Effects of HIV-1 Tat on the enteric nervous

Joy Ngwainmbi

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EFFECTS OF HIV-1 TAT ON THE ENTERIC NERVOUS SYSTEM

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Acknowledgement

Words cannot adequately express the appreciation and gratitude I feel for everyone who has contributed in one way, or another to make this work a success.

First and foremost, I would like to thank my mentor, Dr. Hamid I. Akbarali for accepting me into his lab, training me and for everything. He went far and beyond what was required of a mentor. There was never a day I thought that he did not have my best interest. He provided me with an excellent environment, believed in me, encouraged me and is an example for me to follow. Thank you very much Hamid!

I would also like to thank my committee members: Dr. Dewey, Dr. Hauser, Dr. Logothetis and Dr. Grider for their time, ideas, and support. I really appreciate the time you invested in my future. I would like to thank Dr. Dewey for all his input towards my training. Thank you for providing an excellent environment at the Department of Pharmacology and Toxicology – a place where everyone feels accepted and important. Thank you for all your ideas during our lab meetings. A sincere thank you goes to Dr. Hauser for his help, his expertise in the HIV research field and for all the resources he provided to me.

Special Thanks go to Dr. Joyce Lloyd and Suzanne Barbour. They’ve been there since the beginning of this journey. They were the first people with whom I spoke before joining the PREP program at VCU and they have been there for me throughout this journey. They have been a source of support, encouragement and are examples for me.
I am grateful to all the past and present members of the Akbarali and Dewey labs: Dr. Tricia Smith, Dr. Datta De, Dr. James Kang, Dr. Dwight William, Dr. Bethany David, Dr. Atsushi, Dr. Kensuke, Aravind, Sukhada, Ryan, Essie, Fayez, Jacy, Maciej, Kumiko, and Karan. Thanks guys for being a source of encouragement and being such a support. Thanks Krista for helping us with the mouse work and DAR. Thank you very much Dave for all your help.

Special Thanks also go to Allen. We started this program together and you have been a very good friend. Thank you.

Special thanks go to Dr. Sylvia Fitting who has been a very good friend and “mentor”. Thank you very much for all your help Sylvia.

I would also like to thank the staff of the Department of Pharmacology and Toxicology: Sheryol, Sallie and Pat. I would also like to thank the faculty of the Pharmacology and Toxicology Department for the training they provided to me. Especially Dr. Bettinger for the seminar course.

I would not have had the opportunity to even start this program if not for the help of my ‘big sister’ Michelia Ward. She believed in me. That was my strongest driving force throughout this program. I knew someone believes in me and I could not give up.

I would like to thank my family for their support. I would like to thank my dad for being such a rock. Whenever I called him, he prayed for me, encouraged me. Special thanks go
to my mom for her support. I am what I am today because of the education that my parents
gave me. Special thanks to my sister and her husband: Commy and Silas and brothers:
Godwin, Seth, Paul and Caleb.
I am grateful to my family in Northern Virginia. I would like to thank the Tangwa’s, the
Esunge’s and the Nkaimbi’s for their help and support. I am grateful to my friend Elsie.
Sincere thanks goes to the Guedia’s: Papa Guedia, Alex, Ingrid, Michelle, Isaac, Yolande
and Axelle. I would also like to thank my friends: Dr. Brice Nouthe, Maxwell and
Sandrine. Special thanks go to my Richmond crew: Pa Paul, Ma Bri, Sorelle, Martial,
Anita and Samantha.
I would like to thank my awesome husband, Dr. Gilles Guedia, for his patience, support
and unconditional love. Thank you Darling for letting me achieve my dreams. Thank you
for being an example of hard work and discipline to me.
Last but not least, I would like to thank God Almighty for the strength and grace He has
given me through life. All of these have been possible because of Him. He put all these
amazing people on my path and has guided my life.
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Clarification of contributions

Chapter III and part of chapter V of this dissertation has been published elsewhere
(Ngwinmbi, J., De, D. D., Smith, T. H., El-Hage, N., Fitting, S., Kang, M., Dewey WL.,
The Journal of Neuroscience : The Official Journal of the Society for Neuroscience,
34(43), 14243-14251)

Chapter IV and part of chapter V of this dissertation has been submitted for publication
HIV-1 Tat sensitizes to lipopolysaccharide-induced inflammatory cytokines via TLR4
signaling in enteric glia (in review))

Additional manuscripts that are indirectly related to this dissertation are:

P. E., Hauser, K. F. and Akbarali, H. I. (2015), Sensitization of enteric neurons to
morphine by HIV-1 Tat protein. Neurogastroenterology & Motility. doi:
10.1111/nmo.12514

Akbarali Morphine dependence in single enteric neurons from the mouse colon requires
deletion of β-arrestin2 Physiological Reports Sep 2014,2(9)DOI: 10.14814/phy2.12140

Smith, T. H., Ngwinmbi, J., Grider, J. R., Dewey, W. L., Akbarali, H. I. An In-vitro
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List of Abbreviations

AIDS: Acquired immune deficiency syndrome
CD4: Cluster of differentiation 4
CCR5: Chemokine receptor type 5
CXCR4: Chemokine receptor type 4
CTL: Cytotoxic T lymphocyte.
cART: Combined antiretroviral therapy
CNS: Central nervous system
CpG: Cytosine triphosphate deoxynucleotide guanine triphosphate deoxynucleotide phosphodiester
DNA: Deoxyribonucleic acid
DRG: Dorsal root ganglion
DOX: Doxycycline
EGCs: Enteric glia cells
ELISA: Enzyme-linked immunosorbent assay
ENS: Enteric nervous system
GFAP: Glial fibrillary acidic protein
GI: Gastrointestinal
HIV: Human immunodeficiency virus
IL-6: Interleukin six
IL-1β: Interleukin 1 beta
LMMP: Longitudinal muscle myenteric plexus
LPS: Lipopolysaccharide

mRNA: Messenger ribonucleic acid

MLN: Mesenteric lymph node

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

PAMP: Pathogen associated molecular pattern

RANTES: Regulated on activation, normal T cell expressed and secreted

RNA: Ribonucleic acid

RT-PCR: Real time polymerase chain reaction

SiRNA: Small interfering ribonucleic acid

SIV: Simian immunodeficiency virus

TLR4: Toll like receptor 4

TLR9: Toll like receptor 9

TRIF: TIR-domain-containing adapter-inducing interferon-β

T<sub>H</sub>17: T helper cells producing interleukin 17

Tat: trans-activator of transcription

TTX: Tetrodotoxin

TNF-α: Tumor necrosis factor alpha

VGSC: Voltage gated sodium channels
Abstract

Effects of HIV-1 Tat on the Enteric Nervous System

By Joy K. Guedia, BS

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2015

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Professor, Department of Pharmacology and Toxicology

More than 1.2 million people are estimated to be currently living with the human immunodeficiency virus (HIV) in the United States of America. The gastrointestinal (GI) tract is both a major target and an important component of HIV pathogenesis. The GI processes that are dysregulated during HIV infection are controlled by the enteric nervous system (ENS). Indeed, both clinical and experimental studies have implicated the ENS in HIV and simian immunodeficiency virus (SIV) pathogenesis. In addition to direct viral effects, the HIV virus also indirectly affects the GI tract via cellular and/or viral toxins released by infected cells. Trans-activator of transcription (Tat) is a viral toxin that plays an important role in replication of the HIV virus. While, the HIV virus does not directly infect neurons, Tat has been shown to modulate neuronal function. HIV infection in the gut is accompanied by: translocation of bacteria and bacterial products from the gut lumen to
peripheral blood, immune activation and inflammation. Lipopolysaccharide (LPS) is a major bacterial product that is used to determine the rate of bacterial translocation and to drive inflammation. Despite reports of enteric ganglionitis in SIV infected monkeys and autonomic denervation in the jejunum of HIV patients, little is known of the mechanism underlying enteric neuropathogenesis in HIV and the role of the ENS in HIV pathogenesis. In the present study, we assessed the effects of Tat on enteric neuronal excitability and how Tat and LPS interact in the ENS to bring about inflammation and GI motility problems observed in HIV patients. We show that Tat significantly increased enteric neuronal excitability by modulating sodium channels expressed on enteric neurons. Tat sensitized ENS cells to LPS-mediated increase in pro-inflammatory cytokines via a TLR4-mediated pathway involving MyD88. Mice expressing the tat transgene (Tat+) had faster GI transit rates and significantly higher frequencies of diameter changes in the proximal ileum than controls (Tat-). Tat+ mice were also more sensitive to LPS-mediated decreases in colonic transit rate. This study highlights the role of viral and bacterial proteins in HIV pathogenesis in the gastrointestinal tract and also demonstrates a critical role of the ENS in HIV pathogenesis.
Chapter I

Background

**Human immunodeficiency virus (HIV)**

HIV belongs to the retroviridae family and is the causative agent of acquired immunodeficiency syndrome (AIDS). There are two main types of HIV: HIV-1 and HIV-2. Even though both cause similar clinical symptoms, HIV-1 is more common while HIV-2 is less common, less easily transmitted and has a longer period between infection and disease manifestation. Generally, when the term HIV is used without specifying, people are referring to HIV-1 because it is the most widespread. I will simply refer to HIV-1 as HIV in this study. HIV is further divided into 4 main groups: M, N, O, and P. Group M-the major group is the most frequent comprising about 90% of all infections. Group O-the outlier has mostly been reported in Central and West Africa. Groups P and N are newer, very rare and have been reported in Cameroon (Abecasis et al., 2013; Plantier et al., 2009). Group M presents the most diversity. It is further divided into nine distinct clades (subtypes) and a number of circulating recombinant forms (CRFs). These clades include: A, B, C, D, F, G, H, J and K (Abecasis et al., 2013; Buonaguro, Tornesello, & Buonaguro, 2007).

The HIV virion is made up of a phospholipid envelope from which protrude glycolipids GP41 and GP120. The envelope surrounds a nucleocapside, which encapsulates 2 single stranded RNA. The HIV genome is composed of 9 genes encoding: 3 structural, 2
envelope, and 6 regulatory proteins in addition to 3 enzymes (integrase, reverse transcriptase, and protease) required for viral life cycle (Figure 1). The life cycle of HIV involves the following stages depicted in Figure 2: viral entry, reverse transcription, integration, transcription, translation, budding and maturation (Figure 2).
Figure 1. A schematic representation of the HIV-1 virion structure and gene products encoded by the HIV-1 genomic sequence.
Figure 2. Life cycle of the HIV:
1. **Entry**: HIV binds to a specific type of *CD4 receptor* and a *co-receptor* on the surface of the CD4 cell. HIV fuses with the host cell (CD4 cell) and releases its genetic material into the cell.
2. **Reverse Transcription**: *reverse transcriptase* makes DNA from viral RNA, so it can be integrated into the host DNA.
3. **Integration**: Viral DNA enters the *nucleus* of the CD4 cell and uses an enzyme called *integrase* to integrate itself into the host’s genetic material, where it may “hide” and stay inactive for several years.
4. **Transcription**: When the host cell becomes activated, and the virus uses host’s enzymes to transcribe viral genes.
5. **Assembly**: *Protease* cuts the longer HIV *proteins* into individual proteins. These proteins are later assembled.
6. **Budding and maturation**: Virus buds out of the cell with part of host cell membrane. This outer part covers the virus and contains all of the structures necessary to bind to a new CD4 cell and receptors and begin the process again.
Effects of HIV on the gastrointestinal tract

Gastrointestinal (GI) disease is a major manifestation of HIV and accounts for a majority of presenting symptoms of acquired immune deficiency syndrome (AIDS) worldwide and to a greater extent in developing countries (Brenchley, 2013; Estes et al., 2010). The HIV virus is mostly transmitted sexually through mucosal surfaces. In the GI tract, transmission may occur via the anorectal or possibly via the oral mucosa. Mother to child transmission occurs mainly via the oral route during breastfeeding. Even though breaks in mucosal surfaces can facilitate transmission, transmission has been shown to occur via intact mucosa (Brenchley & Douek, 2008. Rectal and colon mucosa are lined by a single epithelial layer making it easy for transmission. Anal mucosa has dendritic cells (DCs), which do not express CD4 or the chemokine receptor type 5 (CCR5). The mechanism of anal transmission is not well understood. Even though M cells are suggested to play a role in this transmission there is still no definitive study, which confirms this (Brenchley & Douek, 2008).

The HIV virus is recognized by CD4 receptors expressed on T cells and macrophage/monocyte lineage cells along with a co-receptor CCR5 or chemokine receptor type 4 (CXCR4)). CD4/CCR5 positive cells are highly expressed in the gut and are primary targets of the HIV virus (Brenchley & Douek, 2008). Meanwhile macrophage/monocyte lineage cells are important reservoirs contributing to latency.

