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A MODEL OF VIRUS-INDUCED HYPERALGESIA IN RATS

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A dissertation submitted to the Faulty of Health Sciences, University of the Witwatersrand, in fulfillment of the of the requirements for the degree of Master of Science

Johannesburg, 2006

DECLARATION

I declare that the work contained in this dissertation is my own, with all assistance acknowledged. This work has not been submitted before for any degree or examination at any other University.

All procedures used in this dissertation were approved by the Animals Ethics Screening Committee of the University of the Witwatersrand (AESC numbers: 2003/95/4, 2004/45/3 and 2005/16/3). The procedure complied with the recommendations of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann 1983).

.....

(Musi Thabang Skosana)

..... Day of ……2006

ABSTRACT

The link between infection and hyperalgesia has been determined using mainly lipopolysaccharide, which is a bacterial antigen. The relationship between viral infection and hyperalgesia has yet to be fully characterized, with current models of virus-induced hyperalgesia being based on infections by specific neurotrophic viruses. Therefore, the aim of this series of studies was to develop a general model of virus-induced hyperalgesia by injecting a pyrogenic and non-pyrogenic dose of Poly I:C, a synthetic, double-stranded ribonucleotide that is similar to the double-stranded ribonucleotides produced by almost all viruses, into rats' tails.

To identify a pyrogenic and non-pyrogenic dose of Poly I:C, male Sprague Dawley rats were injected subcutaneously in the tail either with saline (n=8) or 100 $\mu g.kg^{-1}$ (n=8) or 1000 $\mu g.kg^{-1}$ (n=8) Poly I:C. Only rats that received 1000 $\mu g.kg^{-1}$ Poly I:C developed fever. Subsequently, in a separate experiment, tail withdrawal latencies to noxious thermal (49° water) and mechanical stimuli (4N blunt force) were recorded for six days after subcutaneous injection of 100 $\mu g.kg^{-1}$ or 1000 $\mu g.kg^{-1}$ Poly I:C or saline in male Sprague Dawley rats. I also took skin tissue samples from the site of injection to determine the histological changes that occur after Poly I:C injection. Thermal hyperalgesia was not elicited by Poly I:C injection. However, biphasic mechanical hyperalgesia developed in the animals receiving 100 $\mu g.kg^{-1}$ Poly I:C, and sustained mechanical hyperalgesia, that lasted for four days, was evident in animals injected with 1000 $\mu g.kg^{-1}$ Poly I:C. Mild sustained inflammation, at the injection site of both doses of Poly I:C, was present even after the hyperalgesia had subsided.

This study provides evidence for a novel model of virus-induced hyperalgesia that is applicable to most cutaneous viral infections. More importantly, this model can be used to further our understanding of the mechanisms that underlie virus-induced pain.

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PRESENTATIONS

The following oral presentation is offered in support of this dissertation.

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LIST OF ABBREVIATIONS

Polyinosinic:polycytidylic acid	Poly I:C
Human immunodeficiency virus	HIV
Herpes simplex virus	HSV
Acquired immunodeficiency syndrome	AIDS
Tumour necrosis factor-α	TNF-α
Interleukin-1	IL-1
Interleukin-6	IL-6
Double-stranded ribonucleic acid	(ds)RNA
Single-stranded ribonucleic acid	(ss)RNA
Toll-like receptor	TLR
Lipopolysaccharide	LPS
Central nervous system	CNS

Chapter 1

Introduction

1.1. Pain in viral infections

1.1.1. Pain: a brief overview

Pain, which is multidimensional in nature, is known to be an unpleasant sensory or emotional experience that may be associated with tissue damage (Blackburn-Munro 2004; Kandel et al. 2000; Woolf 2004). There are four types of pain, namely, nociceptive, inflammatory, neuropathic and functional pain (Woolf 2004). Before proceeding with my discussion on virus-indiced pain, I shall briefly discuss the basic mechanisms underlying nociceptive and inflammatory pain; a discussion of neuropathic and functional pain is beyond the scope of this dissertation. Nociceptive pain is defined as the detection of noxious stimuli, such as noxious heat, cold, mechanical and chemical stimuli, by specific nociceptive nerve endings, resulting in a reflex withdrawal or reaction to the noxious stimulus. It is the population of thinly myelinated peripheral nerves called A-delta and unmyelinated C-fibres that are responsible for transmitting nociceptive information from the periphery to the central nervous system. The transducer ion channels for these fibres are gated by temperature, chemical ligands and mechanical shearing forces (Woolf 2004). Nociceptive pain is acute and adaptive in that it contributes to the survival of the organism by guarding against tissue injury. Disabling of nociceptive pain leads to tissue damage that can manifest itself through self-induced mutilation (Dray 2005; Woolf 2004).

Compared to nociceptive pain, where the pain only occurs in response to a noxious stimulus, inflammatory, neuropathic and functional pain have different causes and are all

characterized by pain hypersensitivity (allodynia and hyperalgesia) and spontaneous pain (Devor 2005; McMahon 2005; Meyer 2005; Woolf 2004). Sensitization is a leftward shift of stimulus-response function in the magnitude of the neural response to stimulus intensity and occurs during inflammatory, neuropathic and functional pain (Meyer RA 2005). Sensitization increases the probability that a stimulus will activate the target receptor or ion channel, by decreasing the threshold, augmenting the response to suprathreshold stimuli and ongoing spontaneous activity (Meyer RA 2005). Allodynia is pain caused by low-intensity innocuous stimuli, such as light touch, that usually do not cause pain. Hyperalgesia is characterized by an exaggerated and prolonged response to a noxious stimulus that is normally painful (Blackburn-Munro 2004; Woolf 2004).

Inflammatory pain occurs during tissue damage, and typically resolves as tissue repair progresses. In some cases, the pain may outlast the period of repair, in which case inflammatory pain is maladaptive (Meyer RA 2005; Woolf 2004). Inflammation causes an enhanced response to suprathreshold mechanical stimuli, spontaneous activity and expanded receptor fields for both A-delta and C-fiber nociceptors, and reduced thresholds for the activation of nociceptors (Meyer RA 2005). Inflammation leads to the release of numerous chemicals, such as bradykinin, histamine, cytokines and prostaglandins from immunocompetent cells and injured cells, that may act directly or indirectly to alter the sensitivity of peripheral nerve terminals. Sensory fibers express three main classes of receptors affected by inflammatory processes, namely the G-protein-coupled receptors, ligand-gated ion channels and the cytokine receptors (Devor 2005; McMahon SB 2005; Meyer RA 2005; Woolf 2004). The principal determinant of peripheral nerve excitability

appears to be phosphorylation of voltage and ligand-gated membrane ion channels (Dray 2005; Woolf 2004).

To cause pain, inflammatory mediators recruit autonomic and sensory systems and cause the activation and sensitisation of the peripheral nociceptors. Some of these mediators directly activate nociceptors (e.g., bradykinins) by depolarization of the neuron (Martin et al. 2003; Meyer RA 2005; Woolf 2004). It is also the accumulation of various mediators together with tissue acidification acting synergistically that is suspected in the production of pain and hyperalgesia (Oprée and Kress 2000).Other mediators (e.g., prostaglandins) act indirectly via inflammatory cells, which in turn release algogenic agents, and also by changing the neuronal sensitivity and excitability of nociceptors to algogenic agents (Martin et al. 2003; Meyer RA 2005; Woolf 2004). The cytokines that are released during inflammation are suggested to excite nociceptors by activating receptor-associated kinases. These kinases phosphorylate ion channels on nociceptors facilitatoring signal transduction and increasing nociceptive sensitivity (Oprée and Kress 2000). Nociceptor hyperexcitability during inflammatory pain underlies the clinical signs of allodynia and hyperalgesia (Dray 2005; Woolf 2004).

In addition to the influence peripheral inflammation has on the development of hypersensitivity to pain, there are changes in the way sensory information is processed by the central nervous system (CNS), which contributes to the hypersensitivity. Initially central sensitisation of nociceptive pathways is caused by increased nociceptor input into the spinal cord, but is sustained by changes in the dorsal horn neurons, which enhance the

release of neurotransmitters, and change synaptic receptor thresholds, kinetics (Martin et al. 2003; Meyer RA 2005; Woolf 2004). These changes are known to involve inflammatory mediators released by activated glial cells in the dorsal horn (Martin et al. 2003; Meyer 2005; Wieseler-Frank et al. 2005; Woolf 2004). Central sensitisation manifests itself as allodynia and hyperalgesia and the spread of sensitivity to non-injured sites (secondary hyperalgesia) (Meyer RA 2005). For inflammatory pain, circulating cytokines maintain central sensitization, and are known to act on glial cells which they themselves can release other proinflammatory cytokines which result in a neuroexcitatory state (Meyer 2005; Watkins et al. 1995b; Wieseler-Frank et al. 2005). A cascade of events, in the dorsal horn, triggers the release of transmitters which change synaptic receptor threshold, kinetics and activation. Also, circulating cytokines in the blood may cause central sensitization by communicating to the brain via entering the circumventricular sites lacking the blood-brain barrier or via the vagal afferent (Watkins et al. 1995b; Wieseler-Frank et al. 2005).The secondary hyperexcitability, in the CNS, is observed due to peripheral inflammation and nerve injury causing neuroglia cells to release inflammatory mediators and cell regulators (Dray 2005; Woolf 2004). Inflammatory mediators can damage nerves and may begin to induce characteristics of neuropathic pain.

Having summarised the basic principles of nociception and inflammatory pain hypersensitivity, in the remainder of this chapter I shall focus on pain caused by viral infections. In the following section, I shall outline the prevalence of three illustrative

examples of virus-induced pain, namely pain caused by Human immunodeficiency virus (HIV), Herpes simplex virus (HSV) and influenza virus infections.

1.2. Models of Viral Pain

In the previous section, I outlined the prevalence of pain caused by three viral diseases. In this section I shall provide information on animal models of viral pain that have been developed to gain a better understanding of mechanisms behind these pains, and therefore, potentially improve our treatment of viral pain. Rodents have been the most widely used animals in these models (Wang and Wang 2003). Similar structures in humans and in animal models, such as the brain, spinal cord and nerves, have been shown to be involved in the processing of nociception, and associated behaviours and therefore, animal models provide useful and essential systems to study and understand the pathophysiological mechanisms of human pain (Blackburn-Munro 2004). The two principal models of viral infections are models of HIV and Herpes virus infections. I shall now discuss animal models of each infection.

