Published in final edited form as:

Neuroscience. 2013 November 12; 252: . doi:10.1016/j.neuroscience.2013.07.046.

Nerve growth factor acts through the TrkA receptor to protect sensory neurons from the damaging effects of the HIV-1 viral protein, Vpr

Christine A. Webber^{1,*}, Jihan Salame¹, Gia-Linh S. Luu¹, Shaona Acharjee⁵, Araya Ruangkittisakul², Jose A. Martinez⁶, Hanieh Jalali⁴, Russell Watts¹, Klaus Ballanyi², Gui Fang Guo⁶, Douglas W. Zochodne⁶, and Christopher Power³

¹Division of Anatomy, University of Alberta, Edmonton, Alberta CANADA, T6G 2H7

²Department of Physiology, University of Alberta, Edmonton, Alberta CANADA, T6G 2H7

³Department of Medicine, University of Alberta, Edmonton, Alberta CANADA, T6G 2H7

⁴Department of Cell and Developmental Biology, Kharazmi University, IRAN, 31979-37551

⁵Department of Physiology, University of Calgary, Calgary, Alberta, CANADA, T2N 4N1

⁶Department of Clinical Neurosciences, University of Calgary, Calgary, Alberta, CANADA, T2N 4N1

Abstract

Distal sensory polyneuropathy (DSP) with associated neuropathic pain is the most common neurological disorder affecting patients with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS). Viral protein R (Vpr) is a neurotoxic protein encoded by HIV-1 and secreted by infected macrophages. Vpr reduces neuronal viability, increases cytosolic calcium and membrane excitability of cultured dorsal root ganglion (DRG) sensory neurons, and is associated with mechanical allodynia *in vivo*. A clinical trial with HIV/AIDS patients demonstrated that nerve growth factor (NGF) reduced the severity of DSP-associated neuropathic pain, a problem linked to damage to small diameter, potentially NGF responsive fibers. Herein, the actions of NGF were investigated in our Vpr model of DSP and we demonstrated that NGF significantly protected sensory neurons from the effects of Vpr. Footpads of immunodeficient Vpr transgenic (*vpr/RAG1*^{-/-}) mice displayed allodynia ($p < 0.05$), diminished epidermal innervation ($p < 0.01$) and reduced NGF mRNA expression ($p < 0.001$) compared to immunodeficient (*wildtype/RAG1*^{-/-}) littermate control mice. Compartmented cultures confirmed recombinant Vpr exposure to the DRG neuronal perikarya decreased distal neurite extension ($p < 0.01$), whereas NGF exposure at these distal axons protected the DRG neurons from the Vpr-induced effect on their cell bodies. NGF prevented Vpr-induced attenuation of the phosphorylated glycogen synthase-3 axon extension pathway and tropomyosin related kinase A (TrkA) receptor expression in DRG neurons ($p < 0.05$) and it directly counteracted the cytosolic calcium burst caused by Vpr exposure to DRG neurons ($p < 0.01$). TrkA receptor antagonists indicated that NGF acted through the TrkA receptor to block the Vpr-mediated decrease in axon outgrowth in

© 2013 IBRO. Published by Elsevier Ltd. All rights reserved.

*Corresponding author: Christine A. Webber Ph.D., Room 530 Medical Sciences Building Division of Anatomy, University of Alberta, Edmonton, AB, CANADA, T6G 2H7 P: 780-248-1886; F: 780-492-0462; webber2@ualberta.ca.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

neonatal and adult rat and fetal human DRG neurons ($p < 0.05$). Similarly, inhibiting the lower affinity NGF receptor, p75, blocked Vpr's effect on DRG neurons. Overall, NGF/TrkA signalling or p75 receptor inhibition protects somatic sensory neurons exposed to Vpr, thus laying the groundwork for potential therapeutic options for HIV/AIDS patients suffering from DSP.

1.1 Introduction

Over 34 million people are living with human immunodeficiency virus (HIV)-1 infection and the acquired immunodeficiency syndrome (AIDS) (www.UNAIDS.org). Although highly active antiretroviral therapy (HAART) has dramatically improved their life-expectancy, the effects of chronic exposure to the virus remain detrimental to these patients. Distal sensory polyneuropathy (DSP) is the most frequent neurologic complication associated with HIV-1 infection, involving over 50% of infected patients (Ellis et al., 2010; Morgello et al., 2004). Debilitating neuropathic pain, paresthesia and gait dysfunction characterize the clinical features of HIV-associated DSP, which can be exacerbated by concurrent HAART (Power et al., 2009, Cornblath and Hoke, 2006). There are no curative therapies for DSP as current analgesics have limited effectiveness and are often poorly tolerated, thus highlighting the urgent need for new treatments (Smith, 2011).

HIV infects the macrophages in the peripheral nervous system. Although not permissive to HIV-1 infection, dorsal root ganglion (DRG) neurons, the primary sensory neurons that relay somatic sensations to the central nervous system, are the principal neural structures responsible for HIV-1 induced neuropathic pain (McArthur et al., 2005). HIV-1 infected macrophages secrete viral protein R (Vpr) which increases both intracellular free calcium levels and membrane excitability at the neuronal soma, and at sufficient levels Vpr reduces neuronal viability (Acharjee et al., 2010). Transgenic *vpr* mice crossed with an immunodeficient background (*vpr/RAG1^{-/-}* mice) to mimic the immunodeficiency of HIV, display mechanical allodynia. Understanding how Vpr exerts its neurotoxic effects on DRG neurons may lead to new therapeutic interventions to block this interaction and thereby protect sensory neurons and their processes from Vpr-induced effects.

A phase II clinical trial showed that local injections of nerve growth factor (NGF) initially caused painful local inflammation for several days post-injection, however over the course of the 18 week trial, it significantly decreased neuropathic pain accompanying HIV-associated DSP (McArthur et al., 2000). In the mature nervous system, NGF is secreted by Schwann cells along the length of the axon to maintain neuronal survival and it is produced by keratinocytes at all peripheral targets to sustain epidermal innervation of the TrkA-expressing (primarily nociceptive) axons comprising approximately 40–45% of all DRG neurons (Huang and Reichardt, 2001; Ernsberger, 2009; Tucker and Mearow, 2008). Moreover, DSP primarily involves smaller caliber axons, likely to include a substantial proportion that express TrkA. In this study, we hypothesized that the footpads of the *vpr/RAG1^{-/-}* mice have decreased NGF expression which may affect nerve innervation of the nociceptive DRG neurons *in vivo*, and thus contribute to the Vpr-induced allodynia. We studied the effect of sub-toxic doses of Vpr on cultured DRG neurons with or without exposure to NGF. As the McArthur et al., (2000) trial showed NGF injection itself caused pain but it caused an overall protection against HIV-induced DSP, we went on to study downstream mechanisms through which the NGF exerts its neuroprotective effects on the DRG neurons, in hopes of discovering pathways that could be targeted for future therapeutic interventions.

2.1 Experimental Procedures

Animal and human tissue sources

Neonatal (day 1–2) and adult (175–200 g) Sprague Dawley rats were obtained from the Biosciences animal facility within the University of Alberta. All protocols were reviewed and approved by the University of Alberta Animal Ethics Committee. All animals were housed and maintained in accordance with the Canadian Council on Animal Care (CCAC) guidelines. Adult rats were sacrificed by carbon dioxide asphyxiation and neonatal rats were placed on ice and decapitated. Embryonic human DRGs were obtained from 15–19 week aborted fetuses obtained with consent (approved by the University of Alberta Ethics Committee) (Acharjee et al., 2010).

In vivo mouse model

The Vpr transgenic mice were generated as described (Jones et al., 2007) in which Vpr was controlled by the *c-fms* (M-CSF receptor) promoter, permitting expression chiefly in monocytoid cells. The Vpr mice were crossed with *RAG1*^{−/−}, immunodeficient mice which do not produce mature B or T cell lymphocytes (Mombaerts et al., 1992) to generate *vpr/RAG1*^{+/−} mice in F1 generation. The F1 *vpr* transgenic animals were then backcrossed to *RAG1*^{−/−} to generate *vpr/RAG1*^{−/−} animals. The animals used in this study were older adult mice (6–8 months old) than those used in previous work (Acharjee et al., 2010).

Neuropathic pain assessment

The *wildtype/RAG*^{+/−} (n=7) and *vpr/RAG1*^{−/−} (n=6) littermates were habituated on an elevated wire mesh and calibrated Von Frey hair monofilaments were applied to the plantar surface of each hind paw in the ascending order of bending force (range: 0.2–10 g) (Acharjee et al., 2010). An average of 5 hairs per paw was recorded and this test was repeated four times.

