# University of Alberta

HIV-1 Vpr mediates neuropathogenesis through loss of neurons and astrocytes

by

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**ABSTRACT**

The HIV-1 accessory protein, viral protein R (Vpr), has been shown previously to mediate the loss of neurons and astrocytes, which contribute to HIV-1 associated dementia (HAD). The aim of the current thesis was to characterize the mechanisms by which Vpr mediates neurodegeneration. Using a monocytoid cell-specific transgenic Vpr (Tg-Vpr) model under the *c-fms* promoter, Vpr was shown to mediate IL-6 suppression ($p<0.05$), cause subcortical synaptic injury ($p<0.001$) loss of excitatory (VAChT, $p<0.001$) and inhibitory (GAD65, $p<0.01$) neurons and activation of caspase-3. Moreover, a decrease in cortical GFAP immunopositive astrocytes ($p<0.05$) as well as a decreased expression of *insulin-like growth factor-1* ($p<0.001$) were observed in association with fine motor skill neurobehavioural abnormalities ($p<0.05$). Soluble Vpr caused a pro-apoptotic effect in human astrocytes detected as mitochondrial potential loss ($p<0.001$) and activation of caspase-3. These findings emphasize the neuropathogenicity of HIV-1 Vpr, providing a potential target for therapy development.

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"Character cannot be developed in ease and quiet. Only through experience of trial and suffering can the soul be strengthened, ambition inspired, and success achieved."
*Helen Keller (1880-1968)*

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka; (I found it!) but 'That's funny …'"
*Isaac Asimov (1920-1992)*

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CHAPTER 1- INTRODUCTION



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## LIST OF SYMBOLS, NOMENCLATURE, ABBREVIATIONS

| | |
|---|---|
| 7AAD | 7-amino-actinomycin D |
| AIDS | acquired immunodeficiency syndrome |
| AIF | apoptosis inducing factor |
| Akt/PKB | protein kinase B |
| ANT | adenine nucleotide translocator |
| Apaf-1 | apoptotic protease-activating factor-1 |
| APOBEC3G | apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G |
| AQP4 | aquaporin 4 |
| ASK1 | apoptosis signal-regulating kinase |
| ATP | adenosine triphosphate |
| ATR | ataxia telangiectasia and Rad3 related |
| AVE | average |
| BBB | blood-brain barrier |
| Bax | Bcl-2 associated x protein |
| Bcl-2 | B-cell lymphoma protein 2 |
| BDNF | brain derived neurotrophic factor |
| BG | basal ganglia |
| (t-)Bid | (truncated) Bcl-2 like inhibitor of death |
| BIV | bovine immunodeficiency virus |
| BMDM | bone marrow derived macrophages |
| CAEV | caprine arthritis encephalitis virus |
| cART | combination antiretroviral therapy |

| | |
|---|---|
| CC | corpus collosum |
| C-Casp-3 | cleaved caspase-3 |
| CNS | central nervous system |
| CSF | cerebral spinal fluid |
| CTL | cytotoxic T lymphocytes |
| CTX | cortex |
| DEPC | diethylpyrocarbonate |
| DNA | deoxyribonucleic acid |
| DDB1 | damage specific DNA-binding protein 1 |
| DSP | distal sensory polyneuropathy |
| EIAV | equine infectious anemia virus |
| ERM | ezrin/radixin/moesin |
| ESCRT-I | endosomal sorting complex required for transport |
| FIV | feline immunodeficiency virus |
| GABA | gamma aminobutyric acid |
| GAD65 | glutamic acid decarboxylase 65 |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GFAP | glial fibrillary acidic protein |
| HAD | HIV associated dementia |
| HB | hindbrain |
| HFA | human fetal astrocytes |
| HFN | human fetal neurons |
| HIV-1 | human immunodeficiency virus type 1 |

