g), Chaplan and colleagues applied the stimulus to the plantar surface of the hind paw as described above. In the absence of a paw withdrawal response to the initially selected filament, the next stronger stimulus was applied; in the event of paw withdrawal, the next weaker stimulus was selected (Chaplan et al., 1994). (In contrast, Carlton and colleagues (1994) randomised the presentation of filaments in their testing protocol). Stimuli were presented in an ‘up down’ fashion oscillating around the response threshold. According to Dixon (1980), optimal threshold calculation by this method requires six responses in the immediate vicinity of the 50% threshold. The 50% response threshold was then calculated using the formula: 50% threshold (g) = (10\(X_f + \delta\))/10 000, where \(X_f\) = value (in log units) of the final von Frey filament used; \(k\) = tabular value for the pattern of positive/negative responses; and \(\delta\) = mean difference (in log units) between stimuli (Chaplan et al., 1994). A disadvantage of this method is that it is highly complex and time-consuming. Fleetwood-Walker and colleagues (1999) assessed hindpaw withdrawal in response to graded mechanical von Frey stimulation in VZV-infected animals using a calibrated set of von Frey nylon filaments (1.5 – 125.9 g) using a modified Chaplan method. The mean withdrawal threshold response (g/unit area) causing two or more reflex paw withdrawals over ten applications of graded filaments was determined. Each stimulus was applied at 1 – 2 second intervals to and repeated three times with a 5 minute interval between tests. Finally, a mean von Frey filament score (1 – 10) representing the
mean withdrawal threshold was plotted against time (Fleetwood-Walker et al., 1999). In contrast, Wallace and colleagues (2003) determined the threshold withdrawal response using a modified Chaplan method, in which the threshold response (force per unit area mN/mm²) was defined by the filament that caused paw withdrawal at least five times in every ten applications (Wallace et al. 2003). The number of positive withdrawal responses is a somewhat arbitrary standard; however, this difference in protocol is important since the measured response threshold is likely to be lower if only two positive responses (Fleetwood-Walker et al., 1999) are elicited, compared to five (Wallace et al., 2003). As a compromise, and since it has additionally been shown that the force required to bend a filament varies between successive depressions (Andrews 1993), I used a protocol in which the threshold response was determined by the lowest nominal filament bending force that elicited active limb withdrawal in at least three out of five applications. However, a potential limitation in my study is that an average PWT (g) was derived by calculating the mean of three threshold responses and since von Frey filaments increase exponentially in nominal bending force, it may be argued that the mean is an inaccurate measure of the actual PWT. Perhaps, the median or mode would be more accurate in this respect (Pappagallo et al., 2000). In addition, the baseline PWTs (g) determined in response to graded mechanical stimuli with calibrated von Frey filaments (Stoelting, Wood Dale, IL, USA) in the study by Garry and colleagues (2005) is dramatically different to those observed in my study i.e. approximately 120 g compared to approximately 40 g (see Chapter 7). In addition to differences in experimental protocol (i.e. the number of stimuli for each filament and the pattern of application), this may reflect differences in a) von Frey filament used (Stoelting versus Alan Ainsworth) since it is reported that von Frey filaments from different sources do not produce identical forces as designed (Bove, 2006); b) calibration and environmental testing conditions (Andrews 1993; Chesler et al. 2002a; Chesler et al. 2002b); c) experimenter variation (Chesler et al. 2002b); or indeed in d) source of animal (Charles River, Wistar versus B&K, Wistar), since we have observed notable differences in baseline PWTs between Harlan, Wistar and B&K, Wistar animals (Chapters 3 and 7).

Finally, for consistent and reliable data, proper application of the von Frey stimulus, whether applied using the electronic device or conventional mechanical von Frey filaments, is required. Specifically, the probe tips must be held perpendicular to the skin surface. If not, the nature of the presenting stimulus may change. It may be argued that the electronic von Frey device is more operator-dependent than conventional filaments since it requires a constant rate of application. However, this in itself has the advantage over using individual
filaments with discrete force levels to evaluate a sensory continuum which can only approximate thresholds at best (Bove, 2006). An important consideration in using the force transducer is the rate of change of the force applied. Too fast an application will lead to inaccurate response times as the pressure will increase beyond the threshold level whilst the animal is in the process of withdrawal. If the rate is too slow, there is potential for interference from other stimuli and spontaneous movements. However, a major advantage of being electronic is that it allows simultaneous real-time display of force applied against time during testing, and so aids learning. Additionally, values obtained from the force transducer are actual withdrawal thresholds, rather than derived (Chaplan et al. 1994). Additionally, a previous study has shown that inter-observer reliability is high with the potential for comparing results from different investigators and laboratories (Moller et al. 1998). In contrast, I observed considerable inter-operator variability with use of the conventional von Frey filaments, such that mean baseline values obtained by experimenter T. P. were 40 – 50 g compared to 15 – 30 g with experimenter F. H. (Chapter 7, Fig. 7.3). Therefore, the electronic von Frey device represents an efficient superior alternative to conventional von Frey filaments. The importance of experimenter variability in assessing reflex behavioural response has previously been highlighted; although differences in experimenter age, gender, and experience level were found not to correlate with the observed differences, rather differential animal handling has been proposed to be responsible (Chesler et al. 2002b). In addition, application of mechanical stimuli should be in the mid-plantar region of the hind paw, since it has been observed that area of the hind paw onto which a stimulus is directed, influences evoked threshold responses (e.g. paw withdrawal responses elicited near the heel are higher than in the mid-plantar region) (Chung 2004; Vierck 2006).

**Limitation of assessment of hypersensitivity to dynamic mechanical stimulus**

In this study, assessment of hypersensitivity to dynamic ‘brush-evoked’ mechanical stimulus was made by determining the paw withdrawal latency to light ‘brushing’ of the plantar surface of the hind paw as described by Field and colleagues (1999a and 1999b). Briefly, a cotton bud stimulus was applied three times to each paw in turn, for up to 15 seconds or until an active paw withdrawal response was observed. Although a somewhat arbitrary threshold, hypersensitivity to dynamic stimulus was considered to be present for paw withdrawal <8 seconds as previously described (Field et al., 1999a and 1999b). Since an identical protocol was employed, this allows direct comparison of findings with Field and colleagues (1999a): Following sciatic nerve chronic constriction injury, dynamic mechanical hypersensitivity (i.e. PWL <8 seconds) was exhibited by 40% of animals 14 days post-surgery, compared to
100% of animals by day 7 post-spinal nerve ligation. In comparison, 66.6% of VZV (Dumas)-infected animals (n = 12) exhibited dynamic mechanical hypersensitivity (i.e. PWL <8 seconds) 14 days post-infection, compared to 100% of spinal nerve transected animals 14 days post-surgery (Fig. 2.8).

Whilst it may be argued that a cotton bud is not the most suitable stimulus to elicit 'brush-evoked' dynamic mechanical hypersensitivity (a soft brush perhaps being more suitable), it is generally agreed that “dynamic mechanical allodynia” describes pain elicited by moving gentle tactile stimuli (Gold et al. 2006). Indeed, a cotton wool stimulus is often employed in quantitative sensory testing in humans (Pappagallo et al. 2000; Rolke et al. 2006; Lang et al. 2006). Alternative dynamic mechanical stimuli that have been used in animal and human sensory testing include a camel hair brush (Carlton et al. 1994; Pappagallo et al. 2000), and an electric toothbrush (Nikolajsen et al. 2000).

It is also essential that the animal is well habituated, not just to the testing environment (i.e. until all exploratory and grooming behaviour has ceased, as the animal must be reasonably still for the maximum duration of stimulus application), but also to the presenting stimulus (i.e. active withdrawal must be differentiated from simple ambulation, which is subject to inter-experimenter variation as described above). Therefore, to improve reliability of testing, five sets of baseline readings were initially taken, and a mean latency threshold calculated. A further potential disadvantage lies in the actual application of the stimulus. The mesh floor onto which animals were placed for sensory testing consisted of 0.8 cm diameter holes through which the cotton bud stimulus was applied to the mid-plantar surface of the hind paw. However, this is clearly a restricted area and may have affected quality of the presenting stimulus. Ideally, the brush stimulus should be a single stroke of approximately 1 – 2 cm in length over the skin (Rolke et al. 2006).

**Limitation of noxious thermal stimulus application**

In this study, the Hargreaves device (1988) was employed to assess reflex withdrawal response to a noxious thermal stimulus, provided by a radiant heat source. This has the advantage over assays such as the ‘hot water tail flick’ in that the animal is unrestrained, thereby avoiding the potential to modulate pain sensitivity in either direction (Jorum 1988). In contrast to assays such as the ‘hot plate’, the Hargreaves testing paradigm also uses an automated detection of the behavioral end-point, sensitive to the nearest 0.1 second (Hargreaves et al. 1988). However, there are potential limitations with this method. First, and
despite a maximal cut-off latency of 21.4 seconds, there is still the potential to cause thermal cutaneous damage, which is why this assay was performed last in the sequence of behavioural tests. Second, there exists the potential for a ‘learned’ response i.e. with repeated stimulus application, an animal can learn to withdraw its paw as soon as the radiant thermal stimulus is applied, or a low level of heat is felt. This decreases reliability of testing. To avoid this, at least 3 minutes was allowed before testing the same paw and only three trials out of a maximum of five were performed. Third, the paradigm is susceptible to experimenter bias; particularly with regard to delivery of the stimulus e.g. thresholds vary depending on region of paw onto which the stimulus is directed (Hargreaves et al. 1988; Chung 2004), and evaluation of the behavioural response since reflex withdrawal responses cannot be discriminated subjectively from avoidance responses in this paradigm (Vierck 2006). In an extensive study of reflex withdrawal thresholds using the Hargreaves device, Chesler and colleagues (2002a) found that the strongest determinant of variability was the experimenter. Moreover, neuropathic rats often hold their injured paw in an abnormal position, avoiding contact of the whole paw with the glass surface. This also reduces reliability of testing. Whilst withdrawal latency (seconds) is a sensitive measure of thermal hypersensitivity (Hargreaves et al. 1988), it could be of interest to examine additional behavioural correlates of thermal hypersensitivity such as the duration of hind paw withdrawal, the velocity of the withdrawal reflex, and the presence or absence of licking. For example, in a rat model of carrageenan-induced peripheral inflammation, Hargreaves and colleagues (1988) observed an increase in the duration of hind paw withdrawal, slower withdrawal of the inflamed paw, and reduced licking behaviour compared to the contralateral limb (Hargreaves et al. 1988). These behavioural correlates were additionally detected long after paw withdrawal latency returned to normal, suggesting that they represent complex behaviours resulting from sensory integration from input at different sites in the CNS (Hargreaves et al., 1988). Moreover, they emphasise the value of a multidimensional measurement of behavioural responses to noxious stimuli. An alternative method used for the application of thermal stimuli in electrophysiological studies involves the use of water jets. For example, Suzuki and colleagues (2006) assessed response to graded thermal stimuli at five different temperatures (35, 40, 45, 48, and 50°C) applied for 10 seconds per stimulus. However, this technique is not suitable in conscious animals. Importantly, patients with neuropathic pain more frequently complain of thermal sensory loss or allodynia, rather than thermal hyperalgesia (Nurmikko, 1995; Fields et al., 1998; Pappagallo et al., 2000). Yet the tests used in this and numerous other animal pain studies assess response to noxious thermal stimulus e.g. 49°C hot water tail-flick or radiant beam of
noxious heat (46 - 49°C) provided by the Hargreave's device. Perhaps a more appropriate study in neuropathic animals would be to assess the reflex withdrawal response to normally innocuous thermal stimuli.

**Limitation of cooling stimulus application**

In this study, hypersensitivity to an innocuous cool stimulus (4°C) was assessed using the acetone drop application technique modified from Carlton and colleagues (Carlton et al. 1994) in which a volume of 0.1 ml acetone was applied to the mid-plantar glabrous surface of each hind paw. However, a limitation of this method is that the response threshold is a derived value (percentage positive withdrawal). Additionally, behavioural response may be subject to considerable experimenter bias. In this respect, I was careful to consistently score only active limb withdrawal, rather than simple ambulation away from the stimulus. However, scoring did not take into account qualitative differences in behavioural response, such as licking, flinching or guarding behaviour of the hind paw, which may be of value (Chapter 5). A further potential limitation concerns the actual application of the stimulus, since in effect a drop of acetone is created at the tip of the 1 ml syringe, and great care is necessary to apply only the acetone without touching the hind paw with the tip of the syringe, as this would change the nature of the presenting stimulus. An appropriate control to determine the contribution of the mechanical component of this stimulus would be to assess response to a drop of water placed in the same way. Finally, the exact temperature of the stimulus may in fact be lower than 4°C due to latent heat of evaporation, resulting in the stimulus of both TRPA1 and TRPM8 receptors. An alternative to the acetone drop technique that has previously been used to assess cold allodynia is hind paw withdrawal latency following immersion in a cold water bath (Carlton et al. 1994). However, this would require restraining the animal and would therefore be influenced by handling and stress response.

**Environmental conditions in the laboratory**

In addition to influencing the accuracy of von Frey filaments, changes in ambient temperature and relative humidity can have undesirable effects on animal behavioural responses (Allmann-Iselin 2000). For example, rodent metabolism or activity may be increased or decreased by changes in ambient temperature (Allmann-Iselin 2000). High temperature and air humidity may also influence susceptibility to infectious agents; whilst low relative humidity induces pathological changes in rats e.g. ring tail (a condition characterised by dry skin and annular constrictions that may result in loss of portions of the tail). The ideal temperature and humidity for rat experimentation is reported to be 20 - 24°C.
and 50 - 60% (Allmann-Iselin 2000). Ambient temperature and relative humidity were therefore routinely recorded at the start of each testing session and efforts were made to maintain these at constant levels (20 - 24°C and 30 - 40% respectively) throughout testing, however, it is evident that laboratory humidity was below adequate. Environmental stress factors in the laboratory such as strong odours, bright light, noise including ultrasound-emitting devices, and activity by humans or other animals (notably mice) that may influence and modify behavioural responses were kept to a minimum (Sales et al. 1999; Chesler et al. 2002a; Chesler et al. 2002b; Wallace et al. 2005; Khasar et al. 2005). In addition, care was taken to ensure that the testing environment was thoroughly cleaned with 1% trigene solution between animals since exposure to stress (olfactory cues) may strongly influence nociceptive behaviour in animals with the potential to modulate pain sensitivity in either direction i.e. 'stress-induced analgesia' or hyperalgesia (Jorum 1988). Therefore, animals that are well handled and adapted to the environment in the test situation are more likely to exhibit consistent and reliable patterns of behaviour.

**Importance of blinding and randomisation**

Although methods of experimental bias reduction (i.e. randomisation to minimise selection and allocation bias, and experimenter 'blinding' to minimise observer bias) (http://www.jr2.ox.ac.uk/bandolier/band80/b80-2.html) were employed in subsequent experiments (Chapters 3 and 4); for logistical reasons, randomisation and blinding were not performed during initial viral strain and dose-response experiments. This is an important limitation since non-randomised studies have been shown to yield larger estimates of treatment effects than studies using random allocation (Schulz et al. 1995; Carroll et al. 1996). Remarkably, lack of, or inappropriate randomisation in human clinical trials (i.e. allocation bias), has been shown to exaggerate treatment effect by as much as 40% (Schulz et al. 1995). Similarly, lack of blinding (i.e. observer bias) has been estimated to exaggerate treatment effect by some 17% (Schulz et al. 1995). Adequate randomisation and blinding are therefore important quality standards in studies with pain outcomes. However, the effects of failure to adequately randomise and blind have not been investigated in animal experiments and may have implications on behavioural outcomes in this study.
2.4.2 Summary

In conclusion, this chapter further characterises the recently developed rat model of zoster-associated hypersensitivity (Fleetwood-Walker et al., 1999). VZV infection in rats is characterised by hypersensitivity to dynamic and static punctate mechanical stimuli (sensitive to viral inoculum concentration), but not to noxious thermal, or cold stimuli. With the exception of the live-attenuated Oka vaccine strain, which failed to produce hypersensitivity in any sensory modality, viral strain overall, did not influence VZV-induced mechanical hypersensitivity. I have demonstrated improved face validity of the model, which will allow greater clinical predictability of efficacy in human randomised controlled trials of PHN in the future.
Chapter 3

Pharmacological Sensitivity Testing
3.1 Introduction

Persistent herpes zoster-associated pain is a significant clinical problem and an area of largely unmet therapeutic need (Hempenstall et al. 2005). Whilst there is a strong clinical evidence base supporting the efficacy of certain analgesic therapies in established PHN, most of these therapies suffer from a narrow therapeutic index which ultimately limits their clinical effectiveness (Hempenstall et al. 2005; Rice and Hill 2006). Numbers needed to treat (NNT), i.e. the number of patients needed to treat with a certain drug to obtain one with a defined degree of pain relief is a useful method for examining analgesic efficacy as it allows clinically relevant comparisons between different treatments (McQuay and Moore 1998; Sindrup and Jensen 1999). However, the percentage of pain reduction or improvement that is clinically meaningful in chronic neuropathic pain conditions is controversial (Hempenstall et al. 2005). Whilst most systematic reviews report efficacy in terms of a 50% reduction in pain intensity (i.e. NNT$_{50\%}$) (e.g. Hempenstall et al., 2005), this measure has only been validated for acute pain (McQuay and Moore 1998). It is suggested that even 30% pain relief may be clinically important in chronic pain conditions (Farrar et al. 2001). However, since the calculation of NNT is based on a comparison of active treatment responders to placebo responders, the precise choice of what level of pain relief defines a responder is somewhat sterile.

A recent meta-analysis of the clinical trial literature (Hempenstall et al. 2005) identified the treatments with the strongest evidence base for efficacy (i.e. combined NNT$_{50\%} < 5.0$) in PHN (Fig. 3.1). Of these, the three most effective orally administered treatments were tricyclic antidepressants (TCAs), NNT$_{50\%}$ 2.64 (95% confidence intervals) (2.1 – 3.54); opioids, NNT$_{50\%}$ 2.67 (2.07 – 3.77); and gabapentin, NNT$_{50\%}$ 4.39 (3.34 – 6.07) (Hempenstall et al. 2005). However, only 30% - 50% of patients were able to obtain more than 50% pain relief and often at the cost of dose-limiting adverse effects (Hempenstall et al., 2005). For the above treatments, the combined number needed to harm for all reported side effects (NNH$_{\text{minor}}$) (95% confidence intervals) was 5.67 (3.34 – 8.58); 3.57 (2.16 – 10.23); and 4.07 (3.15 – 5.74) respectively, whilst the combined number needed to harm for side effects that precipitated withdrawal from study (NNH$_{\text{major}}$) was 16.9 (8.85 – 178); 6.29 (4.16 – 12.8); and 12.25 (7.69 – 30.2) respectively (Hempenstall et al. 2005). The small degree of separation between NNT and NNH reflects the narrow therapeutic index of these treatments.
No single treatment has been shown to be completely effective for all patients with PHN and concomitant use of drugs with different mechanisms of action may offer additional benefit to PHN patients i.e. combination therapy may offer improved efficacy at lower doses, with fewer side effects than with the use of one agent alone (Gilron et al. 2005). However, there is only a very limited evidence base of the additive and synergistic effects of combining mechanistically distinct therapies (Hempenstall et al. 2005;Gilron et al. 2005;Gilron and Max 2005), despite some support from preclinical studies that additive interactions may occur for certain drugs (e.g. gabapentin and morphine) (Shimoyama et al. 1997;Matthews and Dickenson 2002). However, with such combination therapies, patient compliance may also be affected. Furthermore, a significant proportion of patients (60%) are reported to remain refractory to all measures (Kinloch and Cox 2005). A further difficulty in clinical management arises from the fact that there is no single pathophysiology that underlies PHN (Rowbotham 1999). This may explain why response to any single intervention is so often inadequate. Therefore, there is a need for the development of effective drugs that are directed at the mechanisms underlying PHN (i.e. mechanism-based treatment), and that provide more predictable efficacy and improved tolerability in all patients (Fields et al., 1998; Rowbotham, 1999). An animal model of persistent zoster-associated pain that demonstrates good predictive validity i.e. is sensitive to effective analgesic drugs and treatments, but fails to respond to ineffective ones (Blackburn-Munro 2004), will help achieve this. It will also provide a pre-clinical screen for novel analgesic compounds that may be directed at specific mechanisms underlying PHN, thereby allowing individual treatment paradigms to be more effective.
In this chapter, I have further characterised a recently developed rat model of zoster-associated hypersensitivity (Fleetwood-Walker et al. 1999) with the aim of providing improved clinical validity. I have specifically investigated the influence of chronic i.p. administration of a range of clinically efficacious analgesic drugs, including the novel cannabinoid (CB) agonist, WIN55,212-2, and the antiviral agent, acyclovir, on VZV-induced mechanical hypersensitivity. The analgesic efficacy of cannabinoids for analgesia in central neuropathic pain associated with multiple sclerosis, although not yet proven for peripheral neuropathic pain, has been shown in a systematic review of randomised controlled trials (Rice et al. 2006; McQuay et al. 2006). The discovery of the 'endocannabinoid system' and advances in CB pharmacology (including the identification of two CB receptors: CB₁ (neurones) and CB₂ (immune cells); and their endogenous ligands), has renewed interest in the therapeutic potential of CB agents and prompted the development of a range of novel CB receptor agonists and antagonists (Rice and Hill 2006). WIN 55,212-2 is a potent synthetic CB receptor agonist that has previously been shown to reverse nerve-injury-induced hypersensitivity in rodent models of neuropathic pain (Herzberg et al. 1997; Bridges et al. 2001a; Costa et al. 2004), hence its influence in a model of zoster-associated pain was investigated. In order to further demonstrate that this model shows good pharmacological sensitivity and hence predictive validity; I have also investigated the influence of acyclovir, since a small clinical study involving patients with established PHN found no analgesic benefit (Surman et al. 1990).
3.2 Methods

3.2.1 Experimental procedure

Fifty-two adult male Wistar rats (Harlan) (Bicester, U.K.) with a mean weight of 300g (range 240 – 350g), were infected with VZV (strain Dumas) (93% mean cpe; $2.4 \times 10^5$ mean pfu/ml) as previously described (Chapter 2). As the Dumas strain was most easily propagated on HEL cells and reliably produced a high titre of virus in culture (Chapter 2) in addition to inducing consistent behavioural change, this strain was used for all experiments. Prior to viral infection, the baseline threshold value for hind-paw withdrawal in response to punctate mechanical stimulation was measured using the electronic von Frey device (Somedic, type 735) and graded von Frey monofilaments (Alan Ainsworth, London) (as previously described in Chapter 2). Animals were re-tested on day 14 post-infection to establish development of mechanical hypersensitivity, and only animals demonstrating significant (paired t-test, $p<0.05$) reduction in ipsilateral PWT compared to baseline values were retained for pharmacological study. Day 14 was the time from which maximal behavioural changes were consistently observed among animals.

Animals were randomly allocated to the following treatment groups ($n = 5 – 7$ per group) i.e. animals and treatment groups were pre-numbered and paired according to two random number tables: morphine (2.5 mg/kg) (Decosterd et al. 2004); amitriptyline (10 mg/kg) (Vissers et al. 2003; Decosterd et al. 2004); gabapentin (30 mg/kg) (Back et al. 2004; Decosterd et al. 2004; LaBuda and Little 2005); (S)-(+) Ibuprofen (20 mg/kg) (Bonabello et al. 2003; Schafers et al. 2004); WIN55,212-2 (2 mg/kg) (Bridges et al. 2001a; LaBuda and Little 2005); acyclovir (50 mg/kg) (Biron et al. 1982; Bruggeman et al. 1987; Dalziel et al. 2004); or vehicle (40% DMSO/saline or saline/sterile water). The investigator conducting behavioural assessment was also ‘blinded’ to the various treatment groups (i.e. drug/vehicle was prepared in identical syringes by a separate investigator). Each drug was given in a twice daily paradigm (08:00 and 18:00) for four consecutive days (18 – 21 post-infection inclusive) and each animal received only one drug. Doses were selected based on the non-sedative effect and analgesic therapeutic index stated in the cited reference studies. All drugs, including vehicle, were obtained from Sigma, while gabapentin was obtained from Pfizer.
3.2.2 Statistical analysis

Statistical analysis was performed using Sigmastat (Jandel Scientific Software, version 2.0). Power calculation to determine the sample size for each experiment was performed as previously described. All experiments were of suitable power (0.85 – 0.9). For all intra- and inter-group comparisons, the Kruskal-Wallis one way analysis of variance (ANOVA) on ranks was performed followed by the Tukey test (Dunn’s test where sample sizes are unequal) or Dunnett’s test where appropriate. The Tukey (or Dunn’s) test was used for pairwise comparisons between ipsilateral and contralateral paws at each time point while Dunnett’s test was used to compare the ipsilateral (or contralateral) response thresholds at each time point to respective baseline values. Similarly, Dunnett’s test was used for ipsilateral paw comparisons using the PWT at day 14 post-infection as the pre-drug reference to which subsequent thresholds were compared. Significance level was taken at \( p < 0.05 \). Variance is expressed as standard error of the mean (sem).
3.3 Results

VZV-induced mechanical hypersensitivity is sensitive to a range of analgesic drugs

On day 14 post-infection, VZV-infected animals (n = 50) displayed hypersensitivity to punctate mechanical stimulation ipsilateral to infection. At this time, the mean percentage decrease from baseline in ipsilateral PWTs was 28.6% ± 1.4 (assessed using the electronic von Frey device), and 62.1% ± 2.2 (assessed using graded von Frey monofilaments). Two animals did not demonstrate statistically significant (p<0.05, paired t-test) mechanical hypersensitivity and were therefore excluded from pharmacological study (Fig. 3.2).

**Figure 3.2** CONSORT-type flow diagram for exclusion
At the end of the four day drug administration period (i.e. day 21 post-infection), ipsilateral PWTs for morphine-, amitriptyline-, gabapentin-, (S)-(+) ibuprofen-, and WIN55,212-2-treated animals had returned to baseline values: mean ipsilateral PWT on day 21 post-infection was 41.0g ± 0.7 (electronic von Frey device) and 10.1g ± 0.3 (von Frey monofilaments). Therefore, with the exception of acyclovir, treatment by all active drugs, but not solvent was associated with a reversal of mechanical withdrawal thresholds to baseline. However, in acyclovir- and vehicle-treated animals, mechanical hypersensitivity was still evident on day 21 post-infection: mean ipsilateral PWTs were 29.4g ± 1.6 (electronic von Frey) and 5.5g ± 0.8 (von Frey monofilaments); and 27.7g ± 2.4 (electronic von Frey) and 4.4g ± 0.5 (von Frey monofilaments) respectively. By day 25 post-infection, PWTs in morphine-, amitriptyline-, gabapentin-, (S)-(+) ibuprofen-, and WIN55,212-2-treated animals had reverted to pre-drug (i.e. day 14 post-infection) values: mean ipsilateral PWT on day 25 post-infection was 28.7g ± 1.0 (as assessed using the electronic von Frey device) and 5.0g ± 0.6 (as assessed using von Frey monofilaments). Therefore, VZV infection of rats is associated with a mechanical hypersensitivity that is sensitive to a range of analgesic drugs, but not the antiviral, acyclovir (Figs. 3.3 and 3.4).
Figure 3.3 Comparison of the effect of vehicle (pooled saline/sterile water, n = 7 or 40% DMSO/saline, n = 6) versus active drug administration on ipsilateral paw withdrawal thresholds (PWTs) in response to punctuate mechanical stimulation as assessed using the electronic von Frey device in VZV-infected animals. Intraperitoneal drug/vehicle administration (arrow) took place twice daily over four consecutive days. Time course of the effect of A) morphine 2.5 mg/kg (n = 6); B) amitriptyline 10 mg/kg (n = 7); C) gabapentin 30 mg/kg (n = 5); D) (S)-(+)-Ibuprofen 20 mg/kg (n = 6); E) WIN55,212-2 2 mg/kg (n = 6) and F) acyclovir 50 mg/kg (n = 7). (+ p<0.05 statistical difference between vehicle-administered and active drug-administered animals, one way ANOVA followed by Tukey test; * p<0.05 significant difference between response threshold at this time point compared to mean pre-infection baseline threshold, one way ANOVA followed by Dunnett's test).
Figure 3.4 Comparison of the effect of vehicle (as above) versus active drug administration on ipsilateral paw withdrawal thresholds (PWTs) in response to punctuate mechanical stimulation as assessed using conventional von Frey filaments in VZV-infected animals. Intraperitoneal drug/vehicle administration (arrow) took place twice daily over four consecutive days. Time course of the effect of A) morphine 2.5 mg/kg (n = 6); B) amitriptyline 10 mg/kg (n = 7); C) gabapentin 30 mg/kg (n = 5); D) (S)-(+)Ibuprofen 20 mg/kg (n = 6); E) WIN55,212-2 2 mg/kg (n = 6) and F) acyclovir 50 mg/kg (n = 7). (+ p<0.05 statistical difference between vehicle-administered and active drug-administered animals, one way ANOVA followed by Tukey test; * p<0.05 significant difference between response threshold at this time point compared to mean pre-infection baseline threshold, one way ANOVA followed by Dunnett’s test).
3.4 Discussion

In this experiment, I have demonstrated that VZV infection of rats is associated with a mechanical hypersensitivity that is sensitive to a range of clinically useful analgesic drugs, and in addition, the novel CB agonist, WIN55,212-2. Specifically, chronic i.p. administration of gabapentin (30 mg/kg), morphine (2.5 mg/kg) and amitriptyline (10 mg/kg), the three most effective analgesic drugs in the treatment of PHN, (Hempenstall et al. 2005) reversed VZV-induced mechanical hypersensitivity, with return of neuropathic behaviour once drug treatment was stopped. This suggests that VZV infection causes persistent changes in sensory thresholds, and that the mechanisms responsible for maintaining VZV-induced mechanical hypersensitivity are sensitive to pharmacological interference. Although onset of effect of all drugs was observed from the first dose and was sustained for the duration of drug administration, I adopted a chronic (over four consecutive days) twice daily dosing regime, rather than a single dose regime, to better reflect the clinical scenario of treatment in chronic pain.

Gabapentin

My data supports the findings of Garry and colleagues (2005). These authors demonstrated a reversal of VZV-induced mechanical and thermal hypersensitivity following acute administration of gabapentin (100 mg/kg by oral gavage). Although the mechanism of action of gabapentin, a 3-alkylated analogue of gamma-aminobutyric acid (GABA), is not fully understood, it is thought not to interact with GABA receptors or GABA metabolism, and has no effect on Na+ channels; rather it has been proposed to act via a mechanism involving the αδ subunit of the voltage-gated Ca2+ channel (Sindrup and Jensen 1999) through which it modulates neurotransmitter release from primary afferent terminals (Fehrenbacher et al., 2003). Indeed, the antinociceptive activities of a range of gabapentin analogues in experimental models of inflammatory pain have been found to correlate well with their potency at binding to the αδ subunit (Field et al. 1997). Although the possibility of a peripheral site of action cannot be excluded (Yang et al., 2005), the analgesic actions of gabapentin appear to be largely centrally mediated (Abdi et al., 1998; Chapman et al., 1998a; Chapman et al., 1998b; Suzuki et al., 2005).

An up-regulation in the expression of the αδ subunit has previously been demonstrated in rodent DRG after both traumatic peripheral nerve injury (Newton et al. 2001; Luo et al. 2002) and VZV infection (Garry et al., 2005), and this shows close correlation with the onset of
pain behaviours. Using Western immunoblotting, Garry and colleagues demonstrated a significant increase in $\alpha_2\delta_1$ subunit expression in VZV-infected animals compared to sham-infected animals. Using a gene microarray approach, I investigated global changes in DRG gene expression associated with VZV infection in the rat (see Chapter 7). Differential gene expression was profiled in parallel with a model of traumatic peripheral nerve injury at a time of maximum behavioural change (day 14 post-infection/surgery) allowing changes common to both pain models to be compared. However, my microarray data revealed no change in $\alpha_2\delta_1$ subunit expression after viral infection. In contrast, the subunit was found to be significantly ($p<0.05$) up-regulated in both L4 and L5 DRG after spinal nerve transection (1.71 and 5.64 fold respectively). Possible reasons for the discrepancy in our data compared to Garry and colleagues (2005) are discussed in Chapter 7.

It is possible that the increased expression of the $\alpha_2\delta_1$ subunit undergoes redistribution to central axons and/or injured primary afferents and participates in the generation and maintenance of spontaneous ectopic discharge, either through altered calcium channels or via an unknown mechanism (Luo et al. 2002). It is likely that gabapentin acts at least in part by blocking the action of presynaptic calcium channels and consequently reducing transmitter release by reducing the current that flows through presynaptic calcium channels (Rice and Hill 2006). The current evidence base supports the use of gabapentinoids as second-line treatment in PHN (Hempenstall et al. 2005). The more potent analogue of gabapentin, pregabalin (S-(+)-3-isobutylGABA), has now been introduced for the treatment of neuropathic pain and has similar efficacy in PHN: NNT $50\%$: 4.93 (3.66 – 7.58) (Hempenstall et al., 2005).