Footpad innervation

Footpads skin biopsies were removed with a 3 mm punch and placed into 2% paraformaldehyde, lysine and periodate (Sigma Aldrich, Oakville, ON, Canada) fixative for 16–20 h at 4 °C and cryoprotected overnight in 20% glycerol/0.1 M Sorrenson phosphate buffer at 4 °C (as described in Cheng et al., 2010). Epidermal innervations were visualized following antigen retrieval immunohistochemistry. Skin sections of 25 µm thickness were bathed in Sodium Citrate Buffer (10mM Sodium citrate (Sigma Aldrich), 0.05% Tween 20, pH 6.0) for 30 minutes at 92°C. The slides were cooled to room temperature and rinsed 2× five minutes each in PBS and then incubated for 10 minutes in 1% Triton-X. After 3× five minute rinses in PBS, the tissue was blocked for 1 hour at room temperature in PBS containing 10% normal goat serum, 1% bovine serum albumin (Sigma Aldrich), 0.05% NaN₃, 0.3% Triton X-100, 0.05% Tween 20. PGP9.5 (rabbit polyclonal; Cedarlane, 1:200) was applied overnight at 4°C followed by Cy3 secondary antibodies (goat anti-rabbit; Cedarlane, Burlington, ON, Canada; 1:200) application for 1 hour at room temperature. Images were captured using a Zeiss Axioscope fluorescent microscope. To calculate epidermal nerve terminal densities, the number in total axonal profiles were averaged in five adjacent fields of 3–5 sections for a total 15–25 fields per mouse.

Nerve diameter morphology

Sural nerves (which contain only sensory axons) were harvested and processed as described in previous work (Brussee et al., 2008; Zochodne et al., 2001). Samples were fixed in 2.5% glutaraldehyde in 0.025 mol/L cacodylate buffer overnight. Semithin (1 µm) sections of sural nerve were cut on an ultramicrotome (Reichert, Vienna, Austria). Morphometric

analysis was carried out using a Zeiss Axioskop at magnification $\times 1,000$. Computer-assisted image analysis allowed for the determination of number and caliber of intact myelinated fibers (g-ratios were calculated). All morphological measurements were performed using Image J software (National Institute of Health) by a single microscopist unaware of the origin of the samples.

Immunohistochemistry

Lumbar (L4/L5) DRGs were collected from *wildtype/RAG1^{-/-}* or *vpr/RAG^{-/-}* mice and processed for immunohistochemistry as previously described (Christie et al., 2010; Webber et al., 2011). The DRG were fixed in 4% paraformaldehyde and cryoprotected in 30% sucrose before frozen in optimal cutting temperature (OCT; VWR, Mississauga, ON, Canada) and cut to 10 μ M sections. The sectioned tissues were collected onto superfrost-microscope slides (VWR) and rinsed in PBS permeabilized with 0.1% Triton-X 100 for 5 minutes, blocked with 5% horse serum in PBS. The immunolabeling was done serially as the IB-4 antibody solution was devoid of Triton-X-100 (1:1000 dilution of anti-IB-4 lectin (Invitrogen, Burlington, ON, Canada) in 5% horse serum + PBS) overnight at 4°C. The sections were rinsed 3 $\times$ 10 minutes in PBS and incubated for 2 hours in 1:500 goat anti-lectin 594 (Jacksonlabs Immunoresearch Laboratories, West Grove, PA). The sections were then rinsed 3 $\times$ 10 minutes in PBS followed by 1:1000 dilution of rabbit anti-rat TrkA antibody in 0.3% Triton X-100 + 5% horse serum and PBS overnight at 4°C. The DRGs were incubated in Atto 488 secondary antibodies (goat anti-rabbit; Cedarlane; 1:200) secondary antibody for 4 hours, rinsed 3 $\times$ PBS and mounted in polyaquamount (Polysciences Inc., Warrington, PA). We used a fluorescent microscope to visualize the tissue and only DRG soma's with clearly visible nucleoli were measured. We compared the TrkA and IB4-binding expression patterns between the *wildtype/RAG1^{-/-}* or *vpr/RAG1^{-/-}* transgenic littermates to determine if there were differences in sensory neuron populations mediated by chronic Vpr exposure. At least 6 sections were counted for each sample and we studied DRGs from n=7 individual *wildtype/RAG1^{-/-}* and n=7 individual *vpr/RAG1^{-/-}* mice.

Quantitative RT-PCR of epidermal footpads

Total RNA was extracted from tissues using Trizol reagent as per the manufacturer's instructions (Invitrogen). As described previously, total RNA (1 μ g) was treated with DNase (Promega) and converted to cDNA using the Superscript II reverse transcriptase (Invitrogen) (Christie et al., 2010; Webber et al., 2011). All PCR primers were designed using software Primer Express 2.0 (Applied Biosystems, Carlsbad, CA). Primer sequences were as follows: NGF forward mouse 5'-CAAGGCGTTGACAACAGATGA-3'; NGF reverse mouse 5'-CAGCCTCTTCTTGATGCCTTCC-3'; RPLP0 forward mouse 5'-AAGAACACCATGATGCGCAAG-3'; RPLP0 reverse mouse 5'-TTGGTGAACACGAAGCCCA. TrkA forward 5'-ATCTAGCCAGCCTGCACTTTGT-3'; TrkA reverse 5'-TCTGCTCATGCCAAAGTCTCC. TrkA, NGF and RPLP0 products were labelled using SYBR Green (Invitrogen). All reactions were performed in duplicate in an AB1 PRISM 7000 Sequence Detection System (Applied Biosystems) and analyzed using the 2 cycle threshold method. Results are presented as the relative *vpr/RAG1^{-/-}* epidermis mRNA expression normalized to the relative RPLP0 mRNA and compared with *wildtype/RAG1^{-/-}* (defined as 1.0 fold).

Mass culturing of primary DRG cultures

Neonatal rat DRGs were aseptically removed from the spinal columns of day 1–2 Sprague-Dawley rat pups (Acharjee et al., 2010). The ganglia were enzymatically dissociated into a single-cell solution by incubation in L-15 air (Life Technologies, Burlington, ON, Canada) + 1 mg/mL collagenase (Sigma Aldrich) for 25 minutes, and then 1 mg/mL of trypsin (Sigma

Aldrich) for 5 minutes. The solution was then quenched with 10% rat serum (in house serum collection by the Animal Facility at the University of Alberta) in PBS. Ganglia were rinsed with PBS and further dissociated mechanically in L-15 air by gentle trituration with a p200 pipette tip connected to a disposable 2 mL pipette. The resulting cells were filtered through a 70 μ m filter and spun at 800 rpm for 3 minutes. The pellet was resuspended into L-15 air, 2.5% rat serum, 50 ng/mL NGF (Cedarlane laboratories), 1% penicillin/streptomycin and 10 μ M 1- β -D-Arabinofuranosylcytosine (AraC; Sigma Aldrich) to decrease the number of proliferating glial cells. The cells were plated onto collagen coated 35 mm dishes (western blots cultures and calcium imaging), 96-well dishes (in cell westerns), or to the central compartment of Campenot chambers. The medium was changed every 2 days *in vitro*. On day 7, cultures were given L-15 air, 2.5% rat serum with or without NGF (10 ng/mL, 100 ng/mL) as indicated and the experimental conditions were established on day 9 (see below).

Adult rat DRGs (175–200g) were aseptically harvested from all spinal segments and placed in Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F12; Life Technologies) as described previously (Webber et al., 2008; Christie et al., 2010; Andersen et al., 2000). They were enzymatically treated for 40 minutes with 1 mg/mL collagenase in PBS. The softened DRGs were mechanically dissociated by trituration with p1000 and then p200 pipette tips, filtered through a 70 μ m mesh (Fisher Scientific; Toronto, ON, Canada) and centrifuged at 800 rpm for 10 minutes. The single-cell suspension was placed in DMEM/F12 medium including 1:100 N₂ supplement (Gibco), 0.1% bovine serum albumin and 1% penicillin/streptomycin (Invitrogen) with or without NGF (10 ng/mL, 100 ng/mL) and plated onto poly-ornithine (Sigma) and laminin (10 μ g/mL; Invitrogen) coated 96-well dishes. NGF-deprived cultures were deprived of NGF for entire culture period.