Clinical manifestations of HIV in the GI include diarrhea, weight loss and GI ulcers (Bhaijee, Subramony, Tang, & Pepper, 2011). HIV infection is characterized by epithelial cell apoptosis and alterations in genes responsible for epithelial repair and regeneration and
an upregulation of genes associated with inflammation (Estes et al., 2010). Moreover, HIV infection is accompanied by increased depletion of Th17 cells that are responsible for enterocyte homeostasis and production of microbial defensins both of which are important in regulating intestinal mucosa. Alteration in homeostasis may also occur due to influx of cytotoxic T lymphocytes (CTL) (Brenchley et al., 2008; Brenchley, 2013; Klase et al., 2015). Recent studies have shown that there is dysbiosis of gut microbiota during HIV infection. This dysbiosis is strongly linked to bacterial translocation and local activation and inflammation (Ciesielczyk & Thor, 2013; Grenham, Clarke, Cryan, & Dinan, 2011; Klase et al., 2015; Vujkovic-Cvijin et al., 2013). HIV causes immune activation that results in structural changes in the intestinal mucosa causing intestinal epithelial breakdown. Natural hosts of simian immunodeficiency virus (SIV), the African green monkeys and Sooty mangabey show rapid loss of CD4 CCR5 cells but recover implying that initial loss in these cells alone is not sufficient to drive progression to AIDS (Brenchley et al., 2008). Immune activation has been shown to be one factor that contributes to disease progression. Even though the mechanism by which bacterial translocation causes immune activation is unclear, studies have shown strong associations between bacterial translocation and immune activation. (Shan & Siliciano, 2014). With the advent of combined antiretroviral therapy (cART), viral replication is significantly reduced, and patients are living longer, but individuals treated with cART still have a higher mortality rate than uninfected people. This higher mortality rate is associated with continued immune activation that may be due to bacterial translocation (Klatt, Funderburg, & Brenchley, 2013; Sandler et al., 2011a)
Figure 3. Schematic presentation of viral and microbial translocation across epithelial barrier. (1) Uninfected gut epithelium: gut lumen contents separated from gut wall. (2)–(3) HIV infection leads to loss in integrity of epithelial membrane. (4)–(5) Infiltration of HIV virus and gut bacteria into lamina propria and infection of lamina propria cells. (6) Release of Tat by infected cells. (7) Modulation of enteric neurons by Tat.
Lipopolysaccharide (LPS)

The rate of bacterial translocation from the gut lumen to extra intestinal organs can be assessed directly, by measuring the amount of bacterial products or indirectly by measuring the receptors or antibodies to which these products bind (Giulia Marchetti et al., Clin Microbial Rev., 2013). Lipopolysaccharide (LPS) is a component of the cell membrane of gram-negative bacteria that is frequently used to quantify bacterial translocation. It is recognized as a pathogen associated molecular pattern (PAMP) by Toll like receptor 4 (TLR4). LPS is made up of three molecules: A hydrophobic section (Lipid A), a hydrophilic core polysaccharide chain (composed of an inner and an outer core) and a hydrophilic O-antigenic oligosaccharide that is specific to the bacterial type. The Lipid A molecule is responsible for LPS’ biological effects. LPS interacts with several mammalian proteins in order to stimulate the immune system. These proteins include: LPS binding protein (LPB), CD14 and the MD2/TLR4 complex. LBP is a soluble protein, which binds to LPS and facilitates the binding of LPS to CD14. There are two types of CD14: Soluble (sCD14) and membrane bound (mCD14) CD14. CD14 facilitates the binding of LPS to the MD-2/TLR4 complex. Upon binding and recognition of LPS by TLR4, a MyD88 and/or TRIF dependent signaling cascade is activated which results in the release of pro-inflammatory cytokines and/or type I interferons respectively (Lu, Yeh, & Ohashi, 2008a; Plociennikowska, Hromada-Judycka, Borzecka, & Kwiatkowska, 2015a; Rhee, 2014). Also, a non-canonical pathway has been reported in which, cytosolic LPS is able to bind to murine caspase 11 or the human homolog, caspase-4/caspase-5 to induce pyroptosis and antibacterial responses to eliminate the invading pathogen. Excessive activation of this
pathway is thought to lead to septic shock (Hagar, Powell, Aachoui, Ernst, & Miao, 2013; Shi et al., 2014; Yang, Zhao, & Shao, 2015).
Figure 4. Schematic of the basic structure of lipopolysaccharide: LPS consists of three regions: from the bottom, lipid A, core sugars, and O-antigen, which consists of repeating units of oligosaccharides (Ogawa, T. 2007).
Figure 5. Activation of TLR4 by LPS. LBP binds to LPS facilitating its transfer to CD14. CD14 transfers LPS to TLR4/MD-2 complex. The TLR4 receptor oligomerizes and recruits downstream effectors by interacting with TIR domains. MyD88 associates with TIR domain recruiting IRAK, which is activated by phosphorylation. Activated IRAk associates with TRAF6 which signals to activate NF-κB leading to the production of cytokines. A TRIF-dependent pathway can also be activated leading to the internalization of TLR4 and ending up in regulating the production of type I interferons (Plociennikowska, Hromada-Judycka, Borzecka, & Kwiatkowska, 2015b).
**HIV-1 trans-activator of transcription (Tat)**

In addition to direct effects of the HIV virus on the gut, indirect injury occurs as a consequence of cellular and viral toxins released by infected cells. One such toxin is HIV-1 trans-activator of transcription (Tat). Tat is a 14 KDa protein that is one of the first proteins to be expressed after HIV infection and regulates the transcription of the HIV virus. It is an RNA binding protein that binds to trans-activator response elements (TAR) hairpin on the nascent HIV-1 RNA transcript. Upon binding it enhances the processivity of polymerase II and thus stimulates efficient elongation of the viral transcript allowing for full-length transcripts to be produced (Ott et al., 2004). The Tat protein is 86 to 102 amino acids long and is encoded by two exons. Exon 1 encodes the first 72 amino acids, which exhibits full trans-activating activity and contains an arginine-lysine-rich region (amino acid 49-57), which is important for nucleic acid binding of Tat. The other exon encodes the C terminal portion containing about 14 amino acids (73-86) which are not important for trans-activation but play an important role in binding of extracellular Tat to integrins. Intact infected cells have been shown to release Tat (Chang, Samaniego, Nair, Buonaguro, & Ensoli, 1997a). Serum concentrations of Tat are in the nanomolar range in HIV infected individuals even though this concentration is believed to be higher around infected cells due to the fact that Tat is adsorbed on cell surfaces (Goldstein, 1996; Urbinati et al., 2009; Westendorp et al., 1995; Xiao et al., 2000). Extracellular Tat can be taken up by other cells and have been shown to modulate cellular functions (Chang, Samaniego, Nair, Buonaguro, & Ensoli, 1997b; Liu et al., 2000; Ma & Nath, 1997; Sabatier et al., 1991).
Tat has previously been shown to contribute to GI pathogenesis of HIV. Canani et al (Canani et al., 2006) showed that Tat inhibited glucose uptake in enterocytes by SGLT-1 missorting and thus may contribute to diarrhea of unknown origin (Canani et al., 2006). Tat has also been shown to induce calcium-dependent chloride ion secretion in human colonic mucosa and in enterocytes, inhibit enterocyte proliferation and induce enterocyte apoptosis (Buccigrossi et al., 2011; Canani et al., 2003). To the best of our knowledge, this is the first study that assesses the effects of Tat on the enteric nervous system.
**Figure 6. Primary structure of HIV Tat protein.** Region I: Proline-rich region: 1 – 21 residues (red). Region II: Cysteine-rich region: residues 22 – 37 (green). Region III: Core region: residues 38 – 48 (blue). Region IV: Basic region: residues 49 – 59 (orange). Region V: Glutamine-rich region: residues 60 – 72 (purple). Regions I to V are encoded by the first exon. Region VI is encoded by the second exon (Johri, Mishra, Chhatbar, Unni, & Singh, 2011).
The enteric nervous system (ENS)

HIV is characterized by severe GI dysfunction including diarrhea, increased permeability and motility disorders. The GI processes that are affected are regulated by the enteric nervous system (ENS). The ENS consists of enteric neurons and glia arranged in two main ganglionated plexii: the myenteric plexus, found between the outer longitudinal and inner circular muscle and the sub mucosal plexus found between the circular muscle and the mucosa. The myenteric plexus forms a network of neurons that line the entire GI tract around the circumference though it may vary in size, shape and orientation from one part of the GI tract to another and between species. The submucosal plexus on the other hand only forms ganglionated plexuses in the small and large intestines and forms scattered ganglia in the stomach and esophagus, which are not ganglionated (Furness, 2012).

The ENS has been shown to play a role in regulating: blood flow, motility, digestion-by controlling gastric acid secretion, nutrient handling, absorption of fluids, and interacting with the immune and endocrine systems of the gut. (Furness, Oxford Press 2006). While the myenteric plexus is primarily involved with regulating GI motility, the submucosal plexus mostly functions in sensing the gut luminal environment, blood flow and gut epithelial integrity. Indeed, enteric neurons together with enteric glia cells (EGCs) have been shown to regulate gut epithelial membrane integrity and thus regulate inflammation by releasing substances that maintain gut epithelial wall integrity (Chandra et al., 2005; Neunlist et al., 2003; Savidge et al., 2007). Knockout of glia in mice led to significant increases in pro-inflammatory cytokines (Savidge et al., 2007).
Three broad classes of enteric neurons exist: sensory (intrinsic primary afferents (IPANs)), interneurons and motor neurons. Neurons can also be excitatory or inhibitory based on the neurotransmitter secreted by the neuron. The enteric nervous system functions autonomously even though functions such as digestion require input from the central nervous system (CNS). EGCs outnumber enteric neurons and the neuron to glia ratio varies across regions of the ENS and across species (Neunlist et al., 2014). For a long time, enteric glia were thought to mainly function as support cells to enteric neurons but recent studies have shown that enteric glia are involved in several gastrointestinal functions and are severely affected in several pathologies of the GI tract. EGCs when activated play a major role in antigen presentation and cytokine release (Ruhl, Nasser, & Sharkey, 2004).

Both EGCs and neurons have been shown to respond to inflammatory insults and to release cytokines (Coquenlorge et al., 2014; G. von Boyen & Steinkamp, 2010a; G. B. von Boyen et al., 2004). Not only does the ENS regulate inflammation but studies suggest that inflammatory and other insults can result in plasticity of the ENS (Barbara, Stanghellini, Cremon, De Giorgio, & Corinaldesi, 2008; Chaudhury, Shariff, Srinivas, & Sabherwal, 2004; Kamm, Hoppe, Breves, Schroder, & Schemann, 2004; Tonini et al., 2005).

Anderson et al (Anderson et al., 1990) reported intestinal ganglioneuritis in two children infected with HIV implying that the ENS may be involved in HIV pathogenesis. Other clinical case reports also supported widespread involvement of the ENS in HIV pathogenesis(Anderson et al., 1990; Batman, Miller, Sedgwick, & Griffin, 1991a; Griffin et al., 1988). Orandle et al (Orandle, Veazey, & Lackner, 2007) had similar observations in animal models of SIV. They reported that even though the ENS was not a direct target of
SIV, enteric ganglionitis was observed in SIV infected macaques suggesting that the ENS may be playing a role in SIV pathogenesis (Orandle et al., 2007). Despite all of these studies directly implicating the ENS in HIV pathogenesis, little is known about the mechanisms underlying HIV enteric neuropathogenesis.
Figure 7. Organization of the enteric nervous system. Adapted from Furness, 2007
Aims

The overall goal of this project was to study the effects of HIV-Tat on the ENS in order to broaden our knowledge of HIV neuropathogenesis which will help us find new targets for future HIV therapies.

The specific aims were:

Aim 1: To determine the effects of HIV-1 Tat on enteric neuronal excitability

Aim 2: To determine the interaction of Tat with bacterial proteins (the case of LPS) on the ENS

Aim 3: To determine the effects of Tat and it’s interaction with LPS on GI motility
Chapter II

Methodology

All experiments were conducted in accordance with the procedures reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University.