**Morphine**

Morphine exerts most of its analgesic effect at the mu–opioid receptor. The reversal of VZV-induced mechanical hypersensitivity following i.p. administration of morphine (2.5 mg/kg) is consistent with previous studies investigating the effects of systemically and locally administered opioids in various rodent models of neuropathic pain (Hammond 2006). For example, reversal of mechanical and/or thermal hypersensitivity is well documented in models of traumatic peripheral nerve injury (Lee et al. 1995; Truong et al. 2003; Decosterd et al. 2004); and has been reported in a rodent model of streptozocin (a pancreatic islet beta cell toxin)-induced diabetic peripheral neuropathy (Zurek et al. 2001). In contrast, Garry and colleagues (2005), observed no effect on sensory response thresholds following intrathecal (i.t.) administration of the selective mu–opioid receptor agonist, DAMGO (D-Ala$_3$, MePhe$_4$,}
Gly-ol enkephalin) (1 µmol/50 µl saline). Whilst in contrast to my findings, this lack of effect is consistent with previous studies investigating the effect of i.t. administration of opioids, in which action of the drug is limited to the spinal cord. For example, Zurek and colleagues (2001) compared the antinociceptive effect of fentanyl administered via the i.p. and i.t. routes in a model of streptozocin-induced diabetic neuropathy. Interestingly, whilst i.p. fentanyl produced a dose-related antinociceptive effect in neuropathic rats in the electric current, paw pressure and tail flick nociceptive tests; i.t. administration produced little antinociceptive effect in all three tests. Similarly, Lee and colleagues (1995) demonstrated a dose-dependent reduction in traumatic peripheral nerve injury-associated mechanical hypersensitivity by systemic (i.p.) and supraspinal (intracerebroventricular), but not spinal (i.t.), administration of opiates. Thus, it may be that spinal cord opioid systems are ineffective for antinociception following VZV infection, and that supraspinal mu opioid receptors are responsible for the antinociceptive effect of opioids in these animals. This may account for the difference between our study and Garry et al., (2005).

In neuropathic pain, high doses of opioids are often required for effective treatment, narrowing the therapeutic index (Hempenstall et al., 2005). The decreased effectiveness of systemic morphine in neuropathic pain is thought to be caused by loss of opioid potency at peripheral and spinal sites of action (Rashid et al. 2004). Under normal conditions, mu-opioid receptors are mainly expressed on the central terminals of small-diameter unmyelinated DRG neurones. In neuropathic pain states however, the expression of mu-opioid receptors in DRG of peripheral nerve-injured mice has been found to be markedly reduced (Rashid et al. 2004). Thus, this could account for the lower potency of spinal intrathecal morphine in neuropathic pain. Drugs acting at opioid receptors other than the classical mu-receptor may demonstrate efficacy in neuropathic pain. For example, the novel delta-opioid-selective agonist, SB-235,863, has been shown to be effective in animal models of inflammatory and neuropathic pain (Petrillo et al. 2003), though its efficacy in humans has yet to be determined.

Additionally, the microarray data in this study was examined for changes in opioid-related gene expression following VZV infection and SNT (Chapter 7). Whilst I observed no change in gene expression for mu- and delta-opioid receptors in either model, a significant (p<0.05) down-regulation for kappa-opioid and sigma-opioid receptors, opioid binding proteins, and opioid-like receptors, was observed (Table 3).
Table 3 Down-regulation (↓) with fold change of opioid-related genes in DRG following VZV infection and spinal nerve transection (SNT) in the rat.

<table>
<thead>
<tr>
<th>Affymetrix Probe ID</th>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>L4 SNT</th>
<th>L5 SNT</th>
<th>VZV</th>
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<td>Opioid receptor, mu 1</td>
<td>Oprm1</td>
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<tr>
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<td>Oprd1</td>
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<tr>
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<td>↓ 2.71</td>
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<tr>
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<td>Oprs1</td>
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<td>↓ 1.47</td>
<td></td>
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<tr>
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<td>Opioid-binding protein/cell adhesion molecule-like</td>
<td>Opcml</td>
<td>↓ 2.64</td>
<td>↓ 1.52</td>
<td></td>
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<tr>
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<td>Opioid receptor-like</td>
<td>Oprl</td>
<td></td>
<td></td>
<td>↓ 1.54</td>
</tr>
</tbody>
</table>

Tricyclic antidepressants

The current evidence base supports the first-line use of a tricyclic antidepressant (TCA) for orally administered treatment of PHN (Hempenstall et al., 2005). Consistent with efficacy in humans, I have shown that chronic administration of the TCA, amitriptyline (10 mg/kg, i.p.), reverses VZV-induced mechanical hypersensitivity in rodents. However, there is limited evidence demonstrating efficacy in experimental models of neuropathic pain. For example, Decosterd and colleagues (2004) found that amitriptyline (10 mg/kg, i.p.) attenuated stimulus-induced progressive tactile hypersensitivity in the sciatic nerve crush rodent model of neuropathic pain. Progressive tactile hypersensitivity develops months after recovery from nerve crush in response to repeated intermittent low-threshold mechanical stimulation of the reinnervated sciatic nerve skin territory, and represents a model of stimulus-induced pain where external stimuli alter sensory processing to generate pain hypersensitivity-related behaviour that outlasts the initiating stimulus (Decosterd et al. 2004). Similarly, LaBuda and Little (LaBuda and Little 2005) demonstrated only partial reversal of spinal nerve ligation-induced mechanical hypersensitivity following amitriptyline (1.5, 3 and 10 mg/kg, s.c).

It is thought that TCAs interact with monoamine systems (pathways that include mood and emotions as well as pain), and may also modulate opioid systems in the CNS via an indirect serotonergic route (Rice and Hill 2006). However, it is unlikely that the analgesic properties of these agents are solely due to effects on serotonin, as selective serotonin uptake
antagonists (e.g. fluoxetine) appear less useful analgesics than non-selective agents (e.g. amitriptyline). It is probable that the analgesic actions of TCAs require blocking uptake of both serotonin and noradrenaline (Sindrup and Jensen 1999).

**Non-steroidal anti-inflammatory drugs**

Although the clinical efficacy of systemically administered non-steroidal anti-inflammatory-type drugs has not been adequately tested, topical aspirin (NNT$_{50\%}$ 1.83) and topical indomethacin (NNT$_{50\%}$ 5.5) have been shown in a high quality clinical trial to have some clinical efficacy in PHN (Hempenstall et al. 2005). I demonstrated reversal of VZV-induced mechanical hypersensitivity following chronic i.p. administration of the non-selective cyclooxygenase (COX) inhibitor, (S)-(+)-ibuprofen (20 mg/kg) in the rat. This suggests an element of VZV-induced hypersensitivity that may be reversed by COX inhibitors, and is consistent with previous studies in other models of neuropathic pain (Bonabello et al. 2003; Schafers et al. 2004). In contrast, VZV-induced hypersensitivity was not reversed by the acute administration of diclofenac (100 mg/kg by oral gavage) (Garry et al. 2005). This difference is difficult to explain but may reflect differences in potency of the chosen compound, as well as differences in dose, route and duration of drug administration.

**Cannabinoids**

Recent interest has focused on the potential of CBs as novel analgesics in chronic pain (Rice and Hill 2006; Rice et al. 2006). However, the use of CBs in clinical trials of multiple sclerosis pain have revealed efficacy, but they have not been adequately tested in peripheral neuropathic pain. Behavioural and electrophysiological studies in animal models of inflammatory and neuropathic pain have demonstrated potent antinociceptive and antihypersensitivity effects with systemically or spinally administered CBs (Rice 2005). CBs are known to exert such effects through an interaction with at least two distinct receptor subtypes, both coupled to G proteins (involving cyclic adenosine 3',5'-monophosphate (cAMP)-dependent and cAMP-independent mitogen activated protein (MAP)-kinase signaling): CB$_1$ (Matsuda et al., 1990) and CB$_2$ (Munro et al., 1993). CB$_1$ receptors are expressed throughout the pain pathway mainly by neurones of the central and peripheral nervous system (Berdyshev, 2000; Pertwee, 2001). These receptors are specifically located on DRG sensory neurones (Bridges et al. 2003); interneurones of the superficial spinal cord dorsal horn (Farquhar-Smith et al., 2000); and in supraspinal sites (i.e. thalamus, periaqueductal grey, amygdala, and in the rostral ventromedial medulla where they modulate the activity of neurones projecting to nociceptive regions of the spinal cord) (Tsou et al.,
In contrast, CB2 receptors are found exclusively on non-neuronal cells, particularly in immune cells, and are not expressed in the CNS (Berdyshev, 2000). After nerve injury, activity of the endocannabinoid system is increased, and there is evidence to suggest that changes in CB receptors also occur (Berdyshev, 2000; Costa et al., 2004). For example, plasticity of spinal CB1 function (Chapman, 2001), and increased expression of CB2 within the lumbar spinal cord (coinciding with the appearance of activated microglia) (Zhang et al., 2003) after peripheral nerve injury have been reported. Importantly, I observed a significant down-regulation in CB1 receptor gene expression in both L5 and L4 DRG of SNT-operated animals (2.26 and 1.5 fold decrease compared to sham), but no change in expression of the CB2 receptor (Chapter 7). In support of this, Chapman (2001), who observed a loss of inhibitory effect of the CB agonist, HU210, on C-fibre evoked neuronal responses in nerve-injured rats, proposed that CB receptors on C-fibres are functionally down-regulated after peripheral nerve injury, and that an uncoupling of pre-synaptic spinal CB receptors and associated G-proteins may be responsible (Chapman 2001). However, I observed no change in expression of either CB receptor in VZV-infected animals (Chapter 7). This may in part, reflect the limitation of microarrays in detecting small changes in gene expression, or the fact that the L4 and L5 DRG were pooled for VZV animals, thus potentially 'diluting' changes in important genes (discussed in Chapter 7).

There is also the possibility that the antinociceptive and antihyperalgesic effects of CBs may be mediated by receptors other than CB1 and CB2 receptors, for example CB2-like receptors (Pertwee 2001); and in the case of anandamide (prototypical endocannabinoid), transient receptor potential (TRP) channels, specifically TRP vanilloid receptor subunit 1 (TRPV1), which is an ionotropic channel with a key role in both thermal nociception and inflammatory hyperalgesia (Patwardhan et al. 2006).

I examined the efficacy of repeated doses of the experimental CB receptor agonist, WIN55,212-2 (2 mg/kg), in a model of zoster-associated pain and found that it significantly reduced VZV-induced mechanical hypersensitivity (even after i.p. administration of the first dose). My data is consistent with previous studies in rodent models of neuropathic pain in which WIN55,212-2 reversed peripheral nerve-injury induced mechanical and/or thermal hypersensitivity in a dose-related fashion (Herzberg et al. 1997; Bridges et al. 2001a; Wallace et al. 2003; Costa et al. 2004). WIN 55,212-2 is a potent CB receptor agonist that binds to both CB1 and CB2 receptors. Antagonism of its effect by the CB1-selective antagonist, SR141716A, indicates the involvement of the CB1 receptor in traumatic peripheral neuropathy (Fox et al. 2001; Bridges et al. 2001a). (Similar antagonist studies in the VZV...
model would be useful in understanding the mechanisms underlying zoster-associated hypersensitivity). There is good evidence that CBs produce antinociception by acting on central CB1 receptors (Pertwee 2001). Specifically, CBs may be readily antagonised by the CB1-selective antagonist, SR141716A; antisense oligodeoxynucleotides directed against the CB1 receptor; or by drugs expected to impair CB1 receptor signalling (e.g. pertussis toxin) following intracerebroventricular and intrathecal injection, or injection into discrete brain areas (Pertwee 2001). In addition, it has been proposed that an action on CB1 receptors may modulate descending control exerted on spinal nociceptive neurones by the rostral ventromedial medulla (RVM) (Meng et al., 1998). Meng and colleagues investigated the effect of intravenous (i.v.) WIN 55,212-2 on the activity of subpopulations of neurones in the rat RVM projecting to the dorsal horn. These neurones exhibit either a pause in activity (‘off’ cells) i.e. inhibiting spinal nociceptive neurotransmission; or a burst of activity (‘ON’ cells) i.e. facilitating spinal nociceptive neurotransmission, just before the tail is withdrawn in response to a noxious radiant heat stimulus (tail flick test). Both the activity pause of ‘OFF’ neurones and the activity burst of ‘on’ neurones were eliminated by WIN 55,212-2 in anaesthetised rats and this effect was found to be reversed by i.v. injection of SR141716A. Additional evidence that CBs modulate the activity of neurones projecting from brain to spinal cord comes from the suppression of activity (evoked by the application of noxious heat to the hind paw) in wide dynamic range neurones of the lumbar dorsal horn following direct injection of WIN 55,212-2 into the rat cerebral ventricular system (Hohmann et al. 1999). Moreover, it was found that the ability of i.v. WIN 55,212-2 to suppress noxious heat-evoked activity of these neurones could be abolished by spinal transection (a procedure that abolishes descending inhibitory neural input from the brain) (Hohmann and Herkenham 1999). Finally, there is evidence that CBs act on pre-synaptic CB1 receptors on peripheral terminals of primary afferent neurones (Richardson et al., 1998; Hohmann and Herkenham, 1999). Richardson and colleagues (1998) showed that carrageenan-induced thermal hyperalgesia of the rat hind paw could be attenuated by the intraplantar injection of anandamide; and further that this effect was mediated by CB1 receptors located in the hind paw. Similarly, Hohmann and Herkenham demonstrated that cannabinoid receptors undergo anterograde axonal transport from the DRG towards the peripheral terminals of sensory nerves following peripheral nerve injury.

However, the therapeutic potential of CBs is limited by undesirable central effects mediated by CB1 receptors within the brain, particularly a long term increased risk of psychosis and schizophrenia (Rice and Hill 2006; McQuay et al. 2006). Therefore, one approach to avoid such toxicity issues associated with the use of exogenous CBs is to potentiate the action of
endogenous ligands to CB receptors (endocannabinoids) outside the CNS. These are rapidly but transiently produced in neurones by ‘on-demand’ cleavage from membrane phospholipid precursors in response to injury (Piomelli et al. 1998; Pertwee 2001). This might be achieved by developing therapeutic agents from anandamide uptake inhibitors compounds such as AM381 or AM404 (Pertwee, 1999) that activate the endocannabinoid system indirectly by selectively inhibiting the tissue uptake or metabolism of endogenous CBs, and thus increasing their levels at CB receptors (Rice 2005). In addition, inhibitors of fatty acid amide hydrolase (FAAH), a membrane-bound serine hydrolase enzyme that catalyses the hydrolysis of anandamide (itself, a fatty acid amide), may offer some therapeutic potential since FAAH activity is critical to the control of anandamide tissue concentrations. Pharmacological inhibition of this enzyme or its genetic disruption to produce FAAH knockout mice both cause substantial increases in anandamide concentrations measured in rodent brain and spinal cord tissue (Cravatt et al. 2001; Lichtman et al. 2004; Hohmann et al. 2005). FAAH inactivation is reported to decrease responses to acute noxious stimuli (Cravatt et al. 2001; Lichtman et al. 2004) reduce hyperalgesic responses after inflammatory (Lichtman et al. 2004; Cravatt et al. 2004) and neuropathic injury (Chang et al. 2006) and enhance stress-induced analgesia (Hohmann et al. 2005). Another strategy for future drug development in minimising the unwanted central effects of CBs would be to selectively target CB2 or CB2-like receptors; or only those CB1 receptors expressed outside the brain. These drugs should be more selective than direct agonists as they are unlikely to affect all parts of the endocannabinoid system at one time, producing instead effects only at sites where on-going production of endogenous CBs is taking place. A third strategy would be to exploit the synergistic interactions that occur between CBs and opioids for antinociception (Rice 2005). This may reduce the dose, and hence side effect profile of concomitant opioids.

**Acyclovir**

However, administration of the antiviral, acyclovir (50 mg/kg) failed to attenuate VZV-induced mechanical hypersensitivity. This suggests that this model is mirroring aspects of persistent zoster-associated pain rather than AHZ, since whilst antiviral drugs (e.g. valaciclovir and famciclovir) are somewhat effective in decreasing morbidity in AHZ, providing treatment starts early enough after onset of the rash (Coen et al. 2006), a small clinical study involving patients with established PHN found no analgesic benefit from orally administered acyclovir (Surman et al. 1990; Hempenstall et al. 2005). This observation provides the model with some degree of predictive validity, defined as ‘the requirement for an animal model of pain to be sensitive to effective analgesic drugs and treatments (e.g. gabapentin) but fail to respond to ineffective drugs and treatments (i.e. acyclovir)’
(Blackburn-Munro 2004); and hence it appears that the model shows good pharmacological sensitivity. The finding that acyclovir failed to attenuate VZV-induced mechanical hypersensitivity may also provide a mechanistic insight into mechanical hypersensitivity in PHN since it suggests that ongoing viral replication (i.e. lytic infection with subsequent cell destruction) is not necessary for the maintenance of mechanical hypersensitivity. This is further supported by Dalziel et al., (2004) who found that early chronic administration of the antiviral agent valaciclovir (50 mg/kg by oral gavage), had no effect on the development of VZV-induced mechanical hypersensitivity in rodents, suggesting that viral replication is not necessary for the induction of behavioural change following infection with VZV. Importantly however, similar administration of valaciclovir in HSV-1 infected rodents prevented the induction of mechanical hypersensitivity, most likely by inhibiting active HSV-1 replication (Dalziel et al. 2004). These data combined with my observations support the hypothesis that VZV-induced mechanical hypersensitivity is not due to virus replication-induced tissue damage or accompanying inflammation as would be seen during the acute phase of herpes zoster infection. Rather, it has been proposed to be due to an interaction of "resident" VZV infection with the host nervous system (Dalziel et al. 2004).

3.4.1 Summary

Importantly, I have shown that the rat model of zoster-associated pain demonstrates good pharmacological sensitivity, as well as some degree of predictive validity. Further investigation will help determine its true predictive validity allowing it to be used as a preclinical screen for novel analgesic compounds in the future. For example, this study could be extended to examine the influence of additional treatments with proven efficacy in PHN (e.g. capsaicin (NNT<sub>50%</sub> 3.26), an alkaloid derived from chillies that depletes the neurotransmitter substance P from sensory nerves; the sodium channel blocker, lidocaine (NNT<sub>50%</sub> 2.0); and the corticosteroid, methylprednisolone (NNT<sub>50%</sub> 1.13) etc) (Hempenstall et al., 2005). Moreover, the influence of N-Methyl-D-Aspartate (NMDA) receptor antagonists (e.g. memantine, MK 801 etc), which are proposed to block the hyperexcitability following activation of spinal NMDA receptors associated with C-nociceptor sensitisation seen in a subset of PHN patients ('irritable nociceptor group'), could be investigated in this model. This would be interesting as although NMDA receptor antagonists are reported to have poor efficacy in PHN (NNT<sub>50%</sub> 23.86) (Hempenstall et al. 2005), they have been found to have good efficacy in various models of neuropathic pain (Decosterd et al. 2004;Rodrigues-Filho et al. 2004;Yoshimura and Yonehara 2006). An exception to this is in
the paclitaxel (Taxol)-induced neuropathy model in which MK-801 produced no significant reversal of Taxol-induced mechanical hypersensitivity (Flatters and Bennett 2004). Ultimately, the effect of such drugs will improve our understanding of the underlying pathophysiology in zoster-associated pain and allow us to design novel analgesic compounds that are targeted to these mechanisms.
Chapter 4

Integrated Behavioural Paradigm of Pain: Open Field Activity
4.1 Introduction

For patients with persistent neuropathic pain, the experience of pain is intimately linked with psychological co-morbidity, in particular anxiety which may interfere significantly with quality of life (Meyer-Rosberg et al. 2001; Nicholson and Verma 2004; McCarberg and Billington 2006). The hallmark of anxiety disorders is thus a “marked, persistent, and excessive or unreasonable fear” that in humans is reflected in behavioural disturbances, including for example, avoidance, escape and/or hypervigilance (Palanza 2001; Cryan and Holmes 2005). A similar series of behavioural and physiological responses may be observed in animal anxiety, for example, when exposed to unfamiliar environments, an animal may initially display inhibition of exploratory behaviour, freezing, flight, risk assessment behaviour, increase in heart rate, urination, defaecation, and an increase in plasma corticosterone levels (Rodgers et al. 1997). Whilst anxiety-related symptoms have been reported in the majority (55%) of patients suffering from lesion of a peripheral nerve or nerve root (Meyer-Rosberg et al. 2001), anxiety-like behaviour has rarely been assessed in rodent models of neuropathic pain.

In this chapter, I have extended behavioural paradigms beyond stimulus-evoked reflex limb withdrawal tests conventionally employed in pain models, to encompass more complex outcome measures of integrated pain behaviour reflecting neuropathic pain co-morbidities, specifically anxiety-like behaviour in the open field paradigm. This was undertaken in VZV-infected rats in parallel with two conventional models of traumatic peripheral nerve injury (i.e. partial sciatic nerve injury and spinal nerve transection). Further, pharmacological sensitivity testing in the open field paradigm was performed; specifically the response to acute i.p. administration of gabapentin (30 mg/kg) and (S)-(+-)ibuprofen (20 mg/kg) was examined.
4.2 Methods

4.2.1 Animals and surgical procedure

Experiments were performed on adult male Wistar rats with a mean weight of 300g (range 240 - 350g) (B&K, Hull, U.K.) in accordance with British Home Office regulations. VZV infection (strain Dumas, 80% mean cpe) was performed as previously described. Partial sciatic nerve ligation (PSNL) and spinal nerve transection (SNT) was performed under general anaesthesia with isoflurane. For PSNL surgery, the left (ipsilateral) sciatic nerve was exposed just above its trifurcation and ½ - ½ of the nerve trunk ligated with a 7-0 non-absorbable silk suture (Mersilk, Ethicon) (Seltzer et al. 1990). In sham animals, the sciatic nerve was exposed in the same way but not ligated. SNT surgery was performed using a technique modified from Kim and Chung (Kim and Chung 1992; Bridges et al. 2001a). A 2 – 3 cm midline skin incision was made at the level of the iliac crests. Blunt dissection was used to separate the ipsilateral paraspinal muscles from the spinous processes of the L4 to S2 vertebrae. The transverse process of L6 was identified and a hemi-laminectomy performed to expose the L5 and L6 spinal nerve roots. The L5 nerve root was tightly ligated (4-0 Mersilk, Ethicon) and then transected 1 – 2 mm distal to the ligature. Transection of the L5 nerve route was confirmed on post-mortem and only data from these animals was included in the analysis. In sham animals, an identical procedure was performed except hemi-laminectomy; ligation and transection were not executed. Postoperative analgesia consisted of s.c. injection of 0.05ml 0.5% bupivicaine (Antigen Pharmaceuticals, Ireland) to the wound site, followed two hours later by i.p. administration of carprofen (20%, 0.1ml/200g body weight, Pfizer). Animals were housed in individually ventilated colony cages and maintained on a 14:10 hour light/ dark cycle with free access to food and water.

4.2.2 Novel open field

The novel open field apparatus consisted of a square arena (100 x 100 x 35 cm) with opaque black Plexiglas walls and floor that was evenly illuminated to 4 lux (measured in the centre of the arena) (Fig. 4.1). The rat was placed in the centre of the arena and its spontaneous locomotor activity recorded for 15 minutes using an infrared camera (Sanyo VCB 3372). As there is considerable variability in the duration of behavioural testing, ranging from 2 to 20 minutes (Prut and Belzung 2003), pilot studies in naive animals determined the optimal period of scoring to be 15 minutes (data not shown). Total distance
traveled, time spent in the centre of the arena (40 x 40 cm), and entries into the centre were measured using the EthoVision video tracking system (version 2.0, Tracksys Ltd., Nottingham). These primary outcome measures were partly determined from previous studies (Boguszewski and Zagrodzka 2002; Prut and Belzung 2003; Cryan and Holmes 2005). Additional parameters measured were number of faecal boli and urinary emissions.

![Image](image-url)

**Figure 4.1 Open field paradigm** (modified from Cryan and Holmes, 2005)

Importantly, environmental stress factors in the test room that may influence and modify responses; such as odours, noise including ultrasound-emitting devices, and activity by humans (Sales et al., 1999; Chesler et al., 2002), was kept to a minimum. In addition, I endeavored to maintain the temperature, humidity and light at constant levels during testing (24°C, 28%, and 4 lux respectively), and to score behavioural responses at the same time of day (i.e. in the afternoon). Care was also taken to ensure that the testing environment was thoroughly cleaned with 1% trigene solution between animals. Following exposure in the novel open field arena, animals were placed into an intermediate 'holding' cage before being returned to their home cage. This was to avoid any potential confounding effects of 'empathy', since a recent study has reported that pain sensitivity in mice may be modulated solely by exposure to cagemates in pain (Langford et al. 2006a). A similar phenomenon may occur with respect to expressed anxiety, though this has yet to be determined fully (discussed in Chapter 8).

4.2.3 Effect of habituation in the open field

To assess the effect of prior exposure or habituation to the open field apparatus, naïve animals (n = 8) were tested and then re-tested under identical experimental conditions two weeks later as described above.
4.2.4 Assessment of anxiety-like behaviour in the open field

Animals were randomly allocated to the following groups i.e. animals and experimental groups were pre-numbered and paired according to two random number tables: naïve (n = 15); PSNL (n = 10); PSNL sham (n = 11); SNT (n = 11); SNT sham (n = 8); VZV-infected (n = 12) and fibroblast-infected controls (n = 12). Baseline threshold value for hind-paw withdrawal in response to punctate mechanical stimulation was measured prior to viral infection or surgery using the electronic von Frey device (as previously described in Chapter 2). Paw withdrawal threshold (PWT) measurements were repeated on day 14 post-surgery/infection at which time animals were introduced into the novel open field (animals were not previously exposed to the open field arena). Behavioural testing was performed by an experimenter 'blinded' to the individual animal groups (i.e. cage identification labels were removed) on day 14, as this was found to be the time at which maximal mechanical hypersensitivity was reliably established.

4.2.5 Open field pharmacological sensitivity testing

Separate animals were used for pharmacological sensitivity testing in the open field paradigm. Animals were randomly allocated to the following groups i.e. animals and treatment groups were pre-numbered and paired according to two random number tables: SNT (n = 34); SNT sham (n = 28); VZV-infected (n = 32) and fibroblast-infected controls (n = 25). Behavioural testing to punctate mechanical stimulus was performed using the electronic von Frey device on day 14 post-infection/surgery to confirm development of mechanical hypersensitivity in injured animals. Only animals demonstrating a statistically significant (paired t-test; p<0.05) decrease in ipsilateral PWTs on day 14 compared to baseline were retained for pharmacological studies. Animals were then randomly assigned (as above) to receive acute i.p. administration of either gabapentin (30 mg/kg), (S)-(+-)ibuprofen (20 mg/kg) or vehicle (saline or 40% DMSO/saline) 20 minutes before testing in the open field paradigm. Animals were not previously exposed to the open field arena and separate animals were used in each treatment group. In addition, the behavioural investigator was 'blinded' to the various treatment groups (i.e. drugs and vehicle were prepared in identical syringes by a separate experimenter).
4.2.6 Statistical analysis

Statistical analysis was performed using Sigmastat (Jandel Scientific Software, version 2.0). Power calculation to determine the sample size for each experiment was performed as previously described. All experiments were of suitable power (0.8 – 0.9). The paired t-test was used to examine the effects of prior exposure to the open field, and to compare ipsilateral PWTs at baseline to those at day 14 post-surgery/infection within groups. Comparison of open field outcome measures between groups was performed with a t-test (followed where appropriate by the Mann Whitney rank sum test). Significance level was taken at $p<0.05$. Variance is expressed as standard error of the mean (sem).
4.3 Results

*Prior exposure to the open field is associated with intra-animal variability*
While there were no statistical differences in open field outcome measures between tests, there was considerable intra-animal variability in inner zone frequency and duration (Fig. 4.2).

*Development of anxiety-like behaviour in VZV-infected and SNT-operated animals and relationship with mechanical hypersensitivity*
Hypersensitivity to punctate mechanical stimulation developed in all groups examined on day 14 post-injury: SNT- and PSNL-operated animals displayed a 50.7% and 41.7% reduction from baseline ipsilateral PWTs respectively, compared to 22.7% in VZV-infected animals (Fig. 4.3). Compared to sham-operated and fibroblast-infected controls, SNT and VZV-infected animals displayed greater thigmotactic ('wall-hugging') ambulation (Fig. 4.4) and entered the central area of the open field arena less frequently (number of entries: 2.9 ± 0.7 for SNT-operated animals compared to 8.6 ± 1.5 in shams; and 7.1 ± 1.6 for VZV-infected compared to 12.6 ± 1.9 in uninfected fibroblast-injected controls) (Fig. 4.5). Moreover, inner zone frequency was found to be positively correlated with degree of mechanical hypersensitivity in both SNT-operated and VZV-infected animals (Pearson correlation coefficient: 0.72 and 0.63 respectively) (Fig. 4.6). However no difference in behaviour was observed between PSNL-operated and sham animals (Fig. 4.5). Furthermore, SNT and PSNL animals traveled less distance during the 15 minute testing session (5232.1 ± 605.1 cm and 5711.3 ± 285.5 cm respectively) compared to sham-operated animals (8140.1 ± 619 cm and 7492.8 ± 343.9 cm respectively). In contrast, there was no difference in total distance covered by VZV-infected animals compared to fibroblast-infected controls (Fig. 4.5). Finally, no difference in number of faecal boli or urinary emissions was observed between nerve-injured or VZV-infected and respective sham animals (data not shown).
Figure 4.2 Effect of habituation on open field outcome measures in naive rats (i) n = 8; and in (ii) individual animals: A) Frequency into the inner zone of the open field arena; B) time spent in the inner zone; and C) total distance traveled in the arena. Animals were re-tested under identical environmental conditions two weeks after first being introduced into the open field arena. No statistical difference (paired t-test; p>0.05) between first and second exposure was observed, although considerable intra-animal variability may be seen.
Figure 4.3 Development of hypersensitivity to punctate mechanical stimulation (measured using the electronic von Frey device) in the ipsilateral paw of A) SNT (n = 11) animals; B) PSNL (n = 10) animals; and C) and VZV-infected (n = 12) animals. *p<0.05, paired t-test, for PWT comparisons on day 14 post-surgery/infection compared to baseline.
Figure 4.4 A) Open field arena (100cm$^2$) and inner zone (40cm$^2$); B-F) Examples of characteristic track patterns as monitored using an infrared camera.
A. Inner Zone Frequency

B. Inner Zone Duration

C. Total Distance

Figure 4.5 Open field parameters across all groups: naïve (n = 15); sham PSNL (n = 11); PSNL (n = 10); sham SNT (n = 8); SNT (n = 11); uninfected fibroblast-injected control (n = 12); VZV-infected (n = 12). Open field parameters measured during a 15 minute testing session under low level lighting conditions were: A) Number of entries (frequency) into the inner zone (40 x 40 cm zone); B) duration in the inner zone; and C) total distance moved throughout the arena (100 x 100 cm). * p<0.05 compared to naïve animals (t-test, followed by Mann-Whitney rank sum test where normality test failed); # p<0.05 (t-test, followed by Mann-Whitney rank sum test where normality test failed) for sham vs experimental.
Figure 4.6 Relationship between open field inner zone entry and mechanical hypersensitivity. Paw withdrawal threshold (PWT) was measured using the electronic von Frey device. A) VZV (Dumas)-induced mechanical hypersensitivity is positively correlated with anxiety-like behaviour (as reflected by reduced entry into the open field inner zone). Pearson correlation coefficient, R = 0.63 (p = 0.02). ▲ represents individual VZV-infected animals on day 14 p.i. (n = 12) while the dotted line represents the trend between variables. However, there was no correlation in uninfected fibroblast-injected control animals. Mean ± sem percentage decrease in mechanical hypersensitivity for VZV-infected animals on day 14 p.i. was 26.2% ± 3.7, while mean frequency into the inner zone was 7.1 ± 1.6. For uninfected fibroblast-injected animals, there was a 1.1% ± 3.8 increase in mechanical PWTs overall, while mean frequency into the inner zone was 12.6 ± 1.9. B) Mechanical hypersensitivity is positively correlated with anxiety-like behaviour in spinal nerve transected (SNT) animals (n = 11). Pearson correlation coefficient, R = 0.71 (p = 0.01). ■ represents individual animals 14 days post-surgery while the dotted line represents the trend between variables. However, there was no such correlation in sham animals. Mean ± sem percentage decrease in mechanical hypersensitivity for SNT animals on day 14 was 50.1% ± 2.6, while mean frequency into the inner zone was 2.9 ± 0.7. For sham animals, there was a 0.8% ± 1.6 decrease in mechanical PWTs overall, while mean frequency into the inner zone was 8.6 ± 1.5.
Anxiety-like behaviour is reversed by acute i.p. administration of gabapentin (30 mg/kg) and (S)-(+)-ibuprofen in SNT, but not VZV-infected animals

On day 14, SNT-operated (n = 27) and VZV-infected (n = 27), but not respective sham animals, displayed hypersensitivity to punctate mechanical stimulation ipsilateral to the injury: A 51.1% reduction from baseline ipsilateral PWT values was observed in SNT animals compared to a 32.9% reduction in VZV-infected animals (Fig. 4.7). Fourteen animals were excluded from pharmacological studies due to insufficient behavioural change, autotomy or death in the post-operative period (Fig. 4.8). In gabapentin- and (S)-(+)-ibuprofen-administered SNT animals, frequency of entry into and time spent in the central area of the open field arena was increased (Fig. 4.9 and 4.10). This was paralleled by a reduction in the total distance traveled following administration of (S)-(+)-ibuprofen but not gabapentin (Fig. 4.9 and 4.10). Further, vehicle administration had no effect in SNT animals for all parameters examined. In contrast, gabapentin administration was observed to have no effect on open field outcome measures in VZV-infected animals (Fig. 4.9), while (S)-(+)-ibuprofen resulted in a decrease in both frequency of entry into the inner zone and total distance traveled (Fig. 4.10). There was no effect on inner zone frequency following vehicle administration in VZV-infected animals, although administration of 40% DMSO vehicle did result in a reduction in total distance traveled (Fig. 4.10).
Figure 4.7 Development of hypersensitivity to punctate mechanical stimulation (measured using the electronic von Frey device) on day 14 post-surgery/ infection in A) SNT (n = 27), but not sham-operated (n = 26) animals; and in B) VZV-infected (n = 27), but not fibroblast-injected control (n = 25) animals prior to open field pharmacological validation. A 51.5% reduction from baseline in ipsilateral PWTs was observed in SNT-operated animals, compared to 32.9% reduction in VZV-infected animals. * p<0.05 (paired t-test) compared to baseline threshold values.
Animals randomly allocated to receive drug or vehicle (n = 105)

<table>
<thead>
<tr>
<th>Groups</th>
<th>SNT (n = 6)</th>
<th>Sham SNT (n = 6)</th>
<th>VZV-infected (n = 6)</th>
<th>Fibroblast-infected controls (n = 5)</th>
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<tr>
<td>Received saline vehicle (i.p.)</td>
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<td>Received (S)-(+) ibuprofen (20 mg/kg i.p.)</td>
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Figure 4.8 CONSORT-type flow diagram for pharmacological sensitivity testing in the open field paradigm
Figure 4.9 Open field pharmacological sensitivity testing: Effect of acute administration of gabapentin (GP) (30 mg/kg) or vehicle (saline) in A) SNT and sham-operated animals; and B) VZV-infected and uninfected-fibroblast (Hel) control animals 14 days post-surgery/infection. Drug/vehicle (n = 5 – 8 per group) was administered by the i.p. route 20 minutes before testing in the open field. The following parameters were measured during a 15 minute testing session: (i) inner zone frequency; (ii) inner zone duration; and (iii) total distance traveled in the arena. * p<0.05 (t-test) compared to SNT or VZV-infected animals; # p<0.05 (t-test) compared to sham or Hel-injected control animals.
Figure 4.10 Open field pharmacological sensitivity testing: Effect of acute administration of (S)-(−)-ibuprofen (IBU) (20 mg/kg) or vehicle (40% DMSO/ saline) in A) SNT and sham-operated animals; and B) VZV-infected and uninfected-fibroblast (Hel) control animals 14 days post-surgery/ infection. Drug/vehicle (n = 6 – 9 per group) was administered by the i.p. route 20 minutes before testing in the open field. The following parameters were measured during a 15 minute testing session: (i) inner zone frequency; (ii) inner zone duration; and (iii) total distance traveled in the arena. * p<0.05 (t-test) compared to SNT or VZV-infected animals; # p<0.05 (t-test) compared to sham or Hel-injected control animals.
4.4 Discussion

I have examined anxiety-like behaviour as a more complex outcome measure of integrated pain behaviour, thus allowing more accurate examination of the diverse symptomatology of persistent neuropathic pain. In the open field paradigm, virus-infected and SNT-operated animals demonstrated an anxiety-like pattern of ambulation (i.e. thigmotaxis ('wall-hugging') and reduced entry into the central area of the open arena) which may reflect pain-related co-morbidity. Moreover, mechanical hypersensitivity was found to be positively correlated with anxiety-like behaviour. Finally, the acute i.p. administration of gabapentin (30mg/kg) and (S)-(+) ibuprofen (20mg/kg) was found to attenuate anxiety-like behaviour in nerve-injured, but not virus-infected animals.