Human DRG cultures were prepared as described previously (Power et al., 1998) from 15–19 week aborted fetuses obtained with consent (approved by the University of Alberta Ethics Committee). The DRGs were aseptically harvested from all spinal segments in modified Eagle's medium (Life Technologies), enzymatically treated for 40 min with 1 mg/mL collagenase (Sigma) and 0.2 mg/mL DNase (Roche, Mannheim, Germany), followed by 0.25% trypsin (Invitrogen, Burlington, ON, Canada). Trypsin was inactivated by addition of equal volume of DMEM supplemented with 10% FBS, 1% L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 1% dextrose, 1% penicillin and streptomycin, 20 μ g/mL gentamicin, and 0.5 μ g/mL fungizone. The softened DRGs were then mechanically dissociated by trituration, filtered, and centrifuged at 1000 rpm for 10 min. The pellet was resuspended in neuronal medium with NGF (10 ng/mL) and plated in Matrigel-coated plates (BD Sciences, Mississauga, ON, Canada). Ara-C (25 μ M) was added to encourage neuronal enriched cultures. On day 7, NGF was removed from half of the cultures and they were deprived of NGF for 48 hours before the experiment conditions were added on day 9.

Experimental cell culture studies

On day 1 of adult DRG cultures and day 9 of human fetal and neonatal rat DRG cultures, 10 nM or 100 nM human recombinant Vpr (Kinakeet Biotechnology, Midlothian, VA) was added to the medium. The culture endpoint was day 5 (adult rat) and day 11 (neonatal rat and human fetal), respectively. To determine if TrkA receptor activation or p75 receptor inhibition alters the impact of Vpr *in vitro*, we introduced (1–10 μ g/mL) the TrkA receptor agonist, RTA, or p75 receptor antagonist, REX, (both kindly provided by Dr. L Reichardt) to the culture medium in lieu of NGF pre-treatment (day 1 for adult and day 9 or human fetal and neonatal rat DRG cultures).

Compartmented cell culture chambers

Neonatal rat DRG neurons were placed into the central compartment of the Campenot chambers (Campenot et al., 2009) and their axons extended left or right along collagen-coated scratches and underneath Teflon partitions seated on the dish surface with silicone grease, and into the separate fluid environments of distal compartments. The axons fasciculate together, forming cables and were observed under the inverted microscope. The neonatal DRGs were grown for 7 days in the presence of 10 ng/mL NGF (center) and 50 ng/mL NGF (peripheral) and AraC to decrease the number of nonneuronal cells. On day 7, NGF was removed from the central and peripheral compartments of all cultures and on day 9, the proximal axons within the peripheral chamber were axotomized and the experimental conditions were established; (i) 10 ng/mL and 50 ng/mL NGF was added to central and peripheral chambers, respectively (ii) no NGF and no Vpr was added to any compartment, (iii) 100 nM Vpr was added to the central chamber, and (iv) 10 ng/mL and 50 ng/mL NGF was added to central and peripheral chambers, respectively and 100 nM Vpr was added to the central chamber. The length of axon extension was measured from days 9–11 and the progression of daily axon growth and total axon outgrowth was reported. At least 6 chambers per condition were averaged for each sample and this experiment was repeated 5 times.

Cell survival assay

After 72 hours in the presence of 10 nM or 100 nM Vpr, cell survival of 1000 DRG neurons per well of a 96 well plate were assessed using the CellTiter 96 Aqueous Nonradioactive cell Proliferation Assay Kit (Promega, Madison, WI) by following manufacturer's instructions. The colorimetric assay was measured by a spectrophotometer at 490 nm and the ED₅₀ of the controls and test samples were compared to evaluate Vpr's cytotoxicity on DRG neurons.

Immunofluorescence

Neurons were fixed in 4% paraformaldehyde for 10 minutes and then permeabilized with 0.1% Triton-X 100 (Sigma Aldrich) in PBS and blocked for 30 minutes in 5% horse serum (Sigma Aldrich) in PBS (Andersen et al., 2000; Christie et al., 2010; Webber et al., 2008). The axons were processed for fluorescent immunocytochemistry using a 488 nm tagged pan-neurofilament antibody (Sigma Aldrich, 1:100) overnight at 4°C. All samples were imaged in black-and-white using a Zeiss AxioScope with digital camera and Axiovision imaging software (Zeiss).

In cell western analysis

In cell western analysis was used to measure total neurite outgrowth (by quantitative neurofilament expression) of mass cultured neonatal rat, adult rat and human fetal DRG neurons. The cultures were grown on a 96-well plate and at the culture endpoint the neurons were fixed in 4% paraformaldehyde for 30 minutes. The cells were rinsed 3× five minutes in PBS and blocked with LiCor Blocking Buffer (LiCor Biosciences, Lincoln, NE) and then labeled with mouse pan-neurofilament antibody overnight at 4°C. The cells were rinsed 3× five minutes in PBS, incubated for 2 hours in an anti-mouse secondary antibody (680 nM) and its fluorescence was quantitatively measured by LiCor plate-reader.

Calcium imaging

DRG cultures were exposed to 5 μ M Fluo-8L acetoxymethyl ester (ATT Bioquest, Sunnyvale, CA) for 30 minutes and then imaged as previously described (Acharjee et al., 2010). Live-cell imaging was performed using a confocal microscope, equipped with an argon (488 nm) laser, emission band pass filter (490–540 nm), and 20× XLUMPlanF1, NA 0.95 objective. Data acquisition was performed using Olympus Fluoview FV300 or FV1000

software. An increase in fluorescence intensity of Fluo-8L corresponded to an increase in cytosolic calcium. DRG cultures were continuously superfused with extracellular solution containing artificial cerebral spinal fluid (ACSF) containing 127 mM Sodium Chloride (Fischer), 2.5 mM Potassium Chloride (EMD, Darmstadt, Germany), 25 mM Dextrose (Fischer), 1.3 Magnesium Sulfate septahydrate (EMD), 2.5 mM Calcium Chloride (EMD), 25 mM Sodium Bicarbonate (Fischer), and 1.2 mM Sodium diPhosphate Monohydrate (Anachemia, Edmonton, Canada). The ACSF was bubbled with 95% O₂ and 5% CO₂. Bath application of ACSF containing 35 mM KCl for 60 seconds depolarized neurons and subsequently induced calcium rise. This provided a positive control for functioning neurons. ACSF containing 100 nM Vpr was added to DRG cultures for 2 minutes and then washed out by resuming ACSF superfusion. Full frame images (512 × 512 pixels) were acquired at a scanning time of 3s per frame and time course traces of change in fluorescence intensity were generated with FluoView software. Statistical analysis included the measurement of the peak of Fluo-8L intensity from baseline with KCl (before and after Vpr) and Vpr treatment (n=3).

Western blot analysis

Cellular protein was isolated from cultured DRGs protein extraction buffer (250 mM Sucrose, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% Triton X-100 in complete mini protease inhibitor cocktail (Roche), 10 mM sodium orthovanadate (Sigma Aldrich) and 10 mM sodium fluoride (Sigma Aldrich) and Western blot analysis was performed as described (Christie et al., 2010). Briefly, protein concentrations were determined by a BCA Protein Assay kit (Pierce), and 15 µg of protein was loaded into each well and samples were separated by SDS-PAGE using an 8% precast polyacrylamide gel (Biorad; Hercules, CA). Separated proteins were transferred onto PVDF membrane (Biorad) and placed into blocking solution (5% casein (Nestle) in Tris buffered saline (TBST). Primary antibodies anti-TrkA receptor (RTA: 1:1000), anti-p75 receptor (REX: 1:1000), GSK3- α , and phosphorylated GSK3 α (Sigma Aldrich, 1:1000) and a mouse anti- β -actin antibody (Promega, 1:1000) as a loading control. Following secondary antibody exposure (ImmunoPure Goat Anti-Mouse IgG, (H+L) 1:1000), or Goat Anti-rabbit IgG, (H+L), Peroxidase Conjugated (Thermo Scientific; 1:1000) the signal detection was performed by exposing the blot to enhanced chemi-luminescent reagents ECL (Lumi-Light Plus; Roche Diagnostics) and the blots were subsequently exposed on Hyperfilm (Amersham Biosciences) to capture the images of the bands. Image J software measured pixel density and ANOVA statistics were performed using a Dunnett's *post hoc* comparison (p<0.05) (n=3).

Statistical analysis

Statistical analyses were performed with GraphPad InStat version 3.0 (GraphPad Software), using ANOVA, with a Dunnett's (cell survival assay) or Bonferoni (compartmented cell culture, in cell western) *post hoc* comparison. Unpaired *t*-tests with a Dunnett's *post hoc* comparison were used for neuronal count, behavioural tests, calcium imaging, qRTPCR, epidermal nerve counts, DRG neuronal counting, western blot analysis and behavioural analyses. Values of *P* < 0.05 were considered significant. Image J software was used to measure pixel density for western blot analysis.