| | |
|---|---|
| HIV-2 | human immunodeficiency virus type 2 |
| HIVE | HIV encephalopathy |
| IBTX | iberiotoxin |
| ICAD | inhibitor of caspase-activated DNase |
| IGF-1 | insulin like growth factor-1 |
| IκB | inhibitor of kappa B |
| iNOS | inducible nitric oxide synthase |
| IP-10/CXCL10 | interferon gamma inducible protein |
| LAV | lymphadenopathy associated virus |
| LPS | lipopolysaccharide |
| LTNP | long term non-progressors |
| LTR | long terminal repeats |
| MCMD | minor cognitive motor disorder |
| MCP-1 | monocyte chemoattractant protein-1 |
| M-CSF | macrophage-colony stimulating factor |
| MDM2 | murine double minute 2 |
| MVB | multivesicular bodies |
| MVV | maedi visna virus |
| ND | non-demented |
| Nef | negative regulator factor |
| NFκB | nuclear factor-kappa B |
| NFAT | nuclear factor of activated T cells |
| NGF | nerve growth factor |

| | |
|---|---|
| NHE1 | sodium/hydrogen exchanger |
| NMDA | N-methyl-D-aspartic acid |
| PARP-1 | poly(ADP-1 ribose) polymerase-1 |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffer solution |
| PI3K | phosphoinositide-3 kinase |
| PIC | pre-integration complex |
| PMA | phorbol 12-myristate 13 acetate |
| PTPC | permeability transition pore complex |
| PTX | pertusis toxin |
| Puma | p53 upregulated modulator of apoptosis |
| PVR | periventricular region |
| R5 | CCR5 tropic |
| RA | retinoic acid |
| Rev | regulator of expression of the virion |
| RFN | rat fetal neurons |
| RNA | ribonucleic acid |
| RTI | reverse transcriptase inhibitors |
| RT PCR | real-time PCR |
| SEM | standard error of the mean |
| SIV | simian immunodeficiency virus |
| SMUG1 | single-strand selective monofunctional uracil DNA glycosylase |
| S/N | supernatant |

| | |
|---|---|
| STDEV | standard deviation |
| Synap | synaptophysin |
| Tat | transactivator of transcription or tyrosine aminotransferase |
| TCR | T cell receptor |
| TFIIB | transcription factor II B |
| Tg-Vpr | transgenic Vpr |
| TMRE | tetramethylrhodamine ethyl ester |
| TNF-α | tumor necrosis factor alpha |
| TUNEL | terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling |
| UNG | uracil DNA glycosylase |
| VAChT | vesicular acetylcholine transferase |
| VDAC | voltage dependent anion channel |
| VEGF | vascular endothelial growth factor |
| Vif | virus infectivity factor |
| Vpr | viral protein R |
| Vpu | viral protein U |
| Wt | wildtype |
| X4 | CXCR4 tropic |
| Δ | deletion |

# CHAPTER 1- INTRODUCTION

## 1.1 HIV

### 1.1.1 DISCOVERY OF HUMAN IMMUNODEFICIENCY VIRUS

In the late 1970's and early 1980's a new emergent disease, later described as acquired immunodeficiency syndrome (AIDS), came to light as cases of immunocompromised patients (mostly gay men and drug abusers) appeared (112, 205, 293). Cases were grouped based on the symptoms of initial fever, subsequent weight loss and later having unusually low $CD4^+$ T-lymphocyte count, as well as opportunistic infections from fungi, bacteria, viruses and parasites (252, 296, 305). An infectious and cytopathic agent was isolated and initially named lymphadenopathy associated virus (LAV), as it was isolated from the peripheral blood mononuclear cells (PBMCs) of a patient with lymphadenopathy, another sign of the disease (23). Also identified by other laboratories (99), this new emergent virus later became known as human immunodeficiency virus type 1 (HIV-1) for its immune dysfunction capabilities (58). Another AIDS causing virus called human immunodeficiency virus type 2 (HIV-2) was also isolated a few years later in West Africa. There is, however, only about 60% sequence homology between HIV-1 and HIV-2 (54).