Assessment of open field behaviour is a well recognised paradigm for the investigation of anxiety-like traits in rodents (Belzung and Dubreuil 1998; Prut and Belzung 2003; Cryan and Holmes 2005). The arena itself is an anxiogenic environment as the animal is separated from its social group and placed into an open unfamiliar and potentially aversive environment much larger than the animal’s natural environment. In this setting, naïve rats characteristically exhibit a thigmotactic pattern of ambulation. However, to discourage this inherent pattern of behaviour, lighting conditions in the laboratory were reduced (lux 4) and minimal human activity allowed. In this way, stress-induced inhibition of exploration behaviour was decreased and central locomotion into the inner zone increased. Moreover, since it has been suggested that validity of behavioural and hence pharmacological testing is reduced if animals have been habituated to the testing environment (Holmes and Rodgers 2003), I assessed the effect of previous exposure to the open field apparatus. I found that while prior exposure to the open field arena was not associated with significant inter-group variability, it was associated with significant intra-animal variability, specifically in frequency and time spent in the central area of the open field i.e. those animals that had explored more on first exposure, did so significantly less on second exposure, and vice versa. In light of these findings and previous reports, habituation to the open field testing environment was not performed in subsequent tests.

In this study, I assessed anxiety-like behaviour in VZV-infected animals in parallel with two conventional models of traumatic peripheral neuropathy. Compared to naïve and sham animals, SNT and virus-infected animals demonstrated less central locomotion which was positively correlated with degree of mechanical hypersensitivity. This appears to be
consistent with clinical observations in PHN patients (Rowbotham et al. 1996). Rowbotham and Fields (1996) examined thirty-five patients with established PHN and found that the severity of mechanical allodynia was positively correlated with ongoing pain severity. Changes in spontaneous ambulation as detected in the open field may reflect ongoing spontaneous pain in nerve-injured animals. Importantly however, the presence of hypersensitivity phenomena following peripheral nerve injury does not reliably imply the presence of anxiety-like behaviour as demonstrated in PSNL animals, though it may be argued that this could be a reflection of the sensitivity of the paradigm in measuring anxiety-like behaviour. A potential confounding factor in the open field paradigm is that it is based on ambulation and in nerve-injured animals motor function could conceivably be reduced. To examine this I also measured the total distance traveled in the arena and found that in fact SNT and PSNL animals covered less distance compared to sham-operated animals. In contrast, there was no difference in total distance covered by VZV-infected animals compared to fibroblast-infected controls. This suggests that following VZV infection there is an increase in anxiety-like behaviour that is not simply due to motor impairment, and this may reflect more complex co-morbidity behaviour associated with pain conditions. However, there were no differences in open field outcome measures between PSNL and PSNL-sham animals which may reflect differences in the mechanisms mediating pain-induced anxiety behaviour.

Furthermore, I have demonstrated that traumatic peripheral nerve injury-induced anxiety-like behaviour in rats may be reversed by the acute i.p. administration of gabapentin (30 mg/kg) or (S)-(++)-ibuprofen (20 mg/kg). Since gabapentin is documented to have an anxiolytic effect in addition to a primary analgesic effect in addition to a primary analgesic effect (Singh et al. 1996; Pollack et al. 1998; de-Paris et al. 2000), I examined the influence of a purely analgesic compound on anxiety-like behaviour in the open field. Previous studies have shown that gabapentinoids have a robust inhibitory effect on stimulus-independent (spontaneous), as well as evoked neuronal measures (Chapman et al., 1998a, Chapman et al., 1998b; Suzuki et al., 2005). Since changes in spontaneous ambulation as detected in the open field may reflect ongoing spontaneous pain-related behaviour in nerve-injured animals, the data presented in this study is consistent with clinical findings where gabapentin alleviates ongoing pain in addition to mechanical allodynia (Attal et al., 1998). However, no change in behaviour was observed in VZV-infected animals following either gabapentin or (S)-(++)-ibuprofen administration. The reason for this difference is difficult to explain but may reflect the difference in baseline anxiety-like behaviour in VZV and SNT animals i.e. although both groups demonstrated significant
decreases in inner zone entry compared to controls, SNT animals exhibited relatively more anxiety-like behaviour (number of entries into inner zone: 2.9 ± 0.7 for SNT-operated compared to 7.1 ± 1.6 for VZV-infected animals). If this is the case, it may be that higher doses of drug are necessary to observe an effect on VZV-induced anxiety-like behaviour, which may explain why a change in behaviour was not observed in these animals.

4.4.1 Pathways mediating pain-induced anxiety and proposed mechanisms

Pain has both sensory discriminative and affective dimensions which have been proposed to be transmitted to the brain by two parallel spinal pathways. These pathways terminate in discrete brain areas that monitor the quality of the initiating stimulus and show remarkable plasticity (Price 2000; Hunt and Mantyh 2001). The spinothalamic pathway which projects onto the cortex is thought to be concerned with the sensory discriminative qualities of the nociceptive stimulus. It originates primarily from second-order neurones in the spinal dorsal horn and terminates in the ventroposterior and ventrobasal thalamus. The spinoparabrachial pathway is thought to mediate the affective component of the nociceptive stimulus and terminates within the parabrachial area and the periaqueductal grey. These areas in turn project on the hypothalamus and amygdala and are thought to modulate the affective dimensions of pain. It is this pathway that is believed to be responsible for pain-induced anxiety and depression (Price 2000; Hunt and Mantyh 2001). However, the exact neurobiological mechanisms underlying pain-induced anxiety have yet to be determined.

There is much evidence supporting a key role for the amygdala in fear and anxiety (Davis et al. 1994; Narita et al. 2006a). Additionally, the endogenous opioid system has been implicated in anxiety and stress (Narita et al. 2006a). Further, as the amygdala has high levels of endogenous opioid peptides, it was recently proposed that chronic pain-induced changes in opioidergic function in the amygdala may lead to an anxiogenic effect (Narita et al. 2006a). Another mechanism activated under conditions of chronic stress, such as persistent pain and anxiety, is the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis (Ulrich-Lai et al. 2006). In this system, information regarding a stressor is processed by the limbic system (which includes the amygdala). Upon activation, corticotrophin releasing hormone is released from the hypothalamus causing release of adrenocorticotropic hormone (ACTH) from the pituitary, which in turn acts on the adrenal cortex to cause release of glucocorticoids. However, long-term or repeated activation of the HPA axis produces changes in the limbic system and HPA axis. Indeed, chronic pain patients often display
altered glucocorticoid negative feedback and abnormal cortisol levels (Ulrich-Lai et al. 2006). However, it has recently been demonstrated that indices of HPA axis activation do not change following chronic constriction injury of the sciatic nerve in rats (Ulrich-Lai et al. 2006). This contrasts with previous studies employing other types of nociceptive stimuli, e.g. repeated i.p. injections of hypertonic saline was found to increase basal plasma corticosterone levels (Kiss and Aguilera 1993). Therefore, the response to stress in animals may depend largely on the nature of the perceived stimulus.

4.4.2 Limitations and further studies

The open field paradigm is widely used for the assessment of anxiety-like behaviour in animals, however considerable variation in design of the open field environment exists, and this could be explored in further studies (Prut and Belzung 2003; Cryan and Holmes 2005). For example, the arena may take the form of a circular field, rather than a square or rectangular one (Prut and Belzung 2003). Indeed, the first description of the open field test in rats employed a circular arena 120 cm in diameter (Hall 1934). An advantage of this design may be that there are no corners, and so the animal is forced to ambulate more. An additional design factor that could be further investigated relates to size of the arena, since small rodents have an innate aversion to large exposed spaces, probably reflecting predator avoidance in the wild. However, as they are also naturally exploratory animals, this aversion conflicts with a drive to explore novel environments, especially when foraging (Cryan and Holmes 2005). I used a 100 x 100 x 35 cm arena for rats based on the literature (LaBuda and Fuchs 2001a; LaBuda and Fuchs 2001b; Boguszewski and Zagrodzka 2002) and advice from Tracksys Ltd., Nottingham (and a 39 x 39 x 35 cm arena for mice based on advice from Dr. A. Holmes - Chapter 5). It may be argued that an arena that is too large and exposed may actually exaggerate anxiety-like responses and induce startle or freezing responses in rodents. Thus, false positive results (i.e. high anxiety) may be produced and more subtle changes in anxiety-like behaviour may be missed. Importantly, it may be that the optimal dimensions for testing anxiety-like behaviour may be relative to the animal’s natural predator, rather than to the animal itself. Thus, size of the arena is a critical factor in assessing anxiety-like behaviour.

Open field behaviour also depends on the light-dark cycle (Prut and Belzung 2003), and since animals in this study were not housed on a reverse light-dark cycle, it may be relevant to investigate behaviour at different times i.e. it would be desirable to score behaviour during
the night whilst animals are in the active phase of their diurnal cycle. Another limitation to these paradigms, to which there is no obvious solution, is that they involve testing animals individually; and acute social isolation stress may modify behaviour and therefore further impact on the results. Finally, an important limitation of the open field paradigm and indeed the majority of currently favoured paradigms for the assessment of spontaneous anxiety-like behaviour is that they are based on ambulation, and in nerve-injured animals, motor activity could conceivably be reduced. Thus, motor impairment may produce false positive results. It would therefore be useful to explore alternatives to exploration-based tests for anxiety-like behaviour, e.g. the Vogel test (Vogel et al. 1971). This is an example of a punishment-based conflict test in which water-deprived rats are provided with a drinking spout that delivers a mild shock after every 20 licks. Further, it is sensitive to anxiolytics which attenuate the shock-induced suppression of drinking (Cryan and Holmes 2005).

Moreover, exposing a rat to its natural predator, either as a brief unprotected exposure, or protected exposure (i.e. rat is briefly exposed to a room in which a predator was previously present), or by exposing the animal to predator odours (e.g. cat urine), can induce fearful and anxiety-like states which may enhance more subtle types of anxiety-like behaviour (Roy et al. 2001; Adamec et al. 2004; Beekman et al. 2005; Adamec et al. 2006a; Adamec et al. 2006b). Predator stress has been demonstrated to have a long lasting effect on anxiety-like behaviour, specifically on risk assessment behaviour, including flat back approach and stretch attend behaviours orientated toward the threatening stimulus (Roy et al. 2001; Adamec et al. 2004; Beekman et al. 2005; Adamec et al. 2006a; Adamec et al. 2006b). However, predator exposure is thought to model aspects of post-traumatic stress disorder rather that a generalized anxiety disorder.

Other experimental manipulations in the open field may include the presence of objects or food within the arena. It has been shown that exploration can be increased by some factors such as food or water deprivation (Prut and Belzung 2003). Animal gender and strain may also influence anxiety-like behaviour (Millstein and Holmes 2006; Adamec et al. 2006a; Adamec et al. 2006b). For example, Ademec and colleagues (2006b) recently found that female, but not male, mice are made more anxious in the elevated plus maze by exposure to predator odours. The issue of strain is discussed in Chapter 5.

Additional anxiety-related behavioural parameters that may be considered in future studies include sniffing, rearing, grooming and stretch-attend postures. Certainly, sniffing and...
stretch-attend postures are thought to indicate hesitation and risk-assessment behaviour (Adamec et al. 2006b) which is consistent with anxiety behaviour in humans and frequently seen in chronic pain patients (i.e. risk assessment of potential pain-inducing environments or activities). Further, non-behavioural adjunctive measures such as cortisol levels or autonomic functions may be of value as objective measures of anxiety-like behaviour in animals.

Finally, the present study could be extended to employ a broad range of tests of anxiety-like behaviour in animals, including for example the elevated plus-maze (EPM), the light-dark box and the acoustic startle response test. It is likely that differing environments induce different degrees of anxiety, and as there are qualitative differences between anxiety tests, it is further possible that some paradigms may miss subtle changes in animal behaviour, and therefore it is important to use a battery of tests (Cryan and Holmes 2005). However, an important limitation of the majority of currently favored paradigms is that they are based on ambulation, and in nerve-injured animals, motor impairments could produce false positive results. It would therefore be useful to explore alternatives to exploration-based tests for anxiety-like behaviour, e.g. the Vogel test (Vogel et al. 1971). This is an example of a punishment-based conflict test in which water-deprived rats are provided with a drinking spout that delivers a mild shock after every twenty licks. Further, it is sensitive to anxiolytics which attenuate the shock-induced suppression of drinking (Cryan and Holmes 2005).
Chapter 5

Mechanical and cold hypersensitivity in nerve-injured C57BL/6J mice is not associated with anxiety- and depression-related behaviour
5.1 Introduction

There is significant comorbidity between neuropathic pain and various neuropsychiatric disorders, including anxiety and depression (Dworkin and Gitlin 1991; Meyer-Rosberg et al. 2001; Nicholson and Verma 2004; Leo 2005; Mico et al. 2006). The high prevalence of these disorders and associated clinical burden among patients with chronic pain is well recognised, although the evidence for common pathophysiological mechanisms is only now beginning to emerge (Leo 2005; Ulrich-Lai et al. 2006). A recent study investigating the multidimensional burden of neuropathic pain in a cohort of patients suffering from lesion of a peripheral nerve or nerve root demonstrated significantly impaired health-related quality of life (Meyer-Rosberg et al. 2001). Difficulty sleeping, lack of energy and difficulty concentrating were rated more highly than depression- or anxiety-related symptoms (88%, 86%, and 76% of patients reported some degree of symptom severity compared to 70% and 55% of patients reporting some degree of depression and anxiety-related symptoms respectively). However, depression and anxiety were in turn more highly reported than lack of appetite, dizziness and urinary problems (Meyer-Rosberg et al. 2001). Significantly, 35% and 25% of patients reported moderate to very severe symptoms of depression and anxiety (Meyer-Rosberg et al. 2001). Indeed, one study has previously described a prevalence rate for depression approaching 100% among chronic pain patients (Romano and Turner 1985). In turn there is evidence for the deleterious impact of affective illness on both the diagnosis and prognosis of neuropathic pain (Gallagher and Verma 1999; Verma and Gallagher 2002; Leo 2005).

Rodent models have proven valuable in identifying pathophysiological mechanisms and therapeutic targets for emotional disorders (Cryan and Holmes 2005) and for pain disorders (Seltzer et al. 1990; Kim and Chung 1992). However, there have been relatively few studies investigating the potential effects of neuropathic pain on measures of anxiety- and depression-related behaviours in rodents. Kontinen and colleagues (Kontinen et al. 1999) found that spinal nerve ligation-induced neuropathic pain did not alter anxiety-related behaviours in the open field and elevated plus-maze tests in rats tested two weeks following injury. In contrast, a recent study in mice showed that persistent inflammatory pain induced by intraplantar injection of complete Freund’s adjuvant, as well as nerve injury caused by partial sciatic nerve ligation led to increased anxiety-like behaviours on the light-dark exploration test and elevated plus-maze four weeks later (Narita et al. 2006a). Similarly, we
have observed anxiety-like behaviour in rat models of traumatic, viral and cytokine-induced neuropathic pain as assessed in the open field test (Wallace et al. 2005; Hasnie et al. 2007).

The objective of this study was to employ an established murine model of neuropathic pain to further investigate the effects of pain on anxiety- and depression-related behaviours. C57BL/6J mice underwent unilateral partial sciatic nerve ligation and were then tested on the novel open field, elevated plus-maze (EPM) and tail suspension tests at intervals of seven, fourteen, or twenty-eight days post-surgery. I hypothesised that traumatic peripheral neuropathy would be associated with increased anxiety- and depression-related behaviours, and that these effects would be more pronounced as the chronicity of pain increased. The open field and EPM tests are two well-validated paradigms for assessing anxiety-like behaviour and are based upon the fact that small rodents have an innate aversion to exposed, well-lit spaces, however, this aversion conflicts with a drive to explore novel environments (Cryan and Holmes 2005). Similarly, the tail suspension test (TST) (Bai et al. 2001; Cryan et al. 2005) is a pharmacologically-validated test for assessing depression-like behaviour in mice, and is based on the fact that animals subjected to the short-term, inescapable stress of being suspended by their tail, develop an immobile posture. This immobility is due to an inability or reluctance to maintain effort and reflects a learned helplessness or 'behavioural despair' analogous to the clinical observations that depressed patients often lack sustained expenditure of effort reflected in pronounced psychomotor impairments (Cryan and Holmes 2005; Cryan et al. 2005).

This was a collaborative study with Dr. A. Holmes (Laboratory for Integrative Neuroscience, National Institutes of Health, Bethesda, U.S.A). Surgery was performed by F. Hasnie, while behaviour testing was performed jointly with Miss K. Hefner (National Institutes of Health).
5.2 Methods

5.2.1 Animals and surgical procedure

Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) with a mean weight of 25.6 g (range 20.8 – 30.2 g), were randomly allocated to one of two experimental groups (i.e. animals were pre-numbered and allocated according to two random number generated tables): A) partial sciatic nerve ligation (PSNL) (n = 36); and B) sham procedure (n = 36). Nerve-injured and sham animals were behaviourally examined at one of three time points post-operatively: day 7, 14 and 28 (n = 12 per group) i.e. separate groups of animals were used at each time point. Surgery was performed under general anaesthesia with isoflurane. Briefly, the left (ipsilateral) sciatic nerve was exposed just above its trifurcation and 1/3 - 1/2 of the nerve trunk tightly ligated using 7.0 non-absorbable silk suture (Mersilk, Ethicon) (Seltzer et al. 1990; Malmberg and Basbaum 1998). The wound was closed in layers (4-0 Mersilk, Ethicon) and animals allowed to recover. In sham animals, the sciatic nerve was exposed but not ligated. Mice were housed 4 per cage in a temperature- and humidity-controlled vivarium under a 12 h light/dark cycle (lights on 0600 hours). Experimental procedures were performed in strict accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the local Animal Care and Use Committee.

5.2.2 Stimulus-evoked reflex withdrawal tests

Prior to surgery, the baseline threshold value for hind-paw withdrawal in response to punctate mechanical stimulation was measured in conscious animals with graded von Frey filaments (Stoelting Co., Wood Dale, Illinois). Threshold response was defined by the filament that caused active paw withdrawal at least three times in every five applications (Chaplan et al. 1994; Wallace et al. 2003). Baseline measurements were also taken for hind paw withdrawal responses to an acetone drop applied to the plantar surface of each hind paw (Carlton et al. 1994). The response was scored 0 - 3 based on the following scale: 0 = no response; 1 = brief flick of hind-limb; 2 = hind-limb raised for > 1 second; and 3 = biting and licking of hind-paw. For both tests, the average of two readings was calculated and displayed as the mean ± sem. Animals were re-tested on days 6, 13 and 27 post-operatively for responses to von Frey filaments and acetone to establish the development of neuropathy (i.e., development of hypersensitivity to mechanical and cool stimulation). Animals that did not
demonstrate significant ($p<0.05$, paired t-test) reduction in sensory thresholds compared to baseline values were excluded from the study. The investigators (F. Hasnie and K. Hefner) conducting behavioural assessment were 'blinded' to the surgical groups (i.e. a separate investigator placed animals into the testing apparatus).

5.2.3 Novel open field

The novel open field test was conducted on the day following post-operative stimulus-evoked reflex withdrawal testing, as previously described (Boyce-Rustay and Holmes 2006). The apparatus was a square arena (39 x 39 x 35 cm) with opaque white Plexiglas walls and floor that was evenly-illuminated to approximately 20 lux. The mouse were placed in a corner and allowed to freely explore for 15 minutes. Total distance traveled, time spent in the (20 x 20 cm) centre, and entries into the centre were measured using the EthoVision videotracking system (Noldus Information Technology, Leesburg, VA).

5.2.4 Elevated plus-maze

The elevated plus-maze test was conducted three hours following the open field test, as previously described (Handley and Mithani 1984; Boyce-Rustay and Holmes 2006). The apparatus consisted of two open arms (30 x 5 cm; 55 lux) and two closed arms (30 x 5 x 15 cm; 5 lux) extending from a 5 x 5 cm central area and elevated 49.5 cm from the ground (San Diego Instruments, San Diego, CA) (Fig. 5.1). The walls were made from black ABS plastic and the floor from white ABS plastic. A 0.5 cm raised lip around the perimeter of the open arms prevented mice from falling off the maze. The mouse was placed in the centre facing an open arm and allowed to explore the apparatus for 5 minutes. Time spent and entries into the open and closed arms were measured by the Ethovision videotracking system (Noldus Information Technology Inc., Leesburg, VA).
5.2.5 Tail suspension test

The tail suspension test was conducted on the following day, as previously described (Steru et al. 1985; Boyce-Rustay and Holmes 2006). The mouse was securely fastened by the end of the tail to a flat surface that was suspended in a visually isolated white Plexiglas box (40cm³) and manually observed for the presence or absence of immobility (cessation of limb movements) every 5 seconds during the last 4 minutes of a 6 minute session (Fig. 5.2).
5.2.6 Statistical analysis

Statistical analysis was performed using Sigmastat (Jandel Scientific Software version 2.0). Power calculation to determine the sample size for each experiment was performed as previously described. All experiments were of suitable power (0.8). A paired t-test was used to compare ipsilateral or contralateral paw withdrawal response thresholds at baseline to those measured post-operatively, and for ipsilateral compared to contralateral paws at each time point. For statistical comparisons in the integrated behavioural paradigms, a two-way analysis of variance (ANOVA) (followed where appropriate by Newman-Keuls post-hoc tests) was used for all inter-group and intra-group comparisons. Significance level was taken at $p<0.05$. Variance is expressed as standard error of the mean.
5.3 Results

5.3.1 Behavioural reflex sensitivity

All PSNL-injured (n = 36), but not sham-operated animals (n = 36), displayed hypersensitivity to both punctate mechanical and cool stimulation ipsilateral to the injury (Fig. 5.3). In nerve-injured animals, the mean percentage decrease, from baseline, in ipsilateral paw withdrawal threshold (PWT) to punctate mechanical stimulation on days 6, 13 and 27 post-operatively (n = 12 per group) was 65.1%, 67.4%, and 63.9% respectively; while the mean percentage increase in response threshold to a cooling stimulus was 75.9%, 72.6% and 60% respectively. Significant differences in ipsilateral response thresholds were also observed when compared to contralateral and to sham ipsilateral paw withdrawal values. There was no statistical difference in ipsilateral paw withdrawal response thresholds between each time point post injury, nor was a contralateral difference in response thresholds observed at any time.

5.3.2 Novel open field

On day 14 post-operatively, both PSNL-injured and sham-operated animals displayed a significantly higher frequency of entry into the centre of the arena, as compared to days 7 and 28 (Fig. 5.4A). Moreover, on day 14, both PSNL and sham animals traveled a significantly greater distance in the open field arena as compared to days 7 and 28 (Fig. 5.4B). In PSNL-injured animals, a non-significant trend for an increase in the amount of time (expressed as percentage total time) spent in the centre of the open field arena was observed as compared to sham-operated animals (Fig. 5.4C). Furthermore, on day 14 post-surgery, nerve-injured animals exhibited a significantly shorter latency to first enter the centre of the arena as compared to sham animals (Fig. 5.4D). However, no significant change in percentage time spent in the central area, nor latency to enter was observed in nerve-injured animals between time points. Furthermore, PSNL-injured mice displayed no significant difference in the number of entries into the central zone, nor in the total distance travelled as compared to sham-operated animals throughout the experimental period (Fig. 5.4).
5.3.3 Elevated plus-maze

Frequency of entry into and time spent in the open arms of the EPM was observed to be significantly greater in day 14 PSNL-injured animals as compared to sham-operated animals (Fig 5.5A). This was paralleled by day 14 PSNL animals spending significantly less time in the closed arms as compared to shams (Fig 5.5B). Furthermore, on days 7 and 28 post-operatively, PSNL-injured animals spent significantly more time displaying 'risk assessment' behaviour in the central area of the EPM as compared to shams (Fig. 5.5C). Specifically, day 7 animals spent 88.9 ± 5.4 s in the central area, with a frequency of entry of 23.8 ± 0.6 as compared to sham animals (70.3 ± 4.9 s and 20.9 ± 1.3 respectively). Similarly, on day 28, animals spent 85.5 ± 4.2 s in the central area compared to 72.1 ± 4.9 s in shams. While statistical significance was not reached for nerve-injured animals tested on day 14 post-operatively, a similar trend was observed. Finally, at all time points nerve-injured animals travelled a greater distance in the EPM as compared to sham-operated animals (Fig. 5.5D). For example, on day 7 PSNL mice travelled 1331 ± 36.8 cm, as compared to 1225 ± 48.4 cm in shams. There was no significant difference between PSNL animals at each time point.

5.3.4 Tail suspension test

A significant difference in immobility scores between PSNL and sham-operated animals was not observed at any time point. However, with increased duration of nerve-injury or sham surgery, a decrease in % immobility was observed (Fig. 5.6).
Figure 5.3 Behavioural reflex sensitivity. Development of hypersensitivity to (i) punctuate mechanical and (ii) cool stimuli in the ipsilateral hind-paw of partial sciatic nerve ligated (PSNL) (n = 12 per group) versus sham-operated (n = 12 per group) mice on A) day 6; B) day 13; and C) day 27 post-surgery. * p<0.05 (paired t-test) for ipsilateral response threshold compared to ipsilateral baseline values, contralateral values and sham ipsilateral values. At all time points, PSNL injured animals developed significant sensitivity to both tests as compared to sham animals. The mean % decrease, from baseline, in ipsilateral paw withdrawal threshold to punctate mechanical stimulus in all PSNL animals (n = 36) was 65.4%, while the mean % increase in response threshold to a cooling stimulus was 67.3%.
Figure 5.4 Open field test. Outcome measures during a 15 minute testing session were A) frequency of entry into inner zone; B) total distance traveled in the whole arena (40 x 40cm); C) % time spent in the inner zone (20 x 20cm); and D) latency to entry into the inner zone. Data is shown for PSNL and sham operated mice on days 7, 14 and 28 post-surgery. *p<0.05 compared to sham day 14 post-surgery; **p<0.05 compared to PSNL-operated animals on days 7 and 28 post-surgery; +p<0.05 compared to sham-operated animals on days 7 and 28 post-surgery (statistical analysis was performed using the two-way ANOVA followed by post-hoc tests where appropriate).
Figure 5.5 Elevated plus maze test. Outcome measures during a 5 minute testing session were A) (i) frequency of entry into the open arms; (ii) time spent in the open arms; B) (i) frequency of entry into the closed arms; (ii) time spent in the closed arms; C) (i) frequency of entry into the central square; (ii) time spent in the central square; and D) total distance travelled in the maze. *p<0.05 compared to sham; +p<0.05 compared to day 14 sham; **p<0.05 compared to day 7 PSNL; and +++p<0.05 compared to day 7 sham (statistical analysis was performed using the two-way ANOVA followed by post-hoc tests where appropriate).
Figure 5.6 Tail suspension test. Immobility scores were measured on days 8, 15 and 29 post-surgery. No difference in immobility between PSNL and sham-operated animals was observed at any time point. However, with increased duration of nerve-injury or sham surgery, a decrease in % immobility was observed: 58.3%* (± 5.8) and 59%* (± 3.8) on day 28 respectively compared to 71.5% (± 2.4) and 70.4% (± 2.3) on day 7 in respective sham animals. *p<0.05 compared to PSNL days 7 and 14; +p<0.05 compared to sham day 7 (statistical analysis was performed using the two-way ANOVA followed by post-hoc tests where appropriate).
5.4 Discussion

In humans, neuropathic pain is associated with significant co-morbidity, including anxiety and depression, which impact considerably on the overall patient experience and quality of life (Dworkin and Gitlin 1991; Meyer-Rosberg et al. 2001; Worz 2003). To date, reports of investigations of the presence of such co-morbidity behaviour in rodent models of neuropathic pain is limited and somewhat conflicting (Mogil and Crager 2004). This may be because anxiety and depression are heterogeneous disorders with symptoms manifested at the psychological, behavioural and physiological level making them difficult disorders to model in the laboratory. Therefore, in an attempt to improve the clinical validity of a widely used rodent model of traumatic peripheral neuropathy, I employed three well characterised behavioural paradigms to determine whether traumatic peripheral nerve injury-induced neuropathic pain behaviour in male C57BL/6J mice is associated with increased anxiety- and depression-like behaviour.

Firstly, I chose to utilise two well-validated measures of anxiety-like behaviour; the novel open field test and the EPM. As previously discussed, both tests are based upon the fact that small rodents have an innate aversion to exposed, well-lit spaces, probably reflecting predator avoidance in the wild. However, as they are also naturally exploratory animals, this aversion conflicts with a drive to explore novel environments, especially when foraging (Cryan and Holmes 2005). The aversive area takes a different form in each of the tests; open, elevated arms in the EPM and an exposed brightly lit central area in the open field test. It is likely that the differing environments therefore induce different degrees of anxiety, with the EPM tending to be more anxiogenic (Carola et al. 2002). As these tests may therefore detect subtle differences in behaviour, I felt it necessary to investigate behavioural response in both paradigms. In a typical behavioural session, non drug-treated, naïve mice are expected to avoid these aversive areas. This pattern of behaviour has a certain amount of face validity given that anxiety disorders are typified by a pervasive avoidance of a feared situation (Cryan and Holmes 2005). Importantly, these tests are sensitive to clinically employed anxiolytics, including benzodiazepines (Cryan and Holmes 2005). Therefore, these paradigms lend themselves to the investigation of an ongoing pain state which may cause alterations in an animal’s natural pattern of behaviour.