3.1 Results

3.1.1 Effect of chronic Vpr expression in the footpad

As DSP caused by HIV/AIDS primarily involves adult patients who are immunocompromised, we studied the pathogenic effects of HIV-1 gene expression in a transgenic-immunodeficient (*vpr/RAG1*^{-/-}) adult mouse model. Previous studies showed

young adult *vpr/RAG1^{-/-}* mice (1–2 months) displayed mechanical allodynia (Acharjee et al., 2010). To determine if Vpr's effect *in vivo* is robust, we investigated if older mice (6–8 months) also demonstrated allodynia. Indeed, this older cohort of *vpr/RAG1^{-/-}* mice displayed significant mechanical allodynia at their hindpaw footpads as Von Frey hair testing revealed the *vpr/RAG1^{-/-}* mice exhibited lower sensory thresholds ($1.9 \text{ g} \pm 0.2 \text{ sem}$) compared to *wildtype/RAG1^{-/-}* mice ($2.6 \text{ g} \pm 0.3 \text{ sem}$) ($p < 0.05$) (Figure 1A).

Although it is understood that HIV-infected macrophages at the DRG produce Vpr (Acharjee et al., 2010), it is not known if Vpr's effect is at the perikarya, the axon, or at the distal nerve terminal. To delineate Vpr's effect on the sensory neuron *in vivo*, we compared the sensory neuron's DRG cell somas, sural axons at the foreleg, and the hindpaw axon terminals of these *vpr/RAG1^{-/-}* and *wildtype/RAG1^{-/-}* littermate control mice. At the DRG, two populations of nociceptive neurons were defined by immunolabelling (Figure 1B); the TrkA-expressing (peptidergic) neurons, which comprise up to 45% of the DRG population primarily label the A nerve and C nociceptive nerve fibers, and an IB4-immunoreactive antibody was also used to identify the IB4-binding (TrkA-negative, non-peptidergic) C-fiber neurons which comprise up to 30% of the DRG population (Tucker and Mearow, 2008). The less than 10% population of TrkA+, IB4-binding population of DRG neurons were not counted in this study. The mean number of small diameter ($< 20 \mu\text{m}$) nociceptive DRG somas (with visible nucleoli) of the L4 or L5 ganglia of *wildtype/RAG1^{-/-}* ($n=7$) and *vpr/RAG1^{-/-}* ($n=6$) mice were analysed by confocal microscopy. These analyses revealed similar ratios of TrkA-immunoreactive (TrkA+) to IB4-binding (IB4+) neurons ($1.20 \pm 0.15 \text{ sem}$) from the *wildtype/RAG1^{-/-}* versus ($1.03 \pm 0.1 \text{ sem}$) from the *vpr/RAG1^{-/-}* DRGs ($p > 0.05$) (Figure 1C).

Morphological analysis of the sural nerve axons (shown in transverse section) indicated comparable axonal diameter of both the small pain fibers and the larger mechanoreceptors (Figure 1D) between the *wildtype/RAG1^{-/-}* ($n=7$) and *vpr/RAG1^{-/-}* ($n=6$) mice. G-ratios, a measurement of myelin thickness per axonal diameter illustrated the large-diameter axons to be comparable between *wildtype/RAG1^{-/-}* ($0.71 \pm 0.01 \text{ sem}$) and *vpr/RAG1^{-/-}* ($0.70 \pm 0.01 \text{ sem}$) mice (graph not shown). The smaller diameter myelinated axon g-ratios measured $0.63 \pm 0.01 \text{ sem}$ and $0.62 \pm 0.01 \text{ sem}$ for *wildtype/RAG1^{-/-}* and *vpr/RAG1^{-/-}* mice, respectively. Collectively, these studies illustrated that although Vpr is expressed by macrophages found within the DRG, it did not alter the expression ratios between the pain-sensing DRG subtypes at the ganglia and it did not affect the morphology of the proximal axons *in vivo*.

To study axonal innervation of the footpad, the nerve endings were immunolabeled with PGP9.5 antibody and the numbers of nerve terminals endings within the epidermis were counted (Figure 1E, F). The total number of epidermal nerve terminals per 1 mm of epidermis indicated that *vpr/RAG1^{-/-}* mice had an average of 62% fewer nerve endings compared to corresponding *wildtype/RAG1^{-/-}* controls mice (Figure 1F; $p < 0.001$).

As NGF, primarily secreted by keratinocytes at the epidermis, promotes axonal innervation of the TrkA-expressing DRG neurons at the footpad (Huang and Reichardt, 2001), and we demonstrated that these *vpr/RAG1^{-/-}* mice have less epidermal innervation, we went on to investigate if chronic Vpr exposure affected NGF expression at the footpad of these immunodeficient mice. Quantitative RT-PCR analysis demonstrated that transcripts encoding NGF mRNA were significantly suppressed in the epidermal foot pads of *vpr/RAG1^{-/-}* mice compared to *wildtype/RAG1^{-/-}* (Figure 1G; $p < 0.01$). We showed that the high-affinity NGF receptor tropomyosin related kinase (TrkA) receptor mRNA expression was increased in *vpr/RAG1^{-/-}* footpads compared to *wildtype/RAG1^{-/-}* (Figure 1H; $p < 0.05$).

Collectively, these data suggested that chronic Vpr expression in immunodeficient mice caused allodynia possibly due to reduced epidermal NGF levels and epidermal denervation at the footpad.

3.1.2 NGF protected sensory neurons from Vpr-induced axon growth inhibition

Previous studies have shown soluble recombinant Vpr affected neuronal viability of human DRG neurons (Acharjee et al., 2010) however its effect on axonal outgrowth is unknown. To investigate the mechanism by which Vpr targets DRG neurons, their cell bodies were isolated from their distal axons using compartmented cell culture (Campenot) chambers (Figure 2A). Neonatal DRG neurons were placed into the central compartment of the Campenot chambers and their proximal axons (neurites) grew along scratches under the divider and into the peripheral chambers. As neonatal DRG neurons require NGF for survival for the first week *in vitro*, they were initially plated with NGF (10 ng/mL) in the central chamber. On day 7, NGF was removed from both central and peripheral compartments in half of the cultures for 48 hours (this did not affect cell survival compared to the cultures where NGF was present on days 8 and 9, data not shown). On day 9 (following 2 days of NGF deprivation in half of the cultures), the peripheral axons were axotomized to identify a start point for the next 2 days of axonal growth. Axons exposed to Vpr (100 nM) in the central chamber grew significantly less ($0.45 \text{ mm} \pm 0.03 \text{ sem}$) than the NGF-deprived control cultures ($0.63 \text{ mm} \pm 0.02 \text{ sem}$), demonstrating Vpr acts at the DRG somas to significantly hinder distal axon extension DRG neurons (Figure 2B; $p < 0.01$).

As local injection of NGF was shown to significantly decrease DSP symptoms in HIV/AIDS patients (McArthur et al., 2000) and we showed *vpr/RAG1*^{-/-} mice displayed DSP and decreased NGF expression at the footpad (Figure 1G), we went on to investigate if recombinant NGF treatment at the periphery could block the effects of Vpr at the cell somas. Using sister compartmentalized cultures from above, a subset of cultures were treated with 10 ng/mL and 50 ng/mL NGF to their central and peripheral compartments, respectively at the same time as Vpr exposure to the central chamber. Our data illustrated that NGF protected distal axon extension from Vpr-induced neurite growth inhibition. DRG axons from Vpr treated somas grew 43% less ($0.45 \text{ mm} \pm 0.03 \text{ sem}$) than axons extending from DRG neurons treated with Vpr (soma) after NGF pre-treatment (periphery) (Figure 2B; $0.78 \text{ mm} \pm 0.01 \text{ sem}$; $p < 0.01$). In fact, these NGF/Vpr-treated cultures grew to almost 80% of those cultures treated with NGF alone ($0.91 \text{ mm} \pm 0.03 \text{ sem}$) ($p < 0.01$). Evaluation of the longest axons in each culture highlighted the progression of the experimental conditions throughout the two day treatment phase. These data illustrated Vpr progressively hindered neurite extension throughout the 48 hour time course; the longest axons of Vpr-treated cultures grew an average of $1.57 \text{ mm} \pm 0.05 \text{ sem}$ compared the distal axons pre-treated with NGF before Vpr exposure which grew significantly longer ($1.86 \text{ mm} \pm 0.04 \text{ sem}$) (Figure 2C). Thus, NGF protected the DRG sensory neurons from the growth-inhibiting effect mediated by Vpr exposure.

