ABSENCE OF NEUROTOXICITY AND HYPERNOCICEPTION IN RATS ADMINISTERED THE ANTIRETROVIRAL DRUG STAVUDINE

Sibongile Makweya

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DECLARATION

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

All procedures used in this dissertation were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand, Johannesburg. The ethics clearance certificate numbers are AESC: 2006/24/03 and AESC: 2007/06/03.

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Sibongile Makweya

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ABSTRACT

Stavudine (d4T), a nucleoside reverse transcriptase inhibitor (NRTI) used to treat infection by the human immunodeficiency virus (HIV), is associated with the development of peripheral neuropathy and pain in HIV-positive patients. The mechanisms of this toxic neuropathy are poorly understood, primarily due to a lack of relevant animal models of the neuropathological process initiated by d4T. I investigated whether daily oral or subcutaneous administration of d4T produces neuropathological changes. Compared to previous descriptions of mechanical hypersensitivity induced by daily oral administration of d4T to rats at a dose of 50 $mg.kg^{-1}$over a four week period, I found that this dosing regimen did not result in hyperalgesia to blunt and punctuate mechanical stimuli applied to the gastrocnemeus muscle. In agreement with the lack of hyperalgesia, oral administration of d4T at 50 $mg.kg^{-1}$ over a four week period did not induce significant myelinated nerve fibre loss or morphological changes in the sciatic nerve. I then investigated whether administering 100 $mg.kg^{-1}$ d4T subcutaneously, and therefore avoiding first-pass metabolism, to rats for four weeks causes hyperalgesia and neuropathological changes in nerve morphology. Daily subcutaneous injections of d4T at 100 $mg.kg^{-1}$ over a four week period did not induce the development of hyperalgesia to a punctate mechanical stimulus applied to the tail or significant neuropathology. My studies demonstrate that multiple administrations of d4T at 50 $mg.kg^{-1}$ orally or 100 $mg.kg^{-1}$ subcutaneously over a four week period do not induce hyperalgesia or nerve fibre pathology in rats.

Thus, developing a robust animal model of d4T-induced neuropathy may be challenging in the absence of HIV-infection, such that occurs in infected patients.

Key words: HIV, ART, d4T, hypernociception

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LIST OF FIGURES **Page**

LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
ANOVA	analysis of variance
ART	antiretroviral therapy
ATN	antiretroviral toxic neuropathy
gp120	glycoprotein 120
ddC	zalcitabine
ddI	didanosine
DNA	deoxyribonucleic acid
d4T	stavudine
FIV	feline immunodeficiency virus
HAART	highly-active antiretroviral therapy
HIV	human immunodeficiency virus
HIV-SN	HIV-associated sensory neuropathy
HIV-DSP	HIV- distal sensory polyneuropathy
IL	interleukin
i.p.	intraperitoneal
i.v	intravenous
mtDNA	mitochondrial deoxyribonucleic acid
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
PI	protease inhibitor
S.C	subcutaneous

SIV	simian immunodeficiency virus
TNF-α	tumour necrosis factor-α
WHO	World Health Organisation

TABLE OF CONTENTS **Page**

CHAPTER 1

Introduction

HIV-associated Sensory Neuropathy (HIV-SN) is the most common cause of peripheral neurological dysfunction in HIV, and is characterized by distal axonal degeneration (Woolff et al., 2004; Pardo et al.,2001; Polydefkis et al., 2002; Zhou et al., 2007), where there is a loss of small unmyelinated fibres, which is followed by a loss of large myelinated fibres in more advanced cases (Cherry et al., 2003).. This nerve fibre degeneration is accompanied by the release of local inflammatory mediators, which enhances the sensitivity of surrounding, uninjured fibres, contributing to neuropathic pain (Keswani et al., 2002). Furthermore, the regrowth of damaged nerve fibres appears to be impaired with HIV infection (Hahn et al., 2007) and regeneration of injured nerve fibres is rare (McArthur et al., 2005) and regeneration of injured nerve fibres is rare (McArthur et al., 2005).

About 50% of individuals infected with the human immunodeficiency virus (HIV) develop HIV-associated sensory neuropathy (HIV-SN), a peripheral sensory neuropathy that is often painful, and is characterised by length-dependent degeneration of myelinated and unmyelinated nerve fibres (Keswani, et al., 2002; Ferrari et al., 2006). Non-painful peripheral neuropathy may be the result of partial damage to the nervous system and limited C-fibre dysfunction, allowing for normal nociception (Keswani et al., 2002).

HIV-SN is divided into two clinically indistinguishable sub-categories, namely HIV-associated distal sensory neuropathy (HIV-DSP) and antiretroviral toxic neuropathy (ATN), depending on the onset of signs and symptoms relative to

starting antiretroviral therapy (ART) (Cornblath & Hoke., 2006; Ferrari et al., 2006). Key signs of the neuropathy include reduced vibration sense, temperature thresholds, pin-prick sensitivity and deep-tendon reflexes, while key symptoms include pain, numbness and paraesthesias (Smyth et al., 2007, Martiz et al., 2010, Wadley et al., 2011). Our knowledge of how these factors damage sensory neurones is limited, which has impeded the development of effective therapeutic interventions for HIV-SN. Therefore, HIV-SN continues to be a major cause of disability despite the introduction of less neurotoxic treatment regimens, and improved virologic control (Smyth, et al., 2007*).* Much of what is known about the mechanisms of HIV-induced neuropathy and the pain associated with that neuropathy was discovered using animal models. The HIV envelop glycoprotein gp120 is associated with neurotoxic and nociception in rats (Herzberg & Sagen., 2001; Milligan et al., 2000). HIV-gp120 induces an immune response through macrophage infiltration of the peripheral nerve and dorsal root ganglia (Wallace et al., 2007) and activates macrophage and astrocytes in the brain and spinal cord (Herzberg & Sagen, 2001; Wallace et al., 2007), which then release the pro-inflammatory cytokines interleukin (IL)-1, tumour necrosis factor (TNF)-α, IL-6 as well as nitric oxide (NO) in the spinal cord (Herzberg & Sagen,2001; Milligan et al., 2001; Milligan et al., 2000). NO mediates the nociceptive response of spinal dorsal horn neurons in hyperalgesic states induced by inflammation (Keswani et al., 2003b)

While nucleoside reverse transcriptase inhibitors (NRTIs) form an important part of the highly active antiretroviral therapy (HAART), the mechanisms of NRTI-induced toxic neuropathy are still not well understood partly due to the scarcity of robust animal models of the disease, especially of models employing agents still in common use. The purpose of this introduction is to briefly describe the different forms of HIV-SN resulting from both HIV infection as well as NRTI use, and then to focus on describing animal models of NRTI-induced neuropathy and nociceptive hypersensitivity.

1.1 Peripheral neuropathies in HIV-positive patients

1.1.1 Pre-Highly active antiretroviral treatment (HAART) era:

The incidence of HIV-DSP increases with the disease progression, from very low rates during the early stages of infection (1-2%), to high rates at end-stage disease (~35%) (Barohn et al., 1993, So et al., 1988). In the majority of patients developing HIV-DSP, the neuropathy is symptomatic, with numbness and pain being the most common symptoms (Fuller et al., 1993, Tagliati et al., 1999). The prevalence of HIV-DSP in ambulatory HIV-positive individuals, who are not on antiretroviral therapy, and who are attending outpatient clinics is around 14% (Forna et al., 2007).

The polyneuropathy that developed in the Pre-HAART era, and continues to develop in individuals without access to HAART, is thought to result from immune dysfunction within the nervous tissue, which leads to inappropriate release of inflammatory mediators that can be neurotoxic (Kamerman et al.,

2011). These inflammatory mediators increase the sensitivity of surrounding uninjured fibres, thus contributing to neuropathic pain (Keswani et al., 2002). Experimental evidence supporting this mechanism of action includes recent studies by Laast et al (2007), who infected macaque monkeys with simian immunodeficiency virus (SIV), which resulted in neuronal loss in the trigeminal nerve ganglia of the monkeys and reduced conduction velocity in C-fibres, and Zhu et al (2007), who infected cats with feline immunodeficiency virus (FIV), which was associated with reduced axonal density and dying-back of distal nerve fibre endings in the sural nerve.

1.1.2 HAART era:

The Highly active antiretroviral therapy (HAART) is currently the most effective treatment for acquired immunodeficiency syndrome (AIDS), and has resulted in increased patient survival (Dorsey et al., 2009). HAART works by reducing viral load and increasing the CD4 T-cell count, and therefore it was expected that it would reduce the incidence of HIV-SN when it was introduced. But, the prevalence of HIV-SN has not decreased with the introduction of HAART. Indeed, the rate may have increased, with recent reports describing prevalences close to 60% (Wadley et al., 2011; Maritz et al., 2010). The reason for the continued high rate of HIV-SN is the neurotoxicity of some classes of NRTIs for example zalcitabine (ddC), didanosine (ddI) and stavudine (d4T) (Dalakas, 2001). Of all the NRTIs, Zidovudine (AZT) is the only drug which hasn't been implicated as a neurotoxin (Dalakas, 2001). It became clear early after the

introduction of NRTIs that they were neurotoxic, with ddC being the most neurotoxic. For example, monotherapy with ddC alone caused toxic neuropathy within seven to nine weeks of starting treatment in 100% of patients administered a high dose ($\geq$ 0.12 $mg.kg^{-1}$ per day) of the drug (Berger et al., 1993). Whereas a very high dose of ddI ($\geq$ 12$mg.kg^{-1}$ per day), resulted in neuropathy in approximately 50 % of patients (Lambert et al., 1990). Also, the toxicity of the drugs appears to be additive, such that the incidence of ATN is increased when NRTIs are used in combination. The dual administration of ddI and d4T was associated with a 3.5-fold risk of sensory neuropathy compared with ddI alone and a 2.5-fold risk compared to d4T, and therefore more neurotoxic than ddI or d4T alone (Moore et al., 2000).

Early studies on d4T, using d4T doses similar to therapeutic doses, reported that after one year of d4T use, 12-15% of the subjects had developed peripheral neuropathy, with those with the greatest level of immunosuppression individuals being at the greatest risk (Simpson et al., 1995). More recently our laboratory showed that 21% of patients d4T-based HAART developed ATN within three months of initiating therapy, and 41% had developed ATN by six months (Kamerman, personal communication). And, Winston and colleagues (2005) conducted a study in which 6% of HIV-negative individuals taking the 28-day d4T course (post-exposure prophylaxis) developed symptoms of HIV neuropathy, illustrating that even in healthy individuals, d4T is neurotoxic, although at a reduced level.

The continuing problem of HIV-SN is highlighted in a study by Smyth and Colleagues (2007). They conducted a study in which they described the prevalence and risk factors for HIV-SN both in the pre-HAART (1993) and HAART (2001) eras, and reported that the prevalence of this neuropathy increased from 13% in the pre-HAART era to 44% in the HAART era.

Although NRTIs are effective in suppressing HIV-1 RNA levels and increasing CD4 cell counts, they have a potential of decreasing a patients quality of life if they cause symptomatic neuropathy (Simpson et al., 2003).