I have demonstrated that although hypersensitivity to punctuate mechanical and cool stimulation was sustained for 28 days following partial sciatic nerve injury, there was no
evidence for an anxiogenic effect following PSNL in both paradigms examined. In fact, mice that demonstrated an increased sensitivity in stimulus-evoked hind limb reflex tests following PSNL tended to demonstrate evidence of relatively lesser anxiety-like behaviour in that they showed more ‘approach’ behaviour into potentially aversive environments. Specifically in the open field test, PSNL-injured animals consistently spent a greater amount of time in the centre of the open field arena as compared to sham animals, and there was evidence for nerve-injured animals to enter the central area more quickly suggesting a heightened exploratory behaviour and decreased risk assessment. Furthermore, PSNL-injured animals spent more time ‘risk assessing’ in the central area of the EPM, however, no clear differences were seen on further measures of anxiety-like behaviour in the EPM. Thus, these findings therefore do not support our hypothesis that ‘traumatic peripheral neuropathy is associated with increased anxiety-like behaviour and that this effect is more pronounced as the chronicity of pain is increased’.

Secondly, I employed a pharmacologically-validated test for assessing depression-like behaviour in mice; the tail suspension test (TST) (Bai et al. 2001; Cryan et al. 2005). This paradigm is based on the fact that animals subjected to the short-term, inescapable haemodynamic stress of being suspended by their tail, will develop an immobile posture. Immobility in the TST is due to inability or reluctance to maintain effort rather than a generalised hypoactivity and reflects a learned helplessness or ‘behavioural despair’ analogous to the clinical observations that depressed patients often lack sustained expenditure of effort reflected in pronounced psychomotor impairments (Cryan et al. 2005). Further, the TST is validated by its sensitivity to clinically effective antidepressants which cause mice to actively and persistently engage in escape-directed behaviours as compared to non-treated controls (Cryan and Holmes 2005; Cryan et al. 2005). However, we found no evidence for such depression-related behaviour in nerve-injured animals in this test. Once again, these findings disprove our original hypothesis that mice displaying significant pain-behaviour will also show signs of co-morbidity depression-related behaviours. Taken together these data may suggest that conventional murine models of pain do not fully recapitulate the range of symptoms displayed by humans suffering from neuropathic pain, at least to the extent emotional symptoms can be measured in mice.

In contrast to my findings, a recent study in C57BL/6J mice showed increased anxiety-like behaviours on the light-dark exploration test and elevated plus-maze four weeks following intraplantar injection of complete Freund’s adjuvant or partial sciatic nerve ligation (Narita
et al. 2006a). The reason for these discrepancies is not clear but may reflect differences in experimental protocols and paradigm design e.g. it is not clear whether separate animals were used at each of the four time points examined; or whether the same animals were re-tested at different time points, which is likely to reduce validity of behavioural testing (Holmes and Rodgers 2003).

Another potentially salient factor in the study of anxiety and pain in mice is the presence of marked strain differences. Data from strain comparison studies in tests of anxiety demonstrate a significant strain effect on baseline anxiety levels (Millstein and Holmes 2006) and a similar strain difference exists for various tests of nociceptive sensitivity (Mogil et al. 1999a; Mogil et al. 1999b). In many tests of anxiety and nociception, the C57BL/6J strain tends to be in the mid-range of behavioural response, which is why I selected it as being broadly representative for these experiments. It would therefore be of interest to further this study by including the assessment of strains which demonstrate greater or reduced baseline sensitivities in these tests. This may then allow for the detection of a correlation between the anxiety and pain behavioural traits thereby indicating the most suitable strain for studies of pain related anxiety behaviour. For example, in the open field and light-dark exploration tests, the most 'anxious' is the 129S1 strain, which tends to be the most sensitive to von Frey hair stimulation at baseline and develops the greatest magnitude of sensitivity following peripheral nerve injury (Millstein and Holmes 2006). In contrast, C57BL/6J mice, used in this study, tend to show markedly less anxiety-like behaviour as compared to 129S1 mice and likewise show less sensitivity to nociceptive tests at baseline and following peripheral nerve injury (Millstein and Holmes 2006). Therefore, this would suggest a correlation between the level of anxiety associated with strain and the nociceptive response to mechanical stimulation. However, this effect varies depending upon the stimulus used. For example, on threshold withdrawal from a heat stimulus (Hargreave’s test), 129S1 mice are less sensitive than C57BL/6J mice but interestingly following peripheral nerve injury, 129S1 mice develop a significantly greater hypersensitivity as compared to C57BL/6J mice (Mogil et al. 1999a). Therefore, it may be that the baseline anxiety state associated with strain has a greater influence on a persistent neuropathic pain state. In contrast, in an alternative test of ongoing pain, the formalin test, 129S1 mice show reduced pain behaviour as compared to C57BL/6J mice (Mogil et al. 1999a). Therefore, these data make it hard to make a direct comparison between anxiety behaviour and general pain responses in mice. It is of interest to note however, that depending on the test used, the strain baseline anxiety behaviour alters. For example, the strain difference between 129S1 and C57BL/6J mice,
which is clear in the open field paradigm, is not apparent in the elevated plus maze (Millstein and Holmes 2006), which again highlights the fact that these tests are likely measuring different forms of behaviour. Of note is that different mice strains may also cope with open field and EPM anxiogenic conditions using different strategies. For example, C57BL/6 mice have been observed to display high locomotor and exploratory activity in these paradigms, whereas BALB/c mice cope by depressing their locomotor activity and anxiously exploring the environment by stretching and sniffing (Carola et al. 2002). This suggests that the strain used must be taken into consideration when modelling human conditions including pain and anxiety.

Another factor to consider is potential species differences between rats and mice. A number of rat models of pain have been shown to be associated with increased anxiety-like behaviour in the open field paradigm (see Chapter 4) (Wallace 2006; Hasnie et al. 2007). Therefore, when assessing the suitability of integrated behavioural paradigms for the detection of pain behaviour, we must consider the suitability of the tests with regard to species as it appears that there is not only a strain-specific but a species-specific effect on anxiety-related behaviour. My findings suggest that mice, though widely used in paradigms of anxiety and depression, may not be a suitable species for the investigation of neuropathic pain co-morbidity. A better species for this purpose may be found in the rat (Wallace 2006; Hasnie et al. 2007). Here, pharmacologically sensitive anxiety-like behaviour was characterised by increased thigmotaxis and reduced entry into the centre of an open field arena.

Moreover, gender difference may have some impact on behavioural tests of anxiety and depression in animals (Palanza 2001). Certainly, it is known that the incidence of major depression is greater among women than men (Breslau et al. 1995; Frackiewicz et al. 2000), and these differences have been found to extend to the rate of occurrence, the course of the illness, and the treatment response (Kornstein 1997). Similarly gender discrepancy has been recognised in anxiety disorders (Palanza 2001). However, the majority of animal studies are performed in male mice, largely because there is thought to be less variability as one does not have to take into account and control for stages in the oestrous cycle. Therefore, it may be of interest to investigate the influence of gender differences in paradigms of anxiety and depression.
In conclusion, I have shown that in contrast to previous findings (Narita et al. 2006a) C57BL/6J mice displaying nerve injury induced pain-like behaviour, as determined by standard hind paw withdrawal reflex tests, do not display increased anxiety-like or depression-like behaviour for up to 4 weeks following injury. Neuropathic mice tended towards a less anxious- or depressed-like state in all paradigms assessed. This behaviour may be a correlate of altered endogenous systems known to be associated with anxiety and stress states such as the opioid or serotonergic systems. However, the involvement of such mechanisms in the behaviour we have tested is yet to be determined and merit further investigation. This does suggest however, that these tests could be utilised to assess the involvement of such anxiety-related mechanisms in the endogenous control of pain and comorbidity behaviour which may point towards potential therapeutic targets.
Chapter 6

Immunohistochemical Characterisation of Rat Dorsal Root Ganglia and Footpad following VZV Infection
6.1 Introduction

6.1.1 Presence of viral proteins in DRG of VZV-infected animals

VZV is present in a latent form in human sensory ganglia following primary varicella infection (Cohrs et al. 2000). Latent infection is demonstrated by the reactivation of virus many years later, and by the presence of viral DNA and proteins in sensory ganglia (as demonstrated at autopsy) (Croen et al. 1988; Mahalingam et al. 1990; Meier et al. 1993; Lungu et al. 1995; Kennedy et al. 1998; Kennedy et al. 1999). Although the term ‘latent’ is also used to describe the presence of VZV in experimental models; in the context of rodent models, in which we see behavioural changes clearly reflecting an active viral interaction with sensory neurones (Fleetwood-Walker et al. 1999; Garry et al. 2005; Hasnic et al. 2007); it may be argued that if the virus was truly latent or dormant, than such behavioural changes could not occur. Therefore, a more appropriate term in the context of animal models might be to describe the virus as resident.

Latent infection with VZV in humans is traditionally characterized by a highly restricted pattern of viral gene expression (Kennedy et al. 2001). Considerable evidence exists to show that viral genes 21, 29, 62, 63 and 66 (with variable evidence for genes 4 and 18) are transcribed during latent VZV infection in human peripheral ganglia (Meier et al. 1993; Mahalingam et al. 1996; Kennedy et al. 1998; Kennedy et al. 1999; Cohrs et al. 2000; Kennedy et al. 2000; Kennedy et al. 2001; Cohrs et al. 2003). The most frequently detected VZV transcript is that mapping to gene 63, which has been proposed as a hallmark of VZV latency (Cohrs et al. 2000; Kennedy et al. 2000). Expression of VZV IE63 protein has additionally been demonstrated in rodent ganglia (Debrus et al. 1995; Kennedy et al. 2001), as well as in the skin of patients with early symptoms of herpes zoster (i.e. during VZV reactivation) (Debrus et al. 1995). This suggests that IE63 protein may play an important role both in the maintenance of latent infection, and in the control of the infectious cycle (Merville-Louis et al. 1989; Sadzot-Delvaux et al. 1990). Further, IE63 is a tegument protein and is thought to be required for the efficient assembly of infectious virus particles (Hoover et al. 2006). This may be important in cell-to-cell spread of the virus during lytic infection in vivo. IE63 protein has also been shown to affect gene transcription, in particular a down-regulation in the transcription of IE62 protein has recently been demonstrated (Hoover et al. 2006). Similarly, VZV IE62 protein is a major component of the VZV virion and a potent viral regulatory protein that is likely to be among the first viral genes expressed.
and translated during infection or reactivation (Kinchington et al. 1992). As a 'transcriptional activator', IE62 stimulates the transcription of further viral genes thus increasing the infectivity of VZV (Gilden et al. 2003b). This typically nuclear protein localises to the cytoplasm of neurones to maintain latent infection. Its presence in the nucleus is therefore indicative of active viral replication (Gilden et al. 2003b).

Viral protein expression, specifically mRNA transcripts for viral regulatory proteins encoded by genes 21, 62 and 63, has previously been demonstrated in the peripheral nervous system of VZV-infected rats (Sadzot-Delvaux et al. 1995; Kennedy et al. 2001). This was recently followed by the immunohistochemical detection of VZV IE63 protein in ipsilateral lumbar DRG of similarly infected animals (Fleetwood-Walker et al. 1999). Further, immunoreactive labelling of VZV IE63 protein was demonstrated in both the cytoplasm and nucleus of sensory neurones and thought to correlate with the observed hypersensitivity to sensory stimuli. These findings are further supported by Garry and colleagues (2005). The authors demonstrated the presence of VZV IE62 protein in the cytoplasm of both small unmyelinated and large myelinated DRG sensory neurones at the peak of rat behavioural reflex sensory sensitivity (i.e. day 28 following VZV infection). In addition, while no significant contralateral effects were observed following VZV infection, some contralateral staining of VZV IE62 was found. Though the mechanisms by which VZV causes behavioural changes following infection in animals are unknown, it is likely that expression of regulatory viral proteins, such as IE62 and IE63, within the infected neurone may affect the physiological function of that neurone and thus its contribution to influencing central nociceptive processing. The detection of viral transcripts in sensory ganglia in animal studies (Sadzot-Delvaux et al. 1995; Fleetwood-Walker et al. 1999; Kennedy et al. 2001; Garry et al. 2005) is consistent with studies examining human sensory ganglia from VZV-infected individuals in which viral transcripts have been detected on polymerase chain reaction (PCR) and in situ hybridisation on autopsy (Croen et al. 1988; Lungu et al. 1995; Kennedy et al. 1998).

In this chapter, the presence of viral proteins IE62 and/or IE63 in infected tissue was investigated using a previously described immunohistochemical technique (Garry et al. 2005). Initially, the presence of viral proteins during lytic infection (i.e. in cell culture) was examined. Specifically, the presence of VZV IE62 protein in ipsilateral lumbar DRG from VZV-infected and control animals was examined, including the influence of viral strain and inoculum concentration. Finally, I analysed the percentage distribution of IE62 protein in DRG sensory neurones following infection with different viral strains and performed a cell
size distribution analysis to determine whether small or large cells were preferentially infected.

6.1.2 Investigation of cutaneous innervation densities in VZV-infected animals

The persistence of zoster-associated pain long after healing of herpetic skin lesions (Watson et al. 1991) is thought to reflect a change in cutaneous innervation density, specifically degeneration or partial degeneration of the sensory neurone's peripheral terminal arbors (Oaklander et al. 1998). To investigate this theory, Oaklander et al., (1998) examined the density of epidermal and dermal neurites from subjects with PHN, and without PHN after unilateral AHZ. Skin biopsies were evaluated from the site of maximum pain or zoster involvement and from the homologous contralateral site. The authors found that those subjects with PHN had a significantly lower density of sensory neurites in herpes zoster-affected epidermis compared with subjects without pain. Also, neurite loss was more severe in the epidermis than the dermis, which is consistent with sensory loss in these patients. The authors also noted that those subjects with pain had additionally lost half of the neurites in the contralateral epidermis despite the lack of contralateral herpetic lesions or pain.

Experimentally, significant loss of intra-epidermal nerve fibres (IENF) in the hind paw glabrous skin has been demonstrated in rat models of chemotherapy-evoked painful peripheral neuropathy (Siau et al. 2006). Using quantitative immunolabeling against the pan-neuronal marker, protein gene product (PGP) 9.5, that enables quantification of individual epidermal axons, the authors confirmed a 23.9% and 44.4% reduction in epidermal innervation in models of paclitaxel- and vincristine-evoked peripheral neuropathy respectively at time of maximum behavioural change. This finding is paralleled by a similar, though greater, loss of epidermal innervation following partial nerve injury (chronic constriction injury model) and complete sciatic nerve transection (67.5% and 94.5% reduction relative to control respectively) (Siau et al. 2006). In the same way, I hypothesised that intra-epidermal innervation of the ipsilateral hind paw glabrous skin of VZV-infected rats would be reduced following viral infection, and employed a similar quantitative immunolabeling technique to examine density (number per millimeter) of cutaneous sensory neurites in virus-infected animals.
6.2 Methods

6.2.1 Detection of VZV IE63 and 62 proteins in culture.

The presence of viral transcripts IE63 and IE62 was initially examined during lytic infection in cell culture. VZV (strain Dumas) was propagated on HeLa cells until cells exhibited approximately 80% cpe. Virus-infected cells were then trypsinised (0.25% Trypsin, 1Mm EDTA, Gibco Invitrogen) causing cells to detach from the tissue culture flask, before being re-suspended in 1ml sterile phosphate buffer solution. The virus-infected cell suspension was aliquoted onto Teflon coated slides (Van Waters Rogers (VWR) International Ltd., Leicester, U.K.) (100 µl/slide) and left to air dry. Slides were then fixed by submerging in acetone for 15 minutes. Immunofluorescent detection of viral protein was performed using a method modified from Garry et al., (2005). Monoclonal (1:200 dilution) or polyclonal rabbit antibodies (1:250 dilution) directed against VZV IE63 protein (kindly supplied by Dr. C. Sadzot-Delvaux, University of Liège, Belgium), as well as a polyclonal antibody directed against VZV IE62 protein (1:250 dilution) (kindly provided by Prof. P. R. Kinchington, University of Pittsburgh, U.S.A.) were examined. The bound primary antibodies were finally visualised using an appropriate fluorescence-labeled secondary antibody (1:400 dilution) (Garry et al. 2005).

6.2.2 Localisation of VZV IE62 protein in rat DRG.

At the end of the behavioural testing period (day 21 – 30 or day 60 post-infection), virus-infected (all experimental groups, refer to chapter 2) and control animals (n = 3 per group) were killed with i.p. pentobarbitone (100mg/kg Animalcare Ltd., York, U.K) and transcardially perfused with 100ml heparinised saline followed by 300ml 4% paraformaldehyde (PFA) solution. DRG from lumbar segments L4 and L5 ipsilateral to the side of VZV infection were harvested and pooled. Tissue was post-fixed in 4% PFA solution for 2 – 4 hours before being placed in 15% sucrose solution overnight. DRG were then transferred to a 30% sucrose solution and left for 24 hours before embedding in Optimal Cutting Temperature (OCT) compound (VWR). Cryostat sections (10 µm) were taken and tissue was thaw-mounted on poly-L-lysine slides (VWR). Randomly selected DRG sections were pre-incubated in buffer (0.1M PBS, pH 7.4, 0.2% Triton X-100, 4% fish skin gelatin) containing 10% normal goat serum for 1 hour at room temperature, before being incubated with a polyclonal primary antibody directed against VZV IE62 protein (1:250 dilution)
(kindly provided by Prof. P. R. Kinchington, University of Pittsburgh, U.S.A.) diluted in buffer (0.1 M PBS, pH 7.4, 0.2% Triton X-100, 2% fish skin gelatin) overnight at 4°C (Garry et al. 2005). Sections were then washed in buffer and incubated with a goat anti-rabbit secondary antibody (1:1000 dilution) (Alexa Flour 568) for 2 hours at room temperature. Following three washes with 0.1M PB, sections were coverslipped with Vectashield mounting medium (Vector Laboratories, Peterborough, U.K.) and visualised under fluorescence microscopy. Negative control sections were processed as above omitting the primary antisera. VZV-infected fibroblasts in culture were used as positive control, while for negative control, primary antibody was omitted. Co-localization with the neurone-specific nuclear protein, NeuN (1:1000 dilution, Chemicon) was additionally performed and only those cells staining NeuN positive were included in the subsequent analysis.

Quantification of images of immunopositive DRG cells was semi-automated using software-based measurement of fluorescence intensity. Using the image analysis and processing software Leica QWin V3 (Milton Keynes, U.K.), a standardised threshold intensity for detection of VZV and NeuN immunoreactive cells was set at 140 - 255 and 130 - 255 grey scale units respectively and applied uniformly to all DRG images captured under identical illumination and exposure conditions. Outlines of DRG cell profiles containing a NeuN labeled nucleus were drawn over each image to produce an overlay of the nucleated cells. Exclusion of non-nucleated immunopositive cells from the size analysis was designed to prevent inaccurate measurements of elliptical-shaped DRG neuronal soma when measured in cross-section. A version of the nucleated cell overlay was modified using threshold intensity measurements to represent the population of immunopositive nucleated cells. Both overlays were used to count and make area and fluorescence intensity measurements of all nucleated DRG cell profiles as well as immunopositive cells within this group. In this way, the overall percentage and cell size distribution of NeuN labeled VZV positive neurones from randomly selected sections was determined for each group. At least 100 NeuN labeled cells were randomly sampled per animal from serial sections 100μm apart. Soma area of VZV positive neurones was also measured using image analysis and the % of NeuN labeled cells positive for VZV IE62 protein was then calculated.
6.2.3 Investigation of cutaneous innervation densities in VZV-infected animals

Ipsilateral hind paws from PFA-fixed virus (Dumas)-infected and control animals (*n* = 4 per group) were harvested at the end of the behavioural testing period (day 21 post-infection). All virus-infected animals used in this experiment displayed significant (paired t-test, *p*<0.05) reduction in ipsilateral PWTs to a punctate mechanical stimulus compared to baseline values (Fig. 6.7). Cryostat sections (15 µm) from a block of glabrous skin excised from the wide part of the plantar hind paw lying distal to the calcaneous and proximal to the digital tori were thaw-mounted on poly-L-lysine slides as described above. Sections were pre-incubated in 0.1M PBS containing 0.2% Triton-X 100 (PBST) containing 10% normal donkey serum for 1 hour at room temperature, and then incubated in polyclonal rabbit anti-human PGP 9.5 primary antibody (1:1000 dilution) (UltraClone Ltd., Isle of Wight, U.K.) diluted in PBST containing 4% normal donkey serum for 24 hours at 4°C. Primary antibody was omitted for negative control; for positive control, refer to Siau et al. (2006). Sections were washed in PBST and incubated in donkey anti-rabbit IgG secondary antibody labeled with Cy3 (1:300 dilution) (Vector Laboratories, U.K.) for 2 hours at room temperature. Sections were finally washed in 0.1M PBS and coverslipped. Slides were masked and randomised to conceal their identity until data acquisition was complete. Quantification of intra-epidermal nerve fibers was performed using a standard imaging fluorescence microscope (images were converted to black and white for ease of analysis) by a single observer blind as to the animals' group assignment. Using a 20x objective, all ascending nerve fibers that were seen to cross into the epidermis were counted; no minimum length was required and fibers that branched within the epidermis were counted as one (Siau et al. 2006). The length of the epidermal border was measured (Leica QWin V3), and intra-epidermal nerve fiber counts were expressed as 'number per millimeter of epidermal border'. Four randomly selected sections were examined per animal and data was combined (i.e. 4 animals per experimental group).
6.3 Results

6.3.1 Immunohistochemical detection of VZV IE63 and 62 proteins in culture.

Immunofluorescence visualization of the IE63 and IE62 proteins revealed staining in the cytoplasm and nuclei of infected cells in culture (Fig. 6.1), which provides support for previous findings (Debrus et al. 1995).

Figure 6.1 Immunofluorescence visualisation of VZV IE63 (green) and IE62 (red) protein in cell culture. Cells exhibited approximately 80% cpe before staining with either A) monoclonal antibody directed against VZV IE63 protein (1:200 dilution); B) polyclonal antibody directed against VZV IE63 protein (1:250 dilution); or C) polyclonal antibody directed against VZV IE62 protein (1:250 dilution). Images were photographed at x20 magnification, scale bar 50 µm.
6.3.2 Immunohistochemical localisation of VZV IE62 protein in rat DRG.

A. PHN Associated strain
   (i) [Image]

B. Viral strain not associated with PHN
   (ii) [Image]  (iii) [Image]

C. Dumas/High cpe
   (iv) [Image]

D. Dumas/Medium cpe
   (v) [Image]

E. Dumas/Low cpe
   (vi) [Image]

F. Ellen strain
   (vii) [Image]

G. OKA Vaccine strain
   (viii) [Image]
Figure 6.2 Immunohistochemical demonstration of VZV IE62 protein (1:250 dilution) in ipsilateral L4/5 DRG following infection with A) viral isolate associated with development of PHN following AHZ infection at (i) x20 magnification (arrow indicates a VZV positive cell), (ii) x40 magnification demonstrating diffuse cytoplasmic staining, (iii) x40 magnification demonstrating intra-nuclear staining; B) VZV isolate not associated with development of PHN following AHZ infection; C) Dumas strain/ high cpe; D) medium cpe; E) low cpe; F) Ellen strain; G) Oka vaccine strain; and H) Strain Dumas, day 60 post-infection (resolution of mechanical hypersensitivity); and I) uninfected fibroblast control. Images were photographed at x20 magnification, scale bar 50 μm. The arrow indicates cells positive for VZV IE62 protein. These cells generally exhibited diffuse cytoplasmic staining.
Figure 6.3 Percentage distribution of viral protein IE62 in DRG sensory neurones following infection with different viral strains (n = 3 animals per group). Using an immunofluorescence technique, ipsilateral L4 and L5 DRG from VZV-infected animals were pooled and stained using a polyclonal antibody directed against VZV IE62 protein (1:250 dilution). Cells were co-labeled with the neurone-specific nuclear protein, NeuN and only those cells demonstrating a nucleus were included in the analysis. The number of NeuN labeled cells is >100 per group, taken from a series of random sections 100 μm apart (number of sections per group therefore varies until at least 100 NeuN labeled cells had been sampled). The percentage of NeuN labeled cells positive for VZV IE62 protein was then calculated and is displayed above the relevant bar for each group.
Figure 6.4 Analysis of DRG sensory neurone cell size distribution of VZV IE62 positive NeuN labeled cells. Animals were infected with A) viral strain associated with development of PHN; B) Dumas strain and C) Ellen strain. Cells were co-labeled with NeuN and only those cells demonstrating a nucleus were included in the analysis. The number of NeuN labeled cells is ≥100 per group, taken from a series of random sections 100 μm apart (number of sections per group therefore varies until at least 100 NeuN labeled cells had been sampled).
6.3.3 Immunocytochemical investigation of cutaneous innervation densities

Figure 6.5 Representative immunofluorescence and monochrome images of PGP 9.5 positive intra-epidermal nerve fibers from the ipsilateral glabrous hind paw skin of A) uninfected fibroblast-injected control; and B) VZV-infected rats 21 days post-infection. (x20 magnification; scale bar 50 μm). Arrow indicates an individual intra-epidermal fibre; yellow dots highlight the dermal-epidermal junction.
Figure 6.6 Quantification of ipsilateral plantar footpad intra-epidermal nerve fibre (IENF) densities (fibres/mm) in uninfected fibroblast (Hel) injected control and VZV-infected animals 21 days post-infection. A) Four randomly selected sections were examined per animal (n = 4 animals per group; • and A represent individual sections from control and virus-infected animals respectively; horizontal bar represents the mean for each group). B) Mean (sem) IENF densities were 27.0 (1.98) and 11.28 (0.46) fibres/mm for control and virus-infected animals respectively. VZV-infected animals exhibited a 58.2% reduction (p<0.05; t-test) in IENF density compared to control animals.
Figure 6.7 Hypersensitivity to static punctate mechanical stimulation developed in the ipsilateral paw of all VZV-infected animals (n = 4), but not in uninfected fibroblast-injected control animals (n = 4). Mean percentage decrease from baseline ipsilateral PWTs at day 21 post-infection was 30.6% (assessed using the electronic von Frey device). *p<0.05, paired t-test compared to baseline PWT.
6.4 Discussion

In this chapter, I have demonstrated not only the presence of viral protein IE62 in sensory neurones following infection with different viral strains, which further supports the findings of Fleetwood-Walker et al., (1999) and Garry et al., (2005), but also the percentage distribution between viral strains, and a cell size distribution analysis identifying VZV in both small and large cells. Moreover, I report the very interesting and novel finding that VZV-induced mechanical hypersensitivity in rats is accompanied by a partial degeneration of the sensory innervation in the epidermis of hind paw glabrous skin.

Detection of viral proteins in DRG of VZV-infected animals

Whilst much evidence supports the detection of VZV exclusively in the cytoplasm and/or nucleus of sensory neurones in infected human ganglia (Gilden et al. 1987; Debrus et al. 1995; Dueland et al. 1995; Kennedy et al. 1998), few studies have demonstrated the presence of the virus in the cytoplasm of non-neuronal satellite cells (Croen et al. 1988; Meier et al. 1993), or in both (Lungu et al. 1995). In this study, diffuse neuronal cytoplasmic staining of VZV IE62 protein was generally observed and demonstrates that the virus is indeed resident in sensory DRG neurones following intra-plantar infection in the rat. Importantly, the specific presence of intra-nuclear IE62 protein in animals infected with the viral strain associated with development of PHN suggests that in these animals active viral replication is taking place. However, the presence of viral proteins was not observed in non-neuronal cells, which is consistent with the majority of the literature. Furthermore, viral protein expression was not demonstrated following resolution of mechanical hypersensitivity (i.e. on day 60 pi), suggesting that a ‘hit and run’-type mechanism of action for sensory sensitivity may be acting. In addition, the presence of viral protein contralateral to infection was rarely observed (data not shown), and only in those animals exhibiting contralateral behavioural changes, which is consistent with previous findings (Garry et al. 2005).

Numerous studies have confirmed the presence of IE62 and/or IE63 viral protein in the DRG of infected rats and in humans (Sadzot-Delvaux et al. 1995; Kennedy et al. 1998; Kennedy et al. 2000; Kennedy et al. 2001; Kennedy 2002b). It would appear from my study that in general there is a association between the amount of viral protein resident in sensory ganglia and the degree of mechanical hypersensitivity exhibited. For example, the percentage of DRG sensory neurones expressing VZV IE62 protein following infection with high cpe (100%: 6 x 10^5 pfu/ml) virus was 72.5%, compared to 27.2% in animals infected with
medium cpe (35%; $8.6 \times 10^3$ pfu/ml), and 8.2% in animals infected with low cpe (15%; $3 \times 10^2$ pfu/ml) virus (Dumas strain). This is consistent with previous observations by Garry and colleagues (2005). The authors found that VZV IE62 was expressed in >80% of myelinated and unmyelinated sensory neurones. However, in my study, an exception to the proposed relationship between amount of viral protein in DRG and degree of mechanical hypersensitivity exhibited is the viral strain not associated with development of PHN. Whilst infection with this strain resulted in a similar degree of hypersensitivity compared to the Dumas strain, the percentage of DRG neurones expressing IE62 protein was only 5.1%, suggesting that it may not be essential for this viral strain to reach the sensory ganglia for a behavioural change to be observed. Further evidence to support the hypothesis that quantity of virus within sensory ganglia is not an important factor for development of virus-induced hypersensitivity phenomena comes from quantitative PCR analysis in human tissue. These studies have found the burden of VZV in DRG during latent infection is indeed low (Mahalingam et al. 1993; LaGuardia et al. 1999; Cohrs et al. 2000; Levin et al. 2003).

Finally, it appears that both small and large DRG cells equally express the viral protein. This is consistent with the observed thermal insensitivity (small fiber neuropathy) and mechanical hypersensitivity (large fiber involvement). Whilst the role of these viral proteins in the generation of a persistent pain state is as yet unknown, the association of VZV IE62 expression with particular subtypes of nociceptive afferents suggests an influence on cellular mechanisms within these afferents.

**Mechanism of VZV-induced behavioural hypersensitivity**

IE62 protein is a major component of the VZV virion (Kinchington et al. 1992). It is a potent viral regulatory protein that is likely to be among the first viral genes expressed and translated during infection or reactivation. IE62 stimulates transcription of further viral genes thereby increasing the infectivity of VZV (Gilden et al. 2003b). However, the precise mechanism(s) by which VZV infection causes the observed behavioural changes in rats is unknown. One possible theory is for ongoing viral replication within ganglion cells. In support of this theory, we demonstrated the intra-nuclear presence of viral IE62 protein, albeit in a minority of DRG neurones. Furthermore, microarray analysis (Chapter 7) identified genes involved in biosynthesis, viral transport, and transcription which suggests that active replication may be occurring. However, the majority of evidence does not favour this theory. Importantly, productive viral replication has not been demonstrated following VZV infection in rats i.e. viral RNA has not been detected and studies have shown an
absence of cpe when known VZV-infected rat DRG neurones are cultured *ex-vivo* (Sadzot-Delvaux et al. 1995). The hypothesis is further challenged by the findings of Dalziel et al., (2004) in which valaciclovir treatment at the time of viral infection in VZV-infected rats was found to have no effect on the development of mechanical hypersensitivity. This suggests that replicating virus (i.e. lytic infection) is not required for the induction of VZV-induced mechanical hypersensitivity. Additionally, pharmacological sensitivity testing using acyclovir in our study has further demonstrated that active viral replication is not necessary for the maintenance of mechanical hypersensitivity (Chapter 3). Furthermore, tissue damage and cell destruction which are characteristic of ongoing viral replication during reactivation of VZV was not seen. Finally, IE62 was consistently detected within the neuronal cytoplasm rather than within nuclei, supporting findings from previous studies (Fleetwood-Walker et al., 1999; Garry et al., 2005), with the premise that active viral replication is generally not taking place.

Another theory underlying VZV-induced hypersensitivity is that the presence of viral regulatory proteins such as IE62 and/or 63 somehow interfere with the physiological function of infected DRG neurones and so influence central nociceptive processing. Certainly, the increased expression of neuropeptides NPY and galanin, and of ATF-3 observed by Garry et al., (2005) (and supported by our PCR microarray validation data, Chapter 7) indicates damage to large-diameter DRG neurones (NPY and galanin), and axonal damage or cell stress (ATF-3). These functional changes may have implications in the generation and/or maintenance of spontaneous ectopic activity and hyperexcitability commonly seen in persistent pain states. Specifically, VZV-induced sensory neurone axonal damage may result in spontaneous activity at different sites along the primary afferent; up-regulation of excitatory adrenergic receptors on primary afferents; or inflammation along the axon and a hyper-excitable state.