The ability of NGF to promote axonal outgrowth even in the presence of Vpr was confirmed by quantitative measurement of neurofilament immunofluorescence in partially purified mass neuronal cultures (Figure 3). First, we showed the doses of Vpr used in this study did not affect cell survival of adult (Figure 3B) and neonatal (data not shown) rat DRG neurons. We went on to quantify neurofilament expression to assess neurite extension following 3 days of Vpr exposure and we confirmed that Vpr (10–100 nM) significantly decreased neurite extension in both adult rat (Figure 3C) and human fetal (Figure 3E) DRG neurons. Vpr decreased neurite extension of neonatal rat DRG neurons at 100 nM (Figure 3D). NGF pre-exposure of the adult and neonatal rat DRG neurons (100 ng/mL NGF) as well as human fetal DRG neurons (10 ng/mL NGF) protected the neurons from Vpr-induced inhibition of axon growth (Figure 3C–E). Finally, we confirmed that, similarly to the decrease in NGF

mRNA at the footpad of *vpr/RAG1^{-/-}* mice (Figure 1), recombinant Vpr (100 ng/mL) exposure decreased NGF mRNA in the Schwann cells of the DRG culture (Figure 3F). These data indicate that Vpr decreased NGF expression and NGF pre-treatment protected adult and neonatal rat as well as human fetal DRG neurons from Vpr's effect on axon outgrowth *in vitro*.

3.1.3 Vpr decreased activation of signalling molecules and receptors responsible for axonal extension of DRG neurons

To examine the mechanism by which Vpr exerted its effects and NGF wielded its protective actions, western blot analysis was performed on three separate neonatal DRG neuronal lysates following Vpr exposure $\pm$ NGF pre-treatment (Figure 4). Immunoblots revealed Vpr exposure decreased TrkA immunoreactivity which was accompanied by reduced phosphorylated GSK3 (pGSK3) immunodetection, an indicator of inactivated GSK3 which therefore is no longer able to inhibit axon extension in sensory neurons (Zhao et al., 2009) (Figure 4A). Conversely, NGF pre-treatment restored both TrkA and pGSK3 immunoreactivity levels. Quantification revealed the ratio of pGSK3 to total GSK3 was decreased for the Vpr-exposed cultured neurons (Figure 4B; $p < 0.05$). Similarly, Vpr exposure reduced TrkA expression relative to α -actin abundance (Figure 4C; $p < 0.05$). NGF pre-treatment prevented the Vpr-induced decrease in pGSK3 and TrkA protein levels (Figure 4B, C). In addition, p75 receptor abundance was enhanced by Vpr exposure that suggested a trend toward suppression by NGF treatment, albeit non-significantly (Figure 4A, D). These studies highlighted the significance of the pivotal signalling molecules, TrkA receptor and pGSK3 in Vpr-mediated DRG neuronal injury and their susceptibility to the protective actions of NGF. Importantly, these data show Vpr directly affected axon outgrowth signalling pathways and influenced the expression of the TrkA signalling pathway. Importantly, however, it remained to be determined if NGF directly blocked Vpr-induced neurotoxicity of these sensory neurons or if NGF merely promoted neurite extension independent of Vpr exposure.

3.1.4 NGF directly protected sensory neurons from Vpr

An increase in cytosolic calcium is a robust indicator of increased neuronal excitability and occurs in DRG neurons associated with neuropathic pain (Wall and Devor, 1983; Choi, 1992). We previously showed, using Fluo-4 fluorescence dye to measure the cytosolic calcium levels, that Vpr transiently increased intracellular calcium in human fetal and adult rat DRG neurons (Acharjee et al., 2010). To extend these analyses, we demonstrated that neonatal rat DRG neurons, in NGF-deprived control cultures, displayed a transient cytosolic calcium rise following Vpr (100 nM) treatment (Figure 5C, E; supplemental movie). KCl (35 mM; positive control) was transiently added to the cultures before and after Vpr treatment (Figure 5B, D) and the decrease in KCl-induced cytosolic calcium rise following the Vpr treatment is indicative of a prolonged effect of Vpr on the DRG neurons (Figure 5D–F; $p < 0.01$).

Conversely, cultures pre-treated with NGF (50 ng/mL) for 2 days prior to Vpr (100 nM) exposure decreased the Vpr-mediated calcium increase levels (Figure 5I, K, M; $p < 0.01$; supplemental movie). KCl induced a significant calcium rise in these DRG neurons both before and after Vpr treatment suggesting these NGF-protected neurons remained healthy following Vpr exposure (Figure 5H, J, L). Thus, these data indicated that NGF blocked Vpr-induced increase in free cytosolic calcium in DRG neurons, providing insight into the mechanism through which NGF protects these neurons from Vpr.

3.1.5 NGF acts through the TrkA receptor to protect sensory neurons from Vpr

Despite producing a long-term decrease in HIV-induced DSP, NGF caused painful inflammation at the injection site, thus prohibiting this study from continuing (McArthur et al., 2000). Thus as an initial step discovering an alternative to NGF injection to block DSP *in vivo*, we investigated the signalling pathway through which NGF blocked Vpr's effect on the DRG neurons.

NGF acts as a ligand for two distinct receptors on DRG sensory neurons including the TrkA receptor and the pan-neurotrophin receptor, p75, both of which activate specific intracellular signalling cascades within the sensory neurons (Huang and Reichardt, 2001). Activation of the Ras/MAP and PI3K pathway through the TrkA receptor is known to promote cell survival and neurite extension, respectively, in sensory neurons, whereas NGF binding to p75 monomers can activate signalling pathways that lead to apoptosis (Huang and Reichardt, 2001; Frade and Barde, 1998). Thus, we hypothesized that NGF protected DRG sensory neurons from Vpr through engagement of the TrkA receptor and the ensuing activation of protective pathways. This hypothesis was examined by adding anti-rat TrkA antiserum (RTA), a functional TrkA agonist or REX, a p75 antagonist to neonatal DRG neuronal cultures before the Vpr treatment. Treatment with RTA (1–10 $\mu\text{g/mL}$) prevented the neurite inhibiting effects of Vpr (100 nM) in neonatal rat (Figure 6A) and human fetal (Figure 6D) DRG neurons ($p < 0.05$). The REX p75 antagonist, protected both neonatal (1–10 $\mu\text{g/mL}$), and adult rat (10 $\mu\text{g/mL}$) DRG neurons from the Vpr-induced inhibition of neurite outgrowth (Figure 6A–C; $p < 0.05$). Similarly in human fetal DRG neurons, activation of the TrkA receptor (10 $\mu\text{g/mL}$) and antagonism the p75 receptor pathway (10 $\mu\text{g/mL}$) protected these neurons from Vpr ($p < 0.05$). Collectively, these data pointed to NGF binding to the TrkA receptor (and alternatively the inactivation of the p75 pathway) as the neuroprotective mechanism which countered the axon outgrowth inhibitory effects of Vpr.

4.1 Discussion

This study describes how the neurotrophin NGF can prevent injury to sensory neurons mediated by a viral protein, Vpr. We showed *vpr/RAG1*^{−/−} mice displayed allodynia, nerve terminal denervation, and a significant decrease in NGF mRNA expression at the footpad compared to *wt/RAG1*^{−/−} mice. *In vitro*, we demonstrated that pre-treatment with NGF protected cultured DRG neurons from Vpr's ability to inhibit distal axon outgrowth. NGF acted through its TrkA signaling pathway to promote axon outgrowth signaling pathways as well as protect the neuron from a Vpr-induced calcium surge. This study provides potential therapeutic options for HIV/AIDS patients suffering from DSP and our next step will be to provide neurotrophic support at the epidermis *in vivo* to prevent denervation and ultimately DSP in our *vpr/RAG1*^{−/−} mice model.

Our first aim was to define the physiological effect of Vpr on sensory neurons. Although Vpr is expressed by macrophages in the DRG of HIV-infected patients (Acharjee et al., 2010), our study indicated that the effects of Vpr were most evident at the distal axon terminal and not the cell soma or the proximal nerve (Figures 1, 2). Analysis of epidermal innervation showed, similar to skin samples from HIV-1/AIDS patients (Pardo et al., 2001), there was significantly less innervation in the *vpr/RAG1*^{−/−} mice footpads compared to the *wildtype/RAG1*^{−/−} mice (Figure 1). We used compartmented cell culture chambers to design an experiment to mimic the *in vivo* exposure of Vpr at the cell bodies which are at a distance from their axon terminals. The addition of Vpr to the central chamber containing the cell bodies and their proximal axons caused neurite inhibition of the distal axons (Figure 2). To uncover the mechanism through which Vpr affects axonal extension, we showed Vpr increased the level of free cytosolic calcium, an indicator of neuronal toxicity (Figure 5).