Isolation and culture of neurons from the adult mouse myenteric plexus

Cells were isolated as described recently (Smith, Ngwainmbi, Grider, Dewey, & Akbarali, 2013). After sacrificing mice, the ileum was removed and placed in ice-cold Krebs solution (in mM) (118 NaCl, 4.6 KCl, 1.3 NaH2PO4, 1.2 MgSO4, 25 NaHCO3, 11 glucose and 2.5 CaCl2) bubbled with carbogen (95% O2 / 5% CO2). The ileum was divided into short segments and threaded longitudinally on a plastic rod. Strips of the longitudinal muscle containing the myenteric plexus (LMMP) were gently separated using a cotton-tipped applicator. LMMP strips were rinsed three times in 1 ml Krebs and centrifuged (350 x g, 30 s). LMMP strips were then minced with scissors and digested in 1.3 mg/ml collagenase type II (Worthington) and 0.3 mg/ml bovine serum albumin in bubbled Krebs (37 °C) for 1 h, followed by 0.05% trypsin for 7 min. Following each digestion, cells were triturated and centrifuged (350 x g for 8 min). Cells were then plated on laminin (BD Biosciences) and poly-D-lysine coated coverslips in Neurobasal A media containing B-27 supplement, 1% fetal bovine serum, 10 ng/ml glial cell line-derived neurotrophic factor (GDNF, Neuromics, Edina, MN), and penicillin/streptomycin. Half of the cell media was replaced every 2-3 days with fresh neuron media.
All chemicals and reagents were obtained from Sigma Aldrich (St Louis, MO), unless otherwise noted, except cell culture reagents, which were purchased from Gibco (Grand Island, NY) and the HIV Tat\textsubscript{1-86} protein (Immunodiagnostics, Inc.). Male Swiss Webster mice (25-30 g, Harlan Sprague Dawley, Inc.) or male and female doxycycline (DOX)-inducible, HIV-Tat\textsubscript{1-86} transgenic mice (25-30 g) were used. The HIV-Tat\textsubscript{1-86} transgenic mouse model was developed on a C57BL/6J hybrid background and is described in detail elsewhere (Bruce-Keller et al., 2008). Briefly, the construct was generated using a tetracycline (tet) “on” system. Tat expression, which is under the control of a tetracycline responsive, glial fibrillary acidic protein (GFAP)-selective promoter, was induced with a specially formulated chow containing 6 mg/g DOX (Harlan, Product#: TD.09282, Indianapolis, IN), fed to both the Tat\textsuperscript{−} controls and the inducible Tat\textsuperscript{+} mice. The inducible Tat transgenic mice (Tat\textsuperscript{+} mice) used in this dissertation express both GFAP-RTTA and TRE-Tat genes, while control Tat\textsuperscript{−} mice express GFAP-RTTA but not the TRE-Tat gene. Mice were fed with doxycycline (DOX) containing chow for the specified times (3 days - 2 weeks) to induce the expression of the Tat gene. The 3 days – 2 weeks DOX exposure duration is based on findings from previously published studies that show increased astrocyte activation and an increased percentage of neurons expressing active caspase-3 in the striatum, as early as 2 days of exposure to DOX (Bruce-Keller et al., 2008)

**Immunocytochemistry**

Isolated cells cultured for the specified times (4 days - 2 weeks) on coverslips were fixed in 4% formaldehyde for 30 min. Cells were permeabilized with 0.01% Triton X-100 in PBS (30 min), and blocked with 10% goat serum (1 h). Preparations were incubated with
the primary antibody overnight at 4 °C. Primary antibodies used were as follows: neuronal specific anti-βIII-tubulin (rabbit, Abcam ab18207-100, 1:100), anti-GFAP (Santa cruz, 1:500) anti-Na,1.7 (mouse, Stressmarq, 1:300), Na,1.8 (mouse, Stressmarq, 1:300) and TLR4 (rabbit, Santa Cruz, 1:200). Following 3 washes in PBS, cells were incubated with the appropriate secondary antibodies; goat anti-rabbit Alexa 488 Dye (Molecular Probes, 1:1,000, 1 h, RT) and goat anti-mouse Alexa 594 Dye (Molecular Probes, 1:1,000, 1 h, RT). Visualization was performed on an Olympus Fluoview Confocal Microscope and software (v5.0).

**Whole-cell patch clamp**

Myenteric neuronal cells were studied from 1-4 days in culture. Coverslips containing cells were placed in an experimental chamber and perfused (1-2 ml/min) with an external physiological solution containing (in mM): 135 NaCl, 5.4 KCl, 0.3 NaH2PO4, 5 HEPES, 1 MgCl2, 2 CaCl2, and 5 glucose. Patch electrodes (2-4 MΩ) were pulled from borosilicate glass capillaries (Sutter Instruments, CA) and filled with internal solution containing (in mM): 100 K-aspartic acid, 30 KCl, 4.5 ATP, 1 MgCl2, 10 HEPES, and 0.1 EGTA. In experiments to measure sodium channels, K-aspartic acid was replaced by Cs-Aspartate to block any outward potassium currents. Whole-cell patch clamp recordings were made with an Axopatch 200B amplifier (Molecular Devices, CA) at room temperature and pulse generation and data acquisition were achieved with Clampex and Clampfit 10.2 software (Molecular Devices, CA). Neuronal excitability was determined in current clamp mode with current provided in 13 sweeps of 0.5 s duration ranging from –0.03 nA to 0.09 nA in 0.01 nA increments. Current-voltage relationships were determined
in voltage clamp mode, in 16 0.5 s-sweeps beginning at –100 mV and increasing in 10 mV intervals to 50 mV. Current amplitudes were normalized to cell capacitance (pF) to determine current density. The threshold of action potentials was determined as the voltage at which the d (V)/d (T) function deviated from zero. Action potential height was determined by measuring the threshold to the peak of the action potential. Voltage-dependence of steady-state inactivation and activation were determined and fit via Boltzmann’s distribution as described previously (Akbarali & Giles, 1993)

**In-cell western blot assay**

Analysis of Na\(_{\text{v}}\)1.7 and Na\(_{\text{v}}\)1.8 protein expression was performed by in-cell western blot assay. Enteric neurons were plated in 96 well plates. After three days in culture, cells were fixed (4% formaldehyde in 1 × PBS, 20 min at 25 °C), permeabilized (0.1% Triton in 1 × PBS, 4 washes for 5 min each at 25 °C with rotation) and blocked (0.5 × Odyssey Blocking Buffer), for 1 h at 25 °C with rotation. Primary antibodies: anti-Na\(_{\text{v}}\)1.7 (mouse, Stressmarq, 1:300) and Na\(_{\text{v}}\)1.8 (mouse, Stressmarq, 1:300) were diluted 1:100 in 0.5 × blocking buffer (50 µl per well) and allowed to incubate overnight at 4°C. Cells were then washed 3 × with 1 × PBS-Tween (0.1%) for 5 min at 25 °C with rotation, followed by the addition of secondary antibody: rabbit anti mouse, IRDye® 680RD and IRDye® 800RD, Licor Biosciences (diluted 1:200 in 0.5 × blocking buffer) for 1 h at 25 °C. Following three additional wash cycles, wells were aspirated and the plates were scanned using the Licor Odyssey Scanner (Licor Biosciences). Data were normalized to β-III tubulin loading controls and presented as mean ± SEM responses with reference to controls (100% standard).
Gastrointestinal motility studies:

Upper GI transit was determined by feeding age and weight matched tat transgenic mice with DOX chow for two weeks to activate the tat transgene after which they were fasted overnight. On the test day, mice were administered a charcoal gavage, 30 min later, they were sacrificed and the ileum removed carefully without artificially stretching the tissue. The total length and the leading edge of the charcoal meal were measured. Upper GI tract motility was determined by calculating % charcoal transit/ total ileum length. In a separate series of experiments, to avoid the interference of the DOX diet on GI effects, age matched mice were fed with DOX chow for 1 week after which the DOX chow was substituted for a regular chow for 3 weeks. This paradigm was used to avoid confounding effects of the loss of gastrointestinal microbiota by DOX treatment. PCR was performed to confirm expression of the tat gene in the ileum after 3 weeks of DOX removal. Gastric stasis was determined by dissecting out stomachs from age-matched Tat+ and Tat- mice 2 mm proximal to the lower esophageal sphincter and 2 mm distal to the pyloric sphincter. They were then immediately weighed and bathed in oxygenated physiologic Krebs solution. The stomach contents were emptied by flushing, blotted onto paper towel, and weighed again. The rate of gastric stasis was determined using the following equation (Asakawa et al., 2003; Welsh, Enomoto, Pan, Shifrin, & Belik, 2013).

\[
Net\ stomach\ content\ (gastric\ stasis) = \frac{Total\ stomach\ weight - empty\ stomach\ weight}{body\ weight}
\]
Overall GI transit was determined by counting the fecal pellet output. On test day (day 30), mice were placed in clean cages for 2 hours and the fecal pellets were collected and counted. (Anitha, Vijay-Kumar, Sitaraman, Gewirtz, & Srinivasan, 2012). The water content of the caecum was determined by dissecting out the caecum, collecting the caecum content, immediately weighing it, drying it by heating at 60°C for 1 hour and reweighing it. The water content was determined by the following equation:

\[
Caecum \text{ water content} = \frac{Wet \text{ mass} - dry \text{ mass}}{Wet \text{ mass}} \times 100
\]

**Cytokine release**

Cytokine release was measured by Enzyme-linked immunosorbent assay (ELISA) kits (ebioscience, an affymetrix company, San Diego, USA). Supernatants were obtained from mouse primary neuron/glia cultures or from the enteric glia cells (CRL-2690 Rattus rattus) (Rühl, A. 2001) after treatment with HIV-1 Tat, LPS, CPG or a combination of both at the indicated time points and concentrations. Some experiments were also performed on supernatant from cells treated with Tat overnight to detect the cytokines tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and the chemokine RANTES using ELISA (R&D Systems, Minneapolis, Minnesota, USA).

**RT-PCR**

RNA was extracted from the enteric glial cell line (CRL2690 (ATCC), a transformed EGC line isolated from adult rat myenteric plexus) using RNAqueous® Total RNA Isolation Kit (life technologies) or from mouse ileum or the LMMP using TRIZOL (Invitrogen,
Carlsbad, CA, USA) following manufacturer’s protocol. It was then quantified using spectrophotometer (BIORAD Smart Spec Plus). cDNA synthesis and subsequent polymerization was performed in a one-step using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad, Hercules, CA, USA). The reaction mixture (20 µl) contained 200 nM forward primer, 200 nM reverse primer, 1x iTaq universal SyBR Green reaction mix, 1x iScript reverse transcriptase, and 200 ng total RNA. Real time PCR was performed using a MiniOpticon Real-Time PCR System (Bio-Rad, Hercules, CA, USA). Specific primers for IL-1β, IL-6, TNF-a, IL-1β, Na_v1.3, Na_v1.7 Na_v1.8 and Na_v1.9 were used. 18 s RNA was used as the internal control. Experiments were performed in duplicates from three biological samples. The mean normalized fold expression ± SEM were plotted. The primers used are included in table 1.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat IL-6</td>
<td>5’-CCT GGAGTT TGT GAA CAA CT-3’</td>
<td>5’-GGA AGT TGG GGT AGG AAG GA-3’</td>
</tr>
<tr>
<td>Rat TNF-α</td>
<td>5’-TGT CGA TGC CTG AGT GGA T-3’</td>
<td>5’-AGG GAG GCC TGA GAC ATC TT-3’</td>
</tr>
<tr>
<td>Rat 18S</td>
<td>5’-GAT TAA AGT CCT ACG TGA TCT AAG TCC-3’</td>
<td>5’-TTC GTA ACT GGG AGA AAT TGT AAA-3’</td>
</tr>
<tr>
<td>Mouse Na(_v)1.7</td>
<td>5’-GCC TTG TTT CGG CTA ATG AC-3’</td>
<td>5’-TCC CAG AAA TAT CAC CAC GAC-3’</td>
</tr>
<tr>
<td>Mouse Na(_v)1.8</td>
<td>5’ GTG TGC ATG ACC CGA ACT GAT-3</td>
<td>5’-CAA AAC CCT CTT GCC AGT ATCT-3</td>
</tr>
<tr>
<td>Mouse 18S</td>
<td>5’-TCA AGA ACG AAA GTC GGA GG-3’</td>
<td>5’-GGA CAT CTA AGG GCA TCA C-3’</td>
</tr>
<tr>
<td>Mouse IL-6</td>
<td>5’-CTA AA GTC ACT TTG AGA TCT ACT C-3’</td>
<td>5’-TGT CCC AAC ATT CAT ATT GT-3’</td>
</tr>
<tr>
<td>Mouse TNF-α</td>
<td>5’-GTT GTA CCT TGT CTA CTC CC-3’</td>
<td>5’-GTA TAT GGG CTC ATA CCA GG-3’</td>
</tr>
<tr>
<td>Mouse IL-1β</td>
<td>5’-GTA CAA GGA GAA CCA AGC AA-3’</td>
<td>5-TGT TGA AGA CAA ACC GTT TT-3’</td>
</tr>
<tr>
<td>Rat MyD88</td>
<td>Santa Cruz MyD88 (r)-PR: sc-106986-PR</td>
<td>Santa Cruz MyD88 (r)-PR: sc-106986-PR</td>
</tr>
<tr>
<td>Mouse Na(_v)1.3</td>
<td>5’-TTG GCT CCA AAA AAC CTC AG-3’</td>
<td>5’-TTG AGA GAA TCA CCA CCA CA-3’</td>
</tr>
</tbody>
</table>

**Table 1. Primers used for RT-PCR experiments**
**MyD88 SiRNA Knock down**

To determine the role of MyD88 on Tat and LPS interactions, MyD88 SiRNA (MyD88 siRNA (r): sc-106986) was used to knock down MyD88 in the enteric glial cell line (CRL-2690) following the manufacturers protocol. Briefly, $2 \times 10^5$ cells were seeded in 6 well plates for 18-24 hours. At 60-80% confluence, cells were transfected with 2-8µl of the siRNA duplex for 6 hours after which 1mL of culture media containing 2 times FBS and penicillin/streptomycin was added and cultured for 18-24 hours. The transfection media was replaced with fresh culture media and cultured for 24 hours. MyD88 knock down was confirmed by RT-PCR using specific MyD88 primers. The effects of LPS and Tat on TNF-$\alpha$ and IL-6 were determined using RT-PCR.