Most researchers believe that the toxic effects of antiretroviral drugs, in HIV-positive patients, unmasks and enhances previously existing distal sensory polyneuropathy caused by the virus (Keswani et al., 2002). The virus, pro-inflammatory cytokines and chemokines are thought to cause the initial peripheral nerve injury, which then is exacerbated by the toxic effects of antiretroviral drugs, resulting in symptomatic, painful neuropathy (Keswani et al., 2002). The association between the symptomatic development of neuropathy and the start of antiretroviral therapy currently is necessary for the diagnosis of NRTI-induced neuropathy, as the clinical signs and symptoms of NRTI related neuropathy are similar to those of HIV-DSP (Cornblath et al., 2006), but the pattern of this neuropathy is different to the slowly progressive HIV-DSP in that there is a sudden onset of signs and symptoms, typically intense burning discomfort in both feet after six to eight weeks of treatment (Husstedt et al., 2001). The signs include impaired temperature and vibratory sensation, while cramps and pains (typically burning in quality) in the feet and legs (Simpson & Tagliati, 1995). Kokotis and colleagues (2007) showed that, although the clinical symptoms of both kinds of neuropathy are the same, the pathology of toxic neuropathy and distal sensory polyneuropathy may differ. The researchers found that neuropathy caused by the virus was associated largely with a decrease in the conduction velocity of

myelinated nerve fibres, while toxic neuropathy appeared to decrease the conduction velocity mainly of unmyelinated fibres.

The following guidelines were developed to assist differentiating HIV-DSP from ATN, with affirmative answers to any of the questions indicating ATN is likely:

1. Is there is clinical or electrophysiological improvement of symptoms after stopping therapy (NRTI)
2. Is there an association between the onset of symptoms or rapid worsening of neuropathic symptoms after initiating NRTI therapy
3. Is there worsening of neuropathic symptoms after discontinuation of NRTI therapy, followed by clinical improvement of symptoms later on. (Dalakas, 2001).

The neurotoxic side effects of NRTIs are thought to be related to their disruption of mitochondrial function (Kakuda, 2000). NRTIs are derivatives of adenosine, cytidine, guanosine and thymidine, which provide an alternative substrate for DNA polymerases terminating DNA synthesis (Kakuda, 2000). While the drugs target viral DNA polymerase, human DNA polymerase gamma (γ), which transcribes mitochondrial DNA, is also sensitive to the inhibiting effects of the drugs, resulting in impaired mitochondrial DNA synthesis, and ultimately impaired mitochondrial function through failure of the electronic transport chain (Kakuda., 2000).

Given the continued high use of d4T in developing countries, even those like South Africa where the drug is no longer prescribed as first-line therapy, it is

expected that HIV-SN will continue to affect the HIV-positive patients for some time to come. Thus, it is important that treatments be developed, but in order to do that we need good animal models of the drug-induced disease processes causing the neuropathy so therapeutic targets can be identified. For the remainder of the introduction chapter, I will be covering what is known about animal models of NRTI-induced hypernociception and neurotoxicity.

1.2 Models of NRTI-induced pain and neurotoxicity:

1.2.1 Animal models for HIV/ NRTI-induced pain

In an animal study examining pain caused by antiretroviral drugs, Joseph and colleagues (2004) developed a model of painful peripheral neuropathy induced by NRTI, wherein they showed that didanosine (ddI), zalcitabine (ddC) and stavudine (d4T) produced dose-dependent mechanical hypersensitivity as well as allodynia when administered to rats. In their study, a single intravenous injection of the NRTIs ddI, ddC and d4T to rats resulted in a dose-dependent mechanical and thermal hyperalgesia of the hind paw that lasted for twenty days. Hyperalgesia was observed with all of these NRTIs within one day when a dose of 50 $mg.kg^{-1}$ was used, and within three days with the 25 $mg.kg^{-1}$ and 10 $mg.kg^{-1}$ doses.

Although studies on diabetic and chemotherapy-induced neuropathy have shown these neuropathies are mediated, in part, by second messengers such as protein kinase A, nitric oxide synthase, protein kinase G, and mitogen activated protein

kinase in the peripheral terminals of afferent nociceptors (Aley & Levine, 1999), the hypersensitivity elicited by ddC injections was not diminished by inhibitors to any of these second messengers (Joseph et al, 2004). However, drugs which buffered intracellular calcium diminish ddC-induced mechanical hypersensitivity when administered both peripherally and centrally, leading the authors to suggest that altered calcium signaling played a role in the development of NRTI-induced hypersensitivity and that calcium homeostasis was impaired by mitochondrial dysfunction caused by NRTIs (Joseph et al., 2004).

But in subsequent studies, Joseph and Levine (2006) showed that disrupting the mitochondrial electron transport chain and preventing mitochondrial phosphorylation, attenuated ddC induced hyperalgesia and allodynia, a finding that is not in agreement with the proposed mechanism of action of NRTIs, which assumes that NRTIs cause inhibition of mitochondrial DNA synthesis and a subsequent decrease in the production of mitochondrial proteins, which results in a decrease in mitochondrial energy production. Therefore drugs that block the mitochondrial electron transport chain might be expected to exacerbate the effects of NRTIs and potentially enhance the hyperalgesia caused by NRTIs, instead of abolishing NRTI-induced hyperalgesia as shown by Joseph and Levine (2006).

In another model of ATN-induced neuropathic pain developed around a single injection of an NRTI, Bhangoo and colleagues (2007) demonstrated the involvement of CXCR4 in neuropathic pain signaling. In this study, a single

intraperitoneal (i.p) injection of ddC was administered to rats and increased levels of CXCR4 mRNA expression were observed in glial cells of the dorsal root ganglion and peripheral nerves following drug administration. Furthermore, this ddC-induced mechanical hypersensitivity was alleviated by CXCR4 antagonist, thus implicating CXCR4 involvement in the development of the hypernociception (Bhangoo et al., 2007). Interestingly, the changes in hypersensitivity correlated with nerve pathology. Bhangoo and colleagues (2007) sectioned sciatic nerve samples for histological assessment and nerve pathology was observed within three days following a single i.p injection of ddC and consisted of hypertrophy of the myelin sheath and tormaculae (Figure 1).

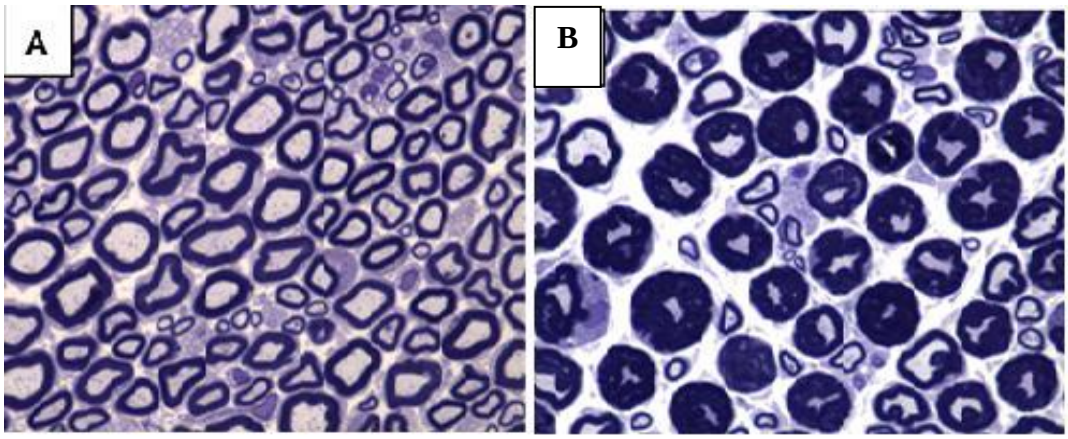

Figure 1. Nerve morphology of the sciatic nerves obtained from a vehicle-treated rat (A) and a rat administered a single i.p injection of ddC (B). From the figure we note the formation of tomaculae in ddC treated rat, hypertrophy of the myelin sheath resulting in the formation of myelin loop (Bhangoo et al., 2007).

In contrast to the findings of Bhangoo and colleagues (2007) study, others have found no significant effect of ddC injection on nerve morphology. For example Siau et al. (2006) found that a single injection of ddC into the tail vein of rats had no effect on intraepidermal nerve fibre density, a measure of axonal die-back that is a characteristic feature of the neuronal damage observed in humans with HIV-SN, in the hind paw. Systemic injections of ddC three times a week for three weeks did however decrease epidermal nerve fibre density in the lateral plantar surface of the hind paw of rats (Wallace et al., 2007b). Thus, it appears that a single intravenous (i.v) injection of ddC may cause morphological changes in peripheral neurones and may result in an increased sensitivity to noxious stimulation, but that a reduction in peripheral nerve fibre density occurs only with continuous NRTI administration.

All the models I have described so far have involved parenteral routes of drug administration and all used ddC. I will now discuss models employing oral routes of drug administration, and studies involving d4T and ddI, the two drugs that, until recently, were still used clinically to treat HIV-infection as first or second-line regimens.

In one of the first studies to employ oral administration of ddC, Anderson and colleagues (1992) found that chronic oral administration of ddC induced a decrease in conduction velocity in the distal sural nerve and structural damage in 28 the sciatic nerve of rabbits. Evidence of neuronal pathology was observed in dorsal root ganglia, the peripheral sensory system, the ventral roots and the

peripheral motor system, and was typified by myelin splitting, intramyelin edema, demyelination and remyelination of axons, and axonal loss. Also, Feldman and colleagues (1992) observed changes in the sciatic nerve and ventral roots structure induced by ddC, typically separation of myelin lamellae, fragmentation of myelin sheaths, demyelinated and remyelinated axons. In a more recent study, Joseph and colleagues (2004) administration ddC orally to rats at a dose of 50 $mg.kg^{-1}$ daily for six weeks; which resulted in mechanical and thermal hyperalgesia in the hind paw from seven days onward. However, a lower dose of 25 $mg.kg^{-1}$ of ddC induced hyperalgesia only after three weeks of daily oral administration. Thus, a in contrast to the rapid onset of hyperalgesia with i.v administration, and high oral bioavailability of NRTIs (Kaul et al., 1999; Kelley et al., 1987), the occurrence of hyperalgesia and allodynia was only evident after one week of daily oral administration of 50 $mg.kg^{-1}$ ddC to rats (Joseph et al., 2004); indicating that the route of administration has significant effect on toxicology, with the oral route having less toxicity. Unfortunately, Joseph and colleagues did not confirm whether the hyperalgesia they measured after administering ddC orally was associated with nerve pathology.

While the evidence supporting and algogenic and *in vivo* neurotoxicity of ddC is good, the evidence for *in vivo* toxicity of d4T and ddI in animals other than humans is poor. Warner and colleagues (1995) failed to observe signs of peripheral neurotoxicity in rabbits given oral ddI or d4T once daily for 24 weeks. In their model, the rabbits were administered doses of the drug far in excess of the

therapeutic doses used in humans (ddI: 250 or 400 mg.kg^{-1} per day depending on body mass; d4T: 30 mg or 40 mg twice daily depending on body mass; WHO, 2004). Yet, even these high doses of ddI and d4T, the drugs failed to induce significant changes in the peripheral nervous system and no change in peripheral nerve conduction or in the histopathology of peripheral or central nerves indicative of neuropathy (Warner et al., 1995). However, when Schmued et al (1996) administered ddI orally, twice daily, for 20 weeks, and observed morphological changes to neurones in the sciatic nerve identical to those described following ddC administration to rabbits (Feldman et al., 1992). For example, the nerve fibres were swollen, while the myelin was split into two distinct sheaths. Pattersons' group (2000) also reported similar observations, which were evident after 15 weeks of daily ddI administration (415 mg.kg^{-1} twice daily), while abnormal nerve fibre morphology was less frequent and only observed after 20 weeks. The authors suggested that the nerves may have been able to adapt to the toxic effects of ddI, making partial recovery during drug administration possible (Patterson et al., 2000), resulting in the improvement in nerve fibre morphology observed after 20 weeks.