Further, we showed Vpr exposure decreased protein expression of the TrkA receptor and pGSK3 (Figure 3), part of the PI3K pathway which regulates axonal outgrowth.

The second major aim of this study was to show that NGF blocked the effect of Vpr *in vitro*. As a phase II clinical trial showed local injection of NGF, a neurotrophic factor that maintains TrkA-expressing sensory axon innervation of the epidermis reduced allodynia of patients suffering from DSP (McArthur et al., 2000), we investigated if NGF protects DRG neurons from Vpr. Neurons treated with NGF before Vpr exposure had significantly higher axonal outgrowth (Figure 2, 3) likely due to levels of pGSK3 and TrkA receptor protein expressions that were comparable with control cultures (NGF-treatment alone) (Figure 4). NGF directly acted on DRG neurons to block the neurotoxic Vpr-induced increase in cytosolic calcium levels (Figure 5). Neurite outgrowth assays confirmed exogenous NGF, TrkA agonism and p75 antagonism protected neonatal and adult rat as well as human fetal DRG neurons from the growth-inhibiting effect of Vpr (Figure 6). It is not clear at this point if the blocking of the p75 pathway directs the endogenous Schwann-cell produced NGF to the available TrkA receptor on the DRG membrane, thus promoting neurite extension, or if other p75 receptor signalling by other binding partners is blocked by the p75 receptor antagonist. Collectively, these data suggest the neuroprotective effect of NGF may be two-pronged; (i) NGF acts through the TrkA pathway (even in the presence of Vpr) to promote neurite extension and (ii) NGF down-regulates the Vpr-induced activation of the growth-inhibiting p75 pathway.

It is likely that Vpr's effect at the distal terminal is primarily on a population of the A (nociceptive) sensory nerve fibers as it is these axons that are NGF responsive and express its two receptors TrkA and p75 (Huang and Reichardt, 2001). NGF maintains axon innervation of TrkA-responsive nociceptive neurons at the footpad and a loss of NGF results in a 'dying-back' of epidermal innervation (Diamond et al., 1992). Indeed, our study showed chronic Vpr exposure within an immunocompromised mouse had significantly less NGF mRNA expression and dieback of pain-sensing distal axons *in vivo* (Figure 1). Therefore chronic Vpr exposure may hinder the NGF-axon terminal interaction at the footpad resulting in the retraction of the NGF-responsive nociceptive neurons. Thus local injection of NGF may re-establish the epidermal footpad innervation and effectively treat *vpr/RAG1*^{-/-} induced mechanical allodynia. In support of this hypothesis, our compartment chamber studies showed that exposure of NGF to the distal axons significantly improved neurite outgrowth of axons whose cell bodies alone were exposed to Vpr (Figure 2).

Although NGF mRNA levels were significantly decreased in *vpr/RAG1*^{-/-} footpads (Figure 1G) there was an increase in TrkA mRNA levels in these mice compared to *wildtype/RAG1*^{-/-} controls (Figure 1H). To understand this paradigm, it is important to know that within the epidermis, NGF is secreted keratinocytes, making these cells primarily responsible for the innervation TrkA-expressing DRG nerve terminals (Albers et al., 1994; Bennett et al., 1998; Di Marco et al., 1993). These NGF-producing keratinocytes express low level TrkA receptor as an autocrine regulator of NGF secretion levels (Pincelli and Marconi, 2000). As our *in vivo* studies showed a decrease in axon innervation at the footpad, and Western blot analysis of cultured DRG neurons demonstrated a decrease in TrkA receptor expression following Vpr expression (Figure 4) the increase in TrkA receptor levels at the epidermis (Figure 1H) is not likely due to axonal TrkA expression. Instead, it is likely that a decrease in NGF levels at the footpad of the *vpr/RAG1*^{-/-} mice (Figure 1G) caused receptor hypersensitivity to TrkA levels within the epidermal keratinocytes. Thus, chronic Vpr exposure decreased NGF receptor expression, which results in a compensatory autocrine response to increase the TrkA receptor expression (Figure 1H). Importantly, other models of DSP, such as Diabetes Mellitus also report a decrease in NGF expression in the epidermis (Anand et al., 1996) and decreased epidermal axonal innervation (Levy et al.,

1992). Similarly in diabetic skin, there is an increase in epidermal TrkA mRNA expression, also thought to be an autocrine compensatory mechanism of these target epidermal cells to the decreased NGF levels (Terenghi et al., 1997).

Our studies showed NGF protected both young and old rat (100 ng/mL), as well as human fetal (10 ng/mL) DRG neurons from Vpr's inhibition of axon outgrowth. The ability of Vpr to induce similar effects on different ages and species of sensory neuron, and the capacity for NGF acting through the TrkA, and not the p75 receptor pathway, to significantly block this effect provides strong evidence that Vpr's effect is robust. Indeed, studying human DRG neurons removes the uncertainties from species differences and provides support for translational research and future therapeutics for HIV1/AIDS-infected patients suffering from DSP.

The *vpr/RAG1*^{-/-} mice had 70% less epidermal innervation of the nociceptive nerve terminals compared to *wildtype/RAG1*^{-/-} mice yet Von Frey filament testing indicated that these mice displayed mechanical allodynia (Figure 1). This observation is similar in mice suffering from diabetes mellitus which display allodynia with decreased nociceptive neurons at their footpad epidermis (Brussee et al., 2008). There are several possible explanations for this behaviour, the simplest being that the remaining nociceptive nerve fibers have a lower pain threshold which when stimulated lead to an allodynic response. We can exclude collateral sprouting of the remaining nociceptive axon terminals as this would have been apparent in our epidermal footpad analysis of free nerve endings (Figure 1). However, it is possible that the absence of nociceptive nerve terminals leads to re-characterization of the larger non-nociceptive A neurons within the epidermis (Brussee et al., 2008; Diamond et al., 1992; Acharjee, et al., 2010). These A mechanoreceptors may become sensitive to the Von Frey filaments at the footpad and release substance P at their synapse within the spinal cord, thus activating second order nociceptive axons.

4.1.1 Conclusion

In conclusion we have shown the NGF pathway can protect DRG sensory neurons from the HIV/AIDS mediated protein, Vpr. We confirmed NGF abrogates Vpr-induced effects. Although the human clinical trial of NGF in HIV induced DSP was apparently positive this line of therapy has not yet been pursued, possibly because of the NGF-induced painful inflammation at the injection site. Thus injection of NGF into the footpads of *vpr/RAG1*^{-/-} mice to observe changes in the Vpr-induced mechanical allodynia will likely be associated with discomfort and therefore not an ideal experiment to pursue. Importantly our study provided additional insight into how NGF protected sensory neurons from Vpr, clearly showing both the activation of the TrkA signalling cascade as well as the inhibition of the p75 pathway is neuroprotective. Thus the pursuit of alternatives to NGF injection, which promote TrkA signalling in a painless, noninflammatory fashion, may be the best strategy to protect sensory neurons from Vpr and HIV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Louis Reichardt for his generous donation of the TrkA and p75 antibodies. We thank Dr. Jennifer Hocking for her helpful review of this manuscript. These studies were supported by the University Hospital Foundation (RES0012374), CANFAR (RES0004428), NSERC Discovery grant (CAW) and the National Institutes of Health (CP). The authors declare no conflicts of interest.