1.2.1. Models of HIV-related pain

The retrovirus HIV, being part of the Lentivirus subfamily, is a virus that thrives within cells of our immune system, ultimately causing immune system failure. By attaching to the CD4 receptor and CCR4/CXCR4 chemokine receptors, the virus infects CD4 T

lymphocytes and macrophages, and then uses these cells to replicate, ultimately destroying them resulting in the progression of the disease correlating with a decrease in CD4 T lymphocytes (Kramer-Hammerle et al. 2005; McArthur et al. 2005; Mogensen and Paludan 2001). Approximately 40 million individuals globally, are infected with HIV/AIDS with about 13000 people contracting the virus daily (WHO, 2005). Pain is prevalent throughout the course of HIV-infection and intensifies with the progression of the disease (Breitbart and Dibiase 2002; Breitbart et al. 1996; Del Borgo et al. 2001; Mogensen and Paludan 2001). The virus itself may cause pain, but secondary infections that occur during the progression of the disease and the pharmocotherapies, such as antiviral treatments, that are prescribed for the disease, also may cause pain (Joseph et al. 2004).

A French survey of 314 patients, reported the incidence of pain in HIV infected individuals to be greater than 60% (Larue et al. 1993). Similar results also have been reported by studies in the United States of America, where 63% of ambulatory acquired immunodeficiency syndrome (AIDS) patients complained of persistent pain (Breitbart and Dibiase 2002). Other studies have reported pain prevalence in HIV infected people ranging from 50 to 90% (Del Borgo et al. 2001; O'Neill and Sherrard 1993). Sub-Saharan Africa has the worst HIV/AIDS epidemic in the world, with a third of the world's HIV infected population living there (Robinson and Gazzard 2005). A recent study conducted in South Africa on terminally ill HIV-positive patients showed that 98% of patients reported pain, with pain being the number one complaint (Norval 2004). If approximately

60-80% of the 40 million individuals infected with HIV/AIDS globally experience pain, between 24-32 million people may be suffering from HIV-related pain.

Body regions affected by HIV-related pains include the lower limbs as the most common site, followed by the mouth and throat region, then headaches and abdominal pain (Breitbart et al. 1998; Hewitt et al. 1997; Hirschfeld 1998; Penfold and Clark 1992). Human immunodeficiency virus has been known to cause pain by initiating neuropathies which occur in 15-50% of HIV-positive patients (Hewitt et al. 1997). A common neuropathy is the distal sensory neuropathy, caused by immunological dysfunction, and inflammatory demyelinating polyneuropathies, that are both acute (e.g., Guillain-Barré syndrome) and chronic (Breitbart et al. 1998; Luciano et al. 2003; O'Neill and Sherrard 1993). Myelopathies and HIV dementia are some of the HIV neuropathies experienced by patients. For myelopathies, the exact mechanisms of how they cause pain are unknown but it is postulated that intralamellar vacuolation occurs in the spinal white matter (McArthur et al. 2005). Also, macrophages are implicated in the development of these pathologies by releasing pro-inflammatory cytokines, such as tumor necrosis factor (TNF), which act on the neurons directly or indirectly or act on glial cells (McArthur et al. 2005). Even though antiviral treatments have been developed for HIV which decrease disease morbidity and mortality (Andoh et al. 1995; Glare 2001; Sasaki 2003), antiviral treatments for HIV infection are known for adverse effects, such as pain (Cinti 2005; Joseph et al. 2004; Lee and Henderson 2001; Stenzel and Carpenter 2000; Newshan et al. 2002).

Milligan and colleagues (2000) established a model of HIV-induced pain in rats. The researchers showed that thermal hyperalgesia and mechanical allodynia occur after intrathecal administration of the human immunodeficiency virus-1 (HIV-1) envelope glycoprotein, gp120, which is an envelope protein that initiates cell entry by interacting with CD4 and chemokine receptors. By using two treatments (fluorocitrate, which selectively blocks astrocyte and microglia metabolism, and CNI-1493, a macrophage deactivator), they were able to show that the spinal cord glia may play a role in the pain states observed after administration of gp120 into the spinal cord. The authors suggested that the gp120 portion of HIV-1 binds to and activates spinal glial cells, which mediate the gp120-induced hyperalgesia and allodynia. The role of inflammatory processes in the spinal cord as mediators of allodynia and hyperalgesia previously had been suggested by others from the same laboratory (Watkins et al. 1995a). Milligan and colleagues (2001) went onto investigate whether glial cells secrete pro-inflammatory cytokines which then mediate the gp120-induced pain state. Indeed, gp120 did induce sections of interleukin-1 (IL-1) and TNF, and administering the respective cytokine blockers attenuated the pain (Milligan et al. 2001). The same group of investigators then showed that spinal cord nitric oxide, stimulated by intrathecal administration of gp120, synthesised by nitric oxide synthase-I, was in part responsible for the activation of the pro-inflammatory cytokines, by increasing mRNA levels, protein expression and protein released (Holguin et al. 2004); blocking nitric oxide production with nitric oxide synthase inhibitors, abolished gp120-induced mechanical allodynia (Holguin et al. 2004).

The model used by Milligan and colleagues (2001), of injecting gp120 intrathecally, provides better understanding of how HIV, by producing inflammation in the CNS, may cause pain, but peripheral pathways also may be involved in HIV-related pain. Chemotactic cytokines, chemokines, released peripherally by immune cells, also have been implicated in producing gp120-induced pain. For HIV to gain enrty into immune cells it must bind CD4 receptors and chemokine coreceptors CCR4 and CXCR4 (Oh et al. 2001). Activation of CCR4 and CXCR4 caused a central and peripheral increase in calcium ions, substance P release, neuronal excitation and resultant allodynia (Martin et al. 2003; Oh et al. 2001).

The viral envelope protein gp120 also has been administered epineurally in rat sciatic nerve, causing allodynia and hyperalgesia (Herzberg and Sagen 2001). The administration of gp120 epineurally caused neuropathic pain and spinal gliosis, the latter being characterized by the excessive proliferation of neuroglia, especially in the interstitium. Glycoprotein 120 causes neuronal death by acting on neurons and glial cell release of neurotoxic substances, such as IL-1, which then disturb the neuronal calcium homeostasis and induce apoptosis (Viviani et al. 2001). Indeed, HIV-1 has been shown to cause sensory axonal degeneration through the induction of pro-inflammatory cytokines (Jones et al. 2005). Therefore, gp120 is able to produce hyperalgesia through peripheral and central mechanisms.

1.2.2. Models of herpetic pain

The Herpes simplex virus (HSV) is phylogenetically old and has evolved with humans (Ellermann-Eriksen 2005). This co-evolution has enabled the virus to cope with the human immune defence system in many cases. The virus belongs to the Alphaherpesvirinae subgroup, has a rapid life cycle and establishes itself within sensory neurons (Ellermann-Eriksen 2005; Mogensen and Paludan 2001). Infection occurs with the cellular uptake of the viral particle via a heparin sulphate chain on a cellular proteoglycans receptor (Ellermann-Eriksen 2005; Mogensen and Paludan 2001). The virus spreads from cell to cell causing an acute immune response, but ultimately the virus establishes a latent infection in sensory neuron ganglia (Dalziel et al. 2004). Currently, the United States of America has a higher HSV prevalence, at about 22% of the population, than Europe (approximately less than 15%) (Smith and Robinson 2002). Herpes simplex virus global prevalence usually reaches 40% amongst teenagers by the age of 15 and rapidly increases to 80% older adults due to increased sexual activity (Smith and Robinson 2002). Also, HSV prevalence rates in developing regions such as Sub-Saharan Africa and the Caribbean are substantially higher at 50% for adults older than 15 years of age. Furthermore, in other regions of the world, HSV infection is on the rise due to socio-economic factors such as poverty (Ellermann-Eriksen 2005). Pain is reported during the primary infection, and during recurrent infections that occur when there is activation of the latent virus in sensory root ganglia (Dalziel et al. 2004; Ellermann-Eriksen 2005).

Herpetic pain states such as post-herpetic neuralgia have been estimated at about 500 000 cases per year in the United States of America (Bowsher 1999), and about 200 000 cases per year in England even though this was thought to be a very conservative estimate (Bowsher 1999; Dalziel et al. 2004). Another study, that was conducted in a large double-blind placebo-controlled clinical trial of acyclovir, an antiviral treatment for HSV infection, in the United Kingdom, reported that about 60% of HSV-infected people had pain in the form of neuralgia (Oster et al. 2005).

Primary infection can be produce more pain than that of secondary infections and may last clinically for three weeks (Dalziel et al. 2004; Mogensen and Paludan 2001). Besides fever and headaches, reactivation of latent HSV results in lesions occurring at areas innervated by infected nerves producing aching and burning sensations (Dalziel et al. 2004; Sasaki 2003). Oster and colleagues (2005) showed that two thirds of subjects with post-herpetic neuralgia complained of their worst pain being moderate to severe, with half of the patients being in constant mild to severe pain (Oster et al. 2005). Also post-herpetic neuralgia, which is classed as neuropathic pain, may last for more than three months with the pain impairing sleep, causing depression and social withdrawal (Dalziel et al. 2004). The mechanisms involving herpetic pain are being investigated but are still largely unknown.

To understand the mechanisms of HSV-1 pain, rats were inoculated intradermally with HSV-1 in the hind limb and behavioural responses to noxious stimuli were examined (Andoh et al. 1995). Hypoalgesia, a decreased response to a painful stimulus, was

produced after administration of HSV-1. Andoh and colleagues (1995) were able to show the presence of paresthesia which was tested for by using the paw-pressure method. The authors postulated that the paresthesia may be caused by the virus disrupting the functioning of neurons. Another model for hyperalgesia and allodynia induced by HSV-1 was established by Takasaki and colleagues (2000b), who showed that inoculation of mice with HSV-1, on the hindpaw, caused mechanical hyperalgesia and allodynia. In this study, the pain states peaked on day five and six post-inoculation, which coincided with a peak in HSV-1 DNA in the dorsal root ganglia. The hyperalgesia and allodynia were most likely a result of herpetic infection of sensory neurons and of HSV-1 propagation in the dorsal root ganglia, causing dysfunction of nociceptive sensory neurons (Takasaki et al. 2000b). Herpes simplex virus-1 inoculation also causes the release of cytokines and prostaglandins in the dorsal root ganglia, which probably contributed to the development of allodynia and hyperalgesia (Takasaki et al. 2000b; Eccles 2005).