**NF-$\kappa$B Assay**

To determine the role of NF-$\kappa$B on Tat and LPS interactions, CRL-2690 cells were treated with the indicated viral and/or bacterial protein for the indicated times (0.5, 1, 2, 4, 6, 16) h. When NF-$\kappa$B is activated, it translocates to the nucleus where it drives gene expression. The nuclear portions were extracted and nuclear/activated NF-$\kappa$B p65 transcript was quantified using NF-$\kappa$B p65 binding ELISA kit (Abcam). The amount of nuclear NF-$\kappa$B represents the amount of activated NF-$\kappa$B.

**Gastrointestinal Motility Monitor (GIMM)**

Colonic motility was measured using the Gastrointestinal Motility Monitor. We slightly modified the methodology reported by Barnes Kyra et al (Barnes, Beckett, Brookes, Sia, & Spencer, 2014). After euthanizing adult Tat transgenic mice, the colon was excised immediately and placed in an illuminated organ bath, continuously perfused with Krebs at
35.5 ± 0.5°C. The Krebs was constantly bubbled with carbogen gas (95% O2/5% CO2). The entire colon containing natural pellets was anchored with pins at the mesenteries of the anal and oral ends to prevent interfering with pellet movement and over-manipulation of the tissue. The remaining mesenteries were gently trimmed. Typically, it took ≤ 5 min from excising the colon until when the recording was started. The number of pellets expelled in 30 min was recorded and was used to determine the rate of colonic transit. The colonic transit rate was computed as the percentage of natural fecal pellets expelled during the recording time (30 min). The ileum was also excised starting immediately proximal to the caecum up to 8 cm proximal to the caecum. It was immediately put into a beaker containing warm Krebs at 35.5 ± 0.5°C. The contents were flushed with Krebs and then placed in an illuminated organ bath, continuously perfused with Krebs at 35.5 ± 0.5°C. The Krebs was constantly bubbled with carbogen gas (95% O2/5% CO2). The ileum was anchored with pins at the mesenteries of the proximal and distal ends to prevent over-manipulating the tissue. The remaining mesenteries were gently trimmed. The ileum was allowed to acclimatize for 5 minutes before the recording was made. Typically it took ≤ 10 minutes from excising the ileum till the recordings were started. Diameter readings were taken every 0.22 mm along the entire ileum segment every 0.0667 seconds for 300 seconds. Using a program designed on EXCEL VBA, diameter changes were determined by the following equation for each ileum segment for 5 min.

\[
\text{Diameter change} = \text{diameter at time (} t + 1 \text{)} - \text{Diameter at time (} t \text{)}
\]

The sum of diameter changes for the first 45 (1 cm) segments most proximal to the caecum was counted as the number of diameter changes of the proximal ileum. The sum of
diameter changes of the last 45 (1 cm) segments immediately proximal to the caecum was considered as the number of changes of the distal ileum.
Figure 8. Gastrointestinal Motility Monitor. Illuminated organ bath containing the tissue to be studied is continuously perfused with Krebs constantly bubbled with carbogen gas. Video recordings are taken with the help of a camera.
**Bacterial translocation**

Bacterial translocation was determined by culturing mesenteric lymph node (MLN), spleen and liver. After euthanizing the mice, MLN, spleen and liver were aseptically collected and weighed separately. They were then placed in separate grinding tubes and homogenized by adding 9 parts of PBS containing 0.1% tween 20 per gram of tissue (e.g. to 0.5 g of MLN add 4.5 mL of PBS containing 0.1% tween 20). 100 µl of homogenate was plated in LB agar. Plates were cultured for 48 hours under aerobic conditions. Colonization was quantified as the number of colony-forming units (CFUs) per milliliter of homogenate.

**Assessment of neuronal pathology.**

Pathology of enteric neurons was assessed by evaluating the morphology of neuronal projections using a Zeiss Axio Observer Z.1 inverted microscope (Carl Zeiss) with an automated, computer-controlled stage encoder with environmental control (37°C, 95% humidity, 5% CO2). Enteric neuron/glia cells were isolated from the ileum and colon and cultured in 12 well plates for 1 week. The cells were then treated with 100 nM Tat, 100 ng/mL LPS or a combination of both for 17 h. For each 12 well plate, six, non-overlapping fields were randomly selected using a 63× objective. Neurons with a rounded cell body and projections were selected from each field. Swellings on axons and dendrites were counted/ unit length by two people who were blinded to the treatment conditions. Repeated-measure analyses were performed on six neurons per treatment per experiment; at least three independent experiments for each treatment were conducted. The data presented is an average of the number of swellings/ unit length obtained by two people and presented as the mean ± SEM.
Chapter III

Aim 1: To determine the effects of HIV-1 Tat on enteric neuronal excitability

This chapter has been previously published elsewhere (Ngwainmbi J, De DD, Smith TH, El-Hage N, Fitting S, Kang M, Dewey WL, Hauser KF, Akbarali HI, J Neurosci. 2014)

Introduction

As described in the introduction, the GI tract harbors 80-90% of the lymphocytes of the body. It presents an important component of acquired immunodeficiency syndrome (AIDS) disease whereby, in addition to chronic diarrhea, alterations in structure and function occur as a result of the destruction of mucosal immunity, enhanced viral replication and prolonged inflammation (Handley et al., 2012; Lackner, Mohan, & Veazey, 2009; Mohan et al., 2012; Orandle et al., 2007; Veazey et al., 1998). In fact, the mucosal CD4+ T-cell destruction within the lamina propria is detectable at earlier times than in peripheral blood (Brenchley et al., 2004; Lim et al., 1993; Mattapallil et al., 2005; Mavigner et al., 2012; Smit-McBride, Mattapallil, McChesney, Ferrick, & Dandekar, 1998). AIDS progression is driven by significant damage to gut associated lymphoid tissue (GALT). Much of our understanding of neuropathogenesis in AIDS arises from work done in the brain while less is known of its effects on peripheral neurons (Cornblath & Hoke, 2006; Johnson et al., 2013a; Lehmann, Chen, Borzan, Mankowski, & Hoke, 2011; Pardo, McArthur, & Griffin, 2001; Simpson & Olney, 1992).
HIV transcription is mainly controlled by Tat. Tat is an early regulatory protein, 86–102 aa in length (14-16 kDa). It plays an important role in viral transcription, replication and has also been implicated in inducing the expression of a variety of cellular genes as well as acting as a neurotoxic protein. It is released by intact infected cells (Chang et al., 1997b). In the CNS, the principal cell types harboring active infection are perivascular macrophages and microglia, and to a lesser extent astroglia (Ellis, Langford, & Masliah, 2007; Gonzalez-Scarano & Martin-Garcia, 2005; Kaul, Garden, & Lipton, 2001; S. A. Yukl et al., 2013). Neurological defects persist even in HIV-infected individuals on combined antiretroviral therapy (cARTs) who are aviremic (McArthur, Steiner, Sacktor, & Nath, 2010). In the gut, as in the CNS, neuronal injury may be an indirect consequence of cellular and viral toxins released by infected cells. Once infected, limiting HIV replication alone may be inadequate to prevent neuronal impairment. With improved survival with cART, the manifestations of chronic exposure to low-levels of viral and cellular toxins associated with HIV-1 infection become increasingly evident—especially in the ileum (S. A. Yukl et al., 2013). Tat production from pre-integration HIV-1 DNA or during the early phase of transcription from integrated proviral DNA is largely unaffected by cART (Kilareski, Shah, Nonnemacher, & Wigdahl, 2009).

Despite clinical evidence of altered gastric motor function and enteric ganglionitis in simian immunodeficiency virus (SIV) infected macaques (Konturek, Fischer, van der Voort, & Domschke, 1997; Orandle et al., 2007), the effects of HIV-1 on the ENS have not been well studied. In this aim, we examined the effects of Tat on enteric neuronal excitability and inflammatory cytokine release.
Results

Tat increased enteric neuronal excitability

Enteric neuronal excitability was assessed using current clamp experiments. The rheobase for action potential generation, number of action potentials evoked and the threshold potential were studied as determinants of neuronal excitability. The average resting membrane potential of neurons was $-50.3 \pm 0.6$ mV ($n=65$) (Table 2). Current injection evoked single action potentials in all control cells and multiple action potentials at rheobase in some cells (6/19). However, when neurons were continuously perfused with 100 nM Tat for up to 30 min, neuronal cells became increasingly excitable with a reduced rheobase and multiple action potentials elicited at rheobase ($n=5$) (Figure 9A). In neuronal cells pretreated with Tat (100 nM) for 17 h- 48 h in culture, the current required to evoke an action potential (rheobase) and the threshold potential for the upstroke of the action potential were significantly lower than controls. Also, 19 of the 20 cells evoked multiple action potentials (Table 2). Increase in neuronal excitability could also be seen by the presence of spontaneous action potentials in 4 cells with 0 nA current injection (Figure 9B). There was no significant difference in resting membrane potential between Tat treated cells and controls (Table 2). To determine that this increase in neuronal excitability was a Tat specific effect, we treated neurons with heat inactivated Tat. These neurons were not significantly different from controls in terms of their excitability (Table 2). Next, we assessed the in vivo effects of Tat on enteric neuronal excitability. Neurons were isolated from Tat transgenic mice (Tat+) and control (Tat-) littermates expressing only the reverse tetracycline trans activator gene (Bruce-Keller et al., 2008). To confirm that Tat was
expressed in the ileum of these mice, RT-PCR experiments were performed on LMMP of both Tat+ and Tat- mice that were treated with DOX for 4 days. The tat gene was highly expressed in Tat+ mice (Figure 9C). Neurons isolated from Tat+ mice after 4 days of DOX treatment, fired multiple action potentials at significantly lower rheobase and threshold potentials than those isolated from Tat- mice (Figure 9D and Table 2). These data suggest that HIV-1 Tat either when perfused directly, treated overnight or exposed in vivo resulted in enhanced neuronal excitability.
Figure 9. Tat increased enteric neuronal excitability: Representative traces showing current clamp recordings of a neuron in the absence and in the presence of 100 nM Tat (A). Increased neuronal excitability in response to 100 nM Tat is evidenced by an increase in the number of action potentials evoked. Action potentials were initiated after 30 pA current injection in the absence of Tat and 10 pA after continuously perfusion with 100 nM Tat, n = 5. Spontaneous action potentials were recorded in 4 neurons post Tat exposure (B). An RT-PCR experiment showing that tat gene is expressed in Tat+ LMMP and absent in Tat- LMMP (C). Neurons isolated from Tat+ transgenic mice were also more excitable than Tat- mice (D). Grey line indicates response recorded at rheobase.
Table 2. Tat mediated increase in neuronal excitability is Tat specific and long lasting. Whole cell patch clamp experiments in current clamp mode on neurons pretreated with 100 nM Tat for 17 h to 48 h. These neurons fired a statistically significant higher number of multiple action potentials and at lower rheobase and action potential thresholds compared to controls. Neurons treated with heat inactivated Tat were less excitable compared to Tat pretreated and as excitable as controls (untreated neurons). Neurons isolated from Tat+ mice were also more excitable that Tat- mice neurons. Significance determined via paired t-test ($p < 0.05$).