Finally, the observed behavioural changes may be due to low grade chronic VZV-induced ganglionitis. This theory is supported by clinical evidence in the field and suggests that inflammatory and neuropathic pain mechanisms may co-exist in PHN (Cohrs et al. 2003). The identification of genes involved in the generation of inflammation i.e. cytokine and chemokine mediated signaling pathways (Chapter 7), and ibuprofen-induced reversal of VZV-induced mechanical hypersensitivity (Chapter 3) also provide support for an inflammatory component to this model.
VZV-induced degeneration of cutaneous sensory afferents

Small thinly myelinated (Aδ) and unmyelinated (C) fibre afferents emerge from the superficial dermal nerve plexus running beneath the epidermis. These small-diameter afferents whose terminals innervate the epidermis and subserve nociception are identified by immunostaining with PGP 9.5, a universal marker for neurones and neuroendocrine cells (Ma and Bisby 2000). I observed a 58.2% reduction relative to uninfected fibroblast-injected control animals in intra-epidermal innervation ipsilateral to viral infection (Figs. 6.5 and 6.6). The magnitude of change is consistent with previous studies examining cutaneous innervation density in experimental models of traumatic- (Basbaum et al. 1991; Munger et al. 1992; Ma and Bisby 2000; Lin et al. 2001; Oaklander et al. 2006a), chemotherapy-induced (Siau et al. 2006), and Human Immunodeficiency Virus (HIV)-associated (Keswani et al. 2006) peripheral neuropathy. This further supports the idea that degeneration of peripheral receptor terminals of somatosensory primary afferent neurones is necessary for the presence of painful peripheral neuropathy.

Loss of IENF (largely Aδ and C fibres) has been documented in PHN (Oaklander 2001), which provides our model with a degree of construct validity, as well as in other small-fibre neuropathic pain syndromes, such as those that accompany diabetes (Sumner et al. 2003), complex regional pain syndrome type-I (Oaklander et al. 2006b), and HIV-associated peripheral sensory neuropathy (Keswani et al. 2006). The functional significance is that these patients suffer pain and deficits in cutaneous pain sensibility. Afferent fibers whose terminal arbors have degenerated develop abnormal spontaneous discharge and hypersensitivity, just as is seen in nociceptors whose axons have been transected. Another aspect of axonal degeneration is sensitisation of neighbouring uninjured afferents. Nociceptor axons that are intact but travel in a peripheral nerve that contains degenerating axons are also known to acquire spontaneous discharge (Wu et al. 2002), and the same phenomenon might occur in terminal arbors that have degenerating neighbours (Siau et al. 2006).

However, the precise mechanism by which VZV causes degeneration of the sensory terminal arbors is not known. In contrast to Wallerian degeneration which underlies ipsilateral losses of distal innervation after axotomy (Djouhri et al. 2006), distal axonal degeneration or "dying back" involves dysfunction of the neuronal cell body, and is a pattern of degeneration commonly seen in small fibre peripheral sensory neuropathies (Siau et al. 2006; Keswani et al. 2006). In the experimental model of chemotherapy-induced painful peripheral
neuropathy, an increased incidence of abnormal mitochondria in sensory axons (Flatters and Bennett 2006) has led to the hypothesis that impaired mitochondrial function may be responsible for degeneration of the axons' terminal arbors (Siau et al. 2006). This theory is supported by pharmacological studies using acetyl-L-carnitine, which has been shown to improve the function of impaired mitochondria, promoting regeneration following nerve injury (Virmani et al. 2004), and preventing paclitaxel- and vincristine-evoked neuropathic pain (Flatters et al. 2006). Thus, it may be that viral specific proteins induce dysfunction of the neuronal cell body and hence distal axonal degeneration through a mechanism that involves mitochondrial dysfunction. The hypothesis for a role for abnormal mitochondria is further supported by evidence relating to diabetic sensory neuropathy, in which this small fibre distal neuropathy is associated with mitochondrial dysfunction and furthermore, with the formation of reactive oxygen species that are observed with activation of the apoptosis cascade (Guo et al. 2004). Importantly, we have demonstrated that there is disregulation of apoptosis-related genes following VZV infection in rats (Chapter 7), and so this presents an attractive hypothesis for VZV-induced IENF loss. Alternatively, a mechanism in which viral specific proteins induce localized axonal toxicity with loss of normal receptor function in the distal axon may be responsible. Since mechanical hypersensitivity is characteristic of rodent VZV infection, the loss of IENF observed in these animals is likely to include Aδ- and C-mechanosensitive nociceptor subtypes. Additionally, partial degeneration of heat-responsive mechanosensitive C-fibres could explain the relative thermal insensitivity seen in these animals.

A potential limitation of our study is that the hind paw contralateral to viral infection was not examined. This would be of interest since examination of skin biopsies using PGP 9.5 immunolabeling has demonstrated that patients with PHN after unilateral AHZ had lost half of the innervation in clinically asymptomatic mirror-image contralateral skin (Oaklander et al. 1998). However, contralgesional neurite loss in PHN patients appears to be without contralateral behavioural correlate because these patients rarely experience bilateral pain after AHZ (Oaklander et al. 1998; Oaklander and Brown 2004). Interestingly, the severity of pain in patients suffering from ipsilateral PHN correlates significantly with the magnitude of contralateral neurite loss (Oaklander et al. 1998). This suggests that contralateral neurite loss may be influenced by the perception of ipsilateral pain (Oaklander and Brown 2004). Contralgesional neurite loss is further supported by evidence from the cadaveric examination of PHN patients (Watson et al. 2000). Watson and colleagues described damage, less severe than that observed ipsilaterally, comprising degeneration of large-diameter fibers,
demyelinated axons, and many thinly myelinated axons, thought to indicate earlier axonal degeneration and regeneration. This was found to occur in nerve trunks and roots and provides evidence that contralesional damage is present proximally as well as distally. These distal changes might also affect central axon terminals of contralesional primary sensory neurones and contribute to deafferentation. VZV-induced contralesional damage was initially attributed to an undetected spread of virus into the spinal cord, or may reflect low level systemic infection/inflammation. However, there is considerable evidence for contralesional effects after unilateral experimental nerve injuries where there is no infection (Koltzenburg et al. 1999). For example, Oaklander and Brown (Oaklander and Brown 2004) recently demonstrated significant and sustained bilateral loss of distal innervation following unilateral nerve injury, specifically a 54% contralesional loss in epidermal innervation following unilateral tibial nerve transection. This suggests that contralesional loss of distal neurites is initiated by ipsilateral axonal injury alone and is likely to involve transcellular signals that link homologous neurones on opposite sides of the body.

In line with IENF loss, PGP 9.5 has been found to be increased in Langerhans cells (LC) in rats after sciatic nerve transection (Hsieh et al. 1996), chronic constriction injury (Lin et al. 2001), and chemotherapy-evoked peripheral neuropathy (Siau et al. 2006). LC are highly branched dendritic macrophages of the mammalian epidermis and have close appositions and thus important interactions with cutaneous neurites (Oaklander et al. 2003). Activated LC display increased synthesis of proinflammatory cytokines. Such mediators are known to sensitize and evoke ectopic spontaneous discharge in intact C-fibre nociceptors, and may represent an additional mechanism for hypersensitivity following distal axonal degeneration (Oaklander et al. 2003; Siau et al. 2006). With regard to VZV, after reactivation the virus is transported distally along sensory axons to produce clusters of cutaneous inflammation, vesicles, and finally necrosis in the skin innervated by the affected neural segment. Within the affected sensory ganglion, the infected neuronal cell bodies degenerate, causing degeneration of their central and peripheral axons as well (Oaklander et al. 2003). The presence of virus in skin and nerves causes recruitment and activation of LC leading to the hypothesis that sensitisation of nociceptive neurites in the skin continues to be a major mechanism of persisting pain (long after disappearance of zoster lesions), particularly for mechanical allodynia in PHN (Oaklander et al. 2003). It would appear then that LC are either not involved, that their involvement is not associated with long-term changes in their numbers, or that their functioning might be altered without proliferating. Alternatively, other
cutaneous cells such as keratinocytes could be the main source of neurite sensitisation. Thus it would be of interest to examine LC in the epidermal skin of VZV-infected animals.

Finally, to further support my initial findings in VZV-infected animals, electron microscopy would allow morphology of the distal plantar nerves to be examined, specifically Remak bundle morphology. A Remak bundle consists of multiple unmyelinated axons ensheathed within a single basal lamina of a Schwann cell. This would be of interest since in an experimental model of anti-retroviral drug (didanosine)-treated gp120 transgenic mice, in which a significant reduction in IENF density was also reported, denervated Schwann cells with empty pockets of collagen within the basal lamina were observed in the distal plantar nerves; indicative of loss of unmyelinated axons (Keswani et al. 2006). Electron microscopy would also allow such observations to be quantified.
Chapter 7

Microarray Gene Profiling
7.0 Introduction

Although the sensory, pharmacological and biochemical features of neuropathic pain in animal models is well characterised; knowledge of the dysfunctional gene expression in these models is still in its infancy. Microarray-based gene expression profiling is an emerging technology that is revolutionising the rate at which information about gene expression can be collected. Until recently, our understanding of the genomics of pain has been led largely by molecular genetic techniques such as the creation of transgenic and knock-out animals. However, these traditional techniques have a number of limitations, notably as single gene assays they do not have the capacity to analyse parallel changes in many thousands of genes at one time (Mogil and Grisel 1998; Mogil 2005). This represents an important limiting factor since it is likely that the complex molecular basis of pain is regulated by a number of genes rather than one candidate gene.

Microarrays are a powerful biological tool that offers efficient genome-wide screening and, as such, has allowed significant advances in the identification of pain relevant genes. Patterns of gene expression have been investigated in various animal models of neuropathic pain, including spinal nerve ligation/transsection (Wang et al. 2002; Valder et al. 2003; Lacroix-Fralish et al. 2006); peripheral axotomy (Costigan et al. 2002; Xiao et al. 2002; Li et al. 2002; Kubo et al. 2002; Yang et al. 2004); and peripheral diabetic sensory neuropathy (Burnand et al. 2004; Price et al. 2006). These studies have identified several hundred differentially expressed genes (including those genes encoding ion channels, signaling peptides, neurotransmitters, vesicular proteins, structural molecules, and receptors), aiding the characterisation of some of the complex pathways that are triggered by nerve insult. However, these studies do not distinguish which genes are directly related to the hypersensitivity phenomena observed in these models from those that are connected with other aspects of the response to a nerve lesion. Nevertheless, the global overview obtained from microarray studies has made it possible to find patterns of gene regulation, and to identify specific genes or groups of genes that show distinct patterns of expression between experimental conditions (Reilly et al. 2004). Thus, the information gained from microarray experiments can be used to look for candidate genes, including novel genes involved in nociception and pain, and will provide an initial screen for future studies (Reilly et al. 2004).

In this chapter, an oligonucleotide Affymetrix Microarray approach was used to globally investigate changes in DRG gene expression associated with VZV infection in the rat. Differential gene expression was additionally profiled in the rat SNT model of neuropathic...
pain and changes common to both pain models were compared. Whilst both models share mechanical hypersensitivity, they represent different ways of insulting a nerve; and since the zoster-associated pain model involves minimal surgery this allows, by comparison with data from a traumatic peripheral nerve injury model, refinement of those genes associated with the development of neuropathic pain. Further, an RNA amplification strategy was used, making it possible to correlate gene expression changes with behaviour in small groups of animals. Finally, two novel targets relevant to zoster-associated pain were identified and their protein products validated using both semi-quantitative polymerase chain reaction (PCR) and immunohistochemistry (IHC).

Microarray experiments require the input of many disparate skills and disciplines and, as such, are inevitably of a multidisciplinary nature. The London Pain Consortium (LPC) (www.lpc.ac.uk) is running a major project devoted to the determination of DRG gene expression changes associated with peripheral nerve injury and bioinformatic analysis of these data. Therefore, much of the work in this study was performed in collaboration with other members of the LPC. Specifically, microarray target preparation (RNA amplification) was performed by Dr. R. Hosseini (University College London), array hybridisation was performed by Dr. N. Jina (Institute of Child Health), while data analysis was performed by Dr. K. Maratou (Institute of Child Health). I was directly responsible for surgery; behavioural characterisation including the supervision of T. Pheby (Imperial College) who performed behavioural assessment of SNT-operated animals; the extraction of RNA from DRG, under supervision of Dr. R. Hosseini; and immunohistochemical target validation. Finally, Dr. K. Okuse (Imperial College) was responsible for PCR target validation including the design of oligonucleotide primers for potential targets.
7.1 Methods

7.1.1 Animals and surgical procedure

Experiments were performed on adult male Wistar rats with a mean weight of 305g (range 250 - 330g) (B&K, Hull, U.K.) in accordance with British Home Office regulations. VZV infection (strain Dumas, 80% mean cpe) and SNT surgery, including respective sham procedures, were performed as previously described (Chapters 2 & 4). Animals were housed in individually ventilated colony cages and maintained on a 14:10 hour light/ dark cycle with free access to food and water.

7.1.2 Stimulus-evoked reflex withdrawal testing

Baseline thresholds for hind-paw withdrawal in response to punctate mechanical stimulation were measured using graded nylon von Frey monofilaments (Alan Ainsworth, London) as previously described (Chapter 2). Animals were re-tested on day 14 post-surgery/infection to establish development of mechanical hypersensitivity following nerve injury, and only animals demonstrating a significant (paired t-test, \( p<0.05 \)) reduction in ipsilateral PWT compared to baseline values were retained for microarray analysis. Day 14 was preferred as this was found to be the time at which maximal mechanical hypersensitivity was reliably established among animals. The experimenters (Dr. F. Hasnie and T. Pheby) were ‘blinded’ to the individual animal groups during testing.

7.1.3 Harvesting of DRG and RNA extraction

Animals were killed with i.p. pentobarbitone (100 mg/kg Animalcare Ltd., York, U.K.). DRG from lumbar segments L4 and L5 on each side were immediately harvested and snap frozen in liquid nitrogen. In order to prevent additional and unwanted changes in gene expression, dissection on ice with minimal tissue damage and time to extraction was undertaken. Importantly, transection of the L5 nerve route in the SNT model was confirmed on post-mortem and only these animals were included in the microarray analysis. Total RNA was then extracted from previously frozen tissue using the RNasy Mini Kit (Qiagen, U.K.) according to the manufacturer’s protocol. Ipsilateral L4 and L5 DRG from VZV-infected (\( n = 8 \) animals; 2 animals per microarray GeneChip®) or uninfected fibroblast-injected animals (\( n = 8 \); 2 animals per chip) were pooled, while lumbar DRG from SNT- (\( n = 16 \); 4 animals...
per chip) or sham-operated (n = 16; 4 animals per chip) animals were kept separate (n = 4 DRG in total per chip). This is because the L4 DRG in the SNT model represents an 'uninjured' data set compared to the transected L5 DRG, and allows the biological response to the injury to be compared to adjacent potentially uninjured neurones. In comparison, the VZV model does not discriminate between an L4 and L5 injury, therefore these DRG were pooled. After isolation, RNA quality was verified by spectrophotometry. Quality control is important to ensure the generation of high quality microarray data (Dumur et al., 2004a). The quantity of RNA in each sample was then determined by measuring optical densities using a spectrophotometer.

7.1.4 Overview of microarray experimental protocol and design

A validated Affymetrix approach using two cycle RNA amplification was employed for target preparation (Fig. 7.1) (Gold et al. 2004; Wilson et al. 2004; Dumur et al. 2004). Briefly, single stranded cDNA was synthesised from extracted RNA samples using reverse transcriptase and an oligo-dT promoter primer. A two cycle cRNA amplification protocol (Affymetrix Small Sample Labeling Protocol vII) was then employed which produced amplified amounts of biotin-labeled complementary RNA (referred to as cRNA). To facilitate efficient and reproducible hybridization, cRNA was first fragmented to 25 - 200 base pair fragments before being hybridised to the Affymetrix Rat Genome arrays (GeneChip® Rat Genome 230 2.0). These chips provide comprehensive coverage of the rat genome as they comprise more than 31,000 probe sets, analysing over 30,000 transcripts and variants from over 28,000 well-substantiated rat genes. Chips were then stained with streptavidin-phycoerythrin, a fluorescent molecule that binds to biotin; and the signal was further amplified. GeneChip® arrays were then scanned with a confocal laser and the distribution pattern of the signal in the array recorded. Images were processed using the Affymetrix GeneChip® Operating Software (GCOS) and data analysed using the Bioconductor package Limma (v1.6) and GeneSpring (v7.2) software.

The microarray experiment was conducted at one time point post-injury (i.e. day 14) and consisted of two conditions per model (i.e. treated animal versus sham) with four replicates per condition (i.e. eight GeneChips: Chips 1 - 4 treated animal; Chips 5 - 8 sham).
Figure 7.1 GeneChip® assay overview employing two cycle amplification (Modified from http://www.ohsu.edu/gmst/amc/amc_technology.html)
7.1.5 Data analyses

Microarray data analysis was performed by Dr. K. Maratou using R v2.3.1 and Bioconductor v1.8 packages as follows: Quality control tests and RMA (Robust Multichip Average) data normalisation was performed on the raw data using SimpleAffy and Affy. The normalised data was filtered, using Genefilter, to remove probe sets with minimal expression levels (i.e., probe sets failing to have a signal higher than log2(100) in 3 or more arrays). Statistical analysis was initially performed using Limma moderated F-statistic with multiple testing correction (fdr). Fdr is a measure of the false discovery rate (i.e. it measures the percentage of genes that are not true positives and therefore is a test of specificity). Initially, fdr less than 0.05 was used to control for multiple testing. However, no significant alteration in expression of probe sets was observed in the VZV model. Therefore, a less stringent criterion using Bayes moderated t-statistic ($p<0.05$) without multiple testing correction was used to identify potentially important genes. (The corresponding fdr at this $p$ value was calculated to be 0.135 and 0.4 for SNT and VZV models respectively). Lists of statistically significant genes were then loaded into GeneSpring (v7.2) software (Agilent Technologies, Cheshire, U.K.) where a second filter to reduce false positive results by removing genes with subtle changes in gene expression (<1.2 fold) was applied. We chose a 1.2 fold change, which is a moderate cut-off, to signify differential expression, because the two cycle amplification protocol used in this study may suppress fold differences (see discussion). Thus, probe sets with at least a 1.2 fold change and $p<0.05$ between sham and treated groups were considered significant. Finally, Venn diagrams were used to cross-compare data between models.

7.1.6 Functional annotation analysis

To identify important biological pathways within the lists of significant probes for each model, Dr K. Maratou used the program MAPPFinder (v2.0) (Doniger et al. 2003), which is part of the GenMAPP (v2.1) application package (Dahlquist et al. 2002), and dynamically links gene-expression data to known gene ontology (GO) hierarchy terms. For each term, MAPPFinder calculates a set of percentages for genes specifically associated with that term; a second set of percentages for the total number of genes associated with that term and all of its sub-categories; and a statistical score ($z$ score). MAPPFinder requires a user-defined criterion for a meaningful gene-expression change and the criteria used were $p$ value $<0.05$ with fold change $>1$ for significant gene expression increase and $p$ value $<0.05$ with
fold change <1 for significant decrease in gene expression. To ease the interpretation of results, the output data were manually filtered to remove terms that represented the same genes. For a process to be included in the results, it was required that the z score was ≥2 and that at least one gene changed significantly. Also, terms that a) comprised of 5 or less genes; or b) had more than 100 genes changed (nested results) were removed, because they were either too specific or too general for the data interpretation.

7.1.7 Microarray validation

Separate VZV-infected animals were used for microarray target validation studies. This was to show that the microarray data could be biologically replicated, rather than simply confirming that the technique was accurate by using the same set of animals. Two novel targets relevant to zoster-associated pain were identified from the microarray analysis and their protein products validated using both semi-quantitative PCR (performed by Dr. K. Okuse) and immunohistochemistry (IHC).
7.2 Results

7.2.1 Microarray experiment

Behavioural reflex sensitivity

On day 14 post-infection/surgery, all VZV-infected (n = 8) and SNT-operated animals (n = 16), but not uninfected fibroblast-injected (n = 8), nor sham-operated animals (n = 16), displayed hypersensitivity to punctate mechanical stimulation ipsilateral to the injury: mean percentage decrease from baseline ipsilateral PWT as assessed using graded von Frey monofilaments was 60.6% and 71.9% respectively (Fig. 7.2). Significant differences (p<0.05) in ipsilateral response thresholds were also observed when compared to contralateral and to sham ipsilateral paw withdrawal values. Behavioural data for individual microarray GeneChips from VZV-infected and SNT-operated animals is illustrated (Fig. 7.3). In five SNT-operated animals, the correct spinal nerve route was not transected on post-mortem examination; therefore these animals were excluded from further study.

![Figure 7.2](image-url)

**Figure 7.2** Punctate mechanical hypersensitivity in VZV-infected (n = 8) and SNT-operated (n = 16), but not sham animals in the ipsilateral paw 14 days after infection/surgery. Mean percentage decrease from baseline ipsilateral PWT was 60.6% and 71.9% respectively. *p<0.05, paired t-test compared to baseline PWT
Figure 7.3 Behavioural data for individual microarray GeneChips as assessed using graded von Frey monofilaments from A) VZV-infected (n = 2 per chip); B) uninfected fibroblast-injected (n = 2 per chip); C) SNT-operated (n = 4 per chip); and D) sham-operated animals (n = 4 per chip). *p<0.05, paired t-test compared to baseline PWT.
Functional annotation analysis

MAPFinder analysis of the statistically significant ($p<0.05$) disregulated genes identified for each model a wide variety of significant ('z' score $\geq 2$) GO terms. For ease of interpretation these terms are presented according to (a) biological process; (b) molecular function; and (c) cellular location. The lists of significant GO terms for the VZV model are illustrated in Tables 7.1 and 7.2, while Tables 7.3 (A & B) and 7.4 (A & B) illustrate the significant GO terms for the SNT model (terms identified in L4 and L5 DRG are listed separately). GO term lists are presented in order of 'z' score, with the most significant being listed first. In the SNT model, 27% of the significantly up- and down-regulated genes were linked to a GO term; while in the VZV model, 37% of the significantly up- and 27% of the significantly down-regulated genes was linked to a GO term.
Table 7.1: Gene Ontology terms associated with Up-Regulated Genes in the VZV Model

(a) Classification According to Biological Process

<table>
<thead>
<tr>
<th>Term</th>
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<tbody>
<tr>
<td>Cytokine and chemokine mediated signaling pathway</td>
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<tr>
<td>Apoptosis</td>
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<tr>
<td>Regulation of apoptosis</td>
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<tr>
<td>Vesicle-mediated transport</td>
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<tr>
<td>Cytoskeleton organisation and biogenesis</td>
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<tr>
<td>Development</td>
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<tr>
<td>Cartilage development</td>
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<tr>
<td>Anti-apoptosis</td>
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<tr>
<td>Regulation of synapse structure and function</td>
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<tr>
<td>Peptidoglycan metabolism</td>
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<tr>
<td>Base-excision repair</td>
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<tr>
<td>Response to organic substance</td>
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<tr>
<td>Regulation of cell growth</td>
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<tr>
<td>Protein amino acid dephosphorylation</td>
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<tr>
<td>Neurone differentiation</td>
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<tr>
<td>Protein kinase cascade</td>
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<tr>
<td>Cell-cell signaling</td>
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<tr>
<td>Cell differentiation</td>
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<tr>
<td>Negative regulation of cell growth</td>
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<tr>
<td>Wnt receptor signaling pathway</td>
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<tr>
<td>Regulation of growth</td>
</tr>
<tr>
<td>RNA splicing</td>
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<tr>
<td>Synaptic transmission</td>
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<td>B cell activation</td>
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<tr>
<td>Sensory perception</td>
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<tr>
<td>Protein amino acid phosphorylation</td>
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<td>Regulation of exocytosis</td>
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<td>Embryonic development</td>
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(b) Classification According to Molecular Function

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<th>Term</th>
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<tr>
<td>Latrotoxin receptor activity</td>
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<td>Single-stranded DNA binding</td>
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<td>Hyaluronic acid binding</td>
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<tr>
<td>Signal transducer activity</td>
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<td>Mannose binding</td>
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<td>Protein tyrosine phosphatase activity</td>
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<tr>
<td>Voltage-gated calcium channel activity</td>
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<td>Steroid hormone receptor activity</td>
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<td>Transcription regulator activity</td>
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<td>GTP binding</td>
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<tr>
<td>Receptor activity</td>
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<tr>
<td>Protein binding</td>
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<td>GTPase activity</td>
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<tr>
<td>Calcium channel regulator activity</td>
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<tr>
<td>C-X-C chemokine receptor activity</td>
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<td>Angiotensin type II receptor activity</td>
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<tr>
<td>Motor activity</td>
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<td>Phosphoprotein phosphatase activity</td>
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<td>Phosphotransferase activity</td>
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(c) Classification According to Cellular Location

- Dynemin complex
- Synapse
- Postsynaptic membrane
- Perinuclear region
- Cytoskeleton
- Microtubule cytoskeleton
- Protein serine / threonine phosphatase complex
- Protein phosphatase type 2A complex
- Microtubule
<table>
<thead>
<tr>
<th>Classification According to Biological Process</th>
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<tbody>
<tr>
<td>Iron ion transport</td>
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<td>Nervous system development</td>
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<td>Neurotransmitter secretion</td>
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<td>Myelination</td>
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<td>Cell adhesion</td>
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<td>Copper ion transport</td>
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<td>Glutamine metabolism</td>
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<td>Response to toxin</td>
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<td>Protein amino acid prenylation</td>
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<td>Superoxide metabolism</td>
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<td>Protein amino acid glycosylation</td>
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<td>Steroid biosynthesis</td>
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<td>Cell-matrix adhesion</td>
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<th>Classification According to Molecular Function</th>
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<tr>
<td>Copper ion transporter activity</td>
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<td>Cytokine binding</td>
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<td>Voltage-gated chloride channel activity</td>
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<tr>
<td>C-X-C chemokine receptor activity</td>
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<td>Receptor inhibitor activity</td>
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<td>Protein-hormone receptor activity</td>
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<td>Taste receptor activity</td>
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<td>C-C chemokine receptor activity</td>
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<tr>
<td>Haematopoietin/interferon-class (D200-domain) cytokine receptor activity</td>
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<tr>
<td>Protein prenyltransferase activity</td>
</tr>
<tr>
<td>Transferase activity, transferring hexosyl groups</td>
</tr>
<tr>
<td>Cobalt ion binding</td>
</tr>
<tr>
<td>Phosphopantetheine binding</td>
</tr>
<tr>
<td>MAP kinase activity</td>
</tr>
<tr>
<td>Galactosyltransferase activity</td>
</tr>
<tr>
<td>Transferase activity, transferring glycosyl groups</td>
</tr>
<tr>
<td>Amino acid-polyamine transporter activity</td>
</tr>
<tr>
<td>ATP binding</td>
</tr>
<tr>
<td>Prenyltransferase activity</td>
</tr>
<tr>
<td>Protein serine / threonine kinase activity</td>
</tr>
<tr>
<td>Rhodopsin-like receptor activity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Classification According to Cellular Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrin complex</td>
</tr>
<tr>
<td>Transcription factor complex</td>
</tr>
</tbody>
</table>
Table 7.3 A: Gene Ontology terms associated with Up-Regulated Genes in the L5 DRG of SNT Animals

(a) Classification According to Biological Process

<table>
<thead>
<tr>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to biotic stimulus</td>
</tr>
<tr>
<td>Defence response</td>
</tr>
<tr>
<td>Protein biosynthesis</td>
</tr>
<tr>
<td>Immune response</td>
</tr>
<tr>
<td>Complement activation</td>
</tr>
<tr>
<td>Antigen presentation</td>
</tr>
<tr>
<td>Antigen processing</td>
</tr>
<tr>
<td>Antigen processing</td>
</tr>
<tr>
<td>Response to wounding</td>
</tr>
<tr>
<td>Feeding behaviour</td>
</tr>
<tr>
<td>Cytolysis</td>
</tr>
<tr>
<td>Complement activation</td>
</tr>
<tr>
<td>Striated muscle development</td>
</tr>
<tr>
<td>Response to stress</td>
</tr>
<tr>
<td>Antigen presentation</td>
</tr>
<tr>
<td>Phosphate transport</td>
</tr>
<tr>
<td>Response to virus</td>
</tr>
<tr>
<td>Chitin catabolism</td>
</tr>
<tr>
<td>Dicarboxylic acid transport</td>
</tr>
<tr>
<td>Neurotransmitter biosynthesis</td>
</tr>
<tr>
<td>Transforming growth factor beta receptor signaling pathway</td>
</tr>
<tr>
<td>N-acetylglucosamine metabolism</td>
</tr>
<tr>
<td>Nucleosome assembly</td>
</tr>
<tr>
<td>Inflammatory response</td>
</tr>
<tr>
<td>Cell adhesion</td>
</tr>
<tr>
<td>L-phenylalanine catabolism</td>
</tr>
<tr>
<td>Peptide cross-linking</td>
</tr>
<tr>
<td>Hormone biosynthesis</td>
</tr>
<tr>
<td>Regulation of immune response</td>
</tr>
<tr>
<td>Transcription from RNA polymerase II promoter</td>
</tr>
<tr>
<td>C21-steroid hormone biosynthesis</td>
</tr>
<tr>
<td>Proteolysis</td>
</tr>
<tr>
<td>Transmembrane receptor protein serine/threonine kinase signaling pathway</td>
</tr>
<tr>
<td>Embryonic development</td>
</tr>
<tr>
<td>Regulation of transcription from RNA polymerase II promoter</td>
</tr>
<tr>
<td>Negative regulation of cell proliferation</td>
</tr>
<tr>
<td>Haemopoiesis</td>
</tr>
<tr>
<td>Anion transport</td>
</tr>
<tr>
<td>DNA replication</td>
</tr>
<tr>
<td>Blood coagulation</td>
</tr>
<tr>
<td>Metabotropic glutamate receptor signaling pathway</td>
</tr>
<tr>
<td>Aromatic amino acid family metabolism</td>
</tr>
<tr>
<td>Regulation of ossification</td>
</tr>
</tbody>
</table>

(b) Classification According to Molecular Function

<table>
<thead>
<tr>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural constituent of ribosome</td>
</tr>
<tr>
<td>MHC class II receptor activity</td>
</tr>
<tr>
<td>rRNA binding</td>
</tr>
</tbody>
</table>
RNA binding
Extracellular matrix structural constituent

Chymotrypsin activity
Receptor binding
Cytokine activity
Chitinase activity
Sodium dicarboxylate symporter activity
Double-stranded DNA binding
Cysteine-type endopeptidase activity
Exonuclease activity
Caspase activity
G-protein-coupled receptor binding
Trypsin activity
Hydrolase activity
Pancreatic ribonuclease activity
Tumor necrosis factor receptor binding
Protein tyrosine phosphatase activity
Peptidase activity
Monoxygenase activity
Protein kinase C binding
NAD+ ADP-ribosyltransferase activity
Oxidoreductase activity
Cysteine-type peptidase activity
Chemokine activity
Protein heterodimerization activity
Receptor activity
Hydrolase activity

(c) Classification According to Cellular Location

Ribonucleoprotein complex
Ribosome
Extracellular region
Extracellular matrix
Extracellular matrix
Large ribosomal subunit
Extracellular space
Nucleosome
Collagen
Basolateral plasma membrane
Chromosome
Chromatin
Table 7.3 B: Gene Ontology terms associated with Up-Regulated Genes in the L4 DRG of SNT Animals

(a) Classification According to Biological Process

Cytolysis
Immune response
Antigen presentation
Antigen processing
Hormone biosynthesis
Response to wounding
Collagen catabolism
Odontogenesis
Inflammatory response
Feeding behaviour
C21-steroid hormone biosynthesis
Gamma-aminobutyric acid signaling pathway
Complement activation, classical pathway
Regulation of immune response
Protein amino acid dephosphorylation
L-phenylalanine catabolism
Peptide cross-linking
Acute-phase response
Cell-cell signaling
Signal transduction
Fatty acid beta-oxidation
Positive regulation of cell proliferation
Chloride transport
Striated muscle contraction
Cell wall catabolism
Neuropeptide signaling pathway
G-protein coupled receptor protein signaling pathway
Chemotaxis
Response to temperature stimulus
Urea cycle
Prostaglandin biosynthesis
Fatty acid transport
Cell surface receptor linked signal transduction
Cell proliferation
Sensory perception
L-serine biosynthesis
Blood pressure regulation
Aromatic amino acid family metabolism
Defence response to bacteria

(b) Classification According to Molecular Function

MHC class II receptor activity
G-protein-coupled receptor binding
Protein tyrosine phosphatase activity
Neurotransmitter receptor activity
Signal transducer activity
Lysozyme activity
Neuropeptide Y receptor activity
Hormone activity
Chemokine activity
GABA-A receptor activity
Cytokine activity
3',5'-cyclic-nucleotide phosphodiesterase activity
Receptor activity
Hyaluronoglucosaminidase activity
Glucuronosyltransferase activity
Calcium-dependent phospholipid binding
Oxidoreductase activity
Growth factor activity
Phosphoinositide phospholipase C activity
Extracellular ligand-gated ion channel activity
Sugar binding
Structural constituent of ribosome
Calcium- and calmodulin-dependent protein kinase activity
Hyaluronic acid binding
Tumour necrosis factor receptor binding
Protein binding
Olfactory receptor activity
Enzyme inhibitor activity
Chymotrypsin activity
Metalloendopeptidase inhibitor activity
Purinergic nucleotide receptor activity
Unspecific monooxygenase activity