Only one model employing oral administration of d4T has been described where Weber et al (2007) developed a rat model of d4T induced hyperalgesia by daily oral administration of d4T at a dose of 50 mg.kg^{-1} to rats which resulted in mechanical hyperalgesia, within three weeks of d4T administration, and the hyperalgesia persisted for up to six weeks. However, hyperalgesia to the thermal

challenge (49 °C water) did not develop and the overall condition of the rats was not affected by the d4T. Unfortunately, they did not assess for the presence of nerve pathology, but a lack of apoptosis and inflammatory cytokine expression in the dorsal horn of the spinal cord, a characteristic feature of neuropathic pain states, indicates that the pain may not have been neuropathic in origin (Weber et al., 2009). Recently, Dorsey et al (2009) developed an animal model of d4T, where a single 50 $mg.kg^{-1}$ i.v injection of d4T resulted in robust tactile allodynia within 24hrs of d4T administration. This allodynia was associated with the expression of *Gan1* gene in cells in the dorsal horn of the spinal cord. Changes in the expression of this gene has been associated with other rare forms of neuropathy, indicating that the allodynia may be neuropathic in origin. Thus, it is unclear whether hypernociception caused by d4T in rodents is neuropathic or not.

The inconsistencies in the effects of NRTIs have on nerve fibres and pain in animal models may be caused by the difference in the toxicity of the NRTIs tested, the duration of NRTI exposure, and the different nociceptive testing methods used. It could also be due to the different routes of administration, as well as by the animal species used. Consequently, the mechanisms of ATN remain poorly understood due to lack of robust and relevant animal models.

1.3 Aims of the Dissertation

Until recently, In South Africa, there were two HIV treatment regimens for both adults and childrens wherein regimen 1 for adults includes d4T and regimen 2 for

children includes ddI: which are both associated with ATN neuropathy (SA HIV treatment guidelines, 2010). Also, antiretroviral treatment is often initiated in patients with a weakened immune system i.e. CD4 T-cell count <200cells.mm^3 irrespective of clinical stage or CD4 count <350cells.mm^3 in patients with TB & HIV and in pregnant women (SA ART treatment guidelines, 2010). Initiating antiretroviral therapy at advanced stages of HIV infection increases the risk of developing ATN neuropathy.

In 2010, the use of d4T and ddI in patients initiating antiretroviral therapy (ART) was stopped (DOH HIV treatment guidelines, 2010). However, the bulk of existing patients remain being treated with these drugs, especially d4T, and often they develop painful HIV-SN. Our understanding of this drug-induced neuropathy, and therefore the treatment of the condition, is compromised due to very few d4T animal models of this neuropathy with which to study the disease process. In our laboratory, Weber and colleagues (2007, 2009) developed a model of NRTI induced mechanical hypersensitivity by administering d4T orally to rats However, they provided no evidence that the hyperalgesia that developed was the result of peripheral nerve damage. Therefore the aims of this dissertation are to develop a robust animal model of stavudine-induced hyperalgesia:

- By looking at the effects of administering d4T via two routes and monitoring the changes in nerve morphology of the sciatic and cocxygeal nerves.
- by looking at the effects of administering d4T via two routes and monitoring the development of hyperalgesia

CHAPTER 2

General methods

2.1 Animals

The methods described in this chapter are those methods that are common to experiment 1 (chapter 3) and experiment 2 (chapter 4). A description of the study specific experimental designs for experiment 1 and experiment 2 are given in chapters 3 and 4, respectively.

I used a total of 55 Sprague-Dawley (200-250)g rats. All rats were housed at an air temperature of ~23°C on a 12:12 h light/dark cycle (lights on at 07h00), with access to food and water *ad libitum*. In Experiment 1: Oral d4T (Chapter 3), rats were housed individually in cages to ensure that the each animal received the correct mass-specific dose of drug administered in a gelatine cube placed in their cage. In Experiment 2: Subcutaneous d4T (Chapter 4), rats were housed in pairs because drug delivery was entirely under the control of the experimenter. All experimental procedures were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand, South Africa AESC: 2006/24/03 and AESC: 2007/06/03.

2.2 Nerve histology

Segments of sciatic (Experiment 1: Oral d4T) and coccxygeal nerves (Experiment 2: Subcutaneous d4T) dissected from anaesthetized rats were immersed in 2% parafomaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 24 hours. Then the tissue was rinsed in phosphate buffer (pH 7.4) and osmicated

in 0.1M phosphate buffer with 2% osmium tetroxide. The tissue was then dehydrated through graded alcohols and embedded in resin. Thereafter, 0.5μm semithin sections of nerves were cut and stained with toloudine blue dye for examination using light microscopy. Nerve sections were photographed at 40X magnification, and two 100 X 100 μm^2 areas per section were printed on 15.6 cm x 16.3 cm (80 $g.m^{-2}$) white paper, and analyzed for nerve pathology using the method described by Flatters (2008). In each of the 100 X 100 μm^2 sections, I counted the number of myelinated axons as well as the number of healthy myelinated axons with signs of pathology. The pathological features I assessed were: splitting, balooning and infolding of the myelin sheath. The values recorded from the two 100 X 100 μm^2 sections from each nerve were then averaged, and the averaged values were then used in all data analysis. Thereafter, I determined the cross-sectional area of the myelinated fibres, and the cross-sectional area of their axoplasm and myelin sheath, by cutting out the respective structures from the printed sheets, and calculating the cross-sectional areas according to the following formula:

$$\frac{\text{weight of paper cut-outs (g)}}{\text{weight of 254 cm}^2\text{ paper sheet (g)}} \times 10\,000\ \mu m^2$$

2.3 Behavioral nociceptive tests

I assessed withdrawal response thresholds to punctate and blunt mechanical stimuli applied to the gastrocnemius muscle (Experiment 1: Oral d4T), and a

punctate stimulus applied to the mid-region on the dorsal aspect of the tail (Experiment 2: Subcutaneous d4T). The punctate stimulus was applied using an electronic von Frey anaesthesiometer (ITTC, Life Sciences, USA), and the blunt stimulus was applied using a somedic analgesiometer (Somedic, AB, Horby, Sweden) with a 0.5 mm diameter rubber probe. The stimuli were applied until limb or tail withdrawal (escape behavior) was elicited, and the average of three measurements, taken one minute apart, was recorded as the withdrawal threshold. When applying the punctuate and blunt stimuli to the gastrocnemius muscle (Experiment 1: Oral d4T), a handler placed a light cloth over the rat and gently restrained the animals body by hand, while the experimenter held the animal's leg and applied the stimulus to the leg (Loram et al., 2007a). When applying the punctate stimulus to the tail (Experiment 2: Subcutaneous d4T), animals were restrained in clear plastic restrainers that restricted body movement but allowed free movement of the tail (Loram et al., 2007b). Animals were habituated to the appropriate method of restraint daily for a week prior to commencing experiments.

2.4 Data analysis

All data are expressed as mean (SD). Changes in withdrawal thresholds over time were described by fitting regression lines to the data When the same type of equation (e.g. linear equation) could be used to describe changes in withdrawal thresholds over time in the control and experimental groups (Experiment 1: Oral d4T), withdrawal thresholds between the two groups were assessed by comparing

the regression line coefficients, intercepts and slopes. When different regression equations were used to describe the changes in withdrawal thresholds between the two groups (Experiment 2: Subcutaneous d4T) then student's t-tests were used. Comparisons of the number of myelinated axons, the number of abnormal axons, and myelin and axoplasm area of myelinated fibres in sciatic nerve and coccxygeal nerve sections were analyzed using one-way ANOVA. The threshold for statistical significance was set at $P<0.05$.

CHAPTER 3

Experiment 1: Oral administration of d4T

3.1 Introduction

Painful peripheral neuropathy continues to be a debilitating side-effect of NRTIs (Kamerman et al., 2012) and this pain is largely unresponsive to current pharmacological treatments for neuropathic pain (Phillips et al., 2010). Until recently in South Africa, the first-line HIV treatment regimen for adults and second-line treatment regimen for children included d4T and ddI respectively (DOH HIV treatment guidelines, 2010). The guidelines have now been amended such that new patients starting therapy do not start on d4T or ddI base regimens. However, the majority of existing patients who are on therapy remain treated with these drugs, especially d4T. Thus, the potential burden of iatrogenic painful peripheral neuropathy remains high. In a recent study looking at the prevalence of HIV-SN in South Africans, Maritz and colleagues (2010) reported the prevalence of the neuropathy was 49% in their study population, and 30% of those were diagnosed with painful neuropathy. The mechanisms underlying the NRTI-induced neuropathies are still unclear and better understanding of these neuropathies will facilitate development of effective treatment for these painful neuropathies.

In our laboratory, Weber and colleagues (2007, 2009) developed a model of NRTI induced mechanical hypersensitivity by administering d4T orally to rats over a six week period. In this study, they found that rats developed hyperalgesia within three week of drug administration and the hyperalgesia remained constant over the remaining three weeks of drug administration (Weber et al., 2007). However,

Weber et al (2007) provided no evidence that the hyperalgesia that developed to orally administered d4T was the result of peripheral nerve damage.

However, others have shown that hypernociception following acute (Bhangoo et al., 2007) and chronic (Wallace et al., 2007a) exposure to ddC is associated with nerve pathology. Therefore the aim of this experiment was to determine whether administering d4T orally to rats for four weeks causes pathological changes in nerve morphology. Although I administered the same dose of d4T, and followed the same dosing schedule, used by Weber et al (2007, 2009), I assessed mechanical hypersensitivity in the hind leg rather than the tail because the sciatic nerve that innervates the hind limb is a larger, more easily accessible nerve than the coccxygeal nerve that innervates the tail, and the nerve pathology described by Bhangoo et al 2007 in response to a single i.p dose of ddC was assessed in the sciatic nerve.

3.2 Methods

The animal husbandry, nociceptive testing techniques, nerve processing, and data analyses used in this experiment are described in Chapter 2.

Experimental procedures

In the first part of this experiment, 20 rats were randomly allocated to one of two groups; 10 rats were allocated to the control group and 10 rats to the d4T group. Animals were familiarized with the handling procedures used during nociceptive

testing for a week before the start of nociceptive testing. During this familiarization time, animals also were trained to eat standard gelatine cubes, which contained no drug, once daily at 14h00 until they ate the entire cube within 15 minutes. After this training period, baseline behavioral nociceptive measurements were made daily for five consecutive days, with all rats continuing to receive standard gelatine cubes containing no drug daily. On day six, animals in the d4T group started receiving gelatine cubes containing 50 $mg.kg^{-1}$ d4T daily for four weeks, while animals in the control group continued to receive gelatine cubes with no added drug daily for four weeks. During this phase of the experiment, behavioral measurements were made once weekly over the four-week period.