What may have also contributed to the allodynia and hyperalgesia was the percentage and type of infected sensory neurons and the continued production of prostaglandins (Takasaki et al. 2000a). In another model of herpetic pain, chronic varicella-zoster virus infection in rats caused both allodynia and hyperalgesia up to 33 days post-infection (Fleetwood-Walker et al. 1999).. The authors stated that immune-mediated damage at the site of injection, in the footpad, may have played a minor role in the causing hyperalgesia and allodynia, while the presence of varicellar-zoster virus in the dorsal root ganglia, which had correlated with the increased sensitivity to sensory stimuli, had probably been the main determinant of the pain hypersensitivity observed. The authors did however

concede that the mechanisms by which varicellar-zoster virus infection caused the observed sensory changes were unknown.

While the models of HIV and herpetic pain I have described provide useful information on the mechanisms of viral pain, these models are specific to particular viruses, which infect the nervous system, and therefore any information gathered from these models may only be applicable to the model investigated (Fleetwood-Walker et al. 1999; Milligan et al. 2000). Hence, a general model of virus-induced hyperalgesia, that is not limited to a particular neurotrophic virus, is needed so that the model may be applicable to most of the viral infections observed. For the model to be applicable to most viruses, if not all, the model should be based on a viral attribute that is common to most, if not all, viruses. Double-stranded RNA which is indistinguishable between live viruses in terms of the toxic response at the organism level (Majde et al. 1998), is produced at some stage of the viral life-cycle in most viruses. Indeed, even single-stranded RNA viruses and DNA viruses produce double-stranded (ds)RNA during infection of host cells (Majde et al. 1998). In single-stranded (ss)RNA viruses, dsRNA is formed as one of the steps during replication and in DNA viruses, dsRNA are formed as complementary mRNA's (Carter and De Clercq 1974; Majde et al. 1998).

1.3. Polyinosinic:polycytidylic acid (Poly I:C)

1.3.1. Characteristics of Poly I:C

Poly I:C is a synthetic double-stranded polyribonucleotide, which is similar to viral polyribonucleotides (Majde et al. 1998). Poly I:C forms a deep, narrow A-form helix that is RNA nuclease resistant, which makes it more resistant to endonuclease and exonuclease degradation than other formulations of viral dsRNA analogues (Majde et al. 1998). This nuclease resistance therefore gives Poly I:C more time to mediate its effects before it is cleared by the immune system. Poly I:C initially was used to induce interferon by activation of macrophages, which are the first line of defense of the host, which at the time was thought to have clinical importance and potential as an antiviral therapy (Chadha et al. 2004; Majde 2000). However, intense work focusing on the potential antiviral effects of Poly I:C, showed Poly I:C to be more toxic than therapeutic (Chadha et al. 2004; Majde 2000).

Poly I:C and other dsRNA activate the immune system through Toll-like receptor 3 (TLR3), which is a transmembrane signaling protein expressed as part of the innate immune system (Fujimoto et al. 2004; Sen and Sarkar 2005). These receptors may be responsible for activating signaling cascades within cells and triggering immune and inflammatory responses to combat infection. Toll-like receptor 3 uses the MyD88-independent pathway to induce interferon and antiviral genes. When TLR's are activated by viruses or dsRNA, they initiate the innate immune response by causing the release of various mediators which activate other TLR's on dendritic cells causing the dendritic

cells to mature, which is needed for a T-cell response (Fujimoto et al. 2004; Sen and Sarkar 2005). Toll-like receptor 3 has been shown to be involved in infections in the mouse with cytomegalovirus, reovirus and influenza (Fujimoto et al. 2004; Sen and Sarkar 2005). Other viruses use different TLR's other than TLR3, such as herpes simplex viruses (TLR9) and respiratory syncytial viruses (TLR4),which initiate different signaling pathways such as the JAK/STAT pathway, initiated by interferon receptor, and RIG-I pathway that is also initiated by the TLR3 independent pathway. These pathways culminate in the induction of a set of genes that are induced by dsRNA as well. Poly I:C induces the activation of the same transcription factors as other viruses that cause the induction of interferon (Fujimoto et al. 2004; Sen and Sarkar 2005).

Interferon is believed to stimulate protein serine/threonine kinase, apoptosis of infected cells and activation of the 2-5A system (Castelli et al. 1998; Majde 2000; Samuel 2001). The active protein serine/threonine kinase affects viral multiplication within single cells by inhibiting mRNA translation through phosphorylation of initiation factor-2α (Castelli et al. 1998; Majde 2000; Samuel 2001). As a means of eliminating infected cells, interferon is believed to stimulate apoptosis in infected cells where generalized cellular RNA degradation occurs by a non-specific mechanism which translates into the shut down of translation and homeostatic mechanisms within the cell, therefore inducing cell death (Castelli et al. 1998; Majde 2000; Samuel 2001). The 2-5A system has also been implicated in antiviral mechanisms by raising 2-5A-dependent RNase and the 2'-5' oligoadenylate synthetases (Castelli et al. 1998). The 2'-5' oligoadenylate synthetase acts to stop viral replication by cleaving dsRNA (Hovanessian 1991). Interferon also acts on

the central nervous system by altering the electrophysiological activity of brain regions, by modulating opiate mediated structures by direct action, involved with pain suppression mechanisms (Dafny and Yang 2005). Besides Poly I:C stimulating interferon, Poly I:C also stimulates the secretion of various cytokines (e.g., IL-6 and TNF) and prostaglandins that are common to influenza and the Epstein-Barr viral infections (Fortier et al. 2004; Majde 2000; Mogensen and Paludan 2001; Eccles 2005), and induces fever, a hallmark of these two viral infections (Krohel et al. 1976). Poly I:C stimulates interferon production through the interferon regulatory factor-3, which is activated by seronine/threonine phosphorylation and leads to transcriptional activation of interferon genes (Mogensen and Paludan 2001; Samuel 2001). Interferon then causes the release of pro-inflammatory cytokine such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and interleukin-6 (IL-6) (Kimura et al. 1994). The sequence of releasing secondary mediators is not known but several studies have speculated the process with interferon, IL-6, TNF-α and IL-1 acting on the hypothalamus and causing a fever (Chuang et al. 1990; Fortier et al. 2004; Krueger et al. 1988; Lindsay et al. 1969; Won and Lin 1988).

1.3.2. Poly I:C-induced fever

Like lipopolysaccharide (LPS), Poly I:C induces the acute-phase response in numerous species (Fortier et al. 2004; Kimura et al. 1994; Mogensen and Paludan 2001; Traynor et al. 2004). Experimentation in rabbits on Poly I:C-induced fever was done to identify whether the effects of Poly I:C observed in humans could be duplicated in rabbits.

Intravenously administration of Poly I:C caused adverse reactions in rabbits that were consistent with humans, namely fever and hypotension, and high levels of serum interferon (Gatmaitan et al. 1980). Hypotension particularly was of interest since it also occurred in humans but was never evident in other species that had been injected with Poly I:C such as monkeys and chimpanzees (Gatmaitan et al. 1980). Hypotension and serum levels of interferon were increased with intramuscular and subcutaneous administrations of Poly I:C (Gatmaitan et al. 1980). Poly I:C fever, induced by Gatmaitan and colleagues (1980) like LPS-induced fever, was biphasic in nature and was antagonized by non-steroidal anti-inflammatory agents such as ketoprofen.

Won and colleagues (1988) went on to demonstrate that Poly I:C administration into the third ventricle or the pre-optic anterior hypothalamus caused a dose-dependent fever in rabbits. Hypothalamic administration of Poly I:C was more effective at inducing fever than the cerebroventricular route, thereby possibly showing that the site of Poly I:C febrigenic action resides in the pre-optic area of the anterior hypothalamus. The attenuation of fever in rabbits by indomethacin and ketoprofen suggested that Poly I:C-mediated fever was mediated by prostaglandins (Won and Lin 1988). Lastly, Kimura and colleagues compared the acute-phase response induced in rabbits by injection of LPS and Poly I:C (Kimura et al. 1994). The authors found that LPS-induced fever to be highest 3 hours after pyrogen administration and that the fever decreased after 6 hours, whilst Poly I:C fever was more extended and resolved only after 24 hours. The authors did not mention when the fever caused by Poly I:C peaked. The LPS and Poly I:C fevers were dose-dependent. Lipopolysaccharide and Poly I:C fever also differed by LPS failing to

induce interferon-α, unlike Poly I:C which did induce interferon-α, and that LPS was able to induce interleukin-1α and interferon-β more rapidly than Poly I:C (Kimura et al. 1994).

There remains a lot of controversy about the exact mechanisms by which Poly I:C mediates fever and the fever response. Chaung and colleagues (1990) showed with the use of a somatostatin antagonist (SS-14) that somatostatin, in the rat hypothalamus, may mediate the fever induced by interferon or its inducer Poly I:C. Another study reported that Poly I:C may act to induce fever through the endogenous release of noradrenaline from the rat hypothalamus (Liu et al. 1989). Fortier and colleagues (2004) suggested that the induction of TNF-α, IL-1 and IL-6 are involved in the initiation of Poly I:C induced fever with IL-6 acting as the circulating signal to the brain. Fortier and colleagues (2004) injected rats intraperitoneally with 750 $\mu g.kg^{-1}$ Poly I:C to create a maximum change in body temperature which caused a fever response that started 2 hours post-injection and was over 8 hours later. Liu and colleagues (1989) used 0.50 μg Poly I:C, intrahypothalamically in rats, whilst Katafuchi et al. (2003) using 3.0 $mg.kg^{-1}$ of Poly I:C, injected intraperitoneally in rats, induced a slow-onset but long lasting fever that began 8 hours after injection, peaked 5 hours post-injection and was still present 7 hours after the peak (Fortier et al. 2004; Katafuchi et al. 2003).

Fever is associated with a whole suite of behavioural changes, one of them being a decrease in activity (Bluthe et al. 2000; Mitchell and Laburn 1997). Like LPS, Poly I:C has been shown to cause a decrease in cage activity (Fortier et al. 2004) and spontaneous voluntary activity, such as running wheel activity (Katafuchi et al. 2003). Besides Poly

I:C having an effect on activity, Poly I:C, like LPS, is known to cause a decrease in food intake and weight loss (Fortier et al. 2004). The authors implicated IL-6, TNF-α and interferon-α in mediating a decrease in food intake by their rats by acting on the satiety region.