(c) Classification According to Cellular Location

Extracellular region
Extracellular space
Basolateral plasma membrane
Striated muscle thick filament
Ribosome
Postsynaptic membrane
Extracellular matrix
Microtubule organizing centre
Nicotinic acetylcholine-gated receptor-channel complex
Table 7.4 A: Gene Ontology terms associated with Down-Regulated Genes in the L5 DRG of SNT Animals

(a) Classification According to Biological Process

- Ion transport
- Potassium ion transport
- Metal ion transport
- Cation transport
- Sodium ion transport
- JNK cascade
- Neurite development
- Cell-cell signaling
- Synaptic transmission
- Neurone differentiation
- Exocytosis
- Synaptic vesicle exocytosis
- Neurotransmitter secretion
- Nervous system development
- Calcium ion-dependent exocytosis
- Response to reactive oxygen species
- Axon guidance
- Chromatin silencing
- Axonogenesis
- Amino acid transport
- Visual perception
- Vesicle-mediated transport
- Microtubule-based process
- Cell motility
- Protein kinase C activation
- Notch signaling pathway
- Calcium ion transport
- Cell differentiation
- Regulation of neurotransmitter levels
- Cytoskeleton organization and biogenesis
- Protein amino acid phosphorylation
- Response to oxidative stress
- Endocytosis
- Sensory perception
- Response to hypoxia
- Development
- Superoxide metabolism
- Tissue development
- Central nervous system development
- Regulation of exocytosis
- Cell surface receptor linked signal transduction

(b) Classification According to Molecular Function

- Ion channel activity
- Cation channel activity
- Voltage-gated ion channel activity
- Potassium ion binding
- Potassium channel activity
Cation transporter activity
Extracellular ligand-gated ion channel activity
Voltage-gated potassium channel activity
Diacylglycerol kinase activity
Receptor activity
Kinase activity
Calcium ion binding
MAP kinase activity
Glutamate-gated ion channel activity
Ionotropic glutamate receptor activity
Nicotinic acetylcholine-activated cation-selective channel activity
Transmembrane receptor protein tyrosine kinase activity
Receptor signaling protein activity
Microtubule motor activity
Calcium- and calmodulin-dependent protein kinase activity
Phosphotransferase activity
Antiporter activity
Metabotropic glutamate receptor activity
Motor activity
Calcium channel activity
Protein kinase activity
Voltage-gated sodium channel activity
cAMP-dependent protein kinase regulator activity
Carbonic anhydrase activity
Sialyltransferase activity
Lipid binding
Amino acid transporter activity
Disulfide oxidoreductase activity

(c) Classification According to Cellular Location

Integral to plasma membrane
Plasma membrane
Synapse
Voltage-gated potassium channel complex
Synaptic vesicle
Postsynaptic membrane
Microtubule
Microtubule associated complex
Nicotinic acetylcholine-gated receptor-channel complex
Trans-Golgi network transport vesicle
Vesicle membrane
Neurone projection
Clathrin coat of trans-Golgi network vesicle
Coated pit
Synaptic vesicle membrane
cAMP-dependent protein kinase complex
Voltage-gated sodium channel complex
Voltage-gated calcium channel complex
Dendrite
Golgi membrane
Membrane fraction
Table 7.4 B: Gene Ontology terms associated with Down-Regulated Genes in the L4 DRG of SNT Animals

(a) Classification According to Biological Process

- Calcium ion transport
- Cation transport
- Striated muscle contraction
- Ion transport
- Myelination
- cGMP biosynthesis
- Visual perception
- Smooth muscle contraction
- Potassium ion transport
- Cartilage development
- Neuropeptide signaling pathway
- Mitosis
- Microtubule-based movement
- Ribonucleoside monophosphate biosynthesis
- Two-component signal transduction system
- rRNA processing
- Embryonic development
- Isoprenoid biosynthesis
- Iron ion transport
- Positive regulation of cell proliferation

(b) Classification According to Molecular Function

- Structural constituent of eye lens
- Isopentenyl-diphosphate delta-isomerase activity
- Calcium channel activity
- Vascular endothelial growth factor receptor activity
- Glutamate-gated ion channel activity
- Ion channel activity
- Bile acid transporter activity
- Cation transporter activity
- Sodium potassium-exchanging ATPase activity
- SH3 domain binding
- Casein kinase activity
- Two-component sensor activity
- Hyaluronic acid binding
- Guanylate cyclase activity
- Receptor activity
- Signal transducer activity
- Sialyltransferase activity
- Fatty acid binding
- Microtubule motor activity
- Serine esterase activity
- Potassium channel activity
- Motor activity
- Acetylglucosaminyltransferase activity
- NAD binding
- G-protein coupled receptor activity
- Phosphoinositide phospholipase C activity
Voltage-gated calcium channel activity
Calcium ion binding
Transporter activity

(c) Classification According to Cellular Location

Striated muscle thick filament
Integral to membrane
Voltage-gated calcium channel complex
Membrane
Microtubule associated complex
Synapse
Postsynaptic membrane
Cytoskeleton
Microarray analysis revealed that of the significantly (p<0.05, 1.2 fold difference) disregulated probe sets in the VZV model, 558 were significantly up-regulated (Appendix A), while 1337 were significantly down-regulated (Appendix B). Significantly more genes were disregulated in the L5, than in the L4 DRG in the SNT model. For the SNT L5 DRG, 3158 probe sets were up-regulated (Appendix C) and 2926 probe sets were down-regulated (Appendix D), while for the SNT L4 DRG, 249 probes were up-regulated (Appendix E) and 306 probes were down-regulated (Appendix F). Complete lists of the differentially expressed probe sets are provided in the attached compact disc (Appendix A – F). Importantly, the number of up-regulated probes in common with both VZV infection and SNT is 6. Similarly, the number of down-regulated probes in common with both models is 26 (Fig. 7.4). These genes are listed below (Tables 7.5 & 7.6).

<table>
<thead>
<tr>
<th>Table 7.5: List of Common Up-regulated Genes Between Models and Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene Title</strong></td>
</tr>
<tr>
<td>Ribosomal protein S15a</td>
</tr>
<tr>
<td>Brain abundant, membrane attached signal protein 1</td>
</tr>
<tr>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>Glia maturation factor, gamma</td>
</tr>
<tr>
<td>VGF nerve growth factor inducible</td>
</tr>
<tr>
<td>Annexin A1</td>
</tr>
</tbody>
</table>

Table 7.5 List of up-regulated probes (n = 6) in common with both VZV infection and SNT. Gene annotations are derived from the Affymetrix NetAffx database. Where a fold change is followed by a question mark, there is overlap in the intensity values between sham and treated animals.
Figure 7.4 Distribution of significant (p<0.05, 1.2 fold difference) probes between models.
<table>
<thead>
<tr>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>L4 SNT</th>
<th>L5 SNT</th>
<th>VZV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcribed locus</td>
<td>---</td>
<td>1.9 ?</td>
<td>2.9</td>
<td>2.0 ?</td>
</tr>
<tr>
<td>WD repeat domain 37 (predicted)</td>
<td>Wdr37_predicted</td>
<td>1.4</td>
<td>1.8 ?</td>
<td>1.3</td>
</tr>
<tr>
<td>Katanin p60 subunit A-like 1</td>
<td>Katn1</td>
<td>1.6 ?</td>
<td>1.6 ?</td>
<td>1.3 ?</td>
</tr>
<tr>
<td>Exportin, tRNA (nuclear export receptor for tRNAs) (predicted)</td>
<td>Xpot_predicted</td>
<td>1.3</td>
<td>1.4</td>
<td>1.3 ?</td>
</tr>
<tr>
<td>Kelch domain containing 1 (predicted)</td>
<td>Klhdc1_predicted</td>
<td>1.4</td>
<td>1.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Transcribed locus</td>
<td>---</td>
<td>1.3</td>
<td>1.4</td>
<td>1.3 ?</td>
</tr>
<tr>
<td>Transcribed locus</td>
<td>---</td>
<td>1.8</td>
<td>2.0 ?</td>
<td>1.5</td>
</tr>
<tr>
<td>Similar to chromosome 20 open reading frame 39</td>
<td>RGD1310753</td>
<td>1.3</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Plastin 1 (I isoform) (predicted)</td>
<td>Pls1_predicted</td>
<td>1.5</td>
<td>1.3 ?</td>
<td>1.5</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase kinase 6</td>
<td>Map2k6</td>
<td>1.5</td>
<td>1.8 ?</td>
<td>1.4 ?</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>1.3</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>Transcribed locus</td>
<td>---</td>
<td>1.3</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>ST3 beta-galactoside alpha-2,3-sialyltransferase 6</td>
<td>St3gal6</td>
<td>1.4</td>
<td>2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Transmembrane protein 19</td>
<td>Tmem19</td>
<td>1.3 ?</td>
<td>1.4 ?</td>
<td>1.5</td>
</tr>
<tr>
<td>Transcribed locus</td>
<td>---</td>
<td>2.2</td>
<td>2.5</td>
<td>1.9 ?</td>
</tr>
<tr>
<td>Unc-5 homolog C (C. elegans)</td>
<td>Unc5c</td>
<td>1.4</td>
<td>1.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Transcribed locus</td>
<td>---</td>
<td>1.5</td>
<td>1.5 ?</td>
<td>1.3</td>
</tr>
<tr>
<td>Transcribed locus, strongly similar to XP_219288.3 PREDICTED: similar to LRRGTO0105 [Rattus norvegicus]</td>
<td>---</td>
<td>1.4 ?</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Transcribed locus</td>
<td>---</td>
<td>1.4</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>1.7 ?</td>
<td>1.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Transcribed locus</td>
<td>---</td>
<td>1.3</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Rap guanine nucleotide exchange factor (GEF) 5</td>
<td>Rapgef5</td>
<td>1.5</td>
<td>1.8 ?</td>
<td>1.3 ?</td>
</tr>
<tr>
<td>G protein-coupled receptor 175</td>
<td>Gpr175</td>
<td>1.2</td>
<td>1.5</td>
<td>1.3 ?</td>
</tr>
<tr>
<td>Solute carrier organic anion transporter family, member 1c1</td>
<td>Slco1c1</td>
<td>2.0</td>
<td>2.7 ?</td>
<td>1.4 ?</td>
</tr>
<tr>
<td>Hyaluronan and proteoglycan link protein 1</td>
<td>Hapln1</td>
<td>1.6</td>
<td>2.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Neuregulin 1</td>
<td>Nrg1</td>
<td>1.4</td>
<td>2.5</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Table 7.6: List of Common Down-regulated Probes Between Models and Fold Change

Table 7.6 lists down-regulated probes (n = 26) in common with both VZV infection and SNT. Gene annotations are derived from the Affymetrix NetAffx database. Unknown genes are annotated as ---; however where the function is not well known or noted in another species, the 'predicted' annotation is given. Where a fold change is followed by a question mark, there is overlap in the intensity values between sham and treated animals.
7.2.2 Microarray validation

*Behavioural reflex sensitivity*

On day 14 post-infection/surgery, all VZV-infected (n = 6), but not uninfected fibroblast-injected control (n = 4) animals displayed hypersensitivity to punctate mechanical stimulation ipsilateral to the injury: mean percentage decrease from baseline ipsilateral PWT as assessed using graded nylon von Frey monofilaments was 66.0% (Fig. 7.5). Significant differences (p<0.05) in ipsilateral response thresholds were also observed when compared to contralateral and to control ipsilateral paw withdrawal values.

![Bar chart](image)

**Figure 7.5** Punctate mechanical hypersensitivity in VZV-infected (n = 6), but not uninfected fibroblast-injected control animals (n = 4) in the ipsilateral paw 14 days after infection. Mean percentage decrease from baseline ipsilateral PWT was 66.0%. *p<0.05, paired t-test compared to baseline PWT
Identification of potential targets

Based on the microarray data, ten genes from the VZV model were proposed as potential targets for validation using semi-quantitative PCR and IHC (Table 7.7). Candidate genes were selected based on increased or decreased expression (>1.5 fold change over that in control DRG), statistical significance (p<0.05) and potential biological significance. Two targets from the list below were then selected for validation studies: Vgf and Kir3.1 (potassium inwardly rectifying channel member 3). Selection was based on the above criteria and the availability of antibody for IHC.

Table 7.7: List of Potential Target Genes for Microarray Validation of the VZV Model

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene title</th>
<th>Direction of Change</th>
<th>Fold Change</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcnj3/Kir3.1</td>
<td>Potassium inwardly rectifying channel member 3</td>
<td>↓</td>
<td>1.86</td>
<td>0.0017</td>
</tr>
<tr>
<td>Vgf</td>
<td>VGF nerve growth factor inducible</td>
<td>↑</td>
<td>1.71</td>
<td>0.00075</td>
</tr>
<tr>
<td>Dapk3</td>
<td>Death associated like kinase</td>
<td>↑</td>
<td>1.76</td>
<td>0.002</td>
</tr>
<tr>
<td>GAT1</td>
<td>GABA Transporter protein</td>
<td>↓</td>
<td>1.71</td>
<td>0.0009</td>
</tr>
<tr>
<td>Kcnab1/KvBeta3</td>
<td>Potassium voltage-gated channel beta member 1</td>
<td>↓</td>
<td>1.7</td>
<td>0.005</td>
</tr>
<tr>
<td>Cxcr4</td>
<td>Chemokine receptor 4</td>
<td>↓</td>
<td>1.69</td>
<td>0.00017</td>
</tr>
<tr>
<td>Kv2.1/Kcnb1</td>
<td>Potassium voltage gated channel</td>
<td>↑</td>
<td>1.68</td>
<td>0.006</td>
</tr>
<tr>
<td>Kcnh2/ERG1</td>
<td>Potassium voltage gated channel member 2</td>
<td>↑</td>
<td>1.63</td>
<td>0.006</td>
</tr>
<tr>
<td>Igfbp2</td>
<td>Insulin-like growth factor binding protein 2</td>
<td>↑</td>
<td>1.56</td>
<td>0.0036</td>
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<tr>
<td>Cldn1</td>
<td>Claudin 1</td>
<td>↓</td>
<td>1.5</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Table 7.7 List of ten potential target genes for microarray validation, ranked by fold change, in the VZV model. Vgf and Kir3.1 are highlighted as they were selected for validation studies using PCR and IHC.
7.2.2.1 VGF

In the VZV model, Vgf was found to be upregulated by 1.71 fold ($p = 0.00075$). No overlap in the intensity values between control and treated animals was observed (which strongly suggests this is not a false positive result). Interestingly, an upregulation was also observed in both L4 and L5 DRG in the SNT model: 3.4 fold ($p = 0.02$) and 3.5 fold ($p = 0.011$) respectively.

**PCR Validation of VGF Peptide**

RNA was isolated from ipsilateral lumbar DRG (L4 and L5 pooled) from VZV-infected animals ($n = 4$) fourteen days after inoculation (as described in section 7.1.3) and subjected to reverse-transcription PCR analysis (Dr. K. Okuse). Primers were designed by Dr. K. Okuse: Forward (5' to 3'): TACCCAGAACGAGGATTGCG; Reverse (5' to 3'): CAACAGTACCGCGGCCAG (Invitrogen, U.K.). A 1.76 fold increase in the expression of VGF peptide was detected by semi-quantitative PCR analysis, consistent with the microarray data.

**Immunohistochemical Validation of VGF Peptide**

PFA-fixed ipsilateral L5 DRG were harvested from VZV-infected animals ($n = 2$) fourteen days after viral infection before being cryostat sectioned (10 µm) and thaw-mounted on poly-L-lysine slides (VWR) as previously described (Chapter 6). Immunofluorescent detection of VGF peptide was performed using indirect Tyramide Signal Amplification (TSA) (PerkinElmer LAS Ltd., Beaconsfield, Buckinghamshire, UK) (I am grateful to Dr. A. Moss, University College London, for his guidance as to the use of this antibody and protocol). TSA is an enzyme-mediated signal amplification method that utilizes the catalytic activity of horseradish peroxidase to generate high-density labeling of target proteins thus allowing immunoreactivity signals many times brighter than those obtained using standard secondary detection to be achieved. Sections were pre-incubated in Tris-Tween buffered saline (TTBS) containing 5% normal horse serum (Vector Laboratories, U.K.) for 1 hour at room temperature, and then incubated with a polyclonal primary antibody directed against the VGF peptide (1:5000 dilution) (Santa Cruz Biotechnology, U.S.A.) diluted in TTBS buffer for 48 hours at 4°C. Sections were washed in PBS (0.1 M phosphate buffer solution) and incubated with a biotinylated horse anti-goat secondary antibody (2.5 µl/ml dilution) (Vector) diluted in TTBS for 90 minutes at room temperature. Sections were again washed in 0.1 M PBS before staining with the Vectastain Elite ABC kit (Vector) for 30 minutes at
room temperature. Biotinylated tyramide solution (13.3 μl/ml dilution) (Invitrogen, U.K.) was then applied for 7 minutes following which slides were washed in 0.1 M PBS before fluorescence staining with Fluorescein Avidin D (1.67 μl/ml dilution) (Vector) for 2 hours. Finally slides were washed in 0.1 M PBS, coverslipped with Vecta Shield (Vector) and visualised under microscopy. Negative control sections were processed as above, omitting the primary antibody. However, as VGF is a novel target, positive control tissue known to express the peptide was not available for study. The expression of VGF peptide in DRG of VZV-infected animals fourteen days post-infection is illustrated (Fig. 7.6). VGF expression was similarly investigated in DRG of SNT-operated animals since peptide expression was similarly found to be upregulated at this time on microarray analysis (Fig. 7.6). Further, expression of VGF peptide was also investigated at day 60 pi at which time mechanical hypersensitivity in VZV-infected animals had resolved (Chapter 2) (Fig. 7.7).

Co-localisation of VGF peptide with VZV IE62 protein was additionally performed fourteen days after VZV-infection (i.e. at time of maximal behaviour change), and sixty days after VZV-infection (i.e. when mechanical hypersensitivity had resolved) (Fig. 7.7 and 7.8). Randomly selected L5 DRG sections ipsilateral to the side of VZV infection were first incubated with VGF antibody using the TSA method as described above. Following the final wash in PBS, sections were immediately pre-incubated in buffer (0.1M PBS, pH 7.4, 0.2% Triton X-100, 4% fish skin gelatin) containing 10% normal goat serum for 1 hour at room temperature, before being incubated with a polyclonal primary antibody directed against VZV IE62 protein (1:250 dilution) (kindly provided by Prof. P. R. Kinchington, University of Pittsburgh, U.S.A.) diluted in buffer (0.1 M PBS, pH 7.4, 0.2% Triton X-100, 2% fish skin gelatin) overnight at 4°C, as previously described (Garry et al. 2005). Sections were than washed in buffer and incubated with a goat anti-rabbit secondary antibody (1:1000 dilution) (Alexa Flour 568) for 2 hours at room temperature. Following three washes with 0.1M PB, sections were coverslipped with Vectashield mounting medium (Vector Laboratories, Peterborough, U.K.) and visualised under standard fluorescence or Confocal microscopy. Negative control sections were processed as above omitting the primary antisera.

I found that VZV infection induced VGF peptide expression in DRG neurones ipsilateral to the injury, which is consistent with both microarray and PCR analysis. It appears that in general, VGF peptide and VZV IE62 protein are co-localised i.e. VGF is only expressed in VZV-infected cells, however, not all IE62 positive cells express VGF peptide (Fig. 7.7). However, these data remain to be quantified. Furthermore, expression of VGF was not seen
once hypersensitivity to punctate mechanical stimulation had resolved (Fig. 7.7). Finally, I found that SNT surgery also induced VGF peptide expression in DRG neurones ipsilateral to the injury (Fig. 7.6), which is consistent with the microarray data. Moreover, on PCR analysis in these animals, a 41.8 fold increase in VGF expression was found.
Figure 7.6 Typical immunofluorescence images showing the presence of VGF peptide (green) in lumbar DRG neurones ipsilateral to A) VZV infection and B) L5 SNT, but not in C) uninfected fibroblast-injected control, nor D) sham-operated animals fourteen days after injury. Expression of VGF peptide was not seen in contralateral lumbar DRG of E) VZV-infected, nor F) SNT-operated animals. Positive- (arrow) and negative- (asterisk) labeled cells are indicated. Scale bar 100 μm.
Figure 7.7 Immunohistochemical co-localisation (iii) of VGF peptide (i) (green) with VZV IE62 (ii) (red) in ipsilateral lumbar DRG cells A) fourteen days after VZV-infection (i.e. at time of maximal behaviour change); and B) sixty days after VZV-infection (when mechanical hypersensitivity had resolved). The arrow indicates a positive-co-labeled cell, while the asterisk indicates a VZV-positive but VGF-negative cell. No staining for either protein was observed at day 60 pi. Scale bar 50 μm.
Figure 7.8 Confocal microscopy co-localisation image of VGF peptide (green) and VZV IE62 protein (red) in a lumbar DRG cell ipsilateral to VZV infection on day 14 post-injury.
7.2.3.2 Kir3.1

In the VZV model, Kir3.1 was found to be down-regulated by 1.86 fold \( (p = 0.0017) \). Additionally, there was no overlap in the intensity values between control and treated animals and multiple 'hits' were observed. Significant disregulation of Kir3.1 was not observed in the SNT model.

**PCR Validation of Kir3.1**

RNA isolated from ipsilateral lumbar DRG (L4 and L5 pooled) from VZV-infected animals \( (n = 4) \) was subjected to reverse-transcription PCR analysis (Dr. K. Okuse). Primers: Forward (5' to 3'): CATGTCGCAACTACACTCC; Reverse (5' to 3'): CATCGATCAGTGCAATG (Invitrogen, U.K.). No change in the expression of Kir3.1 was detected by PCR.

**Immunohistochemical Validation of Kir3.1**

PFA-fixed ipsilateral L5 DRG were harvested from VZV-infected animals \( (n = 2) \) fourteen days after viral infection and cryostat sectioned (10 \( \mu \)m) as previously described (Chapter 6). Immunofluorescent detection of Kir3.1 was performed using a protocol modified from the manufacturer's guidelines (Alomone Labs, Buckingham, U.K.), Bradley et al., (2000), and Dobrzynski et al., (2001). Sections were pre-incubated in buffer (0.1 M PBS, pH 7.4, 0.2% Triton X-100) containing 10% normal goat serum for 1 hour at room temperature, and then incubated with a polyclonal primary antibody directed against Kir3.1 (1:100 dilution) (Alomone Labs) diluted in buffer overnight at 4°C. Sections were then washed in buffer and incubated with secondary antibody (goat anti-rabbit Alexa Fluor 568, Invitrogen) (1:100 dilution) diluted in buffer for 1 hour at room temperature. Finally slides were washed in 0.1 M PBS, coverslipped with Vecta Shield (Vector) and visualised under microscopy. Negative control sections were processed as above, omitting the primary antibody. However, as Kir3.1 is a novel target, positive control tissue known to express the peptide was not available for study, however typical Kir3.1 immunoreactivity was compared to previous studies (Bradley et al. 2000; Dobrzynski et al. 2001).

The expression of Kir3.1 in DRG of VZV-infected and uninfected fibroblast injected control animals fourteen days post-injury is illustrated below (Fig. 7.9). It appears that there may be a
decrease in Kir3.1 expression in VZV-infected compared to control tissue, however this apparent change would need quantification and further investigation. While this is consistent with the microarray data, we observed no change in Kir3.1 expression on PCR analysis.

Figure 7.9 Typical immunofluorescence images showing a slightly reduced presence of Kir3.1 (red) in lumbar DRG neurones ipsilateral to A) VZV infection, compared to that in B) uninfected fibroblast-injected control animals fourteen days after infection. Positive- (arrow) and negative- (asterisk) labeled cells are indicated. Scale bar 50 μm.
7.4 Discussion

The aim of this study was to further characterise a rodent model of zoster-associated pain by globally investigating changes in DRG gene expression associated with VZV infection in the rat. Differential gene expression was profiled in parallel with a model of traumatic peripheral nerve injury (SNT) at a time of maximal mechanical hypersensitivity i.e. day 14 post-injury. Whilst both models share static mechanical hypersensitivity phenomena (mean percentage decrease from baseline ipsilateral PWT as assessed using graded von Frey filaments was 60.6% and 71.9% respectively) (Fig. 7.2), by comparison, the zoster-associated pain model involves minimal surgical trauma. Therefore, this allows the refinement of those genes specifically associated with the development of neuropathic pain. This study is the first to identify global changes in DRG gene expression associated with rodent VZV infection in vivo.

7.4.1 Functional classification of disregulated genes

Classification of significantly (p<0.05, 1.2 fold change) disregulated genes according to gene ontology, and by comparison with the SNT model, enhances our understanding of the mechanisms underlying zoster-associated pain in rodents, and may further be useful in the identification of potential viral targets that can be exploited therapeutically.

*Up-regulated genes*

Significantly up-regulated genes associated with VZV infection include those involved in the generation of inflammation i.e. cytokine and chemokine mediated signaling pathways (Table 1). Such pro-inflammatory mediators are known to be released in response to tissue damage or viral infection, specifically in response to VZV infection (Wang et al. 2005). Further, pro-inflammatory cytokines have been shown to induce or facilitate inflammatory as well as neuropathic pain and are also important in the control of the proliferation, differentiation and function of cells of the immune system (Sommer and Kress 2004). Indeed, the low-level expression of productive-cycle viral genes, such as the IE genes, during viral infection may provide an antigenic stimulus for immune effectors (Kramer et al. 2003). By comparison, a greater number of genes involved specifically in immune and inflammatory pathways (e.g. complement activation, antigen presentation and processing, chemokine activity) were upregulated in the DRG of SNT-operated animals (Tables 3A and 3B).
Additionally, several genes involved in apoptosis (e.g. death-associated protein kinase 3, Dapk3); regulation of apoptosis (e.g. nucleolar protein 3, Nol 3); and anti-apoptosis pathways (e.g. mitogen activated protein kinase 8 interacting protein, Mapk8ip) were found to be significantly up-regulated following viral infection. This is not surprising as it is known that viral infection, including herpesvirus infection (Hood et al. 2003), and replication is associated with modulation of apoptosis (Young et al. 1997; Hood et al. 2003). It has been proposed that virus-induced induction of apoptosis may serve as an efficient mechanism for the dissemination of progeny virus while evading host inflammatory responses (Hood et al. 2003). Moreover, many viruses are known to employ anti-apoptotic strategies to inhibit apoptosis, thereby contributing to the pathogenesis of viral infection by prolonging the survival of lytically-infected cells, or by facilitating the establishment and maintenance of viral persistence (Young et al. 1997). Other viruses may perform both functions, further enhancing survival and spread (Hood et al. 2003). Therefore, since both apoptosis and anti-apoptosis genes were up-regulated in the VZV model, modulation of this actively controlled process of programmed cell death may be at least partially responsible for the pathology associated with VZV infection in rodents. Furthermore, in a recent study examining the ability of VZV to induce apoptosis in dissociated primary human DRG neurones and primary human fibroblasts in culture, Hood et al., (2003) readily detected apoptosis in VZV-infected fibroblasts, but not during productive VZV infection in sensory neurones. Since the ability of VZV to successfully establish a latent infection in sensory neurones is dependent on the neurone remaining viable during this process, the demonstration of an anti-apoptotic function in VZV-infected neurones provides evidence for a mechanism by which the virus ensures the survival of the host cell (Hood et al. 2003). In addition to a potential role during the establishment of latency, it has further been proposed that a neurone-specific anti-apoptotic function may also be important during virus reactivation, since protecting neurones against apoptosis during the critical stages of virus reactivation would allow for greater production of new virions (Hood et al. 2003). However, reactivation-induced neuronal destruction (Watson et al. 1991) is thought not to be a result of virus-induced apoptosis, rather, a consequence of the host cellular immune response. Notably, the viral gene product responsible for the anti-apoptotic phenotype in primary human sensory neurones has recently been demonstrated to be ORF63, the gene encoding the IE63 viral protein (Hood et al. 2006). As ORF63 is expressed in neurones during both productive and latent infection, it may thus play a key role in viral pathogenesis by promoting neurone survival during primary and reactivated infections (Hood et al. 2006).
Traumatic peripheral nerve injury is also known to induce apoptosis in the ipsilateral dorsal horn of the spinal cord (Scholz et al. 2005). By comparison however, no changes in apoptosis-related genes were identified on microarray analysis in the SNT model (though this may reflect differences in tissue examined). Interestingly, Scholz and colleagues (2005) recently demonstrated that the nerve injury-induced apoptosis in spinal cord dorsal horn is slow in onset, occurring at low frequency but persisting for weeks. The observed apoptosis is thought to be triggered by primary afferent input, mediated by glutamatergic transmission, and involving caspase-3 activation in neurones. The cumulative loss of dorsal horn neurones was found to be considerable and included GABAergic interneurones, which may contribute to hypersensitivity phenomena (cumulative loss of >20% of neurones in superficial laminae over 4 weeks). The authors additionally demonstrated a reduction in the number of apoptotic profiles, and attenuation of neuropathic pain-like behaviour following caspase inhibition (Scholz et al. 2005). The influence of anti-apoptotic treatment on VZV-induced pain behaviour could similarly be examined in future studies.

In light of the microarray findings, it would be interesting to examine VZV-infected DRG sensory neurones for evidence of apoptosis. Apoptosis may be observed using the immunohistochemical detection of activated caspase-3, or terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) (Guo et al. 2004). Caspases that participate in the apoptosis cascade are either ‘initiators’ or ‘executioners’. Caspase-3 is an ‘executioner’ enzyme and is considered a key effector mediating the cleavage of other caspase enzymes and preceding the fragmentation of DNA detected by TUNEL (Guo et al. 2004). Whilst TUNEL detects fragmented DNA strand ends (i.e. apoptotic nuclei), it has been reported that in some cells, DNA fragmentation is repairable and not indicative of certain cell death (Guo et al. 2004), therefore apoptosis would ideally be confirmed using a double-label assessment of percentage activated caspase-3 and TUNEL in DRG preparations. Additionally, electron microscopy of VZV-infected sensory neurones may be used to examine for signs of apoptosis i.e. nuclear condensation or fragmentation (Sango et al. 2002). Further, these techniques could be used to assess whether apoptosis differentially affects subpopulations of neurones (and satellite cells) in a region-specific manner, and whether the level of apoptosis changes over time.

The ability of VZV to utilise host cell machinery during infection was also evident from the up-regulation of genes involved in RNA splicing (e.g. WW domain binding protein 11, Wbp11);
base excision repair (e.g. tumor protein 53, Tp53); and cytoskeleton organization and biogenesis (e.g. microtubule affinity-regulating kinase 1, Mark1) (Table 1). It is also reflected by the significant down-regulation of genes involved in ion transport (e.g. voltage-gated potassium channel, KvBeta3); cell adhesion (e.g. myelin basic protein, Mbp); and metabolism (e.g. glutaminase, Gls). Of further note, are those genes involved in neurone differentiation and growth, in particular those encoding cytoskeletal and microtubule-associated motor proteins such as dynein. Dynein was also recently found to be up-regulated following VZV infection in fibroblasts and is thought to have important functions in the transport of varicella zoster virions (Jones and Arvin 2003).

Genes involved in synaptic transmission and neuropeptide signaling (e.g. transient receptor potential cation channel subfamily M member 4, Trpm4; calcitonin-related polypeptide beta, Calcb; galanin receptor 2, Galr2; and protein kinase C alpha binding protein, Prkcaabp) were also found to be up-regulated following VZV infection. Such genes may be important for the generation and/or maintenance of neuropathic pain observed in this model. Of particular interest are the calcitonin-related polypeptide, alpha (Calca) gene that codes CGRPα; and the protein kinase C, epsilon (PKCe), which are up-regulated. CGRP is a marker of nerve growth factor (NGF) sensitive small nociceptive neurones (Snider and McMahon 1998), is up-regulated in invading macrophages following nerve injury (Ma and Quirion 2006) and has a role in the development of thermal hypersensitivity (Mogil 2005). It has also been implicated in the development and maintenance of mechanical hypersensitivity (Jang et al. 2004). PKCe signaling pathways have been implicated in primary afferent nociceptor sensitization (Khasar et al. 1999). In addition, PKC is an important contributor to the development of diabetic hyperalgesia (Ahlgren and Levine 1994), Taxol-induced painful peripheral neuropathy (Dina et al. 2001), painful alcoholic neuropathy (Dina et al. 2000;Dina et al. 2006), as well as inflammatory hyperalgesia (Khasar et al. 1999;Aley et al. 2000;Parada et al. 2005).

**Down-regulated genes**

Of the significantly down-regulated genes associated with VZV infection (p<0.05), these appear to be largely involved in ion transport, receptor activity, neurotransmitter secretion and metabolism. This is supported by similar observations in the SNT model (Tables 4A and 4B). Genes involved in ion transport, specifically in the transport of potassium, sodium and calcium.
ions, in addition to genes involved in neurotransmitter secretion and synaptic transmission were down-regulated following SNT surgery. Such functional changes may have implications in the generation and/or maintenance of spontaneous ectopic activity and hyperexcitability commonly seen in persistent pain states. Of particular interest in the VZV model was the disregulation of potassium (K⁺) channels (Table 7), specifically, the down-regulation of Kir3.1.