In the second part of this study, an additional 15 Sprague-Dawley rats (200-250)g were allocated to one of three groups, where rats in two of the groups were administered either standard gelatine cubes containing no drug (n=5) or 50 $mg.kg^{-1}$ d4T orally in gelatine cubes for four weeks (n=5), and animals in the third group received no treatment (n=5). At the end of the four week period, rats were anaesthetized with a mixture of 100 mg/ml ketamine (Anaket-V; Bayer Animal Health Pty Ltd, Isando, South Africa) and 20 mg/ml xylazine (Chanazine; Bayer Animal Health Pty Ltd, Isando, South Africa) at a ratio of 1:4 injected intramuscularly at a dose of 0.1 ml per 100 g body mass, and the sciatic nerve was dissected free of the surrounding tissue. Ten millimeter section, from immediately proximal to the trifurcation of the nerve trunk was excised and placed in fixing solution. Thereafter animals were administered a lethal overdose of the

anaesthetic. No behavioral measurements were made on these animals, but they were age and weight-matched to animals that were undergoing behavioral testing.

Drugs and method of administration

2',3'-didehydro-3'-deoxythymidine (d4T, Cipla Life Sciences, South Africa) was prepared and administered as described by Weber et al (2007, 2009). Briefly, d4T (50 $mg.kg^{-1}$) was administered once daily as a suspension in flavoured gelatine cubes. The gelatine cubes were prepared by dissolving 7 ml savoury bread spread (Bovril, Unilever, South Africa), 20 g cane sugar, and 12 g unflavoured gelatine powder (Davis Gelatine, Johannesburg, South Africa) in 100 ml warm water. The solution was aliquoted into 3 ml moulds and allowed to set. Standard cubes contained no drug, but d4T containing cubes were prepared by adding powdered d4T to the 3 ml aliquots and vortexing the aliquots before the gelatine set.

3.3 Results

Behavioural nociceptive tests

Changes in withdrawal thresholds to a punctate mechanical stimulus over time are shown in Figure 2. For both groups, the change in withdrawal threshold over time was best described using a linear regression equation, with the withdrawal threshold for the control and d4T groups decreasing over the course of four weeks of the experiment (Control: $y= 259.3-22.07x$, $r^2= 0.92$, $p=0.009$; d4T: $y= 258.2-17.68x$, $r^2=0.92$, $p=0.008$). There was no significant difference in the slope (Control: slope= -22.07 ± 3.63; d4T: slope= -17.68 ± 2.82) or intercept (Control:

intercept= 259.3 ± 8.91; d4T: intercept= 258.2 ± 6.92) of the regression lines between the control and d4T groups. That is, daily oral administration of d4T (50mg.kg^{-1}) did not produce mechanical hypersensitivity to a punctate stimulus applied to the gastrocnemius muscle compared to rats that did not receive any drug.

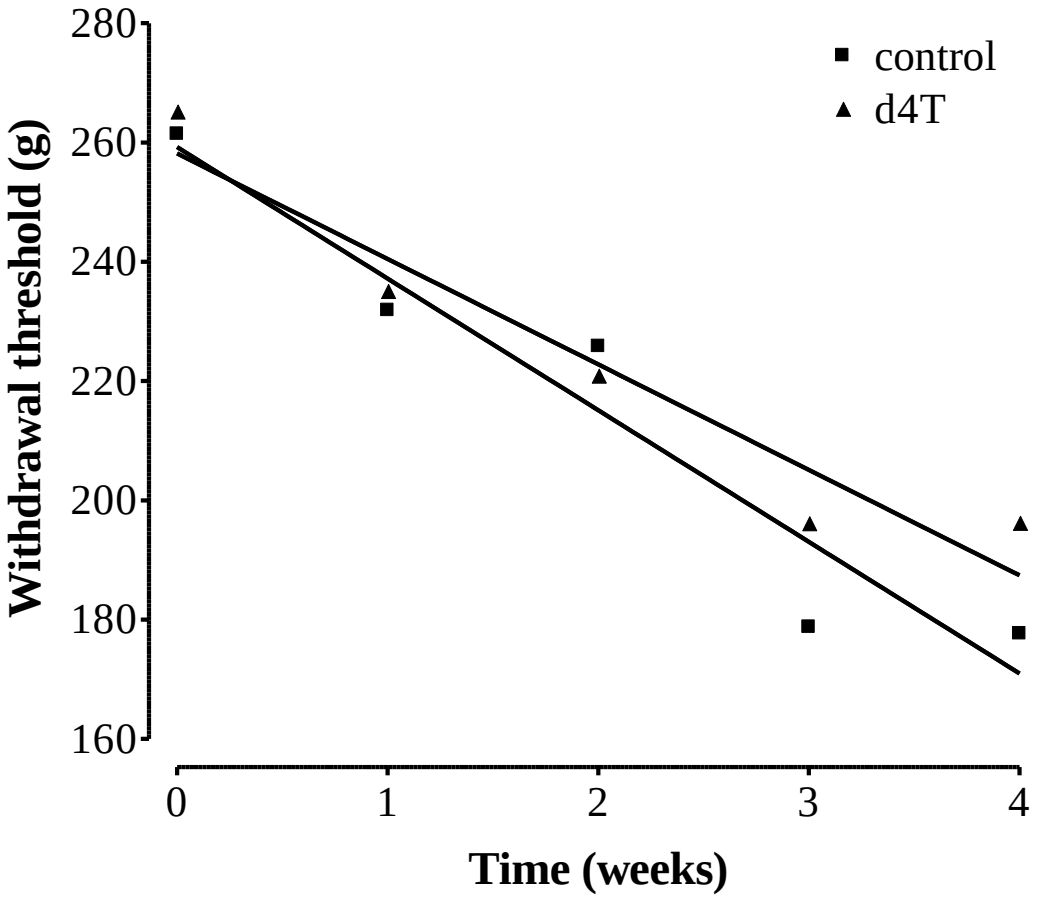

Figure. 2. Mean limb withdrawal thresholds to a punctate mechanical stimulus measured weekly in rats before (week 0), and during (weeks 1 to 4) four weeks of daily oral administration of 50 $mg.kg^{-1}$ d4T (diamonds) or drug-free (squares) gelatine cubes. Withdrawal threshold decreased significantly over time but there were no differences in withdrawal thresholds between the d4T and control groups over the four-week course of the experiment.

Figure 3 shows changes in withdrawal thresholds to a blunt mechanical stimulus. For both groups, the change in withdrawal threshold over time was best described using a linear regression equation, (Control: y= 57.42-5.55x, r^2=0.86, Sy.x= 4.14; d4T: y=56.32-5.54x, r^2= 0.78, Sy.x= 5.29). The withdrawal threshold decreased markedly over the course of four weeks of the experiment in both groups. However, there was no significant difference in the slope (Control: slope= -5.55 ± 1.31, d4T: slope= -5.54± 1.67) or intercept (Control: intercept= 57.42 ± 3.21, d4T: intercept= 56.32 ± 4.10) of the regression lines between the control and d4T groups, showing that d4T administration did not significantly change limb withdrawal thresholds to a blunt mechanical stimulus.

Nerve pathology

Figure 4 shows the appearance of typical cross sections through the sciatic nerve of rats fed 50 $mg.kg^{-1}$ d4T containing gelatine cubes (Figure 4A) or control gelatine cubes (Figure 4B) for four weeks. There was no difference in the total number of myelinated axons (Figure 5A, one-way ANOVA; p=0.70) or the percentage of abnormal axons; for example myelin splitting, oedema or infolding (Figure 5B, one-way ANOVA; p=0.95,) between the no intervention, the control and the d4T groups. Furthermore, daily oral administration of d4T did not alter the axoplasm area per axon (Figure 6A, one-way ANOVA; p>0.05), nor the myelin area per axon (Figure 6D, one-way ANOVA; p=0.76) compared to the no intervention and control groups. Thus, at the drug dose we used, d4T did not induce any significant nerve pathology in myelinated fibres.

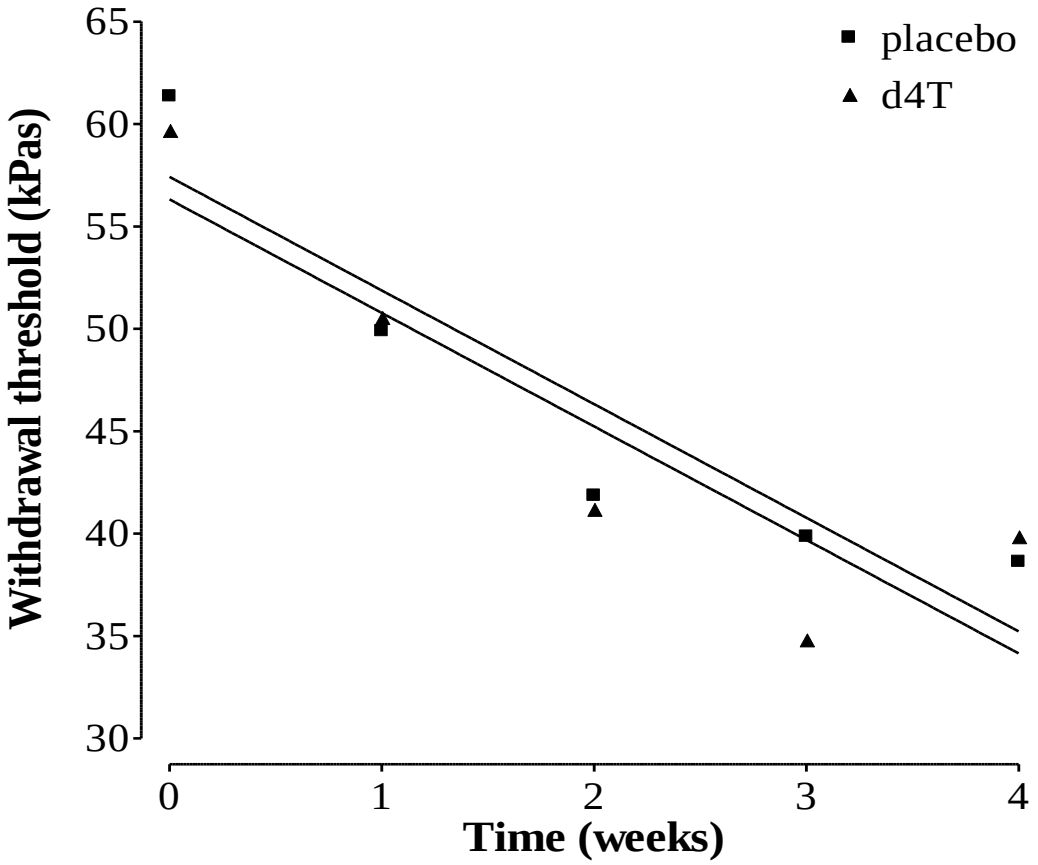

Figure 3. Mean limb withdrawal thresholds to a blunt mechanical stimulus measured weekly in rats before (week 0), and during (weeks 1 to 4) four weeks of daily oral administration of 50 $mg.kg^{-1}$ d4T (diamonds) or drug-free (squares) gelatine cubes. Withdrawal thresholds decreased significantly over time in both groups, but there was no difference in withdrawal thresholds between the d4T and control groups over the four-week course of the experiment.