Because Poly I:C has been shown to mimic the febrile and several behavioural aspects of the acute-phase response to viral infection, and because it is not infective and convenient to use under normal laboratory safety conditions, I think that Poly I:C is a suitable molecule with which to study other sickness behaviour associated with viral infection, namely pain. It is possible then, that Poly I:C is a good choice to develop a model of virus-induced hyperalgesia.

1.4. Infection and hyperalgesia

In an effort to understand the complex relationship between infection and hyperalgesia, various models of hyperalgesia induced by bacterial antigens have been established. Systemic administration of LPS from Gram-negative bacteria has been shown to induce hyperalgesia (Mason 1993; Satyanarayana et al. 2004; Watkins et al. 1994a). The appearance of hyperalgesia in rats administered LPS has been shown to depend on the dose of LPS administered and the testing time for hyperalgesia after injection. For example, low doses of LPS (1 $\mu g.kg^{-1}$) induced monophasic fever and hyperalgesia, but high doses of LPS (10 $\mu g.kg^{-1}$ and 100 $\mu g.kg^{-1}$) only induced hyperalgesia in the first phase of biphasic fever (Abe et al. 2001). Lipopolysaccharide induces the production of

inflammatory cytokines TNF-α, IL-1β and IL-6, which are part of the fever response, these cytokines also have been shown to sensitize nociceptors (Abe et al. 2001).

In addition to the direct effects of these cytokines on nociceptors, infection-induced cytokine production also may produce hyperalgesia by stimulating vagal afferents.Vagal afferents have also been implicated in systemic TNF-α and IL-1β induced hyperalgesia (Morgan et al. 2004; Watkins et al. 1995a; Watkins et al. 1994a; Wieseler-Frank et al. 2004). The vagus nerve, which provides cytokine-to-brain communication, projects to the nucleus raphe magnus which is known to modulate nociception (Morgan et al. 2004; Watkins et al. 1994a). Subdiaphragmatic vagotomy blocks LPS (i.p.), TNF-α (i.p.) and IL-1β (i.p.) induced hyperalgesia (Morgan et al. 2004; Watkins et al. 1995a; Watkins et al. 1994a).

Lastly, whether by humoral or neural mechanism, pro-inflammatory cytokines released during infection may ultimaltely produce prostaglandins in the pre-optic anterior hypothalamus. Prostaglandins, mainly prostaglandin E_2, also are thought to mediate fever and hyperalgesia via the medial pre-optic region of the brain (Heinricher et al. 2004; Hosoi et al. 1997). Receptors for prostaglandin E-type are found within the pre-optic medial region (Heinricher et al. 2004; Hosoi et al. 1997). Application of prostaglandin E_2, in the medial pre-optic region, has been shown to produce hyperalgesia (Heinricher et al. 2004; Hosoi et al. 1997). Heinricher and colleagues (2004) postulated that the rostral ventromedial medulla, which may receive input from the pre-optic medial region, is involved in pain facilitation or inhibition via the use of “on and off” cells. Therefore,

prostaglandin E_2 may activate the on-cells thereby facilitating the hyperalgesia observed and inhibiting the off-cells (Heinricher et al. 2004).

Glial cells within the spinal cord have also been reported to be of importance in cytokine mediated hyperalgesia (Milligan et al. 2000; Wieseler-Frank et al. 2004; Wieseler-Frank et al. 2005). The cytokines act on the glial cells, which then modulate nociception. Functional disruption of spinal glia blocks allodynia and hyperalgesia. It is thought that the cytokines' actions on the glia result in these glia signalling other glia in the spinal cord to release cytokines within the spinal cord. These spinal cytokines are believed to be important in causing the observed pain states (Wieseler-Frank et al. 2005).

While the interaction between infection and hyperalgesia is clear, there is a lack of correspondence between fever and hyperalgesia. For example, Yabuuchi and co-workers (1996) found that at low doses of IL-1β, no fever was induced but the same doses caused hyperalgesia. At higher doses of IL-1β which are febrigenic, analgesia was produced (Yabuuchi et al. 1996). Other studies have also shown that fever and hyperalgesia induced by IL-1β are similarly dissociated by dose and vagotomy (Heinricher et al. 2004). Hosoi et al (1997) also confirmed the dissociation of fever and hyperalgesia following prostaglandin E_2 in the medial pre-optic region, with hyperalgesia obtained only at subpyrogenic doses. Further investigation is needed to understand the mechanism of cytokine induced hyperalgesia (Heinricher et al. 2004)

While the mechanisms mediating fever and sickness behaviour, in terms of hyperalgesia, in response to a bacterial challenge, particularly LPS administration, have been thoroughly investigated and reported on (Mitchell and Laburn 1997), the mechanisms mediating virally-induced hyperalgesia have yet to be fully elucidated. Even though a few viral-pain models exist, they potentially are too specific to that particular virus, and therefore any information obtained from these models will only be applicable to the model investigated.

1.5. Aims

In order to understand the underlying mechanisms of viral pain, improve the therapies that are being prescribed for viral pain, and to understand the complex relationship between viral infection and hyperalgesia, a general model of virus-induced hyperalgesia that is not limited to a particular virus is needed so that the model may be applicable to most of the viral infections reported. Since Poly I:C is a synthetic virus that is similar to double-stranded RNA which is produced by most, if not all viruses, it is a prime candidate in developing a general model of virus-induced hyperalgesia. The aims of this study was to establish a general model of viral hyperalgesia using both pyrogenic and non-pyrogenic doses of Poly I:C.

Chapter 2

Experiments

2. 1. General Methods

2.1.1. Animals

I used 83 male Sprague-Dawley rats that weighed 230-250g at the start of experiments. Animals were housed individually in cages at an ambient temperature of ~23ºC, and on a 12:12 hour light/dark cycle (lights on at 07:00). Food and water were available *ad libitum*.

2.1.2. Drug Preparations

Stock solutions of polyinosinic:polycytidylic acid (Poly I:C, Sigma-Aldrich, USA) were prepared by dissolving appropriate amounts of the immunogen in sterile, pyrogen-free saline, such that 100 $\mu g.kg^{-1}$ and 1000 $\mu g.kg^{-1}$ Poly I:C could be administered intradermally in a 100 µl volume. Aliquots of these stock solutions were frozen at -20ºC, and fresh aliquots thawed immediately before Poly I:C injections.

2. 2. Experiment 1: Pyrogenic properties of poly I:C

2.2.1. Introduction

Since models of infection-induced hyperalgesia use bacterial pyrogens such as LPS, or cytokines released during bacterial infection, such as TNF-α and IL-1β (Yabuuchi et al. 1996), where models of infection-induced hyperalgesia have found a dissociation between fever and hyperalgesia, a new model of virus-induced hyperalgesia also needs to encompass simulated febrile and non-febrile viral infections. Therefore, the viral challenge used will have to include both a pyrogenic and non-pyrogenic dose. Poly I:C is a synthetic viral double stranded RNA that has been used experimentally to simulate virally-induced infection. Poly I:C has been shown to be an appropriate viral challenge since the immune response mimics viral infections and is relatively reproducible (Fortier et al. 2004). However, the route of administration of Poly I:C can affect the febrile response (Fortier et al. 2004). Therefore, before testing for the presence of hyperalgesia after administering Poly I:C, I first needed to establish the pyrogenicity of Poly I:C administered subcutaneously. Then, by using a pyrogenic and non-pyrogenic dose of Poly I:C, I could later (2.3 Experiment 2) determine whether the link between fever and hyperalgesia during simulated viral infection is any different to that reported for simulated bacterial hyperalgesia.

2.2.2. Methods

i) Measurement of body temperature

Rats anaesthetized with ketamine (80 mg.kg^{-1}) and xylazine (40 mg.kg^{-1}) had sterile, wax-coated temperature-sensitive radio telemeters (Mini-Mitter, USA; Figure 1) implanted intraperitoneally seven days before the start of experimentation. Before implantation, telemeters were calibrated in a water bath against a high accuracy quartz-crystal thermometer to an accuracy of 0.1ºC (Quat 100, Heraeus, Germany). The output frequency of each telemeter was monitored by a receiver plate (RTA 500, Mini-Mitter, USA) placed beneath each animal's cage, and transmitted via a peripheral processor (Datacol-3, Mini-Mitter, USA) to a personal computer, where frequencies were converted to temperatures and recorded. Body temperature was recorded every fifteen minutes throughout the experiment.

ii) Experimental procedure

Rats were randomly injected intradermally in the tail either with saline and 100 μg.kg^{-1} Poly I:C (n=7), or saline and 1000 μg.kg^{-1} Poly I:C (n= 6). All injections were administered midway down the length of the tail, on the dorsal surface, in rats restrained in transparent plastic restrainers. All injections took place between 08:15 and 08:45, and animals were released from the restrainers back into their home cages immediately after

injection. Saline and Poly I:C were injected in random order, with at least two weeks between injections.

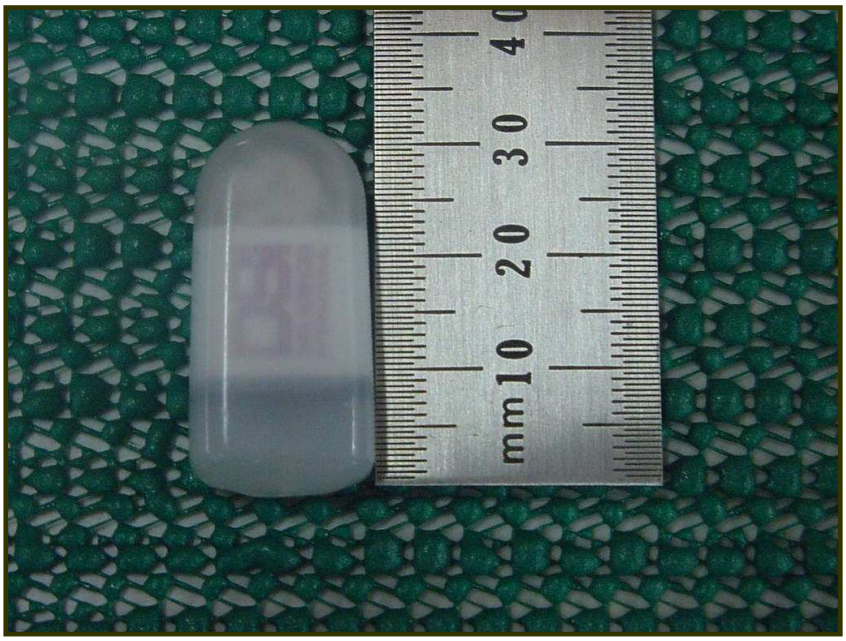

Figure 1. A temperature-sensitive radio telemeter which was inserted into the rat abdomen.

iii) Data analysis

All temperature data are expressed as mean (SD). Temperature data were analysed using a two-way ANOVA with treatment and time as main effects. To reduce the size of the model, hourly averages were used in the analysis (from one hour before injection until eight hours after injection). A Newman-Keuls post hoc test was used to compare hourly means when significant main effects or interactions were detected by the ANOVA using Statistica 5.5 (Statsoft, Inc., Tulsa, USA), was used for all statistical procedures, $\alpha=0.05$.