References

- Acharjee S, Noorbakhsh F, Stemkowski PL, Olechowski C, Cohen EA, Ballanyi K, Kerr B, Pardo C, Smith PA, Power C. HIV-1 viral protein R causes peripheral nervous system injury associated with in vivo neuropathic pain. *FASEB J.* 2010; 24:4343–4353. [PubMed: 20628092]
- Albers KM, Wright DE, Davis BM. Overexpression of nerve growth factor in epidermis of transgenic mice causes hypertrophy of the peripheral nervous system. *J Neurosci.* 1994; 14:1422–1432. [PubMed: 8126547]
- Anand P, Terenghi G, Warner G, Kopelman P, Williams-Chestnut RE, Sinicropi DV. The role of endogenous nerve growth factor in human diabetic neuropathy. *Nat Med.* 1996; 2:703–707. [PubMed: 8640566]
- Andersen PL, Webber CA, Kimura KA, Schreyer DJ. Cyclic AMP prevents an increase in GAP-43 but promotes neurite growth in cultured adult rat dorsal root ganglion neurons. *Exp Neurol.* 2000; 166:153–165. [PubMed: 11031091]
- Bennett DL, Koltzenburg M, Priestley JV, Shelton DL, McMahon SB. Endogenous nerve growth factor regulates the sensitivity of nociceptors in the adult rat. *Eur J Neurosci.* 1998; 10:1282–1291. [PubMed: 9749782]
- Brussee V, Guo G, Dong Y, Cheng C, Martinez JA, Smith D, Glazner GW, Fernyhough P, Zochodne DW. Distal degenerative sensory neuropathy in a long-term type 2 diabetes rat model. *Diabetes.* 2008; 57:1664–1673. [PubMed: 18332094]
- Campanot RB, Lund K, Mok SA. Production of compartmented cultures of rat sympathetic neurons. *Nat Protoc.* 2009; 4:1869–1887. [PubMed: 20010935]
- Cheng C, Guo GF, Martinez JA, Singh V, Zochodne DW. Dynamic plasticity of axons within a cutaneous milieu. *J Neurosci.* 2010; 30:14735–14744. [PubMed: 21048132]
- Choi DW. Excitotoxic cell death. *J Neurobiol.* 1992; 23:1261–1276. [PubMed: 1361523]
- Christie KJ, Webber CA, Martinez JA, Singh B, Zochodne DW. PTEN inhibition to facilitate intrinsic regenerative outgrowth of adult peripheral axons. *J Neurosci.* 2010; 30:9306–9315. [PubMed: 20610765]
- Cornblath DR, Hoke A. Recent advances in HIV neuropathy. *Curr Opin Neurol.* 2006; 19:446–450. [PubMed: 16969153]
- Diamond J, Holmes M, Coughlin M. Endogenous NGF and nerve impulses regulate the collateral sprouting of sensory axons in the skin of the adult rat. *J Neurosci.* 1992; 12:1454–1466. [PubMed: 1556603]
- Ellis RJ, Rosario D, Clifford DB, McArthur JC, Simpson D, Alexander T, Gelman BB, Vaida F, Collier A, Marra CM, et al. Continued high prevalence and adverse clinical impact of human immunodeficiency virus-associated sensory neuropathy in the era of combination antiretroviral therapy: the CHARTER Study. *Arch Neurol.* 2010; 67:552–558. [PubMed: 20457954]
- Ernsberger U. Role of neurotrophin signalling in the differentiation of neurons from dorsal root ganglia and sympathetic ganglia. *Cell Tissue Res.* 2009; 336:349–384. [PubMed: 19387688]
- Frade JM, Barde YA. Nerve growth factor: two receptors, multiple functions. *Bioessays.* 1998; 20:137–145. [PubMed: 9631659]
- Huang EJ, Reichardt LF. Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci.* 2001; 24:677–736. [PubMed: 11520916]
- Jones GJ, Barsby NL, Cohen EA, Holden J, Harris K, Dickie P, Jhamandas J, Power C. HIV-1 Vpr causes neuronal apoptosis and in vivo neurodegeneration. *J Neurosci.* 2007; 27:3703–3711. [PubMed: 17409234]
- Levy DM, Terenghi G, Gu XH, Abraham RR, Springall DR, Polak JM. Immunohistochemical measurements of nerves and neuropeptides in diabetic skin: relationship to tests of neurological function. *Diabetologia.* 1992; 35:889–897. [PubMed: 1397786]
- Di Marco E, Mathor M, Bondanza S, Cutuli N, Marchisio PC, Cancedda R, De Luca M. Nerve growth factor binds to normal human keratinocytes through high and low affinity receptors and stimulates their growth by a novel autocrine loop. *J Biol Chem.* 1993; 268:22838–22846. [PubMed: 7693679]

- McArthur JC, Yiannoutsos C, Simpson DM, Adornato BT, Singer EJ, Hollander H, Marra C, Rubin M, Cohen BA, Tucker T, et al. A phase II trial of nerve growth factor for sensory neuropathy associated with HIV infection. AIDS Clinical Trials Group Team 291. *Neurology*. 2000; 54:1080–1088. [PubMed: 10720278]
- McArthur JC, Brew BJ, Nath A. Neurological complications of HIV infection. *Lancet Neurol*. 2005; 4:543–555. [PubMed: 16109361]
- Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. 1992; 68:869–877. [PubMed: 1547488]
- Morgello S, Estanislao L, Simpson D, Geraci A, DiRocco A, Gerits P, Ryan E, Yakoushina T, Khan S, Mahboob R, et al. HIV-associated distal sensory polyneuropathy in the era of highly active antiretroviral therapy: the Manhattan HIV Brain Bank. *Arch Neurol*. 2004; 61:546–551. [PubMed: 15096404]
- Pardo CA, McArthur JC, Griffin JW. HIV neuropathy: insights in the pathology of HIV peripheral nerve disease. *J Peripher Nerv Syst*. 2001; 6:21–27. [PubMed: 11293804]
- Pincelli C, Marconi A. Autocrine nerve growth factor in human keratinocytes. *J Dermatol Sci*. 2000; 22:71–79. [PubMed: 10674819]
- Power C, Boissé L, Rourke S, Gill MJ. NeuroAIDS: an evolving epidemic. *Can J Neurol Sci*. 2009; 36:285–295. [PubMed: 19534327]
- Campanot, Robert. Cell-cell interactions: a practical approach, Compartmented culture analysis of nerve growth. Oxford UP; 1992.
- Smith HS. Treatment considerations in painful HIV-related neuropathy. *Pain Physician*. 2011; 14:E505–524. [PubMed: 22086103]
- Terenghi G, Mann D, Kopelman PG, Anand P. trkA and trkC expression is increased in human diabetic skin. *Neurosci Lett*. 1997; 228:33–36. [PubMed: 9197281]
- Tucker BA, Mearow KM. Peripheral sensory axon growth: from receptor binding to cellular signaling. *Can J Neurol Sci*. 2008; 35:551–566. [PubMed: 19235438]
- Wall PD, Devor M. Sensory afferent impulses originate from dorsal root ganglia as well as from the periphery in normal and nerve injured rats. *Pain*. 1983; 17:321–339. [PubMed: 6664680]
- Webber CA, Xu Y, Vanneste KJ, Martinez JA, Verge VMK, Zochodne DW. Guiding adult Mammalian sensory axons during regeneration. *J Neuropathol Exp Neurol*. 2008; 67:212–222. [PubMed: 18344912]
- Webber CA, Christie KJ, Cheng C, Martinez JA, Singh B, Singh V, Thomas D, Zochodne DW. Schwann cells direct peripheral nerve regeneration through the Netrin-1 receptors, DCC and Unc5H2. *Glia*. 2011; 59:1503–1517. [PubMed: 21656855]
- Zhao Z, Wang Z, Gu Y, Feil R, Hofmann F, Ma L. Regulate axon branching by the cyclic GMP pathway via inhibition of glycogen synthase kinase 3 in dorsal root ganglion sensory neurons. *J Neurosci*. 2009; 29:1350–1360. [PubMed: 19193882]
- Zochodne DW, Cheng C, Miampamba M, Hargreaves K, Sharkey KA. Peptide accumulations in proximal endbulbs of transected axons. *Brain Res*. 2001; 902:40–50. [PubMed: 11376593]

Highlights

- Viral protein R (Vpr) causes neuropathic pain and footpad denervation *in vivo*
- Vpr increases cytosolic calcium in sensory neurons and decreases axon extension
- Nerve growth factor (NGF) pre-treatment protects sensory neurons from Vpr
- NGF acts through the TrkA pathway to block Vpr's effect on DRG neurons

Figure 1. Effects of chronic Vpr exposure *in vivo*

(A) Behavioral studies confirmed *vpr/RAG1*^{-/-} mice withdrew their hindpaws when an average Von Frey filament weight of $1.91 \text{ g} \pm 0.20 \text{ sem}$ ($n=6$) was applied compared to $2.68 \text{ g} \pm 0.30 \text{ sem}$ in the *wildtype/RAG1*^{-/-} ($n=7$). (B) At the ganglion, immunocytochemistry labeled TrkA-expressing (green) and the IB4-binding (red) nociceptive neuronal subpopulations of both the *vpr/RAG1*^{-/-} and *wildtype/RAG1*^{-/-} mice. (C) Cell counts determined there was no difference in these two populations in the presence of chronic Vpr exposure ($n=6$) compared to the *wildtype/RAG1*^{-/-} ganglia ($n=7$). (D) Transverse section of sural nerve of *wildtype/RAG1*^{-/-} and *vpr/RAG1*^{-/-} mice demonstrated there was no significant difference in g-ratios between the two groups ($p>0.05$; graph not shown). (E) Epidermal axon innervation (PGP9.5 antibody; arrowheads) of the hindpaw footpads from *wildtype/RAG1*^{-/-} and *vpr/RAG1*^{-/-} transgenic mice were measured. Asterisks indicate nerve bundles within the dermis before terminal innervation into the epidermis. (F) Quantification of nerve terminals confirmed an average of $662 \text{ nerve fibers} \pm 42 \text{ sem}$ per 1 mm section *vpr/RAG1*^{-/-} mice footpads ($n=6$) compared to $814 \text{ nerve fibers} \pm 32 \text{ sem}$ in the *wild-type/RAG1*^{-/-} littermate controls ($n=7$). (G) Quantitative RT-PCR of the epidermis indicated a significant decrease in NGF mRNA expression in the *vpr/RAG1*^{-/-} mice compared to *wildtype/RAG1*^{-/-} controls (average relative expression of $0.3 \pm 0.2 \text{ sem}$ and $1.1 \pm 0.17 \text{ sem}$, respectively). (H) TrkA mRNA expression was increased in *vpr/RAG1*^{-/-} mice compared to *wildtype/RAG1*^{-/-} controls ($p<0.05$). All error bars represent SEM and * $p<0.05$ Student's *t* test with a Dunnett's *post hoc* comparison. Scale bar in B, D and E is $20 \mu\text{M}$.