<table>
<thead>
<tr>
<th></th>
<th>Cells with multiple APs/N</th>
<th>Em (mV)</th>
<th>Rheobase (pA)</th>
<th>AP threshold (mV)</th>
<th>AP height</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6/19</td>
<td>-50.6 ± 1.5</td>
<td>20 ± 5</td>
<td>-11.5 ± 2.7</td>
<td>77.7 ± 5.8</td>
</tr>
<tr>
<td>100 nM Tat</td>
<td>19/20*</td>
<td>-49.2 ± 1.2</td>
<td>10 ± 2*</td>
<td>-16.1 ± 1.2*</td>
<td>87.9 ± 3.8*</td>
</tr>
<tr>
<td>Inactivated</td>
<td>1/5</td>
<td>-50.2 ± 1.4</td>
<td>20 ± 6</td>
<td>-13.2 ± 1.4</td>
<td>71.7 ± 8.7</td>
</tr>
<tr>
<td>100 nM Tat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tat-</td>
<td>1/10</td>
<td>-48.9 ± 4.6</td>
<td>24 ± 3</td>
<td>-16.3 ± 1.3</td>
<td>51.9 ± 3.7</td>
</tr>
<tr>
<td>Tat+</td>
<td>11/11*</td>
<td>-52.5 ± 1.0</td>
<td>10 ± 0*</td>
<td>-21.0 ± 0.8*</td>
<td>71.8 ± 2.4*</td>
</tr>
</tbody>
</table>

Table 2. Tat mediated increase in neuronal excitability is Tat specific and long lasting. Whole cell patch clamp experiments in current clamp mode on neurons pretreated with 100 nM Tat for 17 h to 48 h. These neurons fired a statistically significant higher number of multiple action potentials and at lower rheobase and action potential thresholds compared to controls. Neurons treated with heat inactivated Tat were less excitable compared to Tat pretreated and as excitable as controls (untreated neurons). Neurons isolated from Tat+ mice were also more excitable that Tat- mice neurons. Significance determined via paired t-test ($p < 0.05$).
Tat produced a leftward shift of the Boltzmann’s activation curve of sodium channels

Sodium currents were measured in voltage clamp studies with cesium (Cs) in the pipette solution as reported by Smith et al (Smith, Grider, Dewey, & Akbarali, 2012). The current-voltage relationship of sodium currents was shifted to the left upon overnight Tat (100 nM) treatment (Figure 10A). There was no difference in the current density. In order to determine the effects of short-term exposure (17 h) of neurons to Tat on the voltage dependence of steady-state activation/ inactivation of sodium channels, we used a double-pulse protocol in which a variable conditioning pulse was applied from −100 mV to +50 mV in 10 mV increments, for 50 ms followed by a test pulse to 0 mV. For a conventional time- and voltage-dependent Hodgkin-Huxley conductance, the steady-state inactivation curve describes the relative number of available Na+ channels as a function of voltage. The resultant sigmoidal curve was fit to a Boltzmann distribution (Figure 10B). The $V_{0.5}$ of activation, the voltage at which half of the channels are activated, was $-29.08 \pm 0.6$ mV with a slope factor of $4.5 \pm 0.5$ mV for control neurons and $-38.5 \pm 0.8$ mV with a slope factor of $1.9 \pm 1.0$ mV for Tat treated neurons (Figure 10 B). There was a significant leftward shift in the activation curve of sodium channels indicating that channels were activated at more negative membrane potentials. Consistent with this, sodium currents activated at 10 mV more negative (-50 mV) in Tat (100 nM) pretreated cells ($t$-test, $p<0.05$) (Figure 10 A and C) compared to controls (-40 mV). There was no significant change in the inactivation curve upon treatment with Tat. The $V_{0.5}$ for inactivation was $-36.9 \pm 0.8$ mV for controls and $-39.5 \pm 0.1$ mV for Tat treated neurons (Figure 10 B and D).
Figure 10. Tat changes the steady state voltage dependence of activation of sodium channels. Na\(^+\) channel activation/inactivation were examined using whole cell patch clamp experiments in voltage clamp mode and using a double-pulse protocol in the presence and absence of 100 nM Tat and without Tat (control). Cs\(^+\) is present in the internal solution to block outward K\(^+\) current. Current density/voltage curve of controls (untreated cells) and Tat pretreated neurons (A) Boltzmann curve analysis of inactivation and activation of Na\(^+\) indicate a leftward shift of the activation curve in response to Tat (B), indicated by a significant difference in \(V_{0.5}\) of activation (C). No significant difference in \(V_{0.5}\) of inactivation (D). Significance is determined via t-test (\(p < 0.05\)).
Tat selectively modulates sodium channel isoforms

To date, nine mammalian pore forming alpha-subunit isoforms (Na_v1.1-Na_v1.9) of sodium channels have been cloned and functionally characterized with a tenth (Na_x) isoform appearing to be gated by sodium ion concentration. Na_v1.1, Na_v1.2, Na_v1.3 Na_v1.4 and Na_v1.7 are tetrodotoxin (TTX) - sensitive whereas Na_v1.5, Na_v1.8 and Na_v1.9 are TTX-resistant. In the presence of internal Cs^+, fast inward sodium currents were elicited positive to +40 mV from V_h-60 mV. Currents were markedly reduced in the presence of 1 µM TTX but not completely abolished. TTX-sensitive components were obtained by subtracting TTX resistant sodium currents from the total sodium currents (Figure 11A). The mRNA for Na_v1.3, Na_v1.7, Na_v1.8 and Na_v1.9 were examined by quantitative polymerase chain reaction (Q-PCR) of LMMP that were treated with Tat (100 nM) for 30 min or 2 days. There was no difference in Na_v1.7 and Na_v1.8 mRNA expression after 30 minutes of Tat treatment (Figure 11B,C). After 2 days treatment with Tat, the mRNA for Na_v1.7 and Na_v1.8 were significantly enhanced. Na_v1.3 and Na_v1.9 were not significantly different between Tat treated and controls (Figure 11D). These data suggest that Tat significantly increased transcription of sodium channel isoforms upon longer treatment and that Tat’s effect is isoform specific. Similar findings were seen in LMMP preparations of Tat transgenic mice (Figure 11E) where mice containing the tat gene, i.e. Tat+ mice expressed significantly higher Na_v1.7 and Na_v1.8 mRNAs compared to control transgenic mice (Tat-).
Figure 11. Tat transcriptionally modulates Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8. Representative raw traces of voltage-clamp experiments showing the presence of tetrodotoxin (TTX) sensitive and resistant sodium channels in neurons isolated from the adult mouse ileum (A) Q-PCR of LMMP preparation after 30 min Tat treatment. Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8 mRNA levels are not changed (B & C). After long term exposure (2-3 days) to Tat, Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8 mRNA levels are significantly increased but Na\textsubscript{v}1.3 and Na\textsubscript{v}1.9 levels are not affected by Tat pretreatment (D). Significantly higher Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8 mRNA are observed in Tat+ compared to Tat- LMMP (E). Significance determined via ANOVA followed by Bonferroni’s posthoc test ($p < 0.05$).
**Tat up regulates Naᵥ1.7 and Naᵥ1.8 protein levels in enteric neurons**

To examine the protein expression of these channels on enteric neurons, cells were grown on coverslips for 7 days to allow for significant neurite outgrowth. The expression of Naᵥ1.7 and Naᵥ1.8 were determined by immunohistochemistry. Naᵥ1.7 was predominantly expressed on the cell membranes of neuronal cell bodies whereas Naᵥ1.8 were mostly expressed on neuronal projections (Figure 12 A and 12B). To determine the effects of Tat on Naᵥ1.7 and Naᵥ1.8 protein levels, we performed in-cell western blot analyses. Enteric neurons were treated with 100 nM Tat for 3 days after which they were fixed with formaldehyde, permeabilized with 0.1% triton and exposed to Naᵥ1.7 and Naᵥ1.8 specific antibodies. These cells were co-stained with β-III tubulin as a loading control. There was a 15% and 58% increase in Naᵥ1.7 and Naᵥ1.8 protein levels respectively in Tat treated cells compared to controls (Fig 12 C- F).
Figure 12. **Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8 are expressed on enteric neurons and are up regulated by Tat:**

Representative Immunocytochemistry staining showing β-III tubulin (green), neuronal marker and in red Nav1.7 (A) and Nav1.8 (B), sodium channel isoforms. Sodium channel isoforms co-localize with neurons and are membrane bound. In-cell Western blot analysis of isolated enteric neurons, with specific antibodies detecting Nav1.7 and Nav1.8 β-III tubulin was used for normalization. Background wells were incubated with secondary antibody but no primary antibody (C, E).

The histogram represents the average of three sets of quantitative data, demonstrating the percentage induction of Nav1.7 and Nav1.8, respectively upon 72 h treatment with 100 nM Tat. Data were normalized against the β-tubulin signal as mean ± SEM (n = 3). * VS Control (p < 0.05). Analysis for Tat treated wells showed a 15.9% increase in Na\textsubscript{v}1.7, and a 58.4 % increase in Na\textsubscript{v}1.8 expression as compared to the control (D, F).
Tat up regulates sodium current density

The current–voltage relationship of sodium currents showed significantly higher sodium current densities in neurons isolated from Tat+ compared to those isolated from Tat- mice. This higher current density was associated with a loss of voltage-control evident in the current-voltage relationship (Figure 13A). Reducing extracellular Na⁺ was not sufficient to avoid the loss of voltage-control (Figure 13B). Adequate clamp was also not achievable in Tat+ neuronal cells that were in culture for only one day and mostly round in shape (to avoid axonal sprouting artifacts and provide for better spatial clamp), and using wider pipette tips (1.5 – 2 MΩ). This indicated that long-term exposure to Tat in vivo enhanced sodium channel expression making it difficult to adequately clamp these neurons.
Figure 13. Tat+ neurons had higher sodium current densities compared to Tat-. Current density/voltage curves of Tat+ and Tat- neurons at 140 mM (A) and 82 mM (B) bath solution NaCl concentration. Tat+ neuron had higher sodium current densities.
**Tat up regulates pro-inflammatory cytokines**

To determine Tat’s effect on inflammation in the GI tract, pro-inflammatory cytokines were measured in Tat treated and control (untreated) enteric neuronal cells. Cultured neurons/glia co-cultures were exposed to Tat for overnight after which, the supernatant was removed and analyzed by ELISA to quantitatively assess the release of the cytokines TNF-α, IL-6 and the chemokine RANTES. Tat significantly increased RANTES and IL-6 release ($p < 0.05$) but did not have an effect on TNF-α levels (Figure 14 A-C). Similarly, RANTES and IL-6 but not TNF-α expression were enhanced in the ileum of Tat+ ($p < 0.05$). In addition, Tat+ transgenic LMMP preparations had 3 fold higher IL-1β mRNA levels than Tat- (Figure 14D).
Figure 14. Tat selectively upregulates pro-inflammatory cytokines. ELISA assay, assessing pro-inflammatory cytokine release. Tat increases RANTES and IL-6 levels but has no effect on TNF-α levels (A-C). A RT-PCR showing increased levels of IL-1β (D). Significance determined via Student t-test.
Discussion of aim 1

The GI effects of HIV infection include alterations in structural integrity, diarrhea, and motility disorders. These effects are not mutually exclusive and may involve both direct effects of the HIV virus and its proteins and those due to opportunistic infections following breakdown of the epithelial barrier and immunosuppression within the lamina propria. Neuronal injury largely results from the effects of neurotoxic factors, including viral proteins Tat, gp120 and Nef (van Marle et al., 2004). The present study shows that Tat can directly affect enteric neurons. Tat exposure leads to increased neuronal excitability that occurred within 30 min. Two effects of Tat on sodium currents were observed that might account for enhanced excitability, 1) a shift in the activation kinetics of the sodium channel, and 2) enhanced expression of Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8. Tat also enhanced pro-inflammatory cytokine release. To our knowledge, this is the first study that evaluates the effects of Tat on enteric neuronal excitability and also highlights the role of sodium channel expression and kinetics in Tat mediated effects. The direct effects of Tat on neuronal excitability have been previously examined in human fetal brain cells, cultured rat cerebral cortex neurons and in dorsal root ganglia (DRG) neurons (Brailoiu, Brailoiu, Chang, & Dun, 2008). In fetal brain cells, Tat induced membrane potential depolarization that was likely due to activation of the non-selective cation channel. In DRG neurons, the increased excitability may partly result from inhibition of Cdk5, although concentrations of Tat used (20 µM) were much higher than used in our present study (100 nM). The 100 nM concentration was chosen from a range that elicited functional deficits in glia and neurons similar to those occurring in HIV-1, and that are considered to reflect levels seen under
pathological conditions (El-Hage et al., 2005; Nath, Conant, Chen, Scott, & Major, 1999; Perry et al., 2010; Singh et al., 2004). In enteric neurons, we found that increased excitability is seen within 15 min and remains when examined in Tat transgenic mice. However, the mechanisms may differ. After short term exposure (17 h) to Tat, the steady-state voltage dependence of activation of the sodium channel currents is shifted to the left, while at longer times (2 weeks DOX); there is an additional enhancement of sodium current density due to increased expression of Na\(_{v}1.7\) and Na\(_{v}1.8\). The increase in sodium current density resulted in significant loss of voltage control. We hypothesize that this is due to enhanced expression of Na\(_{v}1.8\) that appears to be localized on neuronal projections and thus contributes to the inability to adequately control the voltage (Cummins, Rush, Estacion, Dib-Hajj, & Waxman, 2009).

Voltage gated sodium channels play an important role in regulating neuronal excitability by producing the fast depolarization responsible for the upstroke of the action potential (Ekberg & Adams, 2006; Mantegazza, Curia, Biagini, Ragsdale, & Avoli, 2010). When neurons were exposed to Tat for a longer time (2 weeks DOX), sodium current densities in neurons isolated from Tat+ were significantly higher than those isolated from Tat- mice. These changes in sodium channel expression may have significant consequences in enhanced excitability following bacterial exposure as would occur in AIDS. More recently, Chiu et al. demonstrated that bacterial proteins activate Na\(_{v}1.8\)-expressing nociceptive neurons in the DRG, thus modulating pain sensation (Chiu et al., 2013). Bacterial proteins released following epithelial barrier breakdown may similarly affect intrinsic sensory
neurons within the ENS and thus define a potential site of interaction between HIV and opportunistic bacteria.