2.2.3. Results

Figure 2 shows changes in body temperature of rats injected intradermally with Poly I:C and saline. Restraint and injection of saline and 100 μg.kg^{-1} Poly I:C (Fig 2A) induced a stress hyperthermia in all animals, which lasted for approximately 30min after the injection (main effect of time: $F_{(8,96)} = 44.08$, $P < 0.001$). This short-lived increase in body temperature is the stress hyperthermia associated with handling during injections and has been well documented. After the stress hyperthermia had subsided, no further significant changes in body temperature occurred, and at no time was there a significant difference in body temperature between the two interventions (main effect of intervention: $F_{(1,12)} = 2.09$, $P = 0.174$; interaction: $F_{(8,96)} = 1.20$, $P = 0.309$). All animals injected with saline and 1000 μg.kg^{-1} Poly I:C (Fig 2B) also experienced a stress hyperthermia during restraint and injection. Injection of 1000 μg.kg^{-1} Poly I:C induced an increase in body temperature approximately an hour after the stress hyperthermia had resolved (main effects of time: $F_{(8,80)} = 5.83$, $P < 0.001$; main effect of intervention: $F_{(1,10)} = 25.66$, $P < 0.001$; interaction: $F_{(8,80)} = 8.42$, $P < 0.001$). The increase in body temperature reached a peak amplitude of approximately 38.6ºC approximately four hours after injection of Poly I:C, and remained significantly elevated for three hours after the peak, thus confirming that I used a pyrogenic and a sub-pyrogenic dose of Poly I:C in the nociceptive component of the investigation. Body temperature of all rats returned to pretreatment levels 24h after the Poly I:C or saline administration.

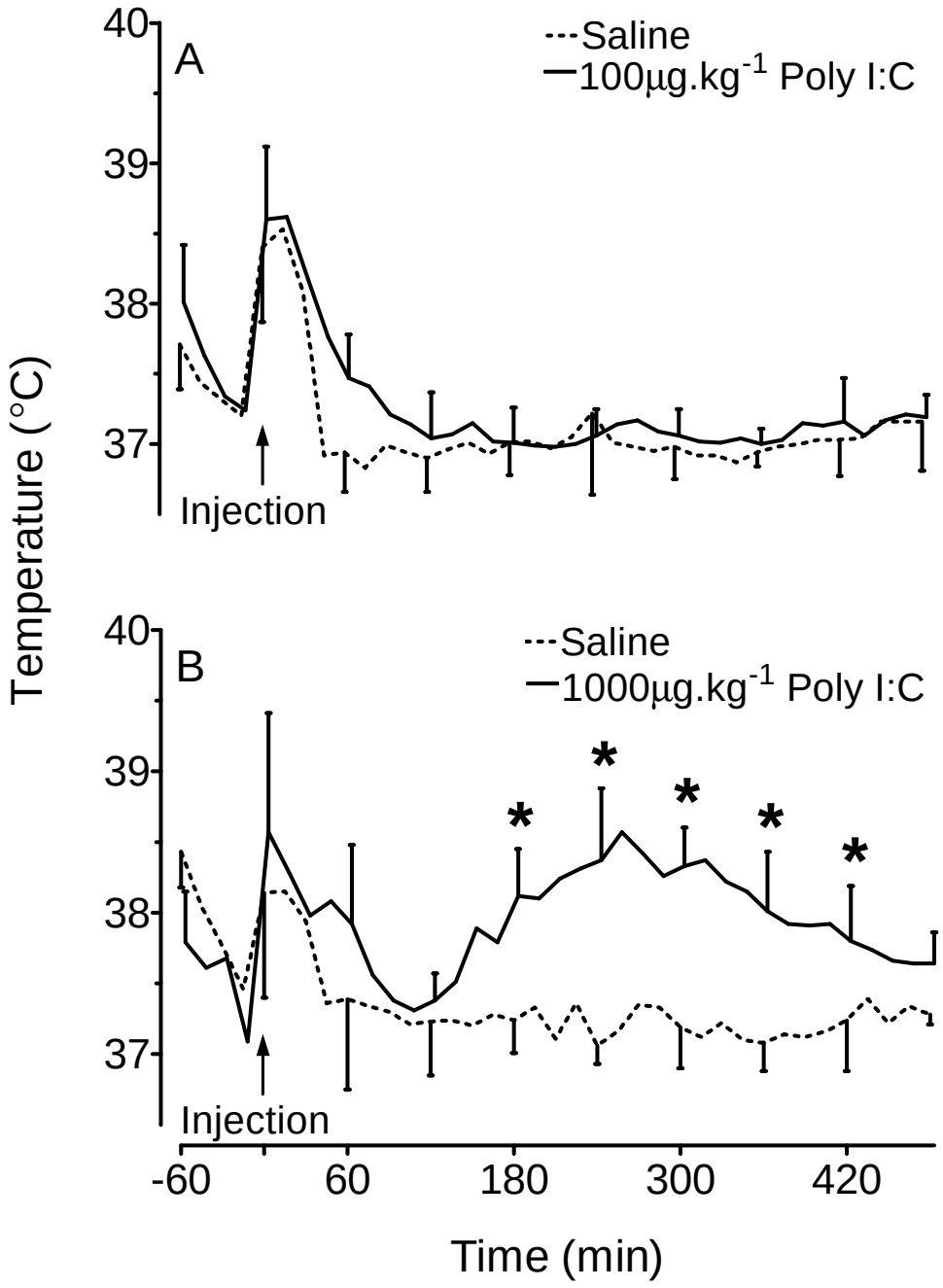

Figure 2: Mean (SD) body temperature of rats injected intradermally either with saline and 100 µg.kg^{-1} Poly I:C (A, n = 7), or saline and 1000 µg.kg^{-1} Poly I:C (B, n = 6). * $P <$ 0.05: saline versus 1000 µg.kg^{-1} Poly I:C.

2.2.4. Discussion

I sought to find a pyrogenic and non-pyrogenic dose of Poly I:C, to use in the development of a Poly I:C-induced model of pain. Two doses of Poly I: C,100 and 1000 $\mu g.kg^{-1}$, that were injected intradermally on the dorsal surface of the rats' tail, were used. I was able to induce a fever with the injection of 1000 $\mu g.kg^{-1}$ Poly I:C. The 1000 $\mu g.kg^{-1}$ Poly I:C caused a monophasic fever, characterized by an increase in abdominal temperature that reached a peak of about 38.6°C and lasted about five hours. Twenty-four hours post injection, no differences in temperatures between saline and Poly I:C injections were observed. The injection of 100 $\mu g.kg^{-1}$ Poly I:C did not cause a fever.

Cytokines such as IL-1, IL-6, TNF-α and interferon are known to be induced by Poly I:C and have been implicated in mediating fever induced Poly I:C (Chuang et al. 1990; Fortier et al. 2004; Kimura et al. 1994; Liu et al. 1989; Luheshi and Rothwell 1996). These cytokines (IL-1, IL-6, TNF-α and interferon) also are known to be pyrogenic themselves and mediate their effects by direct and non-direct actions on the brain, particularly the pre-optic-anterior hypothalamus (Chuang et al. 1990; Luheshi and Rothwell 1996; Krueger et al. 1988; Won and Lin 1988). The exact sequence in which these cytokines are released is controversial however; interferon is thought to be the primary mediator that stimulates the production of IL-1, IL-6 and TNF-α (Chuang et al. 1990; Luheshi and Rothwell 1996; Won and Lin 1988). Therefore, by showing that we have a pyrogenic and a non-pyrogenic dose of Poly I:C, which presumably induce different levels of inflammation at the site of inflammation, we have suitable doses of

Poly I:C to develop a generalized model of virus-induced pain. I have also shown a novel method of Poly I:C fever induction by intradermal injections of Poly I:C in the rats tail.

2.3. Experiment 2: Hyperalgesic properties of Poly I:C

2.3.1. Introduction

Various animal models have been developed to understand the relationship between infection and hyperalgesia (Maier et al., 1993; Abe et al., 2001). Lipopolysaccharide (LPS) from Gram-negative bacteria has been shown to induce hyperalgesia (Mason 1993; Satyanarayana et al. 2004; Watkins et al. 1994a), which probably is linked to its induction of cytokine (Mason 1993; Satyanarayana et al. 2004; Watkins et al. 1994a), such as IL-1 and TNF-α release (Bianchi et al. 1998; Mason 1993; Morgan et al. 2004). The mechanisms implicated for most models involving LPS, or cytokines released after LPS inoculation, for the production of the hyperalgesia involve activation of subdiaphragmatic vagal afferents and more importantly, the synthesis of prostaglandins in the hypothalamus (Abe et al. 2001; Hosoi et al. 1997; Watkins et al. 1995a). However, the link between fever and hyperalgesia is complex, with non-febrile doses of pyrogens inducing hyperalgesia (Abe et al. 2001; Oka et al. 1994), and febrile doses of pyrogens not causing hyperalgesia (Morgan et al. 2004; Oka et al. 1994).

However, the extensive research into fever and hyperalgesia has mainly been concerned with bacterial challenges with little attention given to viral fever and hyperalgesia. I have established a pyrogenic and a non-pyrogenic dose of Poly I:C, therefore the aim of the

second experiment was to determine whether administration of these doses of Poly I:C induced hyperalgesia. I also assessed the extent of peripheral inflammation, at the site of Poly I:C or saline administration and related this tissue inflammation to the hyperalgesia.