### 1.1.2 DISEASE PROGRESSION AND THE CLINICAL DEFINITION OF AIDS

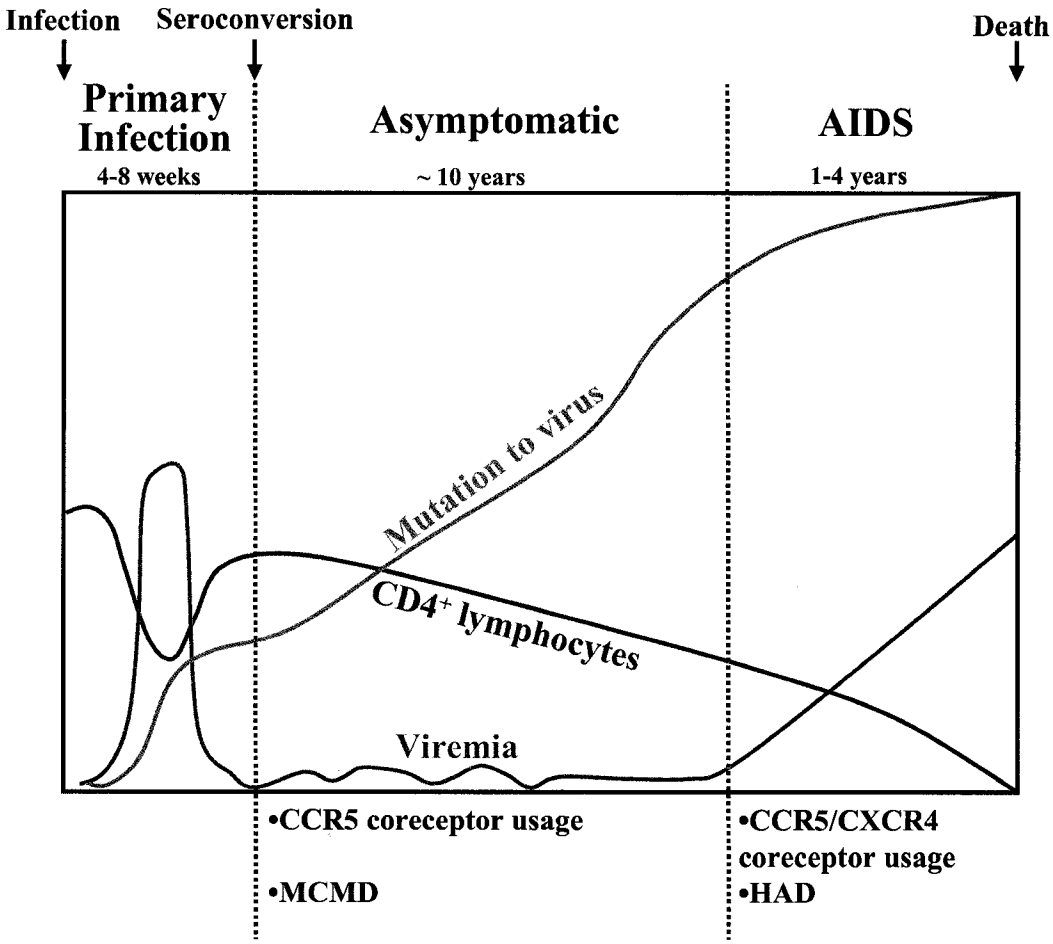
Once a person is infected with HIV-1, the disease caused by the virus is progressive, as shown in **Figure 1.1**, and leads to the final stage of AIDS and eventually to death (reviewed in(328)). Between weeks 4-8 post infection, the patient will go through acute HIV-1 syndrome, during which there is rapid viral dissemination

throughout the organs of the body, inducing a large immune response causing symptoms of fever (67, 124). During this time there is a significant decrease in $CD4^+$ T-lymphocyte cells while there is an increase in viral RNA load (186, 191). Although there is a decrease in $CD4^+$ T cells, a robust adaptive response still exists where antibody seroconversion occurs at the end of this first phase and cellular immunity by $CD8^+$ T cells is induced (30, 164). The second phase of HIV-1 infection is called clinical latency, characterized by a restricted viral replication and the absence of symptoms; however, the number of $CD4^+$ T-lymphocyte cells steadily decreases (213). The duration of this phase varies between individuals depending on the infecting strain and the patient's immune system and susceptibility including initial health, nutrition, age, drug use and efficacy of the current therapy (191, 280, 311). The final phase of HIV-1 infection is the progression to AIDS denoted by a number of properties and symptoms. According to the World Health Organization, the clinical definition of AIDS is declared in a seropositive patient if (1) the patient's $CD4^+$ T-lymphocyte count is less than 200 cells/μl of blood, (2) the patient has a potentially fatal opportunistic infections from viruses, bacteria, fungi and parasites, (3) neoplasm or (4) HIV-1 associated dementia (HAD) (342).