Another factor that has been shown to modulate enteric neuronal excitability is cytokines. IL-6 and IL-1β have previously been shown to increase enteric neuronal excitability (Xia et al., 1999). Experimental and clinical data show that GI inflammation is a serious and common problem in HIV infected individuals (Brenchley, 2013; Kotler, 2005). Higher levels of pro-inflammatory cytokines IL-6, IL-10 and IFN-γ have been reported in HIV patients (Kedzierska & Crowe, 2001). Studies in the CNS showed that Tat increased pro-inflammatory cytokine release by a glia-mediated mechanism (El-Hage, Bruce-Keller, Knapp, & Hauser, 2008). In this study, Tat selectively increased IL-6, IL-10, IL-1β and RANTES release, but had no significant effect on TNF-α levels. The mechanisms underlying Tat-mediated increases in pro-inflammatory cytokine release in the gut is not yet well understood. The increase in cytokine release may also be contributing to and/or exacerbating the increase in neuronal excitability observed in Tat exposed neurons.

Bacterial translocation and immune activation have been reported to underlie the local inflammation observed in HIV patients. The next set of studies were therefore undertaken to assess the role of bacterial proteins and their interaction with HIV-1 Tat on enteric neuronal pathogenesis in HIV AIDS.
Chapter IV

Aim 2: To determine the interaction of Tat with other bacterial products (the case of LPS) on the ENS

This chapter has been submitted for publication (Ngwainmbi; Brun P.; Kang M.; Dewey, W.L.; Hauser, K.F.; Akbarali, H.I. HIV-1 Tat sensitizes lipopolysaccharide-induced inflammatory cytokines via TLR4 signaling in enteric glia (in review))

Introduction

HIV infection is characterized by intestinal mucosal damage leading to increased translocation of bacteria and viruses into the gut wall thereby exposing other cells and organs to bacterial and viral proteins and predisposing to systemic immune activation (Fernandes et al., 2014; Klase et al., 2015; Marchetti, Tincati, & Silvestri, 2013; Vujkovic-Cvijin et al., 2013). Not only is initial HIV pathogenesis affected by the rate of gut bacterial translocation and inflammation, but clinical and experimental data also suggest that increased translocation correlates with progression to AIDS (Kristoff et al., 2014; Sandler et al., 2011b). Even though significant progress has been achieved at developing combined antiretroviral therapy (cART), which is potent, efficacious and has fewer side effects to patients, effective control of gut bacterial translocation still remains a significant problem in HIV patients. In healthy individuals, immune cells in the mesenteric lymph nodes and lamina propria phagocytize these microbes. However, in HIV patients, who have compromised immune systems, these microbes are able to migrate elsewhere to extra-intestinal organs and tissues. Several studies have suggested that microorganisms and microbial products from the lumen of the gut are responsible for immune activation and
inflammation (Sandler et al., 2011b; Vujkovic-Cvijin et al., 2013). One such microbial product that has been used as a tool to quantify the rate of bacterial translocation is lipopolysaccharide (LPS). LPS is a major component of the cell membrane of Gram-negative bacteria. It is recognized by toll like receptor 4 (TLR4), which is expressed on immune and other cell types. LPS triggers the secretion of pro-inflammatory cytokines and can additionally regulate intestinal homeostasis (Guijarro-Munoz, Compte, Alvarez-Cienfuegos, Alvarez-Vallina, & Sanz, 2014; Lu, Yeh, & Ohashi, 2008b).

In the cART era, the pathologic consequences of HIV continue despite diminished viral loads, suggesting that neuroinflammation and tissue injury may result from viral products independent of viral replication, including the viral protein Tat (Ipp, Zemlin, Erasmus, & Glashoff, 2014; Johnson et al., 2013b; Wu, 2004). For example, while the number of infected blood macrophages in HIV-1 infected patients is only moderately increased, recent studies have suggested that it is the reduced phagocytic activity induced by the viral protein, Tat, which likely underlies the development of opportunistic infections (Debaisieux et al., 2015). We have also recently shown that HIV-1 Tat profoundly affects the excitability of myenteric neurons, increases the levels of inflammatory mediators in the myenteric plexus and alters gastrointestinal motility (Ngwainmbi et al., 2014). Furthermore, HIV-1 Tat also sensitized enteric neurons to morphine (Fitting et al., 2015). In this aim, we sought to determine the interactive effects of Tat and LPS on enteric neurons and glia, and what role they plays in inflammation and GI disturbances that are commonly observed in HIV-infected patients. Most of HIV’s effects on neurons are believed to be indirect, i.e., the consequence of bystander effects of viral proteins and host
inflammatory responses (e.g., increased cytokines and oxyradicals) produced by infected immunocytes and glia. There is still no evidence of direct neuronal infection by HIV. We therefore studied the effects of HIV-1 Tat, an HIV regulatory protein secreted by intact infected cells, which has been shown to disrupt neuronal and glial function (Karn, 1999; Nath et al., 1996; Ngwainmbi et al., 2014) on LPS-mediated effects on ENS cells.

**Results**

**Tat sensitizes enteric neuron/glia co-cultures to LPS mediated increase in IL-6, TNF-α and IL-1β.**

To assess the *in vitro* effect of Tat and LPS on pro-inflammatory cytokine expression and release, we used; a) whole longitudinal muscle myenteric plexus (LMMP) preparations, b) primary cultures of neurons and glia isolated from the adult mouse ileum (Figure 15A) and c) the CRL-2690 enteric glia cell line.

We first determined the effect of Tat and LPS on mRNA levels and release of pro-inflammatory cytokines in the mouse ileum LMMP preparation. The LMMPs were treated with 100 nM Tat, 100 ng/mL LPS or a combination of both for 16 h. Neither Tat nor LPS increased TNF-α mRNA but when treated in combination, there was a significant (15 fold) upregulation of TNF-α mRNA levels (Figure 15B). 100 ng/mL LPS but not 100 nM Tat significantly increased TNF-α release. A combination of 100 nM Tat and 100 ng/mL LPS produced a further increase in TNF-α release (Figure 15C). Next, we assessed pro-inflammatory cytokine release in neuron/glia co-cultures isolated from the adult mouse ileum. The cells were treated with Tat (100 nM) and LPS (100 ng/mL) for 16 h. Both Tat and LPS increased IL-6 release and showed further enhanced response when combined
(Tat+LPS) (Figure 15D). Similarly, the Tat/LPS sensitized interaction was also observed when Il-1β was measured (Figure 15E). These data indicate that Tat sensitized the effects of LPS on the release and expression of pro-inflammatory cytokines within the myenteric plexus.
Figure 15. Tat and LPS interaction in neuron-co-cultures isolated from adult mouse ileum. A) Representative confocal microscopic images of enteric β-III tubulin (green) neuron specific marker and glial fibillary protein (GFAP- red) showing the presence of neurons and glia isolated from the adult mouse LMMP. B) mRNA expression of TNF-α of mouse LMMP treated with100 nM Tat, 100 ng/mL LPS or Tat+LPS for 16 h. C) TNF-α release from mouse LMMP treated with100 nM Tat, 100 ng/mL LPS or Tat+LPS for 16 h measured by ELISA D) IL-6 release of mouse neuron/glia co-cultures treated with 100 nM Tat, 100 ng/mL LPS or Tat+LPS for 16 h measured by ELISA. E) IL-1β release of mouse LMMP treated with100 nM Tat, 100 ng/mL LPS or Tat+LPS for 16 h measured by 16 h. n=3 P <0.05. One way ANOVA plus bonferroni’s post hoc test. *Vs CTL, # Vs Tat, ϕ Vs LPS.
Tat sensitizes enteric glia cells to LPS mediated increase in IL-6, TNF-α and IL-1β

To further determine the role of enteric glia in Tat/LPS-mediated increase in pro-inflammatory cytokines, we assessed the effects of Tat and LPS on the CRL-2690 enteric glia cell line. Enteric glia cells were treated with LPS, Tat or a combination of both at various concentrations (10, 100, 1000 ng/mL LPS) for 16 h. mRNA expression and cytokine release were measured. LPS dose-dependently increased IL-6 and TNF-α mRNA levels and this increase was drastically enhanced by co-treating the cells with 100 nM Tat. This sensitized response of enteric glia cells to LPS by Tat was most apparent at lower concentrations of LPS with no further combination effects observed at concentrations of 1 µg/mL LPS (Figure 16 A&B). Furthermore, even though neither 100 nM Tat nor 10 ng/mL LPS affected TNF-α release in enteric glia, there was a significant enhancement of TNF-α and IL-1β release by enteric glia cells when treated in combination with Tat and LPS (Figure 16 C&D).
Figure 16. Tat and LPS interaction in the CRL-2690 enteric glia cell line. A) Normalized fold IL-6 mRNA expression of CRL-2690 enteric glia after treatment with 100 nM Tat or 1, 10, 100 or 1000 ng/mL LPS or Tat+(1, 10, 100 or 1000 ng/mL) LPS for 16 h. B) Normalized fold TNF-α mRNA expression of CRL-2690 enteric glia after treatment with 100 nM Tat or 1, 10, 100 or 1000 ng/mL LPS or Tat+(1, 10, 100 or 1000 ng/mL) LPS for 16 h. C) Release of TNF-α of CRL-2690 enteric glia after treatment with 100 nM Tat, 10 ng/mL LPS or Tat+LPS for 16 h. D) Release of IL-6 of CRL-2690 enteric glia after treatment with 100 nM Tat, 10 ng/mL LPS or Tat+LPS for 16 h. n=3 P <0.05. One way ANOVA plus bonferroni’s post hoc test. *Vs CTL, # Vs Tat, φ Vs LPS.
**Ilea of Tat-expressing (Tat+) mice were more sensitive to LPS induced increase in pro-inflammatory cytokines than Tat- mice.**

To study the *in vivo* effect of Tat and LPS on pro-inflammatory cytokine release, Tat transgenic mice were administered a doxycycline diet for 1 week to induce Tat expression. The diet was replaced with regular chow for 2 weeks to allow for recolonization by gut microbiota and to avoid doxycycline-induced effects. We have previously shown that following, one week of a doxycycline diet, the Tat gene was expressed and remained expressed even after discontinuation of the diet for 3 weeks (Ngwainmbi et al., 2014). The mice were administered LPS (50 µg/mL) in their drinking water for 1 week. RT-PCR and ELISA were used to assess the ilea of these mice for the expression of pro-inflammatory cytokines. In the ilea of Tat+ mice, LPS produced a 2.8-fold increase in IL-1β mRNA compared to a 1.5 fold increase observed in Tat- mice (Figure 17A). Also, LPS produced a 7-fold increase in TNF-α mRNA, which was significantly higher than 1.9-fold increase in Tat- ilea. In ELISA experiments, LPS induced a higher increase of IL-1β protein levels in Tat+ ilea (3.1-fold increase) than in Tat- (2.3-fold increase) (Figure 17C).
Figure 17. Ileum of Tat-expressing (Tat+) mice were more sensitive to LPS induced increase in pro-inflammatory cytokines than Tat- mice. Normalized fold mRNA expression of TNF-α of ileum of Tat transgenic mice (A) or IL-1β (B) mRNA. LPS was administered at a dose of 50 µg/mL in drinking water for 1 week. Tat + mice were more sensitive to LPS induced increase in cytokine expression. C) IL-1β protein measured by ELISA. n=5-6. P<0.05 (students t test) *Vs Tat- or Tat+. 
**Tat and LPS but not Tat+LPS increased Na\(_{v}1.7\) and Na\(_{v}1.8\).**

In aim 1, we showed that Tat mediated increase in neuronal excitability and pro-inflammatory cytokine release was accompanied by a significant upregulation of Na\(_{v}1.7\) and Na\(_{v}1.8\) in enteric neurons. These sodium channel isoforms were expressed on neurons and not on enteric glia. To assess the effects of Tat and LPS on Na\(_{v}1.7\) and Na\(_{v}1.8\), we used whole LMMP. LMMP were treated with 100 nM Tat or 100 ng/mL or Tat+LPS. Both Tat and LPS increased Na\(_{v}1.7\) and Na\(_{v}1.8\) mRNA but a combination of both did not increase Na\(_{v}1.7\) or Na\(_{v}1.8\) any further (Figure 18 A&B). This suggests that the sensitization of LPS- induced increases in pro-inflammatory cytokine release and expression is not be mediated by a direct effect on these sodium channel isoforms.
Figure 18. Tat and LPS increased Na_\text{v}^{1.7} and Na_\text{v}^{1.8} but Tat + LPS did not further enhance Na_\text{v}^{1.7} or Na_\text{v}^{1.8}. A) Normalized fold mRNA expression of Na_\text{v}^{1.7} after treatment with 100 nM Tat, 100 ng/mL LPS or Tat+LPS for 16 h. B) Normalized fold mRNA expression of Na_\text{v}^{1.8} after treatment with 100 nM Tat, 100 ng/mL LPS or Tat+LPS for 16 h. n=3 P<0.05 (students t test) *Vs CTL.
**Tat LPS interaction is Specific and not mediated via TLR9.**

To further determine if the sensitization of pro-inflammatory cytokine expression and release in enteric glia is specific to Tat and LPS, we used CpG an unmethylated dinucleotide, which signals through TLR9 to increase pro-inflammatory cytokines. 100 ng/mL CpG significantly increased release of TNF-α in the CRL-2690 enteric glia cell line, but did not affect IL-1β levels (Figure 19 A&B). When treated in combination with Tat, there was no further increase in TNF-α or IL-1β (Figure 19 A&B). This indicates that the synergistic interaction of viral and bacterial proteins on enteric glia is not be mediated via TLR9.
Figure 19. Tat CpG interaction in the CRL-2690 enteric glia cell line. Normalized mRNA fold expression of IL-1β of CRL-2690 enteric glia (A) or TNF-α (B) after treatment with 100 nM Tat, 10 ng/mL CpG or Tat+CpG for 16 h. n=3 P<0.05(students t test) *Vs CTL.
**Tat LPS interaction is TLR4 mediated.**

Both enteric neurons and glia express TLR4 (Figure 20A&B). To determine the involvement of TLR4 in this enhanced response to LPS by Tat, primary neuron/glia cultures were isolated from TLR4 knockout mice. The primary cultures were treated with Tat and LPS for 16h. Neither 100 nM Tat nor 100 ng/mL LPS nor a combination of both (Tat+LPS) increased IL-1β levels (Figure 20C). The sensitization effect was blocked by knockout of TLR4. This indicates that TLR4 plays an important role in Tat-LPS interactions in the gut.