2.3.2. Methods

All tests of nociception took place with rats placed in transparent plastic restrainers that allowed free movement of the tail, but limited body movement. Before the start of experimentation, rats were accustomed to the restrainers for three hours a day on three consecutive days. On days of nociceptive testing, I placed the rats into restrainers 30 minutes before we measured response latencies to noxious mechanical and thermal challenges.

i) Noxious thermal challenge

I used the method described by Gelgor and colleagues (1986) to test for the presence of hyperalgesia to a noxious heat stimulus in rats injected either with saline or Poly I:C. Briefly, rats' tails were submerged into 49°C water until a co-ordinated motor response (tail-flick) was observed (Figure 3). The time elapsed between submergence and flicking of the tail was measured using a stopwatch. The average of three measurements, taken at least one minute apart, was recorded as the tail-flick latency. Exposure to 49ºC water was limited to 30 seconds to prevent tissue damage (Gelgor et al. 1986). Rats' tails were immersed in 29ºC water for thirty minutes before measurements started, and between

measurements, to remove the confounding effect variations in tail-skin temperature has on tail-flick latency.



Figure 3. The tail of the rat is immersed into 49°C water and the time taken to remove the tail is recorded using a stopwatch.

ii) Noxious mechanical challenge

I used the method described by Vidulich and Mitchell (2000) to test for the presence of hyperalgesia to a noxious mechanical stimulus in rats injected either with saline or Poly I:C. Briefly, a bar algometer (Halda RS, Sweden), which was placed transversely across the injection site, was used to deliver a static force of 4N to rats' tails (Figure 4). The response latency from when the force was applied until the first sign of aversive behaviour, such as the animal attempting to turn around in its restrainer, vigorous

grooming and withdrawing its tail, was measured using a stopwatch. An average of three measurements, taken at least one minute apart, was recorded as the escape response latency. The mechanical challenge was terminated if the rat had not responded within 30 seconds.

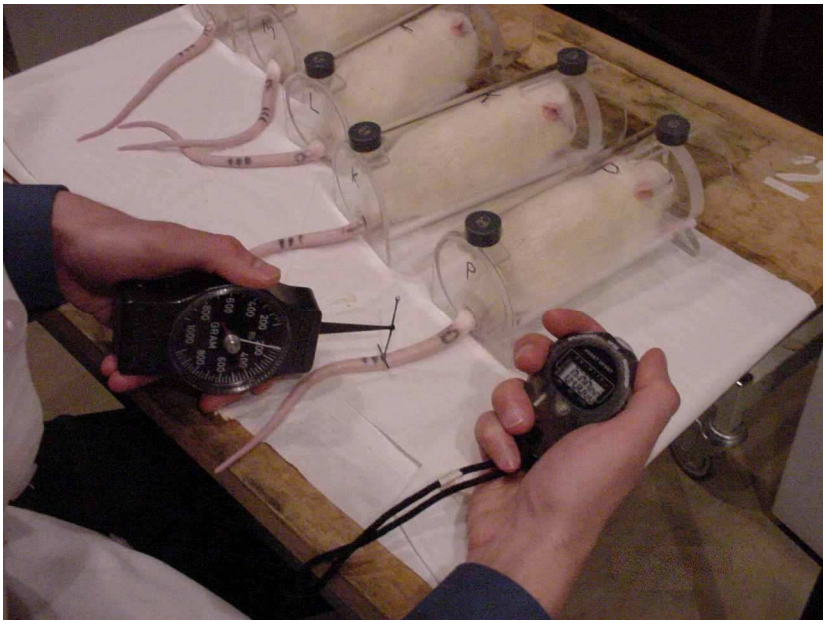

Figure 4. The mechanical stimulus applied to the site of injection at a constant force of 4N using a bar algometer.

iii) Experimental procedure

Rats were injected either with saline (n = 8), 100 μg.kg^{-1} Poly I:C (n = 9), or 1000 μg.kg^{-1} Poly I:C (n = 8). For five consecutive days before rats were injected response latencies to the noxious mechanical and thermal challenges were measured to obtain pre-injection withdrawal response latencies. On the sixth day, animals were injected intradermally with the assigned agent. Animals' response latencies to the noxious thermal and mechanical challenges were then measured three hours after injection and daily thereafter for six days. Injections took place between 08:00 and 08:45, and nociceptive testing always took place between 08:15 and 12:00. The thermal stimulus always was applied before the mechanical stimulus, with at least 30 minutes separating exposure to the thermal and mechanical challenges (Vidulich and Mitchell 2000).

iv) Histology

In a separate experiment, three groups of 15 rats each were injected either with saline, 100 μg.kg^{-1} Poly I:C, or 1000 μg.kg^{-1} Poly I:C. Three animals from each group were killed three hours, one day, four days and ten days after injection, and tail-skin sections (down to, but not including the fascia) were taken from the site of injection. Skin sections were fixed in 10% formalin and embedded in paraffin before been stained with haematoxylin and eosin. The sections then were examined, under a light microscope, by a histopathologist for the presence and severity of inflammation. The fixing and scoring of

the tissues was performed by a consultant pathologist (Dr. B Mitchell, MBBCh (FC) Path SA, Mansfield, UK).

v) Data analysis

All nociceptive data are expressed as mean percentage change in withdrawal response latency from the five-day average of pre-injection values. Repeated-measures ANOVA were used to detect for significant changes in nociceptive response latencies over the six days of the experiment. Zero percentage change from pre-injection values was included in these analyses as a control. When significant effects were detected by the ANOVA, one-sample *t*-tests were used to compare the percentage change in nociceptive response latency at each time interval to zero change (no effect of injection). A Bonferroni correction for multiple comparisons was made. To take the magnitude of the change in nociceptive response latency into consideration, only response latencies that were statistically significant from zero, and where the 95% confidence interval of the mean difference between zero change (no effect of injection) and the measured percentage change in response latency was at least a 20%, were considered biologically significant. These statistical analyses were performed using Prism 4.03 for Microsoft Windows (Graphpad Software, USA), with $P < 0.05$ considered significant.

2.3.3. Results

i) Noxious thermal challenge

Figure 5 shows percentage changes in tail-flick latencies to a noxious thermal challenge after injecting rats intradermally either with saline, 100 μg.kg^{-1} Poly I:C, or 1000 μg.kg^{-1} Poly I:C. There were no significant changes in tail-flick latencies from pre-injection values after injection either of saline ($F_{(7,7)} = 1.58$, $P = 0.164$) or 1000 μg.kg^{-1} Poly I:C ($F_{(7,7)} = 1.82$, $P = 0.104$). For animals injected with 100 μg.kg^{-1} Poly I:C, there were significant variation in tail-flick latencies over time ($F_{(7,8)} = 5.57$, $P < 0.001$), but *post hoc* tests and analysis of the confidence intervals failed to show any significant changes in tail-flick latencies from pre-injection values at each time interval. Across all groups, the mean percentage change in tail-flick latency never decreased by more than 12.5% relative to the pre-injection values (day 4, 100μg.kg^{-1} Poly I:C), and the lower limit of the 95% confidence interval of the mean difference between zero (no effect of injection) and the measured percentage change in tail-flick latency never was greater than 20%. Thus Poly I:C injection did not induce hyperalgesia to a thermal stimulus.

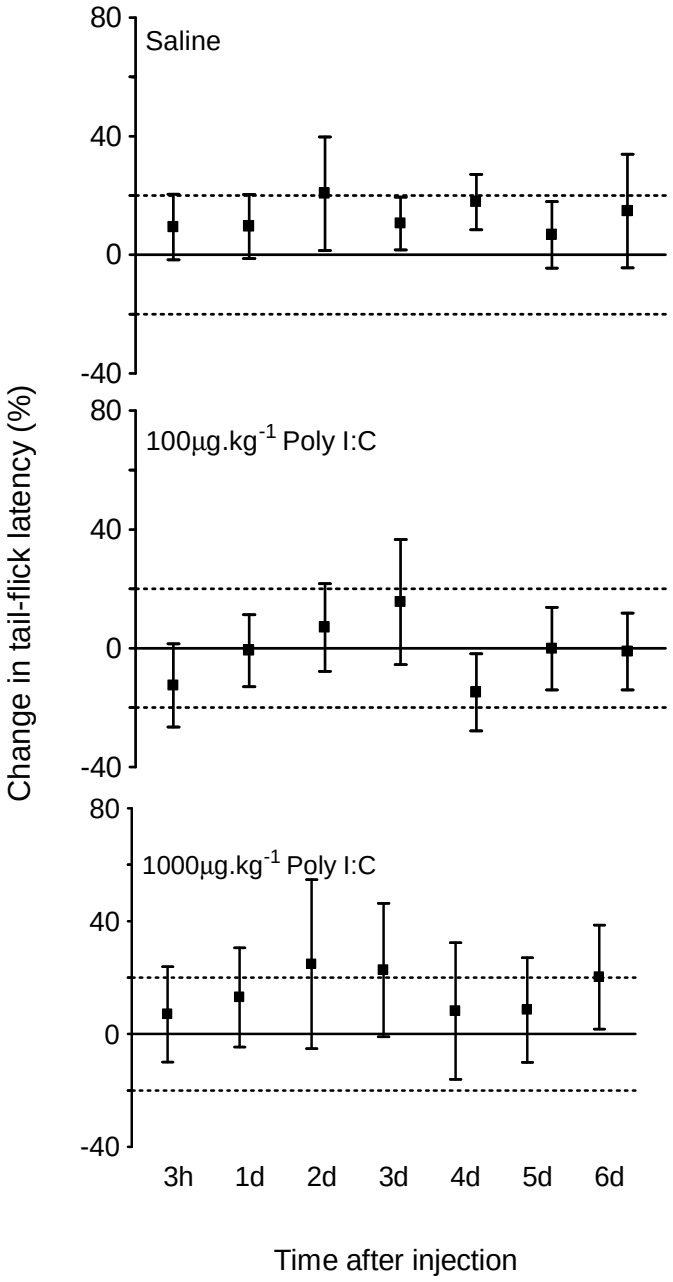

Figure 5 shows percentage changes in tail-flick latencies to a noxious thermal challenge after injecting rats intradermally either with saline (n = 8), 100 µg.kg^{-1} Poly I:C (n = 9), or 1000 µg.kg^{-1} Poly I:C (n = 8). The dotted lines represent the level (20%) at which the measured percentage change in response latency was considered to be biologically significant.

ii) Noxious mechanical challenge

Figure 6 shows percentage changes in escape latencies to a noxious mechanical challenge after injecting rats intradermally either with saline, 100 μg.kg^{-1} Poly I:C, or 1000 μg.kg^{-1} Poly I:C. There were no significant changes in escape latencies from pre-injection values after injection of saline ($F_{(7,7)} = 2.02$, $P = 0.072$). However, injection of 100 μg.kg^{-1} Poly I:C caused significant mechanical hyperalgesia, but only on days one, four and five after injection ($F_{(7,8)} = 13.74$, $P < 0.001$). Injection of 1000 μg.kg^{-1} Poly I:C also caused significant mechanical hyperalgesia, but this hyperalgesia started within three hours of the injection and was maintained for four days ($F_{(7,7)} = 8.12$, $P < 0.001$). On average, the escape latency in animals injected with 1000 μg.kg^{-1} Poly I:C was reduced by about 40%. Thus there was a dose-dependent increase in the duration of the mechanical hyperalgesia induced by Poly I:C.