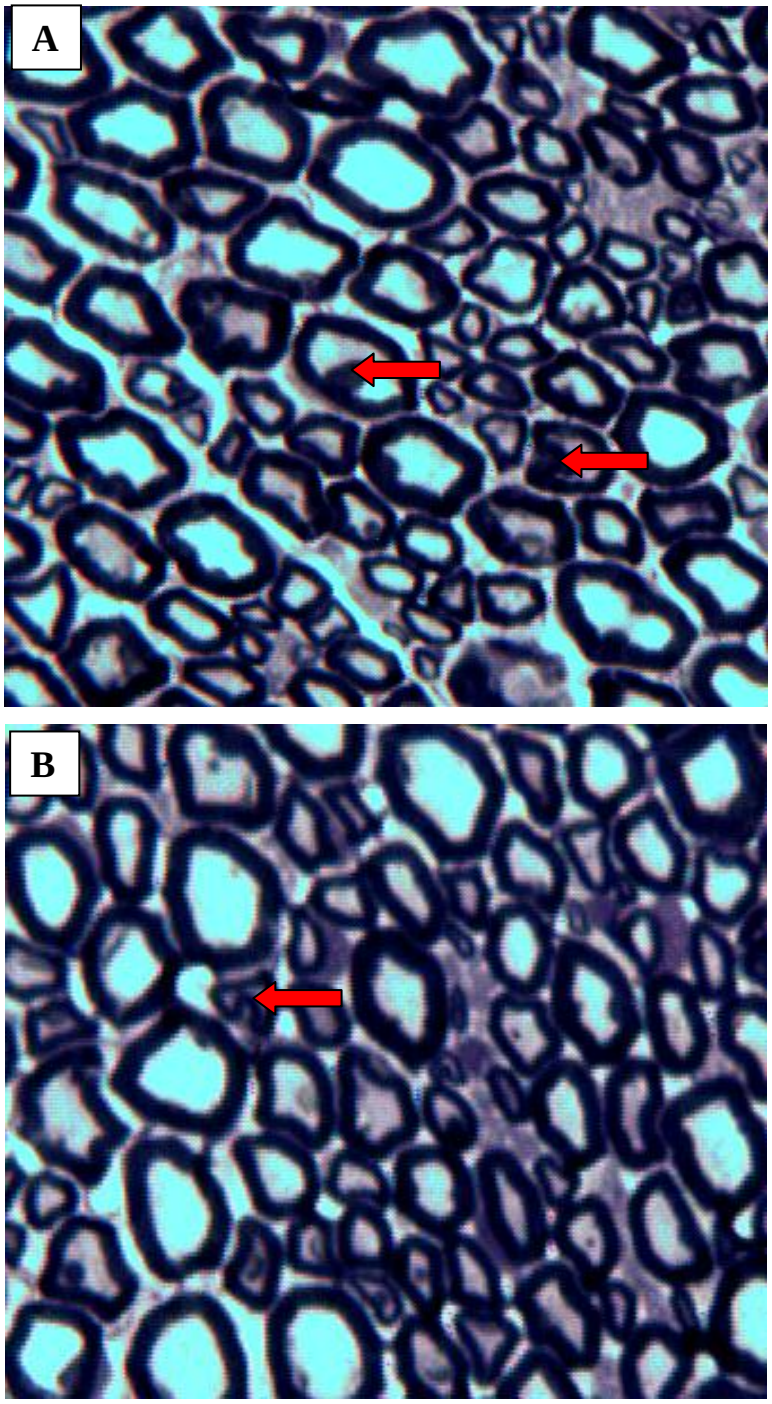

Figure 4. Cross-sections of the sciatic nerve from animals administered 50 mg.kg^{-1} d4T (A) and control (B) gelatine cubes daily for four weeks, (at 40X magnification). No significant myelinated axon loss or fibre pathology was observed in rats administered d4T. The arrows show infolding of the myelin sheath.

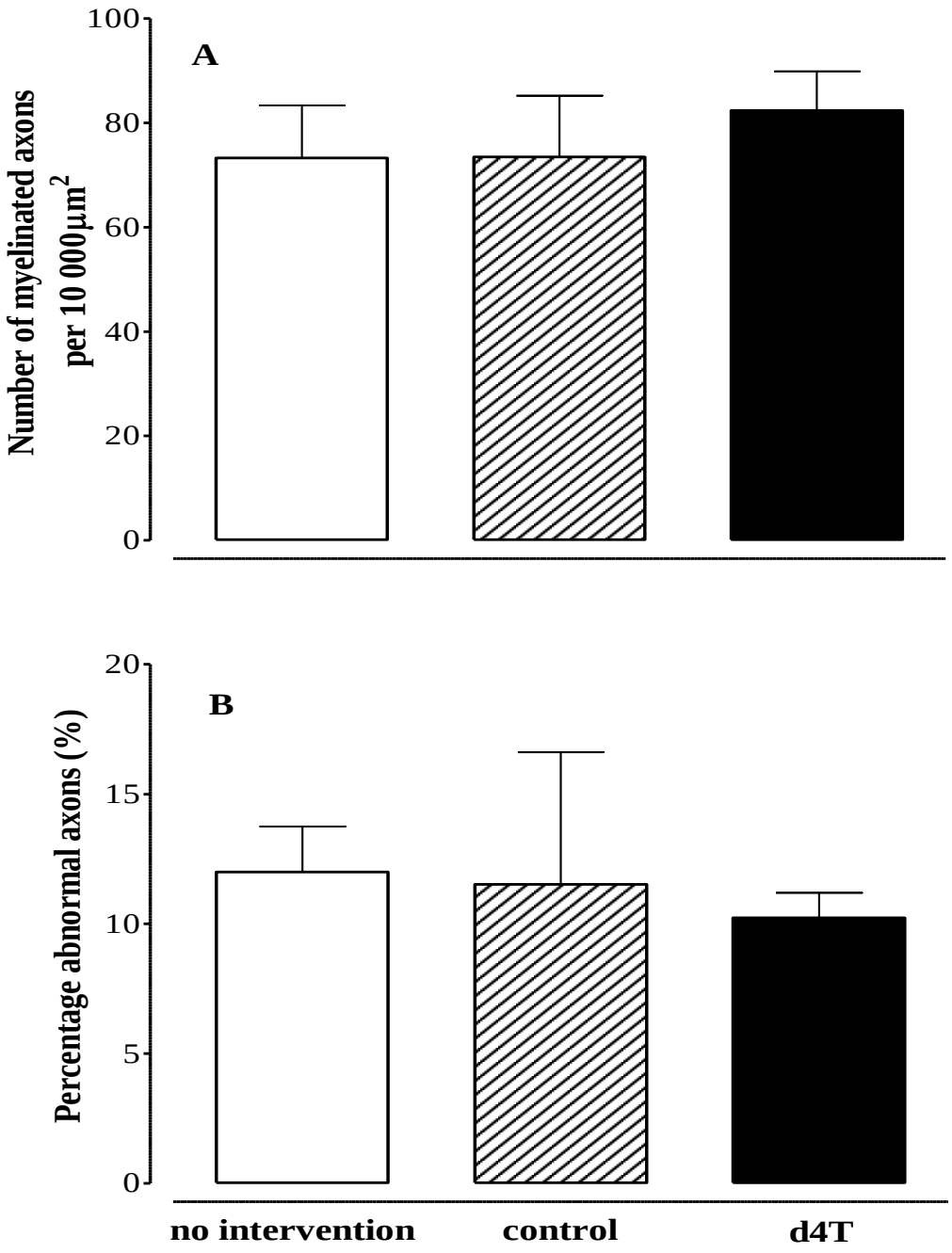

Figure 5. Mean (SD) number of myelinated axons (A) and percentage of abnormal myelinated axons (B) (e.g., ballooning, splitting of myelin sheath) per 10 000 μm^2 cross-sectional area of sciatic nerve. Administration of d4T did not significantly change the number of myelinated axons or the number of abnormal axons compared with the control and no intervention groups

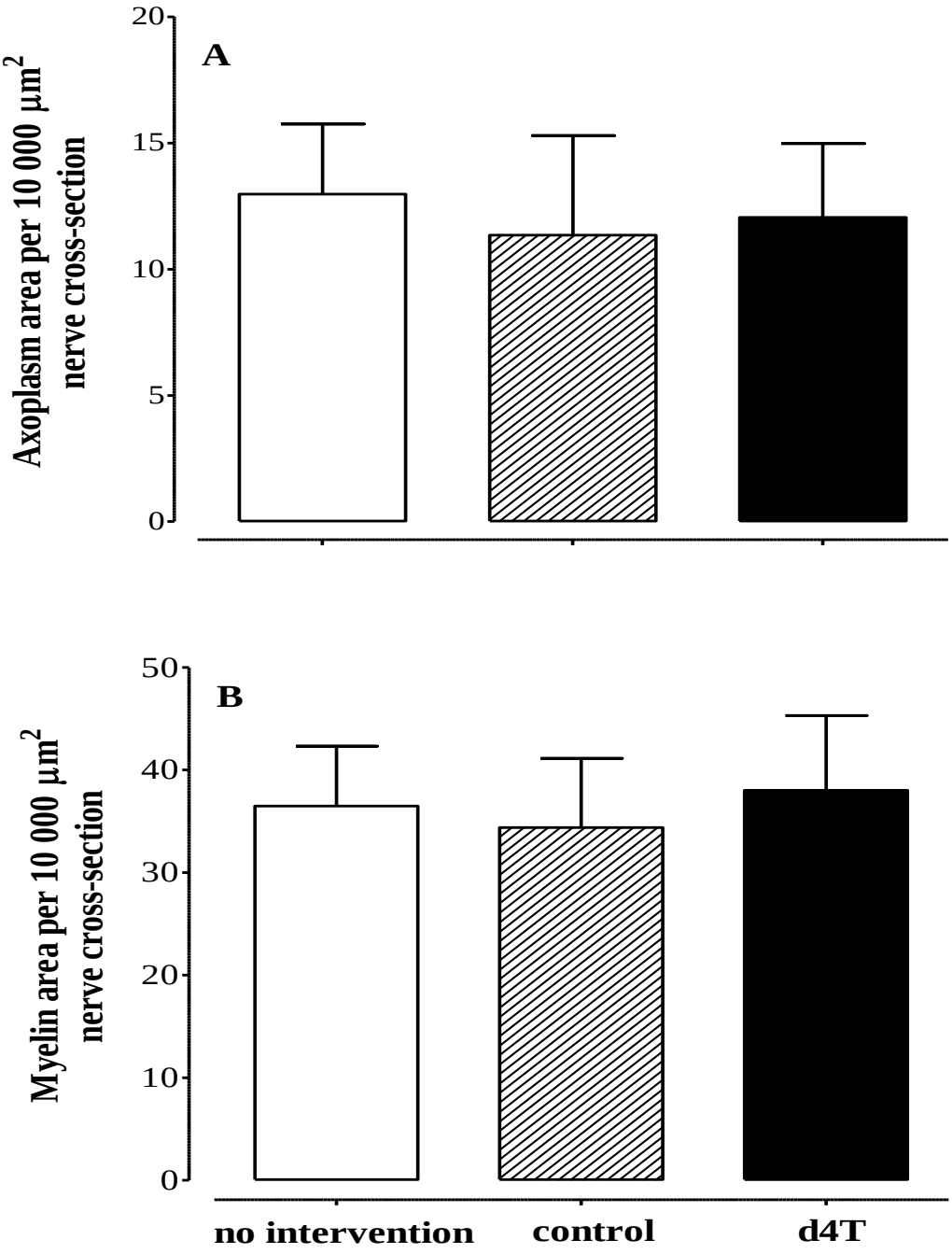

Figure 6. Mean (SD) axopalsmic area per 10 000 µm^2 sciatic nerve cross-section (A) and myelin area per 10 000 µm^2 sciatic nerve cross-section (B). Administration of d4T did not alter the axoplasmic area per 10 000 µm^2 or myelin area per 10 000 µm^2 compared to the control and naive rats.