We then sought to determine the role of MyD88, an adaptor protein downstream of TLR4 signaling. Interestingly, in glia cells treated with siRNA for MyD88, the expression of TNF-α was significantly reduced in basal conditions. The effects of both Tat and LPS were blocked in cells with MyD88 siRNA (Figure 20D). The knock down of MyD88 also prevented the sensitizing effects of the Tat/LPS combination (Figure 20E&G).
**Figure 20. Tat LPS interaction is TLR4 mediated.**

A) Representative confocal microscopic images of enteric β-III tubulin (green) neuron specific marker and TLR4 (red) showing the expression of TLR4 on neurons. B) Representative confocal microscopic images of enteric GFAP (green) glia specific marker and TLR4 (red) showing the expression of TLR4 on enteric glia. C) ELISA showing release of IL-1β by neuron-glía co-cultures isolated from TLR4 knockout mice. Cells were treated with 100 nM Tat, 100 ng/mL LPS or Tat+LPS for 16 h. D) RT-PCR showing knock down of MyD88 in CRL-2690 enteric glia. E) RT-PCR of CRL-2690 enteric glia showing normalized fold expression of TNF-α (F) or IL-6 (G) after treatment with 100 nM Tat, 10 ng/mL LPS or Tat+LPS for 16 h.
We next determined if the sensitization between Tat and LPS could also be observed on NF-κB activation. We found that LPS time dependently activates NF-κB with the highest amount of activation occurring at 4 h LPS treatment (Figure 21A). 100 nM Tat did not significantly increase NF-κB activation; however it sensitized the effects of low concentrations of LPS in inducing NF-κB activation (Figure 21B).
Figure 21. NF-κB activation. A) Optical density reading showing time course of NF-κB activation in CRL-2690 enteric glia treated with 100 ng/mL LPS. Measured as the amount of translocation of NF-κB in the nucleus. n=3, P<0.05(students t test) *Vs CTL. B) Optical density reading showing NF-κB activation in CRL-2690 enteric glia treated with 100 nM Tat and/or (1, 10, 100, 1000) ng/mL LPS with Tat for 4 hours. n=3, P<0.05(two-way ANOVA) ** Vs LPS.
**Effects of Tat and LPS on neuronal pathology.**

Neuron/glia co-cultures were treated with Tat and LPS for 17 h after which the number of dendritic swellings was counted to assess neuronal pathology induced by Tat and LPS (Figure 22 & Figure 23). Both 100nM Tat and 100 ng/mL LPS significantly injured neurons causing significantly higher numbers of dendritic swelling than controls. There was no further increase when these cells were treated with a combination of 100 nM Tat+100 ng/mL LPS. This occurred in neurons isolated from both the ileum and the colon of adult mice.
Figure 22: Representative phase contrast microscopy images of neurons isolated from the adult mouse colon treated with Tat, LPS or Tat+LPS
Figure 23 Representative phase contrast microscopy images of neurons isolated from the adult mouse ileum treated with Tat, LPS or Tat+LPS
Figure 24: **Effects of Tat and LPS on dendritic pathology.** Number of dendritic swellings / unit length in the ileum (A) and colon (B) after indicated treatment for 17 h. mean ± SEM. P<0.05(one-way ANOVA plus Bonferroni's multiple comparisons test) * Vs CTL.
Discussion of aim 2

Microbial translocation in HIV patients is now recognized as an important contributor of HIV pathogenesis. In the present study we demonstrate that the viral protein, HIV-1 Tat sensitizes to LPS-mediated pro-inflammatory cytokine increase and that this increase in inflammation significantly disrupts gastrointestinal motility. We also show that both Tat and LPS significantly alter neuronal morphology leading to increased neuronal swelling. This interaction appears to be mediated via the TLR4 receptor. Our findings point towards a novel interaction between viral and bacterial proteins in the enteric nervous system.

Inflammation is known to directly correlate with the rate of viral replication and progression to AIDS in HIV patients (Kotler, Reka, & Clayton, 1993; Kotler, 2005; McGowan et al., 2004). The cells of the enteric nervous system regulate GI inflammation. Enteric neurons and glia have been shown to undergo structural and functional remodeling during GI inflammation (Brierley & Linden, 2014; Mawe, 2015; Venkataramana, Lourensssen, Miller, & Blennerhassett, 2015). In aim 1 we show that Tat directly modulates the function of enteric neuron/glia co-cultures leading to an increase in pro-inflammatory cytokine release (Ngwainmbi et al., 2014). Also, Tat sensitizes enteric neurons to morphine-mediated decrease in sodium current density (Fitting et al., 2015). In this study, we show that Tat sensitizes LPS-mediated increases in pro-inflammatory cytokines both in vitro and in vivo.

Enteric glia are a major component of the myenteric plexus. They have been implicated in the regulation of gut epithelial membrane integrity (Cheadle et al., 2013; Yu & Li, 2014), mucosal defense against gut bacteria (Matteoli, 2011) and GI inflammation (Cabarros,
Enhanced microbial translocation observed in Tat + mice may allow for potential increase in the inflammatory cytokines by stimulating cells that were not previously exposed to viral and bacterial products. The enhanced release of several pro-inflammatory cytokines by a combination of Tat and LPS in isolated myenteric neuron/glia co-cultures was confirmed in an enteric glia cell line and in the ilea of the Tat + transgenic mice.

Sodium channels have been shown to play an important role in inflammation and pain signaling (Gudes et al., 2015; Hockley et al., 2014). Recently, bacterial components such as α-haemolysin and N-formylated peptide have been shown to excite Na,1.8 expressing neurons and thus modulate inflammation and pain (Chiu et al., 2013). Also, LPS has been implicated in the modulation of sodium channels on microglia following inflammation (Jung, Lee, Rhim, Oh, & Yune, 2013). The majority of sodium channels found in the enteric nervous system are expressed on enteric neurons. Interestingly, in this study we show that even though both Tat or LPS significantly up-regulated Na,1.7 and Na,1.8, Tat+LPS did not further enhance the expression of Na,1.7 and Na,1.8. This might imply that this Tat mediated sensitization is not mediated by their action on sodium channels.

TLR4 is the main receptor for LPS even though recent studies have suggested that caspases may serve as a receptor for LPS when LPS concentrations are high in the cytoplasm (Hagar et al., 2013; Shi et al., 2014). When LPS binds to TLR4, a MyD88
and/or TRIF dependent pathway is activated (Lu et al., 2008b). Activation of the MyD88 dependent pathway leads to downstream activation of NF-κB, which drives the synthesis of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β (Lu et al., 2008b). Moreover, Tat has recently been shown to bind to the TLR4 receptor and to induce cytokine release in a TLR4 dependent manner (Ben Haij, Leghmari, Planes, Thieblemont, & Bahraoui, 2013). The enhancement in inflammatory cytokines observed after treating enteric neurons and/or glia with a combination of Tat and LPS in wild type mice, was absent in the TLR4 knockout mice, and when MyD88 was knocked down by siRNA. LPS concentration-dependently induced NF-κB activation and when treated in combination with Tat activated NF-κβ at a significantly higher level than each concentration. These data suggest that the interaction of Tat and LPS in the enteric nervous system appears to involve the TLR4 receptor. Tat did not further enhance CpG’s effect indicating that the sensitization effect does not involve modulation of TLR9. A possible mechanism may involve allosteric modulation of the TLR4 receptor by Tat, thus enhancing the effect of LPS.
Chapter V

Aim 3: To determine the effects of Tat and its interaction with LPS on GI motility.

Part of this chapter has been submitted for publication (Ngwainmbi, J.; Brun P.; Kang M.; Dewey, W.L.; Hauser, K.F.; Akbarali, H.I. HIV-1 Tat sensitizes to lipopolysaccharide-induced inflammatory cytokines via TLR4 signaling in enteric glia (in review))

Introduction

Motility disturbances have been reported in various regions of the GI tract in HIV patients. Fried RL et al (Fried, Brandt, Kauvar, & Simon, 1994; Zalar, Olmos, Piskorz, & Magnanini, 2003) and Zalar AE et al (Fried et al., 1994; Zalar et al., 2003) reported problems in esophageal motility in HIV patients. Moreover, delayed rates of stomach emptying (Neild et al., 2000) and abnormal small intestinal motility patterns (Batman, Miller, Sedgwick, & Griffin, 1991b) were reported in HIV patients. The mechanisms underlying this disturbed motility patterns in these patients are not yet well understood.

Increase in intestinal inflammation has been shown to profoundly disrupt intestinal motility patterns in various inflammatory diseases (Bassotti & Villanacci, 2012; Bassotti et al., 2014; De Winter, van den Wijngaard, & de Jonge, 2012; Ohama, Hori, & Ozaki, 2007). Mechanisms that have been put forward that could account for this abnormal motility include: changes in neuromuscular signaling, immune cells such as Mast cells and inflammatory cytokine trafficking (Bassotti & Villanacci, 2012; Bassotti et al., 2014; De Winter, van den Wijngaard, & de Jonge, 2012; Ohama, Hori, & Ozaki, 2007).

Furthermore, enteric glia cells have now been shown to not only act as neuronal support
cells but to contribute to GI motility. Studies by Qi R et al (Bassotti, Villanacci, Antonelli, Morelli, & Salerni, 2007; Qi, Yang, & Chen, 2013) showed that enteric glia cell morphology and numbers are affected during GI motility disorders. Enteric glia cells are now considered as major players in GI motility (Bassotti et al., 2007; Qi et al., 2013).

Our studies showed that Tat directly modulates enteric neurons and glia leading to an up-regulation of pro-inflammatory cytokines. In this aim, we sought to determine if these effects of Tat and its interaction with LPS on the enteric nervous system affects GI motility. In this aim, we studied the effects of Tat on gastric emptying rate (by studying stomach stasis), caecum water content, GI transit rate (by counting the number of fecal pellets), colonic transit rate and motility patterns of the mouse ileum using the GIMM.