Figure 2. NGF prevents Vpr-mediated axonal growth inhibition

(A) Schematic diagram of a three-chambered culture in which a teflon divider rests on the floor of a 35-mm culture dish and silicon grease partitions the dish into a central compartment containing neonatal neuronal cell bodies and the proximal axons and two peripheral compartment containing the distal axons. For the first 7 days of growth, NGF was essential for neonatal DRG survival and it was added to all cultures (10 ng/mL to center and 50 ng/mL to peripheral compartments). From days 7–9, NGF was removed from all of the cultures. On day 9, the extending axons in the peripheral chamber were cut at the partition of each culture in order to establish a start point to measure axon extension after 48 hours. DIC images show DRG somas and proximal axons in the central chambers and distal axons in one of the peripheral chambers. (B) Exposure of the central chamber to Vpr (100 μ M) decreased peripheral axonal extension (mean growth of $0.45 \text{ mm} \pm 0.03 \text{ sem}$) compared to

cultures treated at the central (10 ng/mL) and peripheral (50 ng/mL) compartments with NGF alone ($0.9 \text{ mm} \pm 0.03 \text{ sem}$) or no NGF treatment ($0.63 \text{ mm} \pm 0.02 \text{ sem}$). NGF blocked the axon inhibiting effect of Vpr and these axons grew an average of $0.78 \text{ mm} \pm 0.03 \text{ sem}$. (C) Measurement of the longest axon illustrated a progression of Vpr's effect over the treatment phase showing Vpr at the perikarya inhibited distal axon growth ($1.57 \text{ mm} \pm 0.05 \text{ sem}$) and pre-treatment with NGF significantly improves axon extension ($1.86 \text{ mm} \pm 0.04 \text{ sem}$). ** $p < 0.01$; *** $p < 0.001$ ANOVA with a Bonferoni *post hoc* comparison. $n=5$ for all conditions.

Figure 3. NGF protects sensory neurons from Vpr-induced axonal growth inhibition

A–C) Adult rat DRG neurons were plated onto 96-well dishes $\pm$ NGF (10 ng/mL or 100 ng/mL). On day 1, Vpr (10 nM, 100 nM) was added to the cultures and on day 5, quantitative immunofluorescence measured neurofilament expression. D–E) Neonatal rat DRGs (D) and human fetal DRGs (E) were grown in the presence of NGF (50 ng/mL) for 7 days and then NGF was deprived from half of the cultures. On day 9, Vpr (10 nM or 100 nM) was added to the cultures and on day 11, quantitative immunofluorescence measured neurofilament expression. (A) Neurofilament immunoreactivity depicting neurites and somas of cultured adult rat DRG neurons at day 5 without exposure to Vpr. (B) A cell survival assay showed the Vpr doses used in our experiments (1–100 nM) did not cause neuronal death *in vitro*. (C–E) Quantitative neurofilament immunofluorescence showed Vpr (10–100 nM) caused a significant decrease in neurofilament expression in adult rat (C) and human fetal (E) DRG neurons. Vpr (100 nM) decreased neurofilament expression in neonatal rat DRG neurons (D). NGF pre-treatment protected adult and neonatal rat (100 ng/mL) as well as human fetal (10–100 ng/mL) DRG neurons from neurite inhibiting effect of Vpr. * $p < 0.05$ ANOVA with a Dunnett's *post hoc* comparison. Scale bar in A = 20 μ m.

Figure 4. Vpr acts through the PI3K pathway to inhibit DRG neurite extension and pretreatment with NGF blocks this effect

A) Western blot analysis was performed using protein harvested from three separate mass neonatal DRG cultures treated with 50 ng/mL NGF (lane 1), 100 nM Vpr (lane 2), or 50 ng/mL NGF before 100 nM Vpr (lane 3). On the same nitrocellulose membranes, we probed for pGSK3, total GSK3, TrkA receptor and p75 receptor expression. β -actin was labeled for the loading controls. B–C) Densitometry confirmed Vpr significantly decreased the ratio of pGSK3/GSK3 and TrkA receptor expression compared to the control culture treated with NGF alone. Pre-treatment with NGF before Vpr exposure protected these neurons from the Vpr-induced decrease in both pGSK3/GSK3 ratio and TrkA receptor. D) There was a trend towards an increase in p75 receptor expression following Vpr treatment, compared to control which was blocked by pretreatment with NGF. The densitometry of each lane was normalized to its β -actin control and compared to the NGF-alone treatment. This experiment was repeated with three separate experiments. * $p < 0.05$ ANOVA with a Dunnett's *post hoc* comparison.

Figure 5. NGF directly protects DRG neurons from Vpr

A–D) The calcium indicator dye, Fluo-8L-acetoxymethyl ester (Fluo-8L-AM) was added to 9 d-old mass cultured neonatal DRG neurons that were deprived of NGF for 2 days. Confocal imaging of the Fluo-8L labelled rat DRG neurons of control (A–E) and NGF pre-treated (G–K) neonatal DRG neuronal cultures. Images showing the Fluo-8L intensity within the DRG neurons at baseline (A, G) or following 35 mM KCl before Vpr exposure (B, H), 100 nM Vpr exposure (C, I), and 35 mM KCl exposure after Vpr treatment (D, J). Graphic representation of the fluorescent intensity of Fluo-8L of NGF-deprived (E) and NGF pre-treated (K) cultures. The average peak of fluorescent intensity of KCl exposure before and after Vpr exposure in NGF-deprived (F) and NGF pre-treated (L) cultures, as well as during the Vpr treatment (M) was measured (n=3). ** $p < 0.01$; Student's t-test followed by a Dunnett's *post hoc* comparison. Scale bar in A is 75 μm . The movies of these experiments can be found in the supplemental data online.

Figure 6. Activation of the TrkA intracellular signalling cascade and inhibition of the p75 pathway protects the DRG neurons from Vpr exposure

A–B) Neonatal rat DRGs and human fetal DRGs (D) were grown in the presence of NGF (50 ng/mL) for 7 days and then NGF was removed from all of the cultures. The functional TrkA agonist, RTA, or the p75 receptor antagonist, REX, was added to the appropriate cultures from days 7–9 (1 μg/mL, 10 μg/mL RTA or REX). On day 9, Vpr (10 nM or 100 nM) was added to the cultures and on day 11, quantitative immunofluorescence measured neurofilament expression. C) Adult rat DRG neurons were plated onto 96-well dishes in the presence of REX (1 μg/mL, 10 μg/mL). On day 1, Vpr (10 nM, 100 nM) was added to the cultures and on day 5, quantitative immunofluorescence measured neurofilament expression. (A) Exposure of neonatal DRG neurons to a functional TrkA agonist (1–10 μg/mL) protected these neurons from the neurite inhibiting effect of Vpr (100 nM) (n=6). (B) Inhibition of the p75 pathway with a functional p75 antagonist (10 μg/mL) protected neonatal rat DRG neurons from the neurite inhibiting effect of Vpr (n=6). Inhibition of the p75 receptor signalling cascade also protected adult rat (C; 10 μg/mL p75 antagonist) and human fetal (D; 1 μg/mL p75 antagonist) DRG neurons from the neurite inhibiting effect of Vpr (n=4 and n=1, respectively). Activation of the TrkA intracellular signalling cascade (10 μg/mL) also protected human fetal DRG neurons from the neurite inhibiting effect of Vpr (n=1). * p<0.05 ANOVA with a Bonferroni *post hoc* comparison.