### 1.1.3 EPIDEMIOLOGY

Today 40.3 million people are living with HIV-1 infection around the world; 4.9 million of which were infected in 2005 alone and 25 million deaths due to AIDS have been recorded to date. As illustrated by **Figure 1.2**, sub-Saharan African countries are the most affected by the virus as they comprise two-thirds of the total HIV-1 infected

**FIGURE 1.1. THE TEMPORAL INTERRELATIONSHIPS BETWEEN HIV-1 INFECTION, DISEASE AND HOST PROPERTIES.** Stages of HIV-1 disease: primary infection, asymptomatic or clinical latency and AIDS leading to death. Primary infection with HIV-1 includes a sharp increase in viremia and decrease in $CD4^+$ lymphocytes followed by a transient reversal of this effect in the asymptomatic phase; however viremia increases and $CD4^+$ lymphocytes gradually decrease into the last stage of AIDS (186, 191, 213, 342). Progression to AIDS is associated with HIV-1 co-receptor preference from CCR5 to CXCR4/CCR5 associating with increased pathogenicity (15, 49). Some individuals acquire neurological diseases in the later stages of disease. Minor cognitive motor disorder (MCMD) can progress to HIV-1 associated dementia (HAD) (reviewed in (328)). Figure is adapted from (328).



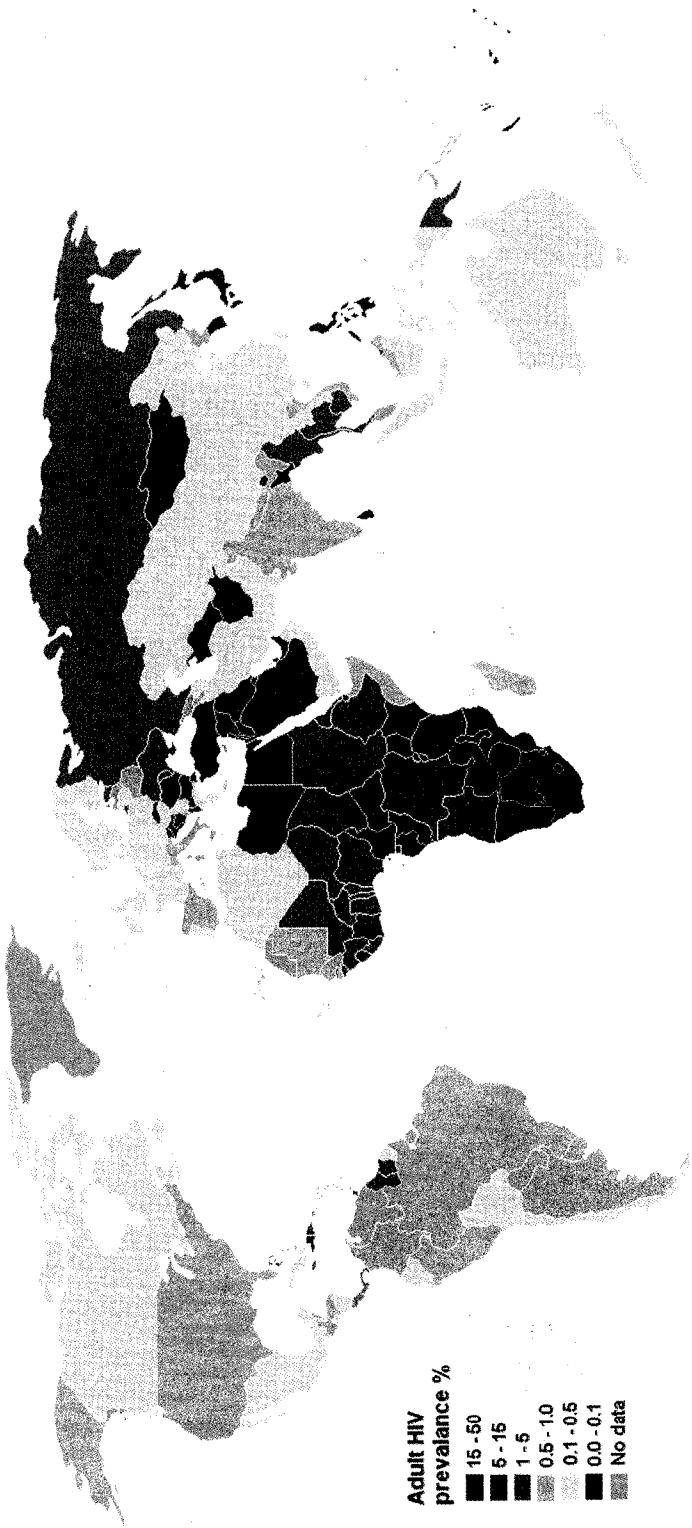
population (reviewed in (28)). In the sub-Saharan African region, life expectancy is currently reduced to 35 years of age, the average adult prevalence of HIV-1 infection is 5.9%, but is as high as 33.4% in Swaziland. In addition, sub-Saharan Africa contains 80% of the world's orphaned children, due to loss infected parents. Additionally, HIV-1 has increasingly become endemic in countries, such as Russia, Estonia, Ukraine, Thailand, Cambodia, the Caribbean, Honduras and Guatemala (USAIDS **www.usaid.gov**. 2006).