3.4 Discussion

The aim of this experiment was to determine whether daily oral administration of d4T produces behavioral and neuropathological changes in the sciatic nerves of rats. Oral administration of d4T at 50 $mg.kg^{-1}$ over a four week period did not induce hyperalgesia or significant myelinated nerve fibre loss or morphological changes in the sciatic nerve. My histological findings are in agreement to the results of Warner and colleagues (1995) who, in the only other study to investigate nerve and behavioral changes following d4T administration, failed to observe signs of peripheral neurotoxicity in rabbits administered oral d4T daily for 24 weeks. In their model, the rabbits were administered d4T (750 $mg.kg^{-1}$ and 1500 $mg.kg^{-1}$) by gastric gavage daily for 24 weeks, and even at these high doses, the investigators still did not observe any mechanical hypersensitivity or neuropathological changes associated with the use of NRTIs.

In my experiment, semi-thin sections of the sciatic nerves of rats administered d4T did not show changes typical of what has been reported by other investigators who administered other NRTIs to rats. For example, Patterson and colleagues (2000) were able to develop a rodent model of ddI induced myelinopathy, with chronic administration of very high doses of ddI (415 $mg.kg^{-1}$, twice daily). However, these dose-dependant changes in myelin architecture were observed only after weeks 15 and 20, far beyond the duration I administered d4T. Although Patterson and colleagues were able to demonstrate changes which occur with chronic administration of NRTIs, the doses used in their study are far beyond the

clinically recommended doses (Schmued et al., 1996; Wientjies and Au., 1992; WHO guidelines, 2003).

Surprisingly, daily oral administration of d4T (50 $mg.kg^{-1}$) did not produce mechanical hypersensitivity to punctuate or blunt stimuli applied to the gastrocnemius muscle. These findings are contrary to those of Weber et al (2007) and Joseph et al (2004) who have both reported the presence of mechanical hyperalgesia following daily administration of d4T at a dose of 50 $mg.kg^{-1}$ respectively. Joseph and colleagues (2004) administered d4T i.v, but Weber et al (2007) used the same dosing route I employed and the presence of hyperalgesia was evident within 3 weeks of 50 $mg.kg^{-1}$ d4T which is much sooner compared to the onset of painful toxic neuropathy in HIV-positive patients, occurring within 6-8 weeks following drug administration (Husstedt et al., 2001). However, a noticeable difference between the original study by Weber et al (2007) tested for the presence of mechanical hyperalgesia in rats using using a bar algometer (a tonic, blunt mechanical stimulus) to the tail, whereas I applied tonic mechanical blunt and punctuate stimuli to the gastrocnemius muscle. The lack of hypersensitivity in my rats may be because of these methodological differences. However, if the hyperalgesia reported by Weber et al (2007& 2009) was caused by drug-induced nerve damage, I still would have expected to see nerve pathology in my rats since the dosing regimen was the same.

In conclusion, I investigated the effects of d4T on healthy rats, using a low dose of d4T. Based on the findings of Weber et al (2007 & 2009), my failure to detect changes in behavior or nerve histology could have been related to my low drug dose or testing behavioral nociception and histology too proximally. Therefore in order to study this further, I administered d4T intraperitoneally to rats and tested for hyperalgesia on the mid-region of the rat tail in experiment 2 (chapter 4).

CHAPTER 4

Experiment 2: Subcutaneous administration of d4T

4.1 Introduction

In our first study, I did not induce hyperalgesia or nerve pathology in rats administered 50 $mg.kg^{-1}$ d4T daily orally for four weeks. This is contrary to Weber and colleagues' (2007) findings that showed that daily administration of 50 $mg.kg^{-1}$ of d4T resulted in mechanical hyperalgesia in the rat tail within 3 weeks of drug administration. Lack of hyperalgesia in our study may be either due to differences in the two anatomical sites of nociceptive testing or differences in the techniques used to test for hyperalgesia. Weber and colleagues used a static, blunt stimulus applied to the tail whereas I used a dynamic blunt and punctate stimuli applied to the gastrocnemeus muscle. Also, antiretroviral toxic neuropathy is a length-dependent neuropathy, therefore by measuring hyperalgesia at the gastrocnemeus level and taking the sciatic nerve biopsies at the level of the thigh, rather than mid-way down the tail, I could have missed the hyperalgesia and nerve pathology.

Although the oral bioavailability of d4T is complete in rats, with 100% of the oral dose reaching the systemic circulation (Kaul et al., 1999), our rats did not develop mechanical hyperalgesia. However, by administering the drug parenterally rather than orally, first-pass metabolism of the absorbed drug is bypassed, which may increase toxicity (Kaul et al., 1999). And only high doses of ddI administered for prolonged periods of time, orally administered d4T have not been shown to induce peripheral nerve damage in animal models of NRTI-induced neuropathy.

Therefore, in this study, I chose to use a higher dose of d4T (100mg.kg^{-1}), administered subcutaneously, measured behavioral responses to a puctate stimulus in the tail and took biopsies from the tail.

4.2 Methods

The animal husbandry, nociceptive testing techniques, nerve processing, and data analyses used in this experiment are described in Chapter 2.

Experimental procedures

Twenty rats were randomly allocated to one of two groups; 10 rats were allocated to the control group and 10 rats to the d4T group. The rats were familiarized with the restrainers for three hours per day for a week before any experimental procedures were done. Baseline behavioral measurements were taken daily for five consecutive days before injections. Thereafter, the rats were injected with 100 mg.kg^{-1} d4T or 1 ml.kg^{-1} saline subcutaneously daily for 28 days and behavioral measurements were carried out weekly over the treatment period. At the end of the period of drug administration, rats were anaesthetized using ketamine (Anaket-V; Bayer Animal Health Pty Ltd, Isando, South Africa) and xylazine (Chanazine; Bayer Animal Health Pty Ltd, Isando, South Africa) and the coccxygeal nerve was dissected free of the surrounding tissue ~20mm distal to the base of the tail, excised and placed in fixing solution. Thereafter animals were administered a lethal overdose of the anaesthetic. Nerve tissue also was collected

from an additional group of age-matched rats (n=5), which had not received any treatment nor had had behavioural measurements made on them.

Drugs and method of administration

In this study, 2', 3'-didehydro-3'-deoxythymidine (d4T, Aspen Pharmacare, South Africa) was suspended in sterile isotonic saline (0.9%) immediately before injections, at a concentration of 100 $mg.ml^{-1}$. Depending on their assigned group, animals were administered either 100 $mg.kg^{-1}$ d4T or an equivalent volume of the saline subcutaneously daily.

4.3 Results

Behavioural nociceptive tests

Figure 7 shows changes in the tail withdrawal thresholds to a punctate mechanical stimulus. The changes in withdrawal threshold over time were best described using second-order polynomial regression equations (Control: r^2= 0.45, Sy.x=20.46; d4T: r^2= 0.60, Sy.x=7.48). But the equations were not good fits for the data so these data were analyzed using the Student t-test to assess whether there was a difference in the tail withdrawal thresholds between the d4T and saline groups. The analysis was performed on the behavioral data collected at the end of each 7-day period (baseline, week 1, week 2, week 3, week 4), and we found that there is no significance difference in the withdrawal thresholds between the experimental and control animals at each time period. That is, daily injections

of d4T (100 mg.kg^{-1}) did not produce mechanical hypersensitivity to a punctate mechanical stimuli applied to the tail.

Nerve pathology

Figure 8 shows the appearance of typical cross sections through the coccxygeal nerve of rats administered daily subcutaneous injections of d4T (100 mg.kg^{-1}) (Figure 8B) or an equivalent volume of saline (Figure 8A) over a four week period. There was no difference in the total number of myelinated axons (Figure 9A; one-way ANOVA; $p>0.05$) or percentage of abnormal axons (e.g. myelin splitting) (Figure 9B; one-way ANOVA; $p=0.38$) between the no intervention, the salineand the d4T groups. Furthermore, daily subcutaneous administration of d4T (100 mg.kg^{-1}) did not alter the axoplasm area per axon (Figure 10A; one-way ANOVA; $p=0.06$) nor the myelin area per axon (Figure 10B; one-way ANOVA; $p=0.12$) compared to the no intervention and salinegroups. Thus, at the drug dose we used, d4T did not induce any significant nerve pathology.

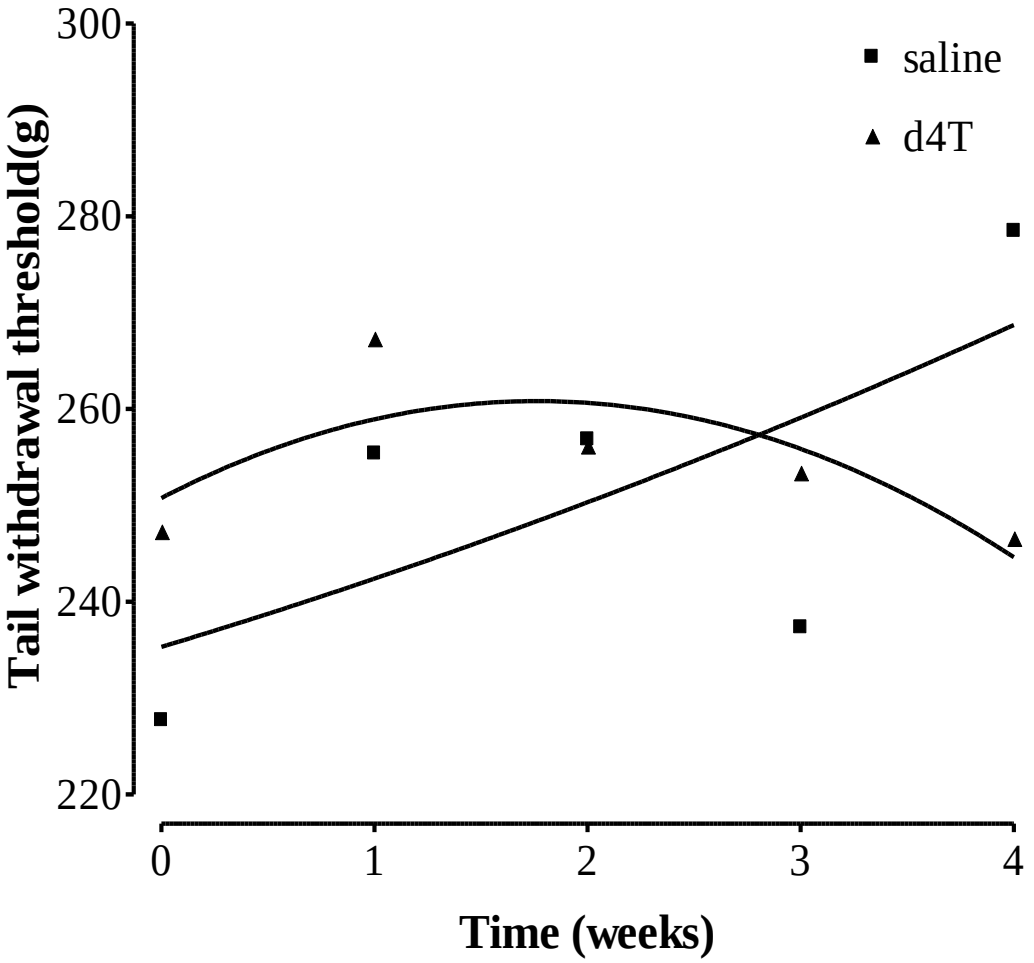

Figure 7. Mean tail withdrawal thresholds in response to punctate mechanical stimulation with an electronic von Frey device measured in rats before (week 0) and during (weeks 1 to 4) four weeks of daily subcutaneous administration of 100 $mg.kg^{-1}$ d4T (diamonds) or an equivalent volume of saline (squares). There was no difference in the withdrawal threshold of the d4T group and control group over the four-week course of the experiment.