Given that Tat and LPS significantly affected neurons and glia and increased pro-inflammatory cytokine levels, we hypothesized that GI motility will be significantly disturbed in mice expressing the Tat gene.
Results

Tat increased GI motility

To determine the effects of Tat on GI motility, upper GI tract motility was measured by calculating % charcoal transit/total ileum length as described in the methods section. The distance travelled in Tat+ mice was modestly but significantly greater than control Tat- mice ($p < 0.05$) (Figure 25A). These initial experiments were conducted on mice that were continuously fed with DOX for 2 weeks to induce the expression of the tat transgene. Continuous treatment with DOX reduces the number of certain types of gut microbiota. So, we decided to replace the DOX diet with regular CHOW in order to avoid the interference of the DOX diet used to induce Tat expression on GI effects and to allow for recolonization of the gut by gut bacteria. In the next set of experiments we used a treatment paradigm in which, age-matched mice were fed with DOX chow for 1 week after which the DOX chow was substituted for a regular chow for 3 weeks. PCR experiments showed that the tat gene expression was maintained after 3 weeks without DOX in Tat+ ileum and absent in Tat- ileum (Figure 25B). There was no significant difference in the rate of stomach stasis in Tat+ mice compared to Tat- mice (Figure 25C). On the other hand, Tat+ mice contained more fluids in their caecum than Tat- mice, which had significantly lower caecum water content than Tat+ mice (Figure 25D). Analysis of the GI transit rate showed that Tat+ mice had a faster stool output rates compared to Tat- mice (Figure 25E).
Figure 25. Tat increased GI motility in Tat transgenic mice. Tat+ mice showed higher upper GI transit rates compared to Tat- (A). GI transit was measured as distance travelled by charcoal meal. Q-PCR showing expression of Tat following 1 week DOX treatment followed by three weeks regular chow diet. Tat was expressed in Tat+ and absent in Tat- mouse ileum after three weeks without DOX (B). Gastric emptying showing no significant difference in stomach stasis rates between Tat+ and Tat- mice (C). caecum water content significantly higher in Tat+ mice than in Tat- (D). Number of stool pellets expelled was significantly higher in Tat+ than Tat- (E). Significance was determined via student T-test.
**Tat+ mice had higher rates of bacterial translocation than Tat- mice.**

To determine the role of Tat and increased pro-inflammatory cytokines on gut bacterial translocation, we assessed the rate of bacterial translocation to mesenteric lymph nodes (MLN), liver and spleen. After replacing the DOX diet with regular CHOW for 1 week, MLN, spleen and liver of both Tat+ and Tat- mice were cultured in aerobic conditions. We found that the MLN, spleen and liver of Tat+ mice had significantly higher bacterial colony forming units (CFU)/mL of tissue homogenates than Tat- controls (Figure 26A, B&C). This data shows significantly higher rates of gut bacterial translocation in Tat+ mice than in Tat- mice. The highest rate of translocation was to the MLN.
Figure 26. Bacterial translocation. Quantification of bacterial colony forming units in (A) MLN, (B) Spleen, (C) Liver. Data plotted as Mean ± SEM. n=3. P<0.05 (students t test) *Vs Tat-
**Tat+ mice were more sensitive to LPS mediated decrease in colonic motility than Tat- mice**

To further examine the functional aspects of the Tat/LPS interaction on GI motility, a video based gastrointestinal motility monitor (GIMM) system was used to generate spatiotemporal maps of the ileum and assess pellet expulsion in the colon.

Figure 27A and B show a typical recording of the spatiotemporal map of ileal contractions from Tat- and Tat+ mice. The spatiotemporal maps revealed significant high frequency, low amplitude contractions throughout the ileum. These contractions occurred across the entire length of the ileum at varying frequencies. The contractions, which we denoted as “ripples”, are similar to the findings in gastrointestinal tissue from embryonic rats reported by Roberts et al (Roberts et al., 2010).

To study the effects of Tat and LPS on these motor patterns in the ileum, the diameter of the ileum was read every 0.22 mm along the entire length of the ileum, every 0.067 sec. The frequency was measured over a 5 min period and divided between proximal and distal segments of the ileum. The proximal ileum of Tat+ mice demonstrated a greater frequency of diameter changes than Tat- mice (Figure 27C). This difference was not observed in the more distal segments of the ileum. In mice treated with LPS, Tat+ mice were more sensitive to LPS-mediated decreases in the number of diameter changes than Tat- mice in the proximal ileum (Figure 27C). In contrast, in the distal ileum, there was no difference in the rate of LPS mediated decrease in diameter changes in Tat- and Tat+ mice (Figure 27C).
We assessed the interactions between LPS and Tat on colonic propulsion. The number of natural colonic pellets expelled in 30 min was recorded and the data presented as percent colonic pellet expulsion rate. There was no difference in the number of pellets between the two groups when removed from the animal (Table 2). Tat- mice expelled 24.3% of their natural pellets, whereas Tat+ mice expelled 92.1% of the natural pellets. LPS treated mice had reduced pellet expulsion rates: 13.3 % in Tat- mice and 18.8% in Tat+ mice indicative of increased sensitivity of LPS in the Tat+ mice (Figure 27D &E).
Figure 27. GI effects of Tat and LPS interaction. Representative spatiotemporal maps of the mouse ileum with the proximal portion zoomed in, to show contraction and relaxation representative of segmented motor patterns in Tat-(A) and Tat+(B). C) Quantification of number of diameter changes in the proximal and distal ileum. LPS was administered at a dose of 50 µg/mL, in drinking water for 1 week. D) Quantification colonic transit measured as rate of expulsion of natural pellets in 30 min. E) Representative pictures showing the number of pellets expelled in Tat+ and Tat- mouse colon. n=5-6. P<0.05 (students t test) *Vs Tat- or Tat+.
There was no significant difference in colon length or number of fecal pellets in Tat+ and Tat- mice.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Length of Colon</th>
<th># of Pellets present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat-</td>
<td>5</td>
<td>5.3 ± 0.5</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>Tat+</td>
<td>5</td>
<td>4.8 ± 0.4</td>
<td>4.2 ± 0.7</td>
</tr>
<tr>
<td>Tat-/LPS</td>
<td>6</td>
<td>5.5 ± 0.4</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>Tat+/LPS</td>
<td>6</td>
<td>5.6 ± 0.4</td>
<td>4.8 ± 0.4</td>
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</tbody>
</table>
Discussion of aim 3

Both increases in neuronal excitability and pro-inflammatory cytokines have been reported to correlate with increased GI motility and to contribute to the diarrhea observed in many GI disorders. GI dysmotilities and diarrhea are persistent problems in the cART era (Mathur, Verma, Makwana, & Sinha, 2013; Mitra, Hernandez, Hernandez, & Siddiq, 2001). Upper GI transit was slightly but significantly higher in Tat+ mice maintained on DOX for two weeks. Since microbiota may affect GI motility, we examined gastric stasis, caecum water content and fecal water content after recolonization of colonic bacteria. Under these conditions, gastrointestinal transit was markedly enhanced. Further studies are required to determine if the increase in caecum water content was due to increased secretion or reduced reabsorption.

The enteric nervous system regulates various motor patterns in the gut. Two main motor patterns are observed in the small intestine. These include peristalsis, which is responsible for propelling the bolus and segmented contractions that mixes the bolus allowing time for digestion and absorption of nutrients (Huizinga et al., 2014). These motility patterns have been shown to be controlled by interaction of the ENS and interstitial cells of Cajal (ICCs) (Huizinga et al., 2011; Huizinga et al., 2014). Gut inflammation has been shown to cause remodeling of the enteric nervous system in various GI diseases (Mawe, Strong, & Sharkey, 2009). We hypothesized that the increase in cytokine release observed will remodel cells of the enteric nervous system and thus affect GI motility patterns. Here, we studied the effects of Tat and LPS on segmented motor activities in the mouse ileum of Tat transgenic mice. We found that Tat+ mice were more sensitive to LPS mediated decreases
in the number of changes in ileum diameter. The proximal ileum was the most sensitive. Colonic transit rates were also significantly increased in the Tat+ mice. In the presence of LPS, the colonic transit time in Tat + mice was significantly reduced compared to the Tat – mice.

These findings suggest that Tat and its interaction with LPS directly modulate enteric neurons and glia, leading to a significant up-regulation of inflammatory cytokines. This up-regulation of inflammatory cytokines together with Tat’s direct effect on these cells significantly modulate GI transit rate and GI motility patterns. Further studies are required to tease apart the role played by each of these players in the GI dysmotility phenotype observed in Tat+ mice.
Chapter VI

Conclusions and perspectives

Taken together, these studies have analyzed the effects of Tat at a single neuronal level and showed that Tat increases enteric neuronal excitability by modulating sodium channels. The increase in neuronal excitability together with an increase in pro-inflammatory cytokine release could account for the increase in GI motility observed in Tat+ mice. These findings correlate with an increase in GI motility and diarrhea observed in HIV infected individuals and explain a possible mechanism by which this may be occurring. The brain-gut axis has also gained recent interest with the findings that many CNS diseases may have an ENS component. For example, GI disturbances and the presence of Lewy bodies in enteric neurons of Parkinson disease patients occur decades prior to advent of motor symptoms (Pan-Montojo et al., 2012). The ileum, in particular, is one of the earliest sites of infection (Levesque et al., 2009) and displays a higher proportion of HIV-infected cells than other regions of the GI tract (S. Yukl & Wong, 2008).

The adult human gut contains about 100 trillion microorganisms that constitute the gut microbiome (Ley, Peterson, & Gordon, 2006). These organisms contribute to gut function by maintaining the gut epithelial wall integrity, fighting against other pathogenic microorganisms, providing metabolic support for the host and mediating inflammatory responses. During HIV infections, there is dysbiosis – down regulation of gut bacteria that are linked to anti-inflammatory effects (Bacteroides) and up-regulation of those that have been shown to be linked to inflammatory states in the GI (Proteobacteria) (Lozupone et al., 2013; Nwosu, Avershina, Wilson, & Rudi, 2014; Vujkovic-Cvijin et al., 2013). This
dysbiosis is also accompanied by increased bacterial translocation. Bacterial and viral proteins pass through the gut wall and can translocate to the general circulation. In addition, other cells of the gastrointestinal tract that were not normally exposed to HIV, viral proteins, bacteria and bacterial proteins can now be exposed to these. In this study, we showed that the HIV protein, Tat, enhanced bacterial translocation to the mesenteric lymph node, spleen and liver. We also showed that Tat and LPS significantly induced the release of pro-inflammatory cytokines in enteric neurons and glia cells. This significant upregulation of pro-inflammatory cytokines was accompanied by profound disruption of GI motility. GI transit rate was significantly faster in mice that expressed the tat transgene than in age and weight-matched controls. Tat modulated the ilea and colon of Tat transgenic mice making them more sensitive to LPS’s effect. Further studies are needed to shed more light on the mechanisms underlying the effects of Tat and LPS on GI motility. Nevertheless, these studies have elucidated a mechanism for the interaction of Tat and LPS on the enteric nervous system leading to the upregulation of pro-inflammatory cytokine release. This mechanism involves the TLR4 receptor and appears to occur via a MyD88-dependent pathway. Further structural and mechanistic studies will enable us to determine if the binding of Tat to the TLR4/MD2 complex modulates the receptor making it more sensitive to LPS-mediated effects on pro-inflammatory cytokine.

Increase in pro-inflammatory cytokines has been shown to further affect gut epithelial membrane integrity. This may lead to further enhancement of bacterial translocation. Since patients who are on cART treatment have reduced rates of viral replication, it is believed that the ongoing inflammation and immune activation may be primarily due to bacterial
translocation. This persistent inflammation in these patients may lead to further complications such as atherosclerosis, neurodegenerative disorders and other end organ complications.

These studies highlight the role of HIV viral and bacterial proteins in HIV pathogenesis in the gastrointestinal tract and also demonstrate a critical role of the enteric nervous system in HIV pathogenesis.
References


Bassotti, G., & Villanacci, V. (2012). Mast cells in intestinal motility disorders: Please also look beyond IBS... *Digestive Diseases and Sciences, 57*(9), 2475-6; author reply 2476. doi:10.1007/s10620-012-2303-4; 10.1007/s10620-012-2303-4


simian immunodeficiency virus infections. *PLoS Pathogens, 6*(8), e1001052.
doi:10.1371/journal.ppat.1001052; 10.1371/journal.ppat.1001052


Proceedings of the National Academy of Sciences of the United States of America, 110(33), 13588-13593. doi:10.1073/pnas.1308673110 [doi]


doi:00126334-200410010-00002 [pii]


doi:10.1371/journal.pone.0034561; 10.1371/journal.pone.0034561


Yang, J., Zhao, Y., & Shao, F. (2015). Non-canonical activation of inflammatory caspases by cytosolic LPS in innate immunity. *Current Opinion in Immunology, 32C*, 78-83. doi:S0952-7915(15)00008-4


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• Travel award : American Society of Pharmacology and experimental therapeutics for Experimental Biology, 2014 and 2015
• Finalist : Young Investigator Award EB 2014 and 2015
• Honorable mention: graduate student presentations at EB 2014

Manuscripts accepted for publication


• Ngwainmbi,J.; Brun P.; Kang M.; Dewey,W.L.; Hauser,K.F.; Akbarali,H.I. HIV-1 Tat sensitizes to lipopolysaccharide-induced inflammatory cytokines via TLR4 signaling in enteric glia (in review)

• Dipanjana Datta De, Sukhada Bhave, Joy Ngwanmbi, Aravind Gade, Hamid Akbarali, Willam Dewey Morphine differentially down regulates µ-opioid receptors in Colon and Ileum (in preparation).

Meetings where seminar or results were presented
Posters:

Talks:
• Effects of HIV-1 Tat protein on enteric neuropathogenesis, Integrative Systems, Translational and Clinical Pharmacology Division Young Investigator Awards Platform Session. Experimental biology, 2014
• “Surviving the First Year of Grad School” PREP/IMSD Mid-Atlantic, North Carolina, May 2014
• HIV-1 Tat sensitizes enteric neurons to bacterial proteins. Integrative Systems, Translational and Clinical Pharmacology Division Young Investigator Awards Platform Session. Experimental Biology, Boston, March 2015

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