### 1.1.4 CLASSIFICATION AND MOLECULAR BIOLOGY

HIV-1 is classified within the *Retroviridae* family, a large group of viruses with unique molecular properties. Besides the *Hepadnaviridae* family, the polymerase, reverse transcriptase, is a hallmark of retroviruses. This enzyme reverse transcribes the positive sense single stranded RNA viral genome to proviral DNA that can later be integrated into the host genome by another distinctive enzyme called integrase, which allows the retroviruses to maintain an immortal status within the cell (reviewed in (97)).

Retroviruses are further classified to *Alpharetroviruses*, *Betaretroviruses*, *Gammaretroviruses*, *Deltaretroviruses*, *Epsilonretroviruses*, *Spumaviruses* and *Lentiviruses* based on their morphology and complexity of their genomes, where the latter four genera are more complex than the previous. HIV-1 is a member of the *Lentivirus* genus, along with other immunodeficiency associated viruses: type 2 human (HIV-2), feline (FIV), simian (SIV) and bovine (BIV), as well as the immune activating betaretroviruses: equine infectious anemia virus (EIAV), caprine arthritis encephalitis virus (CAEV) and maedi visna virus (MVV) (255, 333). *Lentiviruses* can be

**FIGURE 1.2. EPIDEMIOLOGY OF HIV-1 INFECTION.** Different colored areas represent adult HIV-1 prevalence (%) in 2005. Areas with high HIV-1 prevalence include sub-Saharan Africa, Russia, Estonia, Ukraine, Thailand, Cambodia, the Caribbean, Honduras and Guatemala. Taken from UNAIDS (**www.unaids.org**) with full copyright access from GrCampbell.



morphologically distinguished from other genuses by its distinct conical shaped virion structure, surrounded by the typical retroviral envelope (109).

Along with the canonical *gag, pro-pol* and *env* genes that encode the structural proteins, capsid, protease, reverse transcriptase, integrase and envelope, *Lentiviruses* also contain regulatory genes that regulate the viral life cycle and likely facilitate pathogenicity within the host (reviewed in (97)). HIV-1 contains 6 auxiliary genes that have been extensively characterized. These accessory genes include *vif, vpr, vpu, tat, nef* and *rev* (66, 281), which can be further divided into early and late genes. *Tat, rev* and *nef* are expressed early in infection to control viral replication, while the other accessory genes, such as *vif, vpu* and *vpr* are expressed later with the structural genes. *Lentiviruses* can also replicate in differentiated non-dividing cells, particularly in monocytic cells, whereas the other retroviruses cannot (36). In addition, *Lentiviruses* have a similar disease processes including an acute phase, followed by a slow infection, as the name "lenti" implies, which is then followed by death (reviewed in (314)).