Potential biological significance of Kir3.1

K⁺ channels play a major role in the control of all aspects of neuronal excitability and plasticity, and so far are one potential mechanism that has not yet been exploited in the treatment of neuropathic pain (McCleskey and Gold 1999; Wickenden 2002; Wickenden et al. 2004). Four families of K⁺ channels with different structures, functional characteristics and pharmacological sensitivity, may be distinguished in neurones: voltage-gated (Kv), calcium-activated (KCa), inwardly-rectifying (Kir) and two-pore (K2P) K⁺ channels (Miller 2000; Wickenden 2002).

Kir3 channels are G-protein-gated integral membrane proteins, also known as GIRK channels (Ippolito et al. 2005). Of the four GIRK subunits (GIRK1 – GIRK4 corresponding to Kir3.1 - 3.4) identified in mammals, GIRK1 (Kir3.1) is expressed in the greatest range of tissues, forming heterotetramers with other Kir3 subunits in the heart, brain and in endocrine cells (Marker et al. 2004; Ippolito et al. 2005). Transmembrane channels formed by various combinations of these subunits may also be found on nociceptive primary afferents in the periphery and mediate inhibition in the nervous system, contributing to the tone of descending modulatory pathways (Marker et al. 2004). A recent study reported the phosphorylation of the Kir3.1 channel following repeated behavioural stress (forced swim) and nociceptive stimuli induced by inflammation (formalin) and nerve injury (sciatic nerve ligation), resulting in a reduction in channel activity and enhanced neuronal excitability (Ippolito et al. 2005). Further, immunoreactivity for the phosphorylated Kir3.1 channel was demonstrated in dorsal horn glial cells, in addition to neuronal cells. Moreover, a recent study investigating Kir3.1 knock-out mice has demonstrated a role for the Kir3.1 channel in attenuating opioid-mediated antinociception by activating heterotetramers of Kir3.1 and Kir3.2 in the dorsal horn of the spinal cord (Marker et al. 2004). Additionally, roles for Kir3.2 and Kir3.3-containing channels have been identified in the modulation of thermal nociceptive thresholds (Marker et al. 2002; Marker et al. 2004).
A feature of neuropathic pain is neuronal hyperexcitability. The opening of K⁺ channels leads to hyperpolarisation of the cell membrane, which results in a decrease in cell excitability (Passmore et al. 2003). Thus, activation of K⁺ channels theoretically represents a powerful means of reducing excessive neuronal activity. A down-regulation in the expression of these channels is likely to impact on neuronal hyperexcitability and may contribute to the generation of ectopic activity and spontaneous pain. The Kir3.1 channel therefore presents a novel and exciting target for investigation. A down-regulation in Kir3.1 expression was observed in VZV-infected animals on microarray analysis and on IHC.

7.4.2 Genes in common between viral infection and SNT

While far less genes were disregulated following VZV infection compared to SNT surgery (Fig. 7.4), which may reflect the relative subtlety of the injury and the nature of the insult, there were several genes in common with both models (Tables 5 & 6). However, of the six commonly up-regulated genes between models, only three genes also identified in the VZV model were of significant fold change (i.e. >1.5 fold increase compared to fibroblast controls) and include the Finkel-Biskis-Jinkins (FBJ) murine osteosarcoma viral oncogene (Fos), nerve growth factor inducible protein (Vgf), and a non-specific membrane signaling protein (Table 5).

FBJ murine osteosarcoma viral oncogene is homologous to the human oncogene c-fos which is proposed to be a marker of noxious peripheral stimulation (Hunt et al. 1987; Doyle and Hunt 1999; Jergova and Cizkova 2005). C-fos protein is rapidly, though transiently expressed in the superficial layers of the spinal cord dorsal horn following activation of (i) small-diameter cutaneous sensory afferents by noxious heat or chemical stimuli, and (ii) myelinated Aβ and unmyelinated C fibre low-threshold mechanoreceptive afferents by non-noxious dynamic (brush) mechanical stimuli after peripheral nerve injury or inflammation (Hunt et al. 1987; Abbadie and Besson 1994; Wei et al. 1999; Catheline et al. 1999; Jergova and Cizkova 2005; Coggeshall 2005); and has also been demonstrated at the supraspinal level following peripheral nerve injury in rats (Narita et al. 2003). In addition, induction of c-fos protein (Fos) expression by HSV infection has been demonstrated in spinal dorsal horn neurones in mice (Ozaki et al. 1996), while Rahaus and Wolff (Rahaus and Wolff 2003) recently demonstrated an increase in the transcription of Fos following VZV infection in tissue culture. Therefore, Fos expression may be important in the intracellular response to viruses and has been proposed to
play a role in the control of viral transcription (Ozaki et al. 1996). We found that c-fos protein expression was up-regulated in ipsilateral lumbar DRG by 2.5 fold \((p = 0.023)\) following VZV infection compared to 1.9 fold \((p = 0.01)\) and 6.5 fold \((p = 0.000002)\) in both ipsilateral L4 and L5 DRG in the SNT model respectively. However, as there was overlap in the intensity values between control and VZV-infected animals for this gene, it may be that this is in fact a false positive result. Further investigation e.g. immunohistochemical detection of Fos protein in lumbar spinal cord ipsilateral to VZV infection (as induction of c-fos is not seen in the DRG (Hunt et al. 1987) would be necessary to validate this finding. Brush evoked Fos expression is a non-behavioural correlate of the phenomenon of brush-evoked dynamic mechanical allodynia (Catheline et al. 1999). While we have demonstrated hypersensitivity to dynamic mechanical stimulus in VZV-infected animals, immunohistochemical examination of Fos expression following viral infection would further support our behavioural findings. It would be of further interest to ascertain whether the hypothesis of VZV-induced Fos expression can be prevented by pharmacological perturbation.

The most notable of the commonly up-regulated genes appears to be the novel Vgf gene (Table 5). In the VZV model, Vgf was found to be up-regulated by 1.7 fold \((p = 0.00075)\), compared to 3.4 fold \((p = 0.02)\) and 3.5 fold \((p = 0.011)\) in both L4 and L5 DRG in the SNT model respectively. Importantly, this finding was validated using both PCR and IHC. Moreover, in a similar microarray experiment, Costigan et al., (2002) recently demonstrated and validated an up-regulation of VGF peptide by 2.7 fold \((p = 0.0004)\) three days following sciatic nerve transection (axotomy) in the rat. When taken together, these data provide support for developing a hypothesis that VGF plays a fundamental role in the development of mechanical hypersensitivity in the context of neuropathy at the level of the primary afferent neurone. Furthermore, we found that expression of VGF peptide was no longer evident once hypersensitivity to punctate mechanical stimulation had resolved in VZV-infected animals (Fig. 7.7) which again strongly indicates a role for VGF in pain.

Potential biological significance of VGF

VGF (non-acronymic) is a ubiquitous neuropeptide precursor encoded by the vgf gene. It is selectively synthesised in neuronal and neuroendocrine cells and widely expressed in both the peripheral and central nervous systems in the rat (Salton et al. 2000). In addition to abundant
expression in the hypothalamus and brain, VGF mRNA has specifically been detected in the ventral and dorsal horns of the spinal cord, and in dorsal root and sympathetic ganglia where it is present in small to medium diameter sensory neurones. Here, the peptide is synthesised and packaged into vesicles before being transported along the axon and released from the nerve terminal in response to depolarisation (Snyder et al. 1998; Salton et al. 2000). Therefore, VGF peptides may serve as neuromodulators, mediating neuronal communication. VGF peptides may also be found in adrenal medullary, gastrointestinal and pancreatic endocrine cells, suggesting important neuroendocrine functions (Salton et al. 2000). Indeed, the majority of work on VGF has focused on its role in the regulation of energy storage and expenditure (Hahm et al. 1999; Hahn et al. 2002; Levi et al. 2004; Watson et al. 2005; Bartolomucci et al. 2006). Furthermore, structure of the vgf gene is highly conserved from rodent to man and chromosomal mapping has assigned human Vgf to chromosome 7q22 (Salton et al. 2000).

VGF peptide expression is rapidly induced after injury. Specifically, it has been shown to be upregulated in rat DRG 2 – 28 days following axotomy (Costigan et al. 2002; Xiao et al. 2002) and at various time points after spinal nerve injury in the rat spinal cord dorsal horn (Low 2005). VGF may also be important in synaptogenesis and synaptic reorganisation (Costigan et al. 2002; Alder et al. 2003; Low 2005), with some evidence for a role in long-term potentiation and hippocampal synaptic plasticity (Alder et al. 2003). Thus it has been proposed that VGF peptide may have a potential role in the induction and maintenance of central sensitisation (Alder et al. 2003). Moreover, neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) which are themselves important in the development, maintenance and normal functioning of neurones in the nervous system and following injury (Pezet and McMahon 2006), have been shown to induce vgf gene expression in cortical neurones in vitro (Levi et al. 1985; Salton et al. 2000; Alder et al. 2003). (NGF acts as a peripheral pain mediator: it is upregulated following nerve injury and inflammation and sensitises peripheral nociceptive terminals. NGF is retrogradely transported to sensory neurone somata and regulates genes involved in pain processing (Pezet and McMahon 2006). Similarly, BDNF, which is released from the central terminals of nociceptors in response to peripherally produced NGF, acts as a central modulator of pain enhancing central neurone responsiveness (i.e. central sensitisation) (Pezet and McMahon 2006)). Such robust induction of vgf transcription in vitro suggests that expression of this gene might be similarly regulated by neurotrophins in vivo.
Of interest is the potential role of VGF in an anti-depressant-related effect in rodents. It was recently demonstrated that VGF-treated animals spend less time immobile in paradigms of depression (the forced swim and tail suspension tests) compared to vehicle treated controls (Hunsberger et al. 2006). This pattern of behaviour is consistent with an anti-depressant-like response. The authors also observed a depressive phenotype in VGF +/- mice compared to wild-type littermates (Hunsberger et al. 2006).

However, VGF peptide has no known receptor. Whilst there is evidence for an overlap in the mRNA expression patterns of Vgf and Trk (tropomyocin receptor kinase) high affinity neurotrophin-receptors (Snyder and Salton 1998), the precise mechanism of action of VGF and its role in pain has yet to be fully determined. VGF therefore presents a novel and very interesting target for validation and further investigation.

No obvious or well known pain related genes were identified in the commonly down-regulated list between models (Table 6). In fact, of the twenty-six genes commonly down-regulated, the function of eleven of these is completely unknown. Furthermore, nineteen of the genes identified in the VZV model were down-regulated by less than 1.5 fold, and of the remaining seven genes, the function of only one was known (transmembrane protein 19). Thus, it is difficult to draw conclusions from these data.

7.4.3 Differences in gene expression between models

There were notable differences in expression of specific genes or groups of genes between models. For example, our microarray analysis revealed an up-regulation of the nerve injury marker, activating transcription factor-3 (ATF-3) following SNT surgery (a finding similarly verified in previous microarray studies), but not following viral infection. These data are supported by immunohistochemical analysis of injured L5 DRG in which ATF-3 positive neurones were clearly observed following traumatic nerve injury, but not following viral infection (Fig. 7.10).
Figure 7.10 ATF-3 immunofluorescence reactivity (1:300) in ipsilateral L5 DRG of A) SNT; B) VZV; and C) uninfected fibroblast-injected control animals on day 14 post-surgery/ infection. ATF3 reactivity following viral infection is minimal compared to traumatic peripheral nerve injury and appears similar to control animals. Arrow marks an ATF-3 positive cell.
The difference between models may reflect the relative severity of injury (and cell stress) produced. In contrast, and more difficult to explain is that PCR analysis revealed a 1.6 fold increase in ATF-3 expression in DRG ipsilateral to VZV infection. The difference in findings may in part reflect the sensitivity of the microarrays in detecting a change, particularly if subtle; but may also reflect the nature of the different techniques used: PCR involves 33 cycle amplification of RNA and may therefore be more sensitive at detecting small changes in protein expression. Similarly, increased ATF-3 expression, as determined by Western Blotting, in DRG of VZV-infected rats has recently been demonstrated (Garry et al. 2005). This is in contrast to our microarray and IHC findings and may in part reflect amplification of the protein signal; but also differences in the concentration of virus injected, since the authors used a higher viral inoculum concentration (10^7 pfu/ml) in excess of that used in our study (10^4–10^5 pfu/ml); or in the time course of ATF3 expression, since the authors examined infected DRG at day 28 post-infection (as opposed to day 14 post-infection in our study). ATF-3 is thought to be a marker of axonal damage or stress (Tsujino et al. 2000) and its expression thus suggests that such damage may occur following VZV infection. The extent of damage however, may be dependent on time course and concentration of infected virus.

Following peripheral nerve injury, long-lasting changes in the expression of neuropeptides and their receptors in primary sensory neurones are observed. These changes which involve the down-regulation of excitatory peptides (such as substance P and calcitonin gene-related peptide) and the up-regulation of inhibitory peptides (such as galanin), result in a reduction of transmission in the dorsal horn. The release of substance P and other pain neurotransmitters in the spinal cord dorsal horn are inhibited by the neuropeptide, NPY (Naveilhan et al. 2001). Genes encoding the neuropeptides NPY and galanin, which are known to be induced in DRG neurones following peripheral nerve injury (Hokfelt et al. 1994), were found to be up-regulated following SNT surgery (a finding that has been verified and validated in previous microarray studies in nerve-injured animals e.g. Wang et al., 2002). However, no significant change on microarray analysis was demonstrated following viral infection. Despite the microarray data showing no change, interestingly a 2.6 and 30.2 fold increase in the expression of NPY and galanin respectively was found on PCR analysis in VZV-infected animals. This finding supports those of Garry and colleagues (2005). These authors recently demonstrated an increased expression of NPY and galanin protein on Western blotting or IHC in rat DRG neurones ipsilateral to VZV infection.
7.4.4 Comparison with previous microarray studies of peripheral neuropathy

Findings in this study were compared to similar rat oligonucleotide microarray studies investigating gene expression changes in animal models of traumatic peripheral nerve injury (Costigan et al. 2002; Wang et al. 2002; Xiao et al. 2002; Valder et al. 2003). Whilst this study examined the whole rat genome chip, previous studies have examined only partial genome chips that were available at the time. Our data generally agrees with those previously reported in the literature. Specifically, 89% of up-regulated, and 95% of down-regulated genes identified by Wang and colleagues (2002); and 71% of up-regulated, and 80% of down-regulated genes identified by Valder and colleagues (2003), were in common with our SNT data (Appendix G). Genes found to be disregulated included those encoding neuropeptides, receptors, ion channels, and signal transduction molecules. Both of these previous studies used rat RG-U34A arrays containing 8799 probe sets and published lists of differentially expressed genes showed greater than two-fold difference with $p<0.05$. They compared ipsilateral to contralateral pools of L4, L5, and L6 DRGs. The percentage of significantly disregulated genes linked to a GO term in the SNT model was also consistent, which provides another level of validation of our experimental results. Since our SNT model is a variation of the spinal nerve ligation model, and differential gene expression was examined at the same time point post-injury, our data may be compared to these datasets. The good correlation between the SNT results and published observations substantiates the accuracy of our data.

However, there are very few microarray studies specifically investigating VZV infection in rodents (Jones and Arvin 2003), and none to date investigating VZV-induced gene expression changes in vivo. For example, Jones and Arvin (2003) explored transcriptional changes in cellular genes following VZV infection in human T lymphocytes in vitro and human skin xenografts ex vivo (i.e. the cell types targeted during the pathogenesis of primary VZV infection). Classification of significantly disregulated genes (i.e. fold change $>2$) 48 hours after viral infection included those involved in immune and stress responses, apoptosis, signal transduction, and other basic cell functions. This appears to be consistent with our data. In addition, DNA microarrays have been used to study viral gene expression and virus-induced changes in cellular gene expression associated with productive and latent infection of another alphaherpesvirus (HSV) in cell culture (Stingley et al. 2000; Kramer et al. 2003). Specifically, Kramer et al., (2003) identified significant changes in the expression of immune response genes,
consistent with the presence of infiltrating immune and inflammatory cells in latently infected ganglia (Chen et al. 2000; Kramer et al. 2003); and in the expression of neuronal genes following HSV infection in mouse trigeminal ganglia, including several genes with roles in the regulation of neurotransmission and signaling, e.g. those encoding voltage-gated K+ channels. This largely appears to be consistent with our findings.

7.4.5 Limitations of microarray and further study

Although microarray technology offers enormous potential advantages, several factors could potentially limit the confidence with which results are interpreted. We used a two cycle amplification protocol to generate the microarray targets. This approach was taken to avoid pooling DRGs from large number of animals, which has both ethical and experimental variability problems. The two cycle amplification protocol has been shown to produce reproducible results with high correlation between amplified and non-amplified RNAs (Saghizadeh et al. 2003; Klur et al. 2004; Li et al. 2005). However, RNA amplification has also been found to cause slight distortion (mainly decrease) of the expression ratios (Diboun et al. 2006) and failure to detect transcripts from the low intensity range (van Haaften et al. 2006). Therefore, our data may suffer from a slight increase in false negative results. This may offer an explanation as to why the comparison between microarray and PCR data revealed that for the VZV infection model (which exhibits subtle gene changes) the microarrays failed to detect differential expression for certain genes e.g. Npy, Vip, Atf3 and Pap. Furthermore, the threshold fold-difference between probe sets that reveals real regulation and biological significance is not known and arbitrarily set. Indeed, accuracy of fold difference may be lost in a two cycle amplification strategy. Therefore, it may be argued whether fold-difference is the most sensitive measure for detecting changes in gene expression, as genes with low expression levels may not be detected. There also remain other important concerns regarding reproducibility and experimental variability in microarray studies (Costigan et al. 2002).

I have demonstrated that only a proportion of rat DRG cells harbor VZV following infection (Chapter 6), so that even large differences in gene expression in individually infected cells might give rise to very small differences in expression on a whole-ganglion basis. Further, our microarray data reflects changes in gene expression in the entire DRG, which is a heterogeneous tissue, composed of many cell types, including a range of neuronal phenotypes and support cells.
Thus, this study cannot link changes to specific cell types in the DRG. *In situ* hybridisation and/or immunohistochemical experiments are required to further clarify this point. Pooling of DRG may further ‘dilute’ important changes in gene expression and patterns of gene expression may be missed.

I focused on one time point in this study when mechanical hypersensitivity is well established, and thus the genes that we have identified are potentially important for the maintenance of mechanical hypersensitivity. To fully identify candidate genes for hypersensitivity generation and maintenance, this study would have to be repeated at a number of time points which is prohibitively expensive given the current technology. It may be that some genes are up-regulated early on but down-regulated later, and therefore investigating only one time point may miss such changes in gene expression. Further, it may be that certain genes are more important in the induction of hypersensitivity phenomenon and are thus only detectable early after viral infection or nerve injury, while other genes may be specifically associated with the maintenance of neuropathy and are only expressed at later time points. Additionally, it may be of interest to examine gene expression changes following infection with different viral strains, as viral factors, rather than host factors may be more important in development of hypersensitivity phenomenon following VZV infection. Finally, it may also be of interest to profile differential gene expression in spinal cord or brain for a more global overview. Ultimately, it is necessary to complement microarray studies with other molecular genetic techniques, such as antisense knockdowns and siRNAs. These techniques may be useful in further investigating candidate genes identified by microarray screening.

### 7.4.6 Summary

A global picture of gene regulation in animal models of persistent pain is needed to better understand the complex molecular mechanisms underlying neuropathic pain. Microarray is an invaluable technology that allows changes in gene expression to be globally investigated. The identification of specific genes or groups of disregulated genes following VZV infection suggests that viral transport is occurring and host cell machinery is being used. Further, genes involved in the regulation of apoptosis may serve to prolong survival of lytically-infected cells, or facilitate the establishment of viral persistence in DRG sensory neurones. In common with traumatic peripheral nerve injury, genes encoding ion channels and signaling molecules that
contribute to the excitability of neurones, and genes involved in immune and inflammatory pathways were also disregulated. This study has helped to further our understanding of the mechanisms underlying zoster-associated pain. However, functional studies are now needed to address the important question of whether these genes are truly involved in the generation and/or maintenance of neuropathic pain or whether they are incidental markers. In this way, we will identify potential targets for developing novel therapeutics to treat zoster-associated pain.
Chapter 8

General Conclusion
Hypothesis

Infection of rat DRG with VZV is associated with behavioural, pharmacological and gene correlates of neuropathic pain.

Progress in elucidating the underlying pathophysiology of persistent herpes zoster-associated pain and related co-morbidity, in addition to appropriately targeted drug development has been hindered by the lack of a suitable animal model. This thesis refines and further characterises a recently developed rat model of zoster-associated hypersensitivity (Fleetwood-Walker et al. 1999), and examines behavioural and gene correlates of zoster-associated pain. Thus, this thesis aims to provide improved face and predictive validity of the model, and ultimately greater clinical predictability; and also to identify novel mechanistic and therapeutic targets using gene microarrays. Together these experiments and will contribute to our understanding of the mechanisms underlying zoster-associated pain.

8.1 Summary of Findings

8.1.1 Behavioural correlates of zoster-associated pain

Rodent VZV infection of the hind-limb was characterised by a chronic resolving pattern of hypersensitivity to punctuate and dynamic mechanical stimulation (suggestive of static and brush-evoked mechanical allodynia respectively), and a notable absence of hypersensitivity to noxious thermal and cooling stimulation in all viral strains examined (with the exception of the live-attenuated Oka vaccine strain in which no behavioural change was observed). This provides the model with a degree of face validity since dynamic mechanical allodynia is a prominent feature in PHN and thermal sensory thresholds are often preserved or raised in PHN patients (Nurmikko 1995;Rowbotham and Fields 1996;Fields et al. 1998;Pappagallo et al. 2000). The apparent lack of thermal hypersensitivity in our model is in contrast to the original study by Garry and colleagues (2005). However, it is important to note that the thermal hypersensitivity effect demonstrated by these authors is very small, especially when compared to the magnitude of mechanical hypersensitivity, and particularly when the y axis scale is considered. In addition, significant thermal hypersensitivity was only observed at very high viral concentrations, in
excess of those used in our study. Again, the effect size is small when compared to the magnitude of mechanical hypersensitivity.

Overall, there were no consistent differences in response between viral strains, which suggests that viral strain is not a critical factor in development of zoster-associated pain. Further, VZV-induced mechanical hypersensitivity was sensitive to the concentration of virus injected and to a range of clinically useful analgesic drugs. This provides the model with a degree of predictive validity and will provide a tool for pre-clinical screening of novel analgesic drugs. In addition, there was a clear relationship between hypersensitivity phenomenon and anxiety-like co-morbidity behaviour (thigmotaxis) as assessed in the open field paradigm. Virus-infected, but not control animals demonstrated an anxiety-like pattern of ambulation which was positively correlated with mechanical hypersensitivity, but not influenced by analgesics. This may reflect pain-related co-morbidity as well as on-going pain rather than evoked sensitivity, and provides the model with a further degree of face validity.

8.1.2 Gene correlates of zoster-associated pain

A gene microarray approach was used to profile differential gene expression in VZV-infected animals, in parallel with a model of traumatic peripheral nerve injury. By way of comparison, the zoster-associated pain model involves minimal surgical trauma which allows the refinement of those genes specifically associated with the development of mechanical hypersensitivity associated with neuropathic pain. The identification of specific genes or groups of disregulated genes following viral infection of sensory ganglia provides an insight into the mechanisms underlying zoster-associated hypersensitivity in rodents. Firstly, it appears that viral transport is occurring and host cell machinery is being used. This is further supported by the immunohistochemical localisation of intra-nuclear IE62 protein which suggests that active replication is taking place in some cells. Second, disregulation of genes involved in the modulation of apoptosis may serve to prolong survival of lytically-infected cells, or facilitate the establishment of viral persistence in sensory neurones, the latter being consistent with the presence of IE62 protein mainly in the neuronal cytoplasm. In common with traumatic peripheral nerve injury, genes encoding ion channels and signaling molecules that contribute to the excitability of neurones; and genes involved in immune and inflammatory pathways, which further facilitate persistent pain states, were also disregulated. Finally, an upregulation of the
novel vgf gene was observed. Since VGF peptide is known to be induced by neuronal injury and is up-regulated in models of traumatic peripheral nerve injury, this suggests a common molecular mechanism in the generation and maintenance of neuropathic pain states.

8.2 Study Limitations

Although the experiments performed were appropriate and overall prove the above hypothesis, there are a number of shortcomings which can be identified with the benefit of hindsight and some these are summarised below.

8.2.1 Rodent VZV infection

A fundamental limitation in this animal model is a relative lack of standardisation in the precise measurement of the concentration of virus injected into each animal. Whilst cytopathic effect is a standard and widely used microbiological technique that quantifies the destruction of normal fibroblast cell architecture caused by viral lytic infection, and was the method employed in the original study by Fleetwood-Walker et al., (1999), it has an element of subjectivity and provides only an indirect indication of the concentration of infectious virus in the inoculum. To refine this, viral plaque assay was additionally performed in some experiments. Although this method provides a more objective measure, it is extremely time-consuming and importantly provides only a retrospective estimate of viral concentration, as the actual number of infectious viral particles per plaque is unknown. Ideally, the exact concentration of infectious virus at the time of injection should be known, and should be consistent among animals; although a more important measure may actually be the number of virions in each DRG neurone at specific time points post-infection. In this respect, the study by Garry and colleagues (2005) is superior i.e. viral titre and percentage infectious cells was determined prior to animal inoculation: 500 µl of VZV-infected fibroblast cells were thawed from vapour phase liquid nitrogen and diluted in saline 1:10 before being centrifuged for 5 minutes at 1000xg. The virus-cell pellets were then resuspended in saline to display titres of $3.2\times10^7$ plaque-forming cells/ml (64% infectious cells).
8.2.2 Appropriate randomisation and blinding of all experiments

Although methods of experimental bias reduction were employed during pharmacological sensitivity studies (i.e. randomisation to minimise allocation bias and experimenter ‘blinding’ to minimise observer bias); for logistical reasons blinding was not performed during initial viral strain and dose-response experiments. The importance of randomisation and blinding relates to experimental bias, specifically the process of randomisation is important in eliminating selection and allocation bias, while blinding avoids observer bias (http://www.jr2.ox.ac.uk/bandolier/band80/b80-2.html). Moreover, non-randomised studies have been shown to yield larger estimates of treatment effects than studies using random allocation (Schulz et al. 1995; Carroll et al. 1996). Remarkably, the degree of exaggeration of treatment effect when randomisation is inappropriate (i.e. allocation bias) may be as much as 40% (Schulz et al. 1995). Similarly, lack of blinding (i.e. observer bias) has been estimated to exaggerate treatment effect by some 17% (Schulz et al. 1995). Adequate randomisation and blinding are therefore important quality standards in studies with pain outcomes. However, the effects of failure to adequately randomise and blind have not been investigated in animal experiments and may have implications on behavioural outcomes in this study.

8.2.3 Open field paradigm

There is considerable variability not only in design of the open field environment, specifically with regard to shape and size, but also in experimental protocol. Scoring of behaviour may start either immediately or several minutes after the animal has been placed in the apparatus, and may continue for a variable period of time. In addition, husbandry, litter size, animals’ age, prior handling, ambient odours, noise, light intensity, and whereabouts the animal is placed in the field (i.e. centre versus periphery e.g. placing the animal into the centre of the arena may induce a ‘freeze’ response, and therefore a more intense level of anxiety that may adversely influence subsequent behaviour) may all differ and so influence experimental outcome. This variability also makes it difficult to compare one study with another. We employed a square arena but noted that animals would often rest in the corners of the apparatus and be reluctant to ambulate. In contrast, a circular arena, as described in the original open field test in rats (Hall 1934) is perhaps more likely to encourage ambulation. An additional factor
critical in design of the open field is size of the arena. We used a 100 cm$^2$ arena, based on the literature (LaBuda and Fuchs 2001a; Boguszewski and Zagrodzka 2002) and advice from Tracksys Ltd., Nottingham. However, great variation in open field design exists (Prut and Belzung 2003) and it may be argued that an arena that is too large and exposed may actually exaggerate anxiety-like responses and induce startle or freezing responses in rodents. Thus, false positive results (i.e. high anxiety) may be produced and more subtle changes in anxiety-like behaviour may be missed.

Another limitation to these paradigms, to which there is no obvious solution, is that they involve testing animals individually; and acute social isolation stress may modify behaviour and therefore further impact on the results. In a similar line, a potential limitation in animal behavioural studies of pain relates to the presumed ability of an animal to recognise and understand the emotion of another i.e. to demonstrate ‘empathy’. Empathy may thus be described as ‘physiological state-matching that occurs as a result of attending to another’s condition’ (Langford et al. 2006a; Langford et al. 2006b). This process does not necessarily require higher-order cognitive functioning, and may exist in all mammals. Whether this is true empathy (believed to be exclusive to humans and primates) or a hard-wired lower type of empathy, called the emotional contagion effect, remains to be determined. Certainly, in evolutionary terms, an animal that feels distress when it sees a familiar animal in pain is an animal that will be more vigilant to threats in the environment, thus enhancing its chance of survival in potentially dangerous situations. Langford and colleagues (2006a) recently reported the modulation of pain sensitivity in mice produced solely by the real-time exposure of cagemates in pain. Specifically, the observation of a cagemate in pain (abdominal constrictions ‘writhing’ induced by the i.p. administration of 0.9% acetic acid) was found to alter pain sensitivity in an entirely different modality (decreased PWL i.e. hypersensitivity to noxious thermal stimulation) in non-injected animals, suggesting that nociceptive mechanisms in general become sensitised. The authors also observed bi-directional modulation of pain by social factors in the formalin test (1% or 5%) such that mice were found to exhibit more or less pain behaviour depending on the formalin dose administered to their cagemate (i.e. increased pain behaviour in mice receiving low dose formalin whilst observing cagemates injected with high dose formalin). Thus, in the open field paradigm, we have endeavoured to reduce confounding of behavioural data with regard to ‘empathy’ by using a ‘holding’ cage before returning animals to their home cage following exposure in the novel open field environment.
Another important limitation of the open field paradigm and indeed the majority of currently favoured paradigms for the assessment of spontaneous anxiety-like behaviour is that they are based on ambulation, and in nerve-injured animals, motor activity could conceivably be reduced. Thus, motor impairment may produce false positive results. It would therefore be useful to explore alternatives to exploration-based tests for anxiety-like behaviour, e.g. the Vogel test (Vogel et al. 1971). This is an example of a punishment-based conflict test in which water-deprived rats are provided with a drinking spout that delivers a mild shock after every 20 licks. Further, it is sensitive to anxiolytics which attenuate the shock-induced suppression of drinking (Cryan and Holmes 2005). Examples of further behavioural and non-behavioural tests are discussed below (8.3.3).

Finally, open field behaviour also depends on the light-dark cycle (Prut and Belzung 2003), and since animals in this study were not housed on a reverse light-dark cycle, it may be relevant to investigate behaviour at different times i.e. it would be desirable to score behaviour during the night whilst animals are in the active phase of their diurnal cycle.

8.2.4 Environmental enrichment

A potential limitation related to assessment of anxiety-like behaviour in exploratory paradigms such as the open field is the impact of environmental enrichment of the home cage. Enriched environments are generally characterised by the presence of objects that provide physical and tactile stimulation and the opportunity for social interaction in large laboratory cages; whereas deprived environments are devoid of physical and social stimulation. The relative contribution of social versus physical enrichment has also recently been investigated (Elliott and Grunberg 2005). It is well known that animals reared in enriched environments versus deprived environments display a greater degree of curiosity and exploratory behaviour in addition to enhanced cognitive performance (Gardner et al. 1975; Widman and Rosellini 1990). Though animals in this study were housed in groups of 3 – 5 in standard laboratory cages, no environmental enrichment was provided. It is possible that this may have negatively impacted on behavioural responses in the open field test, an idea previously suggested by Gardner et al., (1975). In contrast, Elliott and Grunberg (2005) found that the effects of social, rather than physical, enrichment were more important in altering open field activity in rats.
8.2.5 Pharmacological sensitivity testing

In pharmacological characterisation of the zoster-associated pain model, a twice daily dosing regime for all drugs was adopted to better reflect the clinical scenario of chronic pain, and to maintain blinding. In addition, doses were selected on the basis of analgesic therapeutic index and lack of sedative effects as stated in the reference studies. However behavioural deficits (e.g. catalepsy, ataxia, sedation) were not directly evaluated in this study. A further limitation in pharmacological sensitivity is that the duration of drug administration and pre-administration times in the reflex nociceptive tests and in the open field test did not strictly match. In the former, a chronic dosing regime was employed i.e. twice daily (08:00 and 18:00) injections over four consecutive days in which animals were tested 2 hours after the morning dose, compared to an acute single dose regime 20 minutes before testing in the open field. The reason for this was to allow comparisons to be made with similar single dose pharmacological studies in the open field literature. In a separate study (not detailed here), the acute i.p. administration of gabapentin (30 mg/kg) and (S)-(+) Ibuprofen (20 mg/kg) 20 minutes before reflex behavioural testing was found to attenuate VZV-induced mechanical hypersensitivity (Figure 8.1). Indeed, during pharmacological sensitivity testing of the zoster-associated pain model, I found that VZV-induced mechanical hypersensitivity was completely reversed 2 hours after administration of the first dose of either drug (doses as above) (Chapter 3). It would also be of interest to examine animals at this time point in the open field paradigm. Whilst the effect of gabapentin (30 mg/kg i.p.) 20 minutes before reflex behavioural testing was not investigated in SNT animals in this study, the effect of acute i.p. administration of this dose has been examined in the spared nerve injury model in rats (Decosterd et al. 2004). However, the authors did not observe a significant change in paw withdrawal response thresholds to either mechanical or noxious thermal stimuli at this time, which suggests that the latency of the analgesic and anxiolytic effects of gabapentin are independent, and/or that it may not be necessary to demonstrate a reversal of hypersensitivity behaviour for a change in anxiety-like behaviour to be observed. This study would be improved by examining the influence of a range of analgesic and anxiolytic drugs at different doses on both reflex behavioural testing and on a range of behavioural anxiety paradigms.
Figure 8.1 Effect of acute i.p. administration of A) Gabapentin (GP) (30 mg/kg) (n = 5); or B) (S)-(−)-Ibuprofen (IBU) (20 mg/kg) (n = 6) 20 minutes before reflex withdrawal testing to static punctate mechanical stimulation (asssed using the electronic von Frey device) in the ipsilateral paw of VZV-infected animals. Animals (n = 12) were infected with VZV (Dumas, 80% cpe) and mechanical hypersensitivity was confirmed 14 days post-infection (i.e. *p<0.05; paired t-test; compared to ipsilateral baseline). Mean reduction in ipsilateral PWT was 34.3%. One animal was excluded from pharmacological testing as it failed to demonstrate statistically significant behavioural change (i.e. p>0.05). Animals were randomly allocated to individual drug groups. Separate animals were used for each drug and testing was performed ‘blinded’ by a single experimenter.