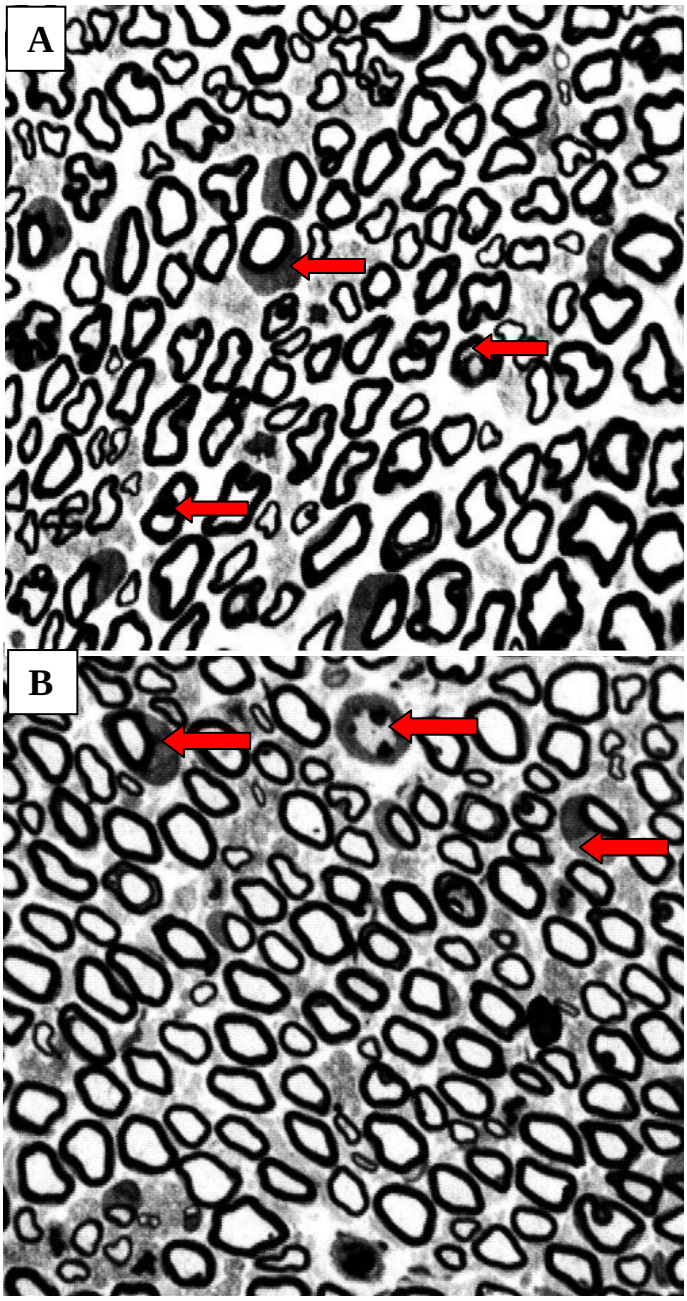

Figure 8. Cross-sections of the coccyxgeal nerve from animals administered 1 $ml.kg^{-1}$ saline (A) and 100 $mg.kg^{-1}$ d4T (B) injections (40X magnification). No significant myelinated axon loss or fibre pathology was observed in rats administered d4T. The arrows show infolding and balooning of the myelin sheath.

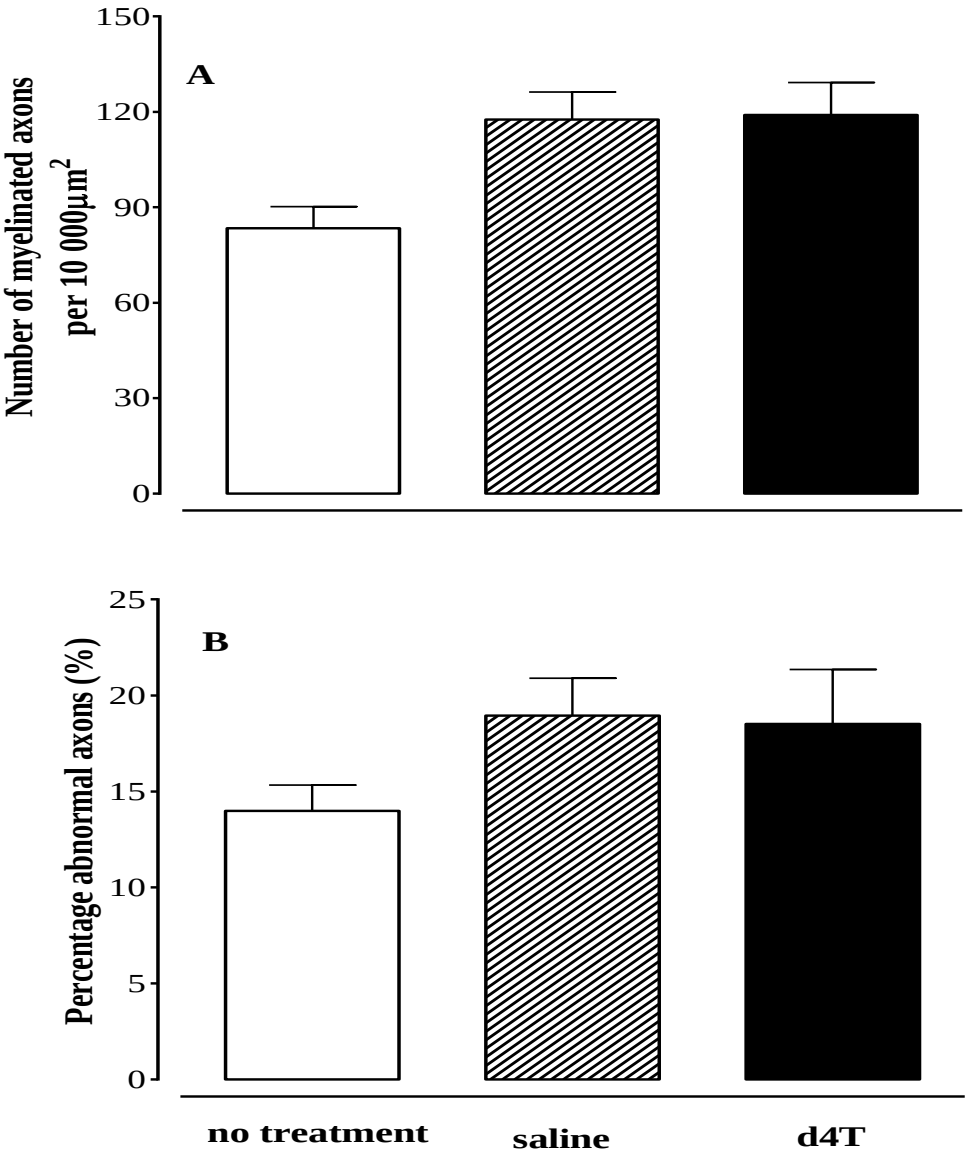

Figure 9. The total number of axons (A) and percentage of abnormal axons (B) (i.e. ballooning, splitting of myelin sheath) per photo (10 000 μm^2). Values are expressed as mean (SD). d4T (100 $mg.kg^{-1}$) administration does not alter the number of axons or number of abnormal axons compared with the saline and naive groups ($P>0.05$).

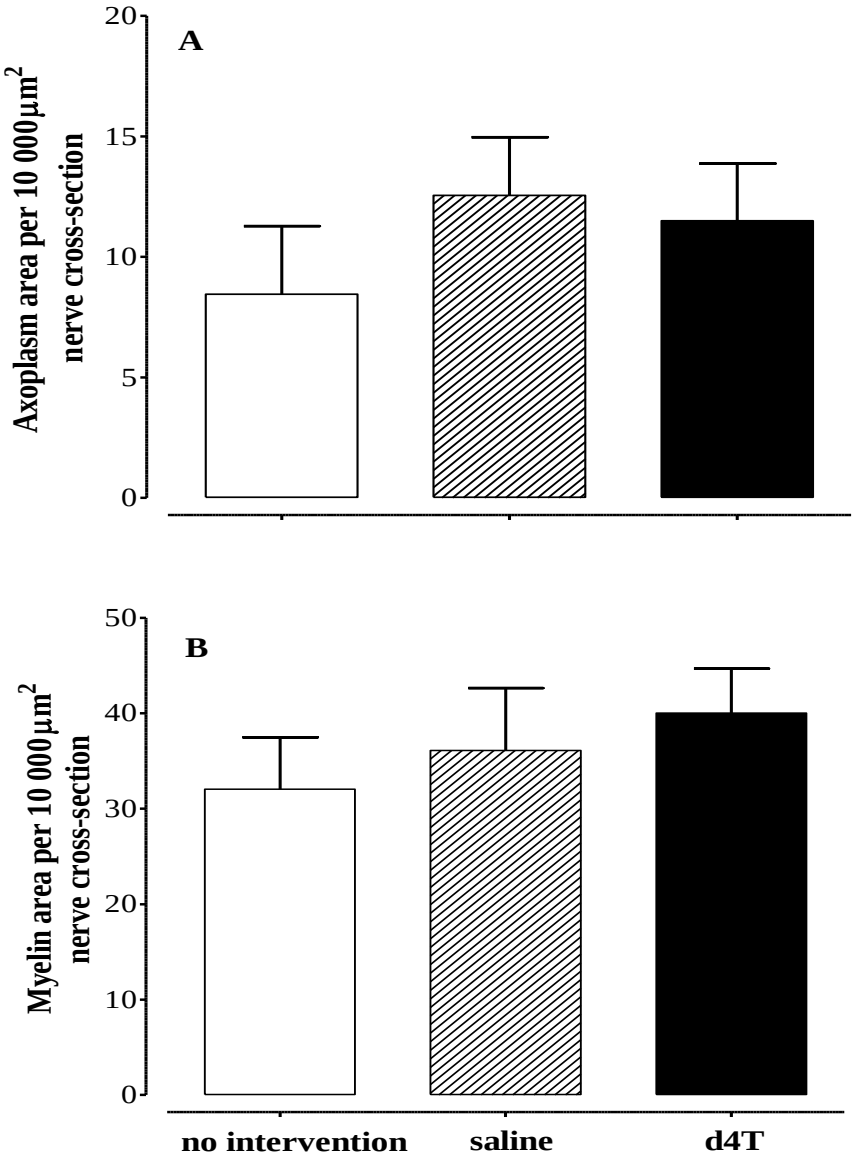

Figure 10. Axopalsmic area per 10 000 µm^2 (A) and myelin area per 10 000 µm^2 (B). Values are expressed as mean (SD). D4T (100 mg.kg-1) administration did not alter the axoplasmic area per cell or myelin area per cell compared to the saline and naive rats (P>0.05).