### 1.1.5 CELL TROPISM

The chief cells that are infected with HIV-1 are $CD4^+$ T-lymphocytes, monocyte/macrophages and dendritic cells. While the primary receptor for HIV-1 is CD4 (193), found on both $CD4^+$ T-lymphocytes and macrophages, researchers observed different cell tropisms for different isolates of the virus, in which one isolate might infect $CD4^+$ T cells better than macrophages and vice versa (49). Other unique cellular tropisms have also been noted, such as infection of astrocytes, a glial cell type in the brain that are $CD4^-$. Though there is no full replication of the virus, it is thought that

astrocytes may act as a reservoir for different strains of HIV-1 (312). The virus also requires a co-receptor for binding. The two predominant co-receptors are the chemokine receptors, CXCR4 and CCR5, for $CD4^+$ T cell tropic viruses and macrophage tropic viruses, respectively (7, 52, 72, 74, 76, 90).

### 1.1.6 LIFE CYCLE AND THE FUNCTIONS OF HIV-1 GENES

Once the HIV-1 virion encounters the target cell, it binds to CD4 through the viral envelope glycoprotein encoded by *env*. The structural gene *env* produces a polyprotein, gp160 that is cleaved by furin, a cellular protease, to generate gp120 and gp41 (71). gp120 initially interacts with CD4, thus creating a conformational change in gp120. This change allows further binding of gp120 to the chemokine coreceptor, CXCR4 or CCR5, creating a stable interaction with the cell membrane (364). The other component of the viral protein envelope, gp41 is then activated and mediates a fusion event between the virus envelope and the host cell membrane allowing the capsid to enter the cell (44, 310, 338). In addition to this receptor mediated entry, HIV-1 gp120 can bind microglia, the resident brain macrophage, via the mannose receptor, independent of CD4 and the co-receptor; however, the virus does not complete the viral life cycle and thus no productive infection occurred here (320).

Once the capsid is within the host cell, reverse transcriptase, encoded by *pol*, immediately produces the proviral DNA that is released from the capsid and transports to the nucleus within the preintegration complex (PIC) consisting of the viral nucleocapsid encoded from *gag* and the accessory protein, Vpr (118, 331). Integrase, also expressed from *pol* gene, can then mediate insertion of the proviral DNA into random sites of the

host genome (282). At this point, the virus can stay dormant within the cell (ie, in cell reservoirs) or the viral gene expression can occur through transactivation of the long terminal repeat (LTR). The LTR contains a strong promoter within a region called U3, whereby expression occurs through recruitment of polymerase machinery by inflammatory transcription factors, such as NFκB and NFAT, as well, HIV-1 viral proteins Tat and Vpr facilitate expression from the LTR (reviewed in (172)). Expression of early and late genes of HIV-1 depends on creation of different mRNA splice variants of HIV-1. HIV-1 Rev modulates which variants will be formed by transporting spliced and unspliced variants out of the nucleus to be translated (89). **Figure 1.3** shows the different splice variations occurring and what proteins are produced (reviewed in (225)). In the cytoplasm, viral proteins will follow different pathways for translation, for example, e*nv* will be translated through the ER-golgi pathway and end up at the budding sites, the plasma membrane or multivesicular bodies (MVB), conversely, the *gag-pol* will be translated by free ribosomes and eventually transport to the gp160 containing membrane (68, 346). g*ag*-encoded proteins function to recruit the HIV-1 dimer RNA genome and other proteins to be included within the virion to the budding site, and once at the membrane, the p6 domain of Gag induces budding through the interaction with cellular ESCRT-I proteins, creating an immature virion particle that subsequently becomes an infectious mature virion through proteolytic cleavage of Gag and Pol once released from the cell (reviewed in (97)). The life cycle of HIV-1 is depicted in **Figure 1.4**.

**FIGURE 1.3. HIV-1 GENOMIC ORGANIZATION AND SPLICE VARIANTS.** Proviral genome containing the long terminal repeat (LTR) is depicted in green; the 3 structural and enzyme genes common to all retroviruses are in blue: *gag*, *pol* and *env*; and the accessory genes in orange: *vif*, *vpr*, *vpu*, *tat*, *nef* and *rev*. Gene expression can be divided into early and late gene expression depending on the presence of Rev. Host splicing machinery will multiply spliced transcripts creating *tat*, *rev* and *nef* mRNA. When Rev accumulates, gene expression shifts to the late phase, where Rev facilitates export of unspliced and single spliced transcripts creating *gag*, *gag/pol* and genomic RNA as well as *vif*, *vpu*, *vpr* and *env* mRNA, respectively. Diagram represents a simplified version of splice variants, where the splice donor site exists at the 5' end and the splice acceptor site occurs just before the gene of interest, creating variations of multiple and single spliced transcripts (reviewed in (225)). Figure 1.3 is adapted from (225).