8.2.6 Microarray experiment

Although microarray technology offers enormous potential advantages, several factors could potentially limit the confidence with which results are interpreted. Firstly, the threshold fold-difference between probe sets that reveals real (biological) regulation is unknown and arbitrarily set. Further, accuracy of fold difference may be lost in a two cycle amplification strategy. Indeed, it may be argued whether fold-difference is the most sensitive measure for detecting changes in gene expression, as genes with low expression levels may not be detected. In addition, there remain other important concerns regarding false positive and negative detection rates (since multiple correction was not used in the analysis), reproducibility, and experimental variability (Costigan et al. 2002). Second, we have demonstrated that only a proportion of rat DRG cells harbor VZV following infection (Chapter 6), so that even large differences in gene expression in individually infected cells might give rise to very small differences in expression on a whole-ganglion basis. Further, our microarray data reflects
changes in gene expression in the entire DRG, which is a heterogeneous tissue, composed of many cell types. Pooling of DRG may further ‘dilute’ important changes in gene expression.

8.3 Further Studies

8.3.1 Enhancing open field experiments

It may be argued that the open field arena on its own is not a provocative enough test for the measurement of anxiety-like behaviour. Therefore, it may be necessary to consider higher level anxiety provocation tests in which the ‘approach-avoid’ conflict is enhanced. For example, exposing a rat to its natural predator, either as a brief unprotected exposure, or protected exposure (i.e. rat is briefly exposed to a room in which a predator was previously present), or by exposing the animal to predator odours (e.g. cat urine), can induce fearful and anxiety-like states which may enhance anxiety-like behaviour (Roy et al. 2001; Adamec et al. 2004; Beekman et al. 2005; Adamec et al. 2006a; Adamec et al. 2006b). Although this is thought to model aspects of post-traumatic stress disorder rather than a generalized anxiety disorder, it may be an interesting experiment in neuropathic animals. Predator stress has been demonstrated to have a long lasting effect on anxiety-like behaviour, specifically on risk assessment behaviour, including flat back approach and stretch attend behaviours orientated toward the threatening stimulus (Roy et al. 2001; Adamec et al. 2004; Beekman et al. 2005; Adamec et al. 2006a; Adamec et al. 2006b). Further, spontaneous exploratory behaviour in the open field may also be increased by the presence of objects or food within the arena. For example, it has been shown that exploration can be increased by food or water deprivation (Prut and Belzung 2003). While these paradigms have not yet been examined in neuropathic animals, it is important to balance the degree of anxiety provocation in these tests with possible effects of stress-induced analgesia.

8.3.2 Additional paradigms for the assessment of anxiety-like behaviour

No one test provides the ideal model for assessing anxiety-like behaviour in animals. Additionally, different paradigms induce different degrees of anxiety and each test comes with idiosyncrasies and limitations (Cryan and Holmes 2005). Therefore, it is important to use a battery of tests. Such an approach to assessment of behaviour will provide strong support for a
true positive anxiety-related phenotype. The EPM (as described and discussed in Chapter 5) and the light-dark exploration-emergence tests (discussed below) (Cryan and Holmes 2005) are suitable paradigms for further investigation of spontaneous anxiety-like behaviours in VZV-infected animals. Notably however, tests such as the place escape/avoidance paradigm (PEAP) (LaBuda and Fuchs 2000; Pedersen and Blackburn-Munro 2006) which measure stimulus-evoked responses to aversive sensory stimuli may not be appropriate for measuring spontaneous behaviours. In addition, it may be argued that they simply confirm findings from reflex withdrawal studies, only using a more complicated approach. Rather, it seems that paradigms that measure innate behaviour (e.g. open field) are more appropriate to the study of spontaneous behaviours in animals. Operant behaviours may be useful, but in the context of a learned, conditioned place preference (e.g. to analgesic drugs), rather than to aversive stimuli.

**Light-dark exploration-emergence test**

A pilot experiment investigating the response of naive animals in the light-dark exploration-emergence test (light-dark box) was conducted by F. Hasnie and L. Low (University College London). Adult male Wistar rats (B&K, Hull, UK) (n = 16) with a mean weight of 268.7 g (range 250 – 280 g) were introduced into the novel light-dark box. I hypothesised that naive animals would prefer the relative safety of a dark environment over a bright, exposed, and potentially aversive environment; and would display risk assessment and emergence patterns of behaviour.

Briefly, the apparatus consisted of two compartments (each 45 cm³) connected by an open doorway (12 x 10 cm). The ‘light’ compartment was open to the environment and consisted of white Plexiglas walls. A 40 watt tungsten bulb positioned 50 cm above the apparatus provided uniform lighting to approximately 100 lux. The ‘dark’ compartment (<4 lux) consisted of black Plexiglas walls and was covered with a black infra-red permeable Plexiglas lid. Four risk assessment zones at the doorway between compartments were outlined; light and dark ‘inner’ zones measuring 12 cm in diameter (corresponding to the width of the doorway) and light and dark ‘outer’ zones measuring 32 cm in diameter (Figure 8.2). A black spot placed on the animal’s head, equidistant from each ear allowed risk assessment behaviour to be measured. The rat was placed into the dark compartment facing away from the aperture and allowed to explore the apparatus for 10 minutes. The primary outcome measure was percentage time spent in the
light compartment (all four paws marking entry), while secondary outcome measures were latency to entry and number of entries into the light compartment (all four paws); and time spent in each risk assessment zone (head marking entry). Behavioural parameters were scored from a digital video recording after the test session (EthoVision, v2.0).

**Figure 8.2** Schematic of light-dark box and risk assessment zones (1 – 4) (red)

Table 8 illustrates the behavioural outcome measures. Naïve animals spent 21.2% of time in the light compartment (Fig. 8.3), of which over half of their time (11.9%) was spent assessing risk (Fig. 8.4). In contrast, animals spent more time (33.7%) risk-assessing in the dark zone before entering the light compartment.

<table>
<thead>
<tr>
<th>Light-Dark Box Behavioural Outcome Measures</th>
<th>mean (n = 16)</th>
<th>sem</th>
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<tbody>
<tr>
<td>Percentage time in light compartment</td>
<td>21.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Frequency of entry into light compartment</td>
<td>6.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Latency to entry into light compartment (s)</td>
<td>123.5</td>
<td>32.4</td>
</tr>
<tr>
<td>Percentage time in dark box outer risk assessment zone (1)</td>
<td>20.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Percentage time in dark box inner risk assessment zone (2)</td>
<td>13.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Percentage time in light box inner risk assessment zone (3)</td>
<td>5.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Percentage time in light box outer risk assessment zone (4)</td>
<td>6.0</td>
<td>0.7</td>
</tr>
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</table>

**Table 8** Light-dark box behavioural outcome measures in naïve animals
Figure 8.3 Percentage time spent in light and dark boxes. Naive animals (n = 16) spent 21.2% of the time in the light compartment and 78.8% of time in the dark compartment.

Figure 8.4 Percentage time spent in inner and outer risk assessment zones in light and dark boxes. Naive animals (n = 16) spend more time risk-assessing in the outer zone of the dark box.

Whilst these preliminary results are generally consistent with findings from previous studies, (Hughes et al. 2004; He et al. 2006), it is not possible to make direct comparisons as there is great variation in design of the light-dark paradigm and in experimental protocols (Bilkei-Gorzo et al. 1998; Adamec 2001; Bannerman et al. 2003; Hughes et al. 2004; He et al. 2006; Hernandez et al. 2006). This study will be extended to examine spontaneous anxiety-like behaviour in nerve-injured and VZV-infected animals in parallel with the open field and EPM tests. Further, it will be of interest to examine animals at various time points after injury; and to assess the influence of clinically efficacious analgesic compounds in an effort to indicate their association with the presence of a pain-like state.
8.3.3 Further behavioural and non-behavioural outcome measures for the assessment of anxiety-like behaviour in rodents

Of note, the open field test was originally designed to score defaecation as a measure of ‘emotionality’ in animals (Hall 1934). However, we observed no significant differences in faecal or urinary boli in neuropathic compared to control animals. Other behavioural outcome measures for the assessment of anxiety-like behaviour may include sniffing, rearing, grooming and stretch-attend postures. Certainly, sniffing and stretch-attend postures are thought to indicate hesitation and risk-assessment behaviour (Adamec et al. 2006b) which is consistent with anxiety behaviour in humans and frequently seen in chronic pain patients (i.e. risk assessment of potential pain-inducing environments or activities).

The shock-probe defensive burying test for anxiety-like behaviour is an attempt to combine ethological- and punishment-based approaches (such as the Vogel test). (Cryan and Holmes 2005). This task, originally developed and validated in rats (Treit 1990), is based on the observation that rodents exposed to an electrified probe will bury the probe with cage sawdust, presumably as an innate response to prevent further contact with the aversive stimulus. The amount of burying behaviour can be quantified and is reduced by anxiolytic treatment (Treit 1990). Similarly, mice have been observed to spontaneously bury novel marbles placed in their home cage in an anxiolytic-sensitive manner (Njung'e and Handley 1991). Although in this paradigm, the stimulus is not obviously noxious, it is still viewed as potentially aversive by virtue of its novelty. In the same way, it would be interesting to compare burying behaviour in neuropathic and sham animals.

Furthermore, fear and anxiety-like responses in rodents have been related to certain characteristic ultrasound vocalisation (USV) frequencies, typically 20 – 30 kHz (Sanchez 2003). In rats, stressful/anxiety-provoking manipulations such as inescapable foot shock, air puffs, withdrawal from stimulant drugs and exposure to an aggressive conspecific or predator, may induce such USVs in an anxiolytic-reversible manner (Sanchez 2003). Indeed, USV during social interaction in an arthritic model of persistent inflammatory pain (Calvino et al., 1996) and in relation to nociception, such as that induced by electrical stimulation of the tail, has also been reported (Jourdan et al., 1995). However, in a recent study investigating USV in three well-established rat models of somatic, visceral and neuropathic pain, USV was found not to be
associated with other direct behavioural indices of pain, suggesting that the use of USV monitoring as an integrated behavioural measure of persistent pain, particularly neuropathic pain, may not be reliable (Wallace et al. 2005) USV monitoring may, however, be a valid technique in stress- and anxiety-related behavioural studies, and there may be additional benefits in ensuring that background ultrasound levels remain constant in order to standardise testing conditions. Importantly, Wallace and colleagues (2003) observed alterations in rodent behaviour associated with the presence of ultrasound emissions in the laboratory. In fact, ultrasound noise is known to be produced by a variety of laboratory equipment (Sales et al., 1999) including computers, digital timers and television monitors. We endeavoured to keep this effect to a minimum in all behavioural tests, especially during assessment in the novel open field to which animals had not previously been habituated.

Finally, non-behavioural adjunctive measures such as core body temperature, cortisol levels or autonomic functions as measured by radiotelemetry may also be of value as objective measures of anxiety-like behaviour in animals (Cryan and Holmes 2005).

8.3.4 Assessment of other pain-related co-morbidities in rodents

In addition to anxiety and depression, various other pain-related co-morbidities e.g. disturbances in sleep, social functioning, concentration, and appetite may negatively impact on quality of life in persistent pain states (Meyer-Rosberg et al. 2001), and therefore merit further investigation in animals. For example, Monassi et al., (Monassi et al. 2003) recently demonstrated social and sleep-waking changes following traumatic peripheral nerve injury in rats. Briefly, disturbances in the sleep-wake cycle were assessed by the invasive implantation of frontal lobe electroencephalograph (EEG) and dorsal neck muscle electromyograph (EMG) electrodes, while disturbances in social/behavioural interaction were assessed using the ‘resident-intruder model’. In the majority of nerve-injured animals, an increase in time spent awake during both the light and dark cycles and a decrease in slow-wave sleep in addition to a decrease in dominant behaviour patterns and an increase in both non-social behaviour (i.e. self-grooming) and risk-assessment behaviour, suggested a more anxious and hyper-vigilant state. This provides a degree of face validity to persistent pain states (Meyer-Rosberg et al. 2001). Similarly, a recent study in mice that were selectively bred for high levels of immobility in the TST were found to exhibit lighter more fragmented sleep and decreased rapid eye movement
sleep latency (El et al. 2003). Whilst these abnormalities resemble those observed in depressed patients, such studies using neuropathic animals would be an interesting direction for future investigation. Similarly, it may be possible to specifically model the increased vigilance associated with increased anxiety in persistent pain states using acoustic startle response (Pulliam and Plotsky 2006). In a recent study, Pulliam and Plotsky (2006) administered 50 millisecond pulses at 95, 110 and 125 decibels, each 10 times, 30 seconds apart, as an index of increased anxiety-like behaviour and hypervigilance in socially defeated rats (the effects of psychological stressors were measured in rats using the resident-intruder paradigm of social defeat). Response to acoustic startle in neuropathic animals could certainly be investigated in similar experiments, thus contributing to improved face validity of persistent pain.

Additionally, pain-related disturbances in concentration and learning may be assessed using behavioural tests for learning and memory that have been adapted from mice e.g. the ‘social transmission of food preference test’ (in which animals demonstrate memory for an odour-cued food that had been sampled on the breath of a cagemate 24 hours previously); or the ‘Morris water maze test’ (in which spatial memory is assessed), though the latter may not be appropriate in a motor-impaired nerve-injured animal (Holmes et al. 2002). Suitable tests have yet to be performed in rats.

8.3.5 Further assessment of spontaneous pain behaviour

*Conditioned place preference*

Spontaneous pain behaviour may additionally be examined in paradigms such as the ‘conditioned place preference’ (CPP) which is sensitive to clinically useful analgesics (Maldonado et al. 1997; Johansen et al. 2001). CPP involves classical conditioning mechanisms in which analgesia is paired to distinctive environmental stimuli. We conducted pilot experiments investigating the influence of gabapentin conditioning in naive and PSNL-injured animals and hypothesised that neuropathic animals would demonstrate an analgesia-induced place preference i.e. they would spend more time in that chamber paired to active drug treatment.
Experiments were performed on adult male Wistar rats (Harlan, Bicester, U.K.) (n = 17) with a mean weight of 310 g (range 290 – 325 g). Reflex withdrawal testing and PSNL surgery was performed by F. Hasnie as previously described (Chapters 2 & 5). Development of mechanical hypersensitivity in nerve-injured animals (n = 9) was confirmed fourteen days after injury (data not shown) at which time animals were introduced into the CPP paradigm. Animals were not previously exposed to the apparatus. The novel CPP apparatus consisted of three Perspex chambers (each 45cm³) (Figure 8.5). A neutral chamber allowed access to two separate conditioning chambers that were individually paired to distinct olfactory (cinnamon versus peppermint) and visual (monochrome horizontal versus vertical stripes) cues. The paradigm involved three phases: preconditioning, conditioning and testing. In the preconditioning phase, the animal was placed in the middle of the neutral chamber and allowed free access to all chambers for 20 minutes. No preference for any of the chambers was initially demonstrated. The conditioning phase consisted of 30 minute sessions over six consecutive days in which animals were randomly allocated to be conditioned to gabapentin (30 mg/kg i.p.), paired with one chamber; and/or vehicle (saline), paired with the other chamber, on alternate days. Separate groups of animals were used. In the testing session, animals received no injection and were allowed free access to all chambers. During this time, their preference for each chamber (i.e. time spent) was evaluated. During conditioning and testing, the experimenter (T. Pheby) was blinded to the treatments.

Figure 8.5 Three chamber conditioned place preference (CPP) apparatus
Figures 8.6 and 8.7 illustrate the influence of vehicle or gabapentin (30 mg/kg i.p.) conditioning on naïve and PSNL-injured animals respectively. (Sham-operated animals were not investigated in this initial experiment). Gabapentin conditioning did not result in an analgesia-induced place preference in naïve or nerve-injured rats, possibly due to an ineffective dose. However, nerve-injured animals did show a preference for the vehicle-paired chamber over the neutral chamber, which cannot easily be explained. One possible theory may relate to the confounding effects of olfactory cues (discussed below), as what we may have been seeing was merely a preference for one olfactory cue over another.

In light of the findings and discussions with Dr. A. Holmes and Tracksys Ltd., Nottingham, we are considering modifying the CPP paradigm before further experimentation. While it may be argued that the neutral chamber serves as a ‘hallway’ or ‘ante-chamber’ allowing the animal to make more of a choice without being forced to remain in one or other of the conditioning chambers; it may also unnecessarily complicate the set-up and actually ‘dilute’ results. Therefore, we will use a two chamber set-up in future experiments. Second, rodents have a heightened sense of smell; therefore the use of olfactory cues may present further complications as they may be too pungent resulting in a cross-over or dissemination of scents. In addition, olfactory cues should neither be aversive, nor appealing; rather neutral, and this is challenging to ascertain in a rodent. Therefore, we will use tactile cues (i.e. subtle non-aversive differences in floor texture) in addition to the visual cues provided by the monochrome stripes. In addition, we will consider lengthening the period of conditioning, and will examine a range of analgesic agents in both VZV-infected and nerve-injured animals.
Figure 8.6 Influence of A) vehicle (n = 4); or B) gabapentin (30 mg/kg i.p.) (n = 4) conditioning on naïve animals in the CPP paradigm. Animals did not show a preference for any chamber on preconditioning. Conditioning with gabapentin did not induce a place preference in naïve rats.
Figure 8.7 Influence of A) vehicle (n = 3); or B) gabapentin (30 mg/kg i.p.) (n = 6) conditioning on PSNL-injured animals in the CPP paradigm. Animals did not show a preference for any chamber on preconditioning. Conditioning with gabapentin did not induce a place preference in nerve-injured rats. However, nerve-injured animals chose the vehicle-paired chamber in preference to the neutral chamber (* p<0.05, t-test).
8.3.6 Non-behavioural correlates of dynamic mechanical allodynia

Immunohistochemical revelation of the protein product of the early-immediate gene c-fos is observed in the dorsal horn of the spinal cord after persistent activation of nociceptors following peripheral nerve injury or inflammation (Hunt et al. 1987; Abbadie and Besson 1994; Buritova et al. 1997; Wei et al. 1999; Catheline et al. 1999; Jergova and Cizkova 2005; Coggeshall 2005); and pharmacological prevention of this phenomenon has been used for the evaluation of novel analgesics (Abbadie and Besson 1994; Buritova et al. 1996; Chapman et al. 1996). Fos expression may be evoked by the light brushing of the skin of neuropathic, but not naive or sham-operated animals and is probably a correlate of the phenomenon of brush-evoked dynamic mechanical allodynia (Buritova et al. 1997). This approach could be used to ascertain whether Fos expression is indeed evoked in a model of zoster-associated pain, and whether it can be prevented by analgesic intervention. This will further support my behavioural data (Chapter 2) and validate the microarray findings (Chapter 7).

8.3.7 Age-related impact of VZV infection on animal behaviour

The number of older people in the population is increasing and there is evidence that chronic painful conditions including neuropathic pain states are associated with advancing age (Novak et al. 1999; Pickering et al. 2006). Certainly, herpes zoster and PHN are more prevalent in the elderly population (Nurmikko 1995; Novak et al. 1999); with age being a principal risk factor in the development of zoster-associated persistent pain (Nurmikko 1995; Coen et al. 2006). The age-related increase in susceptibility to neuropathy may be a result of neurochemical and neuroanatomical changes, such as degeneration of endogenous neural inhibitory systems or increasing spontaneous involution and abnormalities in DRG sensory neurones (Gagliese and Melzack 2000). However, few studies have been done to assess the impact of nerve injury-induced neuropathic pain in old and particularly senescent animals and this may prove a useful further investigation in the zoster-associated pain model. Several authors have reported a non-linear evolution of pain behaviour in response to peripheral nerve injury with age, specifically a peak of pain at mid-life and a decline of pain in old animals (Novak et al. 1999; Gagliese and Melzack 2000; Cruce et al. 2001). For example, Pickering et al., (2006) recently compared the evolution of pain in senescent rats (37 – 39 months) to old (20 – 22 months) and young (4 – 6 months) animals after a chronic constriction of the sciatic nerve. Whilst no significant age
discrepancies in response to a noxious thermal (paw immersion test) stimulus was detected prior to nerve injury, significantly higher thresholds in response to mechanical stimulation (paw pressure test) were reported in young animals compared to old and senescent animals. Following nerve injury, significant hyperalgesia to mechanical and thermal tests was not detected in the senescent cohort of animals, whereas the response of young animals was found to be severe, especially with mechanical stimuli, and was still present as in the case of old animals, at 28 days post-surgery. Importantly, it has further been reported that old rats differ from young rats in emotional behaviour and in a higher anxiety level when assessed in the open field and EPM paradigms (Boguszewski and Zagrodzka 2002). Thus, it would be interesting and more clinically relevant to investigate the impact of age on VZV-induced hypersensitivity and anxiety-like behaviour.

8.3.8 Influence of gender and strain

It is well known that gender differences in pain (Wiesenfeld-Hallin 2005; Torrance et al. 2006), and in the prevalence of anxiety (Breslau et al. 1995) and depression (Haley et al. 1985; Frackiewicz et al. 2000), exist in humans. Specifically, a higher prevalence of chronic pain states and greater pain sensitivity has been observed among women compared with men, with pain threshold and pain tolerance also known to vary with the stage of the menstrual cycle (Wiesenfeld-Hallin 2005). Furthermore, differences between men and women in the spatial pattern and intensity of response to acute pain have been highlighted in brain imaging studies. Animal gender has similarly been shown to influence nociceptive sensitivity (Mogil et al. 2003; Wiesenfeld-Hallin 2005), as well as anxiety- and depression-like behaviour (Palanza 2001; Cryan et al. 2005; Millstein and Holmes 2006; Adamec et al. 2006a). For example, among rodents, females are more sensitive than males to noxious stimuli and have lower levels of stress-induced analgesia; while male rodents generally have stronger analgesic response to μ-opioid receptor agonists compared to females (Wiesenfeld-Hallin 2005). In addition, Adamec et al., (2006b) recently found that female, but not male, mice are made more anxious in the elevated plus maze by exposure to predator odours. There is much evidence for the importance of strain differences in rodents, specifically differences in baseline nociceptive sensitivity and in predisposition to neuropathic pain following neural injury (Mogil et al. 1999a; Lovell et al. 2000; Valder et al. 2003); in sensitivity to pharmacological agents (Belzung and Agmo 1997; Wilson et al. 2003a; Wilson et al. 2003b), and in anxiety- and depression-related behaviour.
(Balcells-Olivero et al. 1998; Bai et al. 2001; Carola et al. 2002; Cryan et al. 2005; Augustsson et al. 2005; Millstein and Holmes 2006) have been reported in both mice and rats. Therefore, it would be valuable to investigate the influence of gender and strain differences on pain-related behaviours in neuropathic rats.

8.3.9 Detection of viral proteins

In addition to immunohistochemical analysis, quantitative PCR would provide further confirmation of the presence of viral proteins in DRG and would importantly allow viral load or burden of VZV to be examined and compared at different time points following infection, or following infection with different strains or viral concentrations. Interestingly, quantitative PCR analysis in human tissue has demonstrated that the ganglionic burden of VZV during latent infection is low (Mahalingam et al. 1993; LaGuardia et al. 1999; Cohrs et al. 2000; Levin et al. 2003). Additionally other tissues could be analysed for the presence of viral proteins e.g. sciatic nerve and spinal cord.

8.3.10 Additional microarray experiments

Due to the limitations discussed previously, it would be beneficial to study a time course of gene expression to examine dynamic gene changes i.e. how particular genes are disregulated with time. It may be that some genes are up-regulated early on but down-regulated later, and therefore investigating only one time point may miss such changes in gene expression. Further, it may be that certain genes are more important in the induction of hypersensitivity phenomenon and are thus only detectable early after viral infection or nerve injury, while other genes may be specifically associated with the maintenance of neuropathy and are only expressed at later time points. Additionally, it may be of interest to examine gene expression changes following infection with different viral strains, as viral factors, rather than host factors may be more important in development of hypersensitivity phenomenon following VZV infection. Further, it may also be of interest to profile differential gene expression in spinal cord or brain for a more global overview. Finally, it is essential to complement microarray studies with other molecular genetic techniques, such as transgenesis and antisense knockdowns. These traditional techniques may prove useful in further investigating candidate genes identified by microarray screening.
8.3.11 Electrophysiological studies

The most prominent electrophysiological abnormality observed in animal models of traumatic peripheral nerve injury is ectopic discharge originating from the site of injury and from the DRG of injured afferent fibers (Kajander and Bennett 1992; Gabay and Tal 2004). Ectopic activity is thought to be responsible for spontaneous pain behaviour and also contributes to central sensitisation and the maintenance of persistent pain (Gabay and Tal 2004). *In vivo* electrophysiology of the spinal cord and DRG in VZV-infected animals will therefore allow the electrophysiological correlates of neuropathic pain to be examined, and will further our understanding of the mechanisms underlying zoster-associated pain. Pharmacological sensitivity testing would also be performed.

8.4 Conclusion

This study describes a rat model of zoster-associated pain, and is characterized by a chronic resolving mechanical hypersensitivity that is sensitive to clinically useful analgesic drugs in the treatment of neuropathic pain (Hempenstall et al. 2005; Rice and Hill 2006). Furthermore, the demonstration of anxiety-like behaviour in virus-infected animals may reflect pain co-morbidity and hence the affective component of persistent pain. However, whilst the model shares aspects of both AHZ and PHN, it is a direct model of neither. Though it may be argued that the time course of the observed behavioural change is consistent with that of an acute zoster attack in humans, it is an over-simplification to state that this is a model of AHZ on the basis of time course alone. The most salient feature suggesting that it is not a model of AHZ is that there are no cutaneous eruptions. Additionally, the administration of the anti-viral acyclovir, which has proven efficacy in the treatment of AHZ (Dworkin et al. 2006) failed to attenuate hypersensitivity phenomenon, which further suggests that this is not a direct model of AHZ. Furthermore, reactivation of latent virus and associated tissue damage does not occur. The lack of construct validity for either AHZ or PHN reflects the fact that VZV has a restricted host range and is not a pathogen in rodents. Despite this, aspects of AHZ and PHN are being modelled. With reference to PHN, infected animals (in which ipsilateral DRG sensory neurones are positive for VZV IE62 protein) display hypersensitivity to mechanical, though not thermal, stimuli, which is consistent with observations in humans (Fields et al. 1998; Pappagallo et al.
In addition, the hypersensitivity changes are attenuated/reversed by pharmacological agents known to have efficacy in PHN (Hempenstall et al. 2005). Thus, this model demonstrates considerable face validity and predictive validity and more appropriately models zoster-associated pain.

However, the mechanisms by which VZV infection causes the observed behavioural changes in rats are unknown. One possible theory is for ongoing viral replication within ganglion cells. In support of this theory, we demonstrated the intranuclear presence of viral IE62 protein, albeit in a minority of DRG neurones. Furthermore, microarray analysis identified genes involved in biosynthesis, viral transport, and transcription which suggests that active replication is occurring. However, the majority of evidence does not favour this theory. For example, productive viral replication has not yet been demonstrated following VZV infection in the rat i.e. viral RNA has not been detected and studies have shown an absence of cpe when known VZV-infected rat DRG neurones are cultured ex-vivo (Sadzot-Delvaux et al. 1995). The hypothesis is further challenged by the findings of Dalziel et al., (2004) in which valaciclovir treatment at the time of viral infection in VZV-infected rats was found to have no effect on the development of mechanical hypersensitivity. This suggests that replicating virus (i.e. lytic infection) is not required for the induction of VZV-induced mechanical hypersensitivity. Additionally, pharmacological sensitivity testing in this model has further demonstrated that active viral replication is not necessary for the maintenance of mechanical hypersensitivity (Chapter 3). Further, tissue damage and cell destruction characteristic of ongoing viral replication during reactivation of VZV was not seen. Finally, though viral protein was detected within the nucleus of some DRG neurones, we generally observed cytoplasmic staining which is consistent with previous studies (Fleetwood-Walker et al., 1999; Garry et al., 2005) and with the premise that active viral replication is generally not taking place.

Numerous studies have confirmed the presence of IE62 and/or IE63 viral protein in the DRG of infected rats and in humans (Sadzot-Delvaux et al., 1995; Sadzot-Delvaux et al. 1995; Kennedy et al. 1998; Kennedy et al. 2000; Kennedy et al. 2001; Kennedy 2002a). It may be that expression of these regulatory proteins somehow interfere with the physiological function of infected DRG neurones, and in this way are able to influence central nociceptive processing. The increased expression of neuropeptides NPY and galanin, and of ATF-3 observed by Garry et al., (2005) (and supported by our PCR data) indicate damage to large-diameter DRG neurones (NPY and
galanin), and axonal damage or cell stress (ATF-3). VZV-induced axonal damage may cause spontaneous activity at different sites along the primary afferent; or an up-regulation of excitatory adrenergic receptors on primary afferents; or inflammation along the axon and a hyper-excitability state. Certainly the microarray data identified genes specifically involved in ion transport, receptor activity, and neurotransmitter secretion which would support this theory. Such functional changes may have implications in the generation and/or maintenance of spontaneous ectopic activity and hyperexcitability commonly seen in persistent pain states.

From quantitative PCR analysis in human tissue, it is known that the ganglionic VZV burden during latent infection is low (Mahalingam et al. 1993; LaGuardia et al. 1999; Cohrs et al. 2000; Levin et al. 2003), which suggests that quantity of virus within the DRG is not an important factor for development of the virus-induced behavioural changes that would occur on reactivation. In our study, mechanical hypersensitivity in the rat developed from day 4 post-infection. However, it is not known how quickly the virus is able to undergo retrograde transport to the DRG and whether it is present in ganglion cells by this stage. It may be that it is not necessary for the virus to be in the DRG for the observed behavioural changes to develop. We have demonstrated the presence of viral protein in the ipsilateral sciatic nerve on preliminary PCR (data not shown). Thus, it may be that expression of viral proteins here is enough to initiate hypersensitivity changes that progresses as the virus continues retrograde transport until a maximum response is reached in the DRG. Indeed, a ‘hit and run’ mechanism may be responsible, and is supported by the fact that viral protein was not detected on resolution of mechanical hypersensitivity. Finally, the observed behavioural changes may be due to low grade chronic VZV-induced ganglionitis and is supported by clinical evidence (Gilden et al. 2003a). Inflammatory and neuropathic pain mechanisms may therefore co-exist in PHN. The identification of genes involved in the generation of inflammation i.e. cytokine and chemokine mediated signaling pathways, and ibuprofen-induced reversal of VZV-induced mechanical hypersensitivity support an inflammatory component to this model.

In conclusion, this thesis has refined and further characterised a rat model of zoster-associated hypersensitivity to provide improved clinical validity and predictability. It will prove useful in elucidating the pathophysiology of herpes zoster-associated pain.
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Publications That Have Arisen From This Thesis

“Further characterization of a rat model of varicella zoster virus-associated pain: Relationship between mechanical hypersensitivity and anxiety-related behavior, and the influence of analgesic drugs”.

Hasnie FS, Breuer J, Parker S, Wallace V, Blackbeard J, Lever I, Kinchington PR, Dickenson AH, Pheby T, Rice AS.

“Mechanical and cold hypersensitivity in nerve-injured C57BL/6J mice is not associated with fear-avoidance- and depression-related behaviour”.

Hasnie FS, Wallace VC, Hefner K, Holmes A, Rice AS.
British Journal of Anaesthesia (May 2007)