4.4 Discussion

The aim of this experiment was to determine whether administering d4T subcutaneously to rats for four weeks causes hypernociception and neuropathological changes in nerve morphology. Daily injections of d4T at 100 $mg.kg^{-1}$ over a four week period did not induce the development of any neuromorphological pathology in myelinated fibres in the coccxygeal nerve. Also, the absence of this nerve fibre pathology correlated with the absence of hyperalgesia in our rats. Our study demonstrates that multiple injections of d4T at 100 $mg.kg^{-1}$ over a four week period do not induce nerve fibre pathology or hyperalgesia in rats.

In our study, semi-thin sections of the coccxygeal nerves of rats administered d4T did not show changes associated with antiretroviral toxic neuropathy. On the other hand, Bhangoo and colleagues found that a single injection of ddC ($25mg.kg^{-1}$) resulted in neuropathological changes in the peripheral nerves as early as post-injection day 3, and these changes were present for about 48 days (Bhangoo et al., 2007). Bhangoo and colleagues observed changes such as hypermyelination (tormaculae formation) in sciatic. Interestingly, in their model, a single intraperitoneal injection of ddC ($25mg.kg^{-1}$) produced mechanical hyperalgesia post-injection, as demonstrated by a significant decrease in paw withdrawal threshold from as early as post-injection day 3 (Bhangoo et al., 2007). The

mechanical hypersensitivity produced by ddC administration was evident between weeks two to four post-injections and was sustainable for up to 43 days post initial exposure (Bhangoo et al., 2007). These findings are in agreement with those of Joseph and colleagues (2004) who administered three single intravenous doses (i.e. 10, 25 or 50 $mg.kg^{-1}$) of ddC, ddI and d4T to three different groups of rats and found that a single dose of these drugs resulted in a significant reduction to paw-withdrawal threshold as early as post-injection day 1 for rats administered 50 $mg.kg^{-1}$ or day 3 for rats administered 10 and 25 $mg.kg^{-1}$ of these drugs.

Although other researchers have been able to successfully develop models of NRTI toxicity and hyperalgesia, most of them have used NRTIs that have greater neurotoxicity than d4T, ddC and ddI, which are no longer used as first-line management of HIV infection. Patients with HIV-SN have evidence of dying back sensory axonal degeneration, with the most distal portions of long fibers being affected first. Keswani and colleagues (2003b) developed a model of HIV neuropathy using GFAP-gp120 transgenic mice, which expressed a viral coat protein gp120 in all tissues, and these mice were administered ddI (25mg.d-1) for four weeks. Mice developed hypernociception, but proximal sciatic nerve sections obtained from the wild-type and gp120 transgenic mice with and without exposure to ddI did not show any evidence of Wallerian-like degeneration and there were no changes noted in the morphology of myelinated axons (Keswani et al., 2006). Moreover, Wallace et al (2007) found no evidence of neural stress

marker ATF-3, a marker that is increased, following neuronal damage, in the dorsal root ganglia neurons in rats administered 50 mg/kg^{-1} ddC intraperitoneally and had developed hypernociception. Thus, development of hypernociception following NRTI administration does not necessarily mean that there will be overt axonal pathology.

CHAPTER 5

Conclusion

Stavudine (d4T), an NRTI often used as first-line treatment for HIV infection, is associated with peripheral neuropathy and pain in HIV-positive patients (Wadley et al., 2011; Maritz et al., 2010). The mechanisms of d4T-induced toxic neuropathy and pain are not well understood, partly because of a lack of robust animal models of the disease process. To date, very few studies have examined the effects of d4T on peripheral nerves and the associated hyperalgesia in animals. Instead most researchers have focused their attention on the more toxic NRTI ddC, which is no longer prescribed for HIV management. Therefore, the primary aim of my dissertation was to investigate whether acute daily oral or subcutaneous administration of d4T produces neuropathological changes associated with the administration of NRTIs.

Daily oral administration of d4T at 50 $mg.kg^{-1}$ over a four week period did not induce significant nerve fibre loss or morphological changes in myelinated fibres in the sciatic nerve. Congruent with the absence of neurotoxicity, daily administration of d4T at a dose of 50 $mg.kg^{-1}$over a four week period did not result in hyperalgesia. The behavioral data are contrary to the results of Weber et al (2007) who observed mechanical hyperalgesia in rats within three weeks, following daily oral administration of 50 $mg.kg^{-1}$ d4T. In the second experiment, I investigated whether administering 100 $mg.kg^{-1}$ d4T subcutaneously to rats for four weeks caused neuropathological changes in myelinated fibres in the coccxygeal nerve. As with the lower dose of d4T, d4T at a dose of 100 $mg.kg^{-1}$

over a four week period, did not induce neuronal loss or myelin abnormalities. And, the absence of this nerve fibre pathology correlated with the absence of hyperalgesia in our rats. Based on these findings, it appears that developing a robust animal model of d4T-induced neuropathy may be challenging in the absence of underlying HIV-infection, and may require exposure to d4T for a longer period or higher doses than what I studied.

Failure to develop a model of d4T-induced neuropathy in otherwise healthy rats in our study is not surprising, since this has seldom been achieved successfully. Previously in our laboratory, Weber et al (2007) demonstrated that hyperalgesia to a mechanical stimulus developed within three weeks of daily oral d4T administration, something I failed to repeat in my series of studies. But they did not determine whether the hyperalgesia was caused by peripheral nerve damage, so it is unclear whether the hyperalgesia was indeed neuropathic in origin. Indeed they found no changes in dorsal horn apoptosis, a feature of neuropathic pain states, so it is unlikely that the hyperalgesia was neuropathic in origin (Weber et al., 2009). Furthermore, in a study done by Warner and colleagues (1995), there were no drug-induced light or electron microscopic changes in the peripheral nerves of rabbits treated with ddI, d4T or AZT for 24 weeks. In another study, ddC produced peripheral neuropathy in cynomolgus monkeys at doses ranging between 0.2-10mg.kg-1 body weight twice daily for up to 38 weeks (Anderson et al., 1992); but, d4T failed to produce any evidence of neuropathy in cynomologus

monkeys at doses of 60, 200 or 5600 mg.kg^{-1} body weight per day over a 1 year period (Warner et al., unpublished data). Thus, even if greater doses of d4T are used, or the drug is used for a longer period of time than the four weeks I administered the drug, I believe that peripheral nerve damage and hypernociception may still not be produced in rats. If the neurotoxicity of d4T is low when administered to experimental animals, how is it that the drug is so strongly associated with HIV-SN in patients being treated with the drug. The most likely reason for greater toxicity in patients is that patients have underlying nerve damage caused by the virus infection (Kokotis et al., 2007; Keswani et al., 2002), making them more susceptible to the additional neurotoxic load of d4T than healthy rodents.

In South Africa, there are two recommended treatment therapies which contain at least 2 NRTIs and either a NNRTI or PI. Until recently, the first and second line treatment therapies included d4T and ddI respectively (National Antiretroviral Treatment Guideline, first edition, 2004), which are potent NRTI and are associated with ATN. Although the guidelines have been amended, such that new patients starting therapy do not start on d4T or ddI base regimens; however, the bulk of existing patients remain being treated with these drugs.

Currently, the mechanisms of antiretroviral toxic (ATN) neuropathy are not fully understood as it is difficult to develop a model of NRTI without HIV infection or

exposure to Human Immunodeficiency Virus–glycoprotein 120 (HIV-gp120) in *in vivo* rodent models of HIV-SN. In a model of ddC-induced HIV-gp120 induced nerve damage and hypernociception, Wallace and colleagues (2007) demonstrated that perineural administration of HIV-gp120 sensitizes rats to the static punctate mechanical stimulus. HIV infection is associated with an increased amount of circulating activated macrophages and pro-inflammatory cytokines (Keswani et al., 2002). These enter the dorsal root ganglion (DRG) and peripheral nerves in large numbers and upon entering the DRG, they release chemokines and cytokines which promote neurotoxicity (Keswani et al., 2002; Keswani et al., 2003a). Also, within the DRG, some of the secreted viral proteins (e.g. Tat, gp120) are thought to be toxic to DRG neurons, thus resulting in a "dying back" pattern of axonal degeneration (Keswani et al., 2002). Some researchers have hypothesized that the development of antiretroviral toxic neuropathy (ATN) is due to the "unmasking" of clinically silent pre-existing neuronal damage due to HIV (Kokotis et al., 2007; Keswani et al., 2002). In another model, Laast and colleagues (2007) inoculated macaques with Simian Immunodeficiency Virus (SIV) in order to better understand the immunological responses associated with HIV infection and the signaling process that ultimately produces symptomatic HIV-SN. In their model, they observed a significant decrease in neuronal density within the trigeminal nerve, which was inversely proportional to macrophage infiltration that seemed to suggest that macrophage infiltration plays a key role in causing nerve damage in HIV infected patients (Laast et al., 2007; Laast et al., 2011). It is likely that there

is a concomitant need for HIV infection as well as abnormalities in the peripheral nervous system that are requirements for the development of ATN (Keswani et al., 2002).

Thus, while developing models of d4T is academically interesting, and would provide a way to better understand the neurotoxicity of the drug as such, the drug is typically used in individuals infected with HIV, except in cases of post-exposure prophylaxis. Therefore any attempt to better understand the pathophysiology of ATN induced by d4T should in the future be based around models employing simulated HIV infection. For example, administering d4T to transgenic mice expressing constitutive gp120, monkeys or cats infected with SIV or FIV respectively, or rodents exposed to exogenous gp120. Only through such models will be able to learn about the true mechanisms underlying d4T-induced ATN. And, I believe that we need to continue investigating the mechanisms of this neuropathy, despite new regulations removing d4T as a first-line treatment (DOH, 2010), because the majority of South Africans, and the majority of individuals in other developing countries, who are receiving treatment have been exposed to d4T, and any exposure to the drug increases the risk of developing ATN (Cherry et al., 2010). Despite the change in first-line treatment regimen, there remains a high risk for continued high rates of d4T-related ATN going forward. Also, only by determining the underlying mechanisms of d4T- related ATN will we possibly identify possible therapeutic targets for the treatment of the

painful symptoms of this neuropathy, which has remained resistant to standard therapies for painful polyneuropathies (Phillips et al., 2010)

CHAPTER 6

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