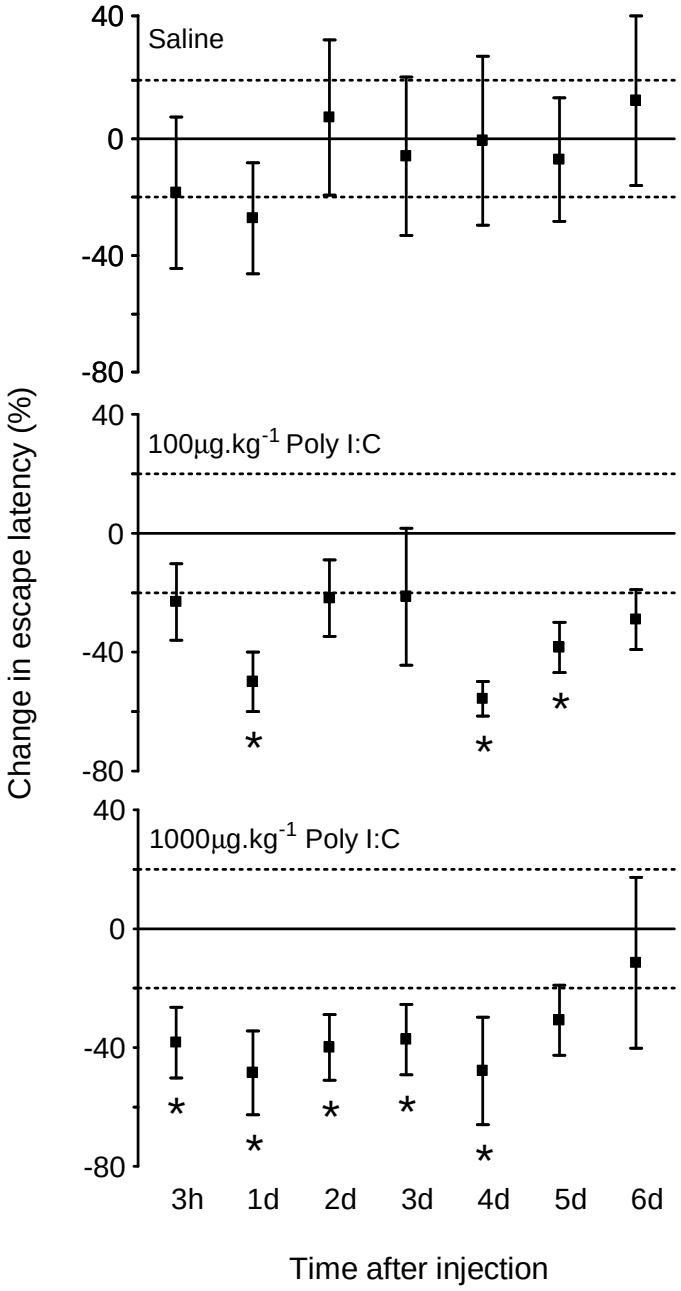

Figure 6 shows the percentage changes in escape latencies to a noxious mechanical challenge after injecting rats intradermally either with saline (n = 8), 100 µg.kg^{-1} Poly I:C (n = 9), or 1000 µg.kg^{-1} Poly I:C (n = 8). The dotted lines represent the level (20%) at which the measured percentage change in response latency was considered to be biologically significant.

iii) Histology

Figure 7 shows representative samples of tail-skin tissue sections taken from the site of either saline, 100 $\mu g.kg^{-1}$ Poly I:C, or 1000 $\mu g.kg^{-1}$ Poly I:C injection three hours, one day, four days and ten days after injection. Irrespective of the injection, all animals developed mild inflammation within three hours of injection. However, the inflammation had resolved 24 hours later in the saline injected animals. On average, mild inflammation was present in tissue samples taken one, four and ten days after injecting rats with 100 $\mu g.kg^{-1}$ Poly I:C. In rats injected with 1000 $\mu g.kg^{-1}$ Poly I:C, the median level of inflammation increased from mild, three hours after injection, to moderate on days one and four after injection. By day ten only mild inflammation, with fibrosis was present. Thus there was a dose-dependent increase in the level of inflammation after Poly I:C injection.

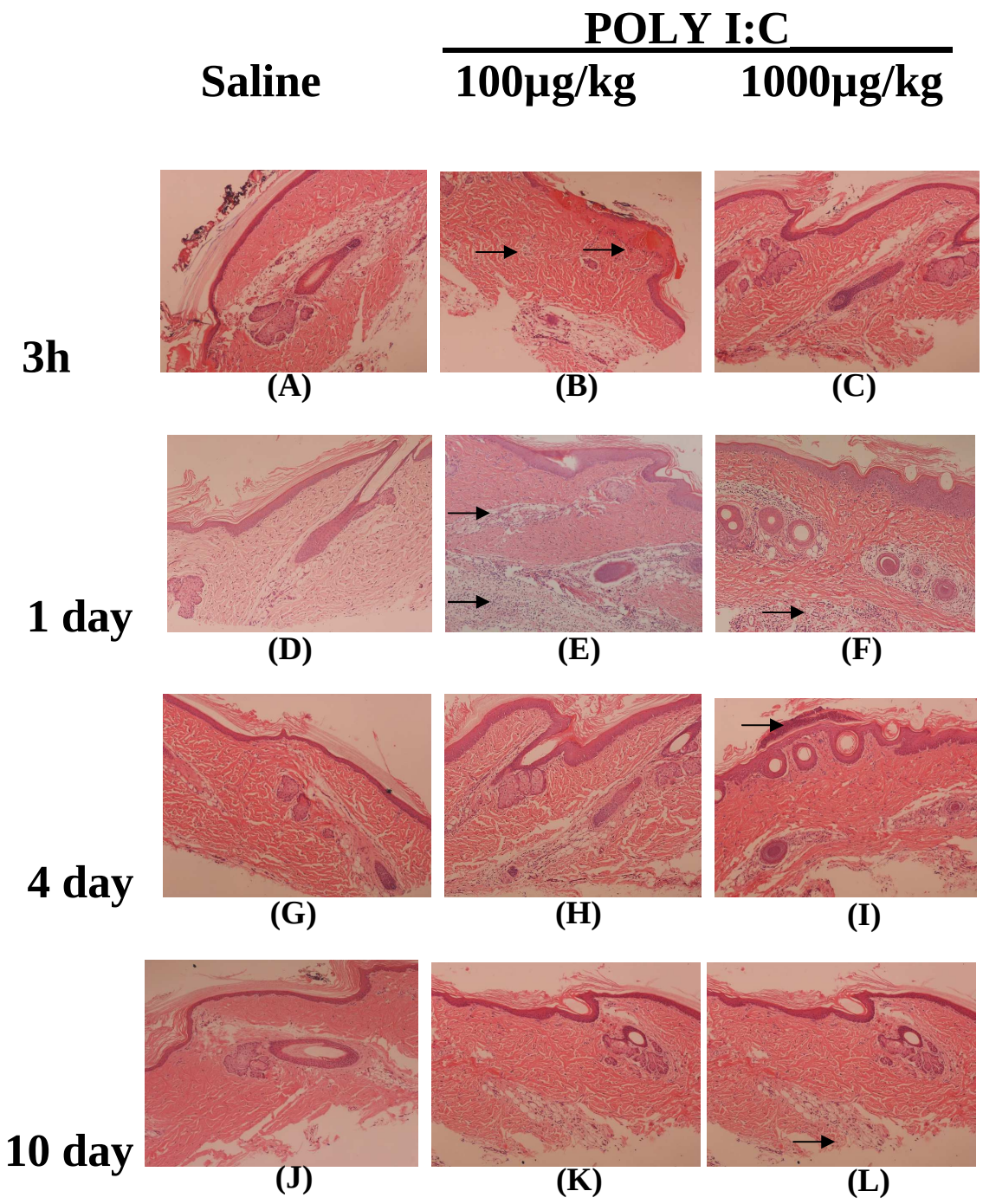

Figure 7. Inflammatory changes in rats' tails 3 hours (3h), 1 day, 4 days, and 10 days after intradermal injection either with saline (n = 15), 100 $\mu g.kg^{-1}$ Poly I:C (n = 15), or

1000 µg.kg^{-1} Poly I:C (n = 15). All sections were stained with eosin and haematoxylin, and photographs were taken at 100x magnification, with the epidermis orientated at the top of the image. Samples taken 3 hours after injection of saline, 100 µg.kg^{-1} Poly I:C or 1000 µg.kg^{-1} Poly I:C all showed the presence of mild inflammation, characterized by the presence of neutrophils, lymphocytes and plasma cells (indicated in B). Samples taken 1 day, 4 days and 10 days after saline injection showed no signs of inflammation, except for one sample taken 1 day after saline injection, which showed the presence of moderate inflammation. Two samples taken 1 day after injecting rats with 100 µg.kg^{-1} Poly I:C showed the presence of mild inflammation, while severe inflammation was found in one sample, as evident by the presence of neutrophils, lymphocytes and plasma cells (indicated in E). One day after injection of 1000 µg.kg^{-1} Poly I:C, mild, moderate and severe inflammation was present in each of the three samples respectively, with necrosis being present in the sample with signs of moderate inflammation (indicated in F). Four days after 100 µg.kg^{-1} Poly I:C injection, two samples had no signs of inflammation, and one sample had mild inflammation, with lymphocytes and plasma cells present. There was moderate inflammation in two samples, and mild inflammation in one sample taken 4 days after injection of 1000 µg.kg^{-1} Poly I:C, with the sample shown in the figure demonstrating the presence of moderate inflammation with focal fibrinopurulent exudates (indicated in I). Mild inflammation with cell infiltrate was evident in all samples taken 10 days after 100 µg.kg^{-1} Poly I:C injection. Ten days after injection of 1000 µg.kg^{-1} Poly I:C, two tissue sample showed signs of mild to moderate inflammation with mild fibrosis (indicated in L). The histopathology was done at the Kings Mill Hospital.