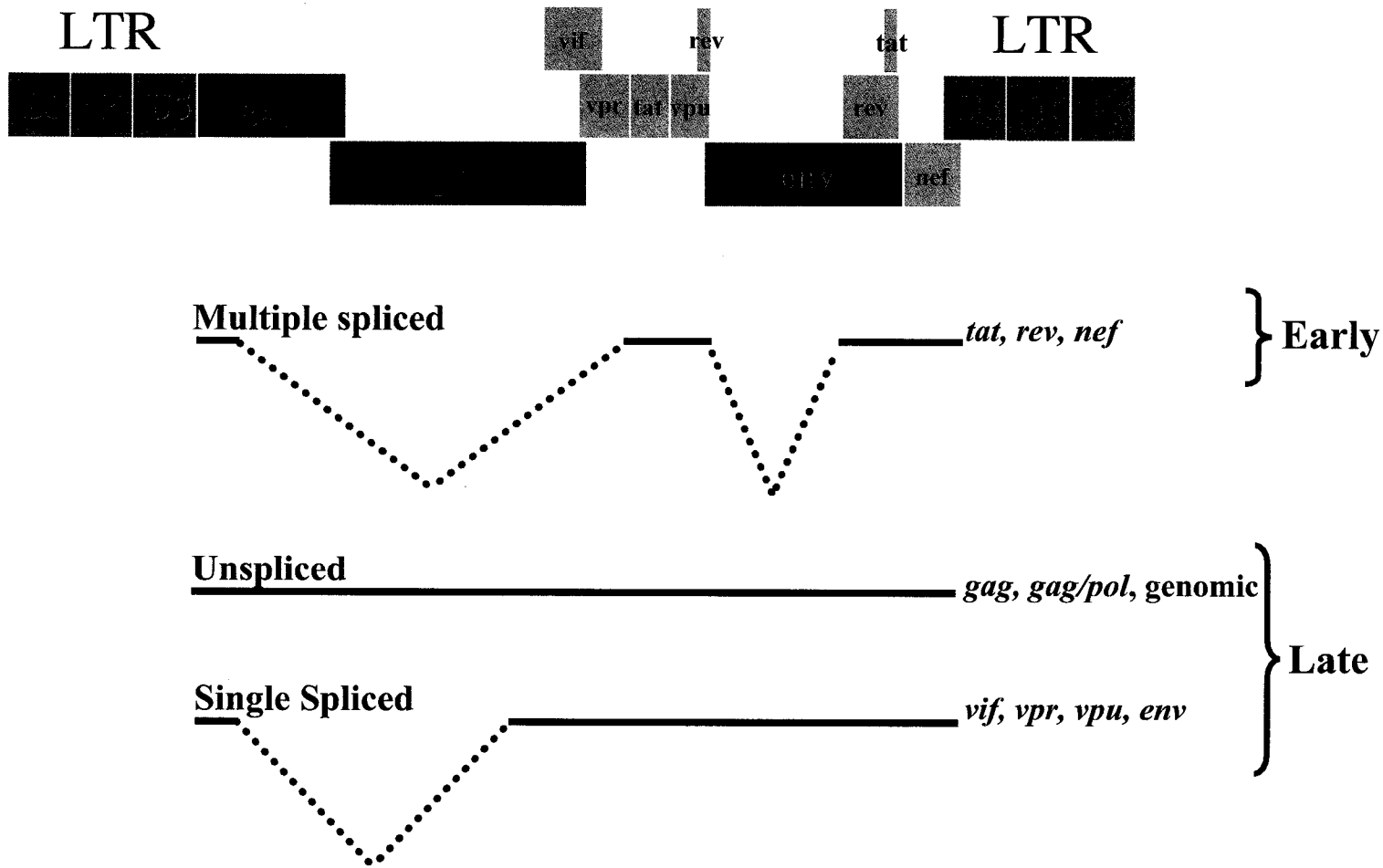