2.3.4. Discussion

I have described the behavioural and histological characteristics of a novel model of virus-induced hyperalgesia, produced by infusing a pyrogenic and non-pyrogenic dose of Poly I:C intradermally in the rats' tail. After 1000 $\mu g.kg^{-1}$ Poly I:C administration, primary mechanical hyperalgesia was induced that started three hours after injection and persisted for four days. Injection of the non-pyrogenic 100 $\mu g.kg^{-1}$ dose of Poly I:C also induced primary mechanical hyperalgesia. However, compared to the response of the 1000 $\mu g.kg^{-1}$ dose, the hyperalgesia took longer to develop, and the response was biphasic in nature, such that there was significant hyperalgesia only on day one after injection and on days four and five after injection. Histologically, there was a dose-dependent increase in the inflammatory response at the site of Poly I:C injection, which may explain the sustained hyperalgesia in animals injected with the higher dose of Poly I:C. Irrespective of the dose of Poly I:C injected, tail-skin tissue still was inflamed after the mechanical hyperalgesia had subsided. Surprisingly, neither dose of Poly I:C produced significant thermal hyperalgesia.

The absence of thermal hyperalgesia in my study may be a consequence of the method I used to test for thermal hyperalgesia. The presence of thermal hyperalgesia was tested for using the method described by Gelgor et al (1986), which involves submerging the tail into 49ºC water. Other authors who showed that viral antigens induced thermal hyperalgesia, such as Milligan and colleagues (2000) who injected gp120 molecules and, Fleetwood-Walker and colleague (1999) who inoculated rats with HSV-1, used the

Hargreaves test, which measures response latencies to a radiant heat source. Herzberg and Sagen (2001) also found the presence of thermal hyperalgesia when using a radiant heat source after directly exposing peripheral nerves to gp120. However, differences in the ability of different methods of testing for thermal hyperalgesia do exist. For example, Weber and colleagues (2005) who also submerged rats' tails into 49ºC water, found no thermal hyperalgesia after incision, but others using a radiant source have found thermal hyperalgesia post-operatively. The lack of thermal hyperalgesia in my animals may imply that at the site of injection, no sensitisation of primary afferent nociceptors occurred; thermal hyperalgesia is believed to be caused by sensitization of Type 1 A-fiber mechano-heat-sensitive nociceptors (Campbell 2005; Meyer 2005). An alternative reason for lack of thermal hyperalgesia in my animals is that Poly I:C may simply not produce thermal hyperalgesia, an aspect which may need further investigation. Congruent with my findings, however, are those of Sasaki et al (2003) who found no thermal hyperalgesia in mice infected with Herpes Simplex Virus, even though they used a radiant heat source as the thermal stimulus.

Although inflammation is present in my study, no thermal hyperalgesia was identified, which is different to other models of inflammatory pain such as carrageenan and complete Freund's adjuvant being injected into the hindpaw (Woolf et al. 1997; Cunha et al. 2000). Inflammatory mediators, such as TNF-α and IL-1, are released and act directly on receptors, or act indirectly by releasing further inflammatory mediators, such as prostaglandins, that act on the neurons themselves (Kidd and Urban 2001; Woolf et al. 1997). Mechanical hyperalgesia, also has been reported after administration of HIV-1

gp120, Herpes Simplex Virus and Varicella-Zoster Virus in both rats and mice (Andoh et al. 1995; Fleetwood-Walker et al. 1999; Milligan et al. 2000; Takasaki et al. 2000a). Mechanical hyperalgesia may be mediated by chemical signals such as TNF-α and prostaglandin E_2 that modulate the sensory fibre terminals (Cunha et al. 2000). The presence of inflammatory cell infiltrate correlating with the presence of mechanical hyperalgesia in the tail, may indicate that in my study, the mechanical hyperalgesia is induced by inflammatory mediators, such as TNF-α (Woolf et al. 1997). However, the presence of the inflammatory cell infiltrate lasted longer than the hyperalgesia induced by the pyrogenic and non-pyrogenic doses of hyperalgesia. I do not know why hyperalgesia would resolve despite persistent tissue inflammation. Full elucidation of the underlying mechanisms in Poly I:C-induced hyperalgesia will probably require characterization of the peripheral and central production of inflammatory cytokines other than Poly I:C inoculation.

While I did measure for the presence of primary hyperalgesia, I did not assess whether injection of Poly I:C induced secondary mechanical hyperalgesia and was thus unable to indicate whether there was any central sensitisation of nociceptive pathways by Poly I:C. Central sensitisation involves the augmentation of pain pathways, with input from low-threshold mechanoreceptors, within the spinal cord (Woolf 2004). Secondary mechanical hyperalgesia should be assessed as opposed to secondary thermal hyperalgesia since secondary hyperalgesia only exists for mechanical but not for thermal stimuli (Campbell 2005; Meyer 2005).

Dispite the disassociation of inflammation and hyperalgesia observed, both pyrogenic and non-pyrogenic doses of Poly I:C produced mechanical hyperalgesia in the tail. The non-pyrogenic dose exhibited maximum hyperalgesia on days 4 and 5 after injection. My results are similar to other virus models of hyperalgesia where maximum hyperalgesia was exhibited on days four to six, peaking on day five after inoculation/injection regardless of the virus used (Fleetwood-Walker et al. 1999; Takasaki et al. 2000a; Takasaki et al. 2000b). The biphasic nature of the mechanical hyperalgesia observed after the administration of the non-pyrogenic in my study is similar to the biphasic hyperalgesic response observed after IL-1β administration into the cerebral ventricle (Zelenka et al. 2005). However, my study is the first to illustrate viral mechanical hyperalgesia following injection of a non-pyrogenic of Poly I:C.

The difference in mechanical hyperalgesia produced by of the pyrogenic and non-pyrogenic dose of Poly I:C is that the pyrogenic dose produced hyperalgesia which was sustained from the third hour after injection until the fourth day after injection. The earlier onset of the mechanical hyperalgesia for the pyrogenic dose of Poly I:C may be a response to the fever induced by that dose. The 1000 $\mu g.kg^{-1}$ Poly I:C dose produced a fever starting three hours after intraperitoneal injection or intradermal injection, as in our study, and peaking four and a half hours after injection (Fortier et al. 2004). Poly I:C-induced fever and possibly mechanical hyperalgesia may in part, be mediated by pro-inflammatory cytokines such as TNF-α and IL-1β. In my study, inflammation as seen by histological changes after injection of both the pyrogenic and non-pyrogenic doses of Poly I:C, may contribute to the presence of mechanical hyperalgesia.

Histological changes obtained after HIV-gp120 administration around a peripheral nerve, revealed inflammatory cell invasion into the nerve trunk to be short lasting (5 days), suggesting that the initial hyperalgesia and allodynia observed was from the inflammatory response, but that the sustained hyperalgesia was mediated by other mechanisms (Herzberg and Sagen 2001). In my study, the Poly I:C fever and initial mechanical hyperalgesia may be mediated by TNF-α and other inflammatory mediators at the site of injection , but the prolonged hyperalgesia may be through centrally mediated mechanisms. A cytokine profile along with behavioural and histological data should be done not only to corroborate the function of non-inflammatory mechanisms in prolonged hyperalgesia but also to ascertain whether a link exists between the fever response, behavioural response, and histological changes.

In conclusion, I have successfully developed an animal model of virus-induced hyperalgesia using the rat's tail. Both non-pyrogenic and pyrogenic doses of Poly I:C induced primary mechanical hyperalgesia. The model encompasses both pyrogenic and non-pyrogenic viruses, and uses the rats' tail that interferes less with the animals' activity. The described model may be used to further elucidate the pathophysiological mechanisms involved with viral infections in general. Understanding these mechanisms could yield effective drug treatments against pain associated with viral infections.

Chapter 3

Conclusion

The lack of appropriate animal models to better understand the underlying mechanisms of viral pain, and the inadequacy of viral pain management are hurdles in dealing with viral pain. The acute models of viral pain that have been created have been specific for certain viral infections, such as HIV and herpes virus infections, so that any information and solutions gathered from these models is only applicable to that specific viral infection. Therefore, the objective of my dissertation was to develop a generic model of virus induced pain. The synthetic analogue of viral dsRNA, Poly I:C, was used, which may enable us to understand the general mechanisms that are associated with viral infections.

In comparison to other virus-specific models, mechanical hyperalgesia observed in my study, lasted 5 days at most, where Fleetwood-Walker and colleagues (1999), who used a live virus, induced mechanical hyperalgesia that lasted for 33 days. For Milligan and colleagues (2000), the mechanical hyperalgesia observed in their study lasted for only 100 minutes and the thermal hyperalgesia, lasted for 120 minutes. In my study, I observed no thermal hyperalgesia, but observed mechanical hyperalgesia that lasted for an average of four and a half days (between the pyrogenic and non-pyrogenic dose). Therefore, the mechanical hyperalgesia in my model lies between that described by Milligan et al. (2001) and Fleetwood-Walker et al. (1999).

The mechanisms responsible for the mechanical hyperalgesia observed in my study may in part be due to cytokines which are known to be released by Poly I:C. In truth, Poly I:C's mechanisms for inducing mechanical hyperalgesia may be the basis for hyperalgesia observed in other viral-specific models of pain. Since the dose and route of

administration is important for fever production after Poly I:C administration, the route of administration may be an important factor as well for the mechanical hyperalgesia. My findings also provide further evidence that fever and hyperalgesia do not necessarily occur concomitantly after an immunological challenge. Thus, my study has been able to demonstrate a viral infection that causes hyperalgesia, in rats, with no consequence of whether fever is produced or not. Also, the signs I measured after Poly I:C administration, in terms of fever and hyperalgesia, mimic general signs and symptoms that are associated with the sickness syndrome reported after viral infections such as influenza. A cytokine profile during the fever and hyperalgesic responses following Poly I:C injection is needed to confirm the role that cytokines may play in both fever and hyperalgesia. This profile would also reveal the sequence of cytokine release during the responses observed.

The model of virus induced pain described in this dissertation provides the platform for future studies on the mechanisms of viral pain. Therefore, for possible future studies, a cytokine profile is needed to elucidate the messengers that are responsible for the hyperalgesia observed. Also, to better the model, further studies using analgesics need to be done. Indeed, the elucidation of the underlying mechanisms of viral pain could lead to the improvement of current therapies and the development of new therapies designed to alleviate viral pain in humans.

Chapter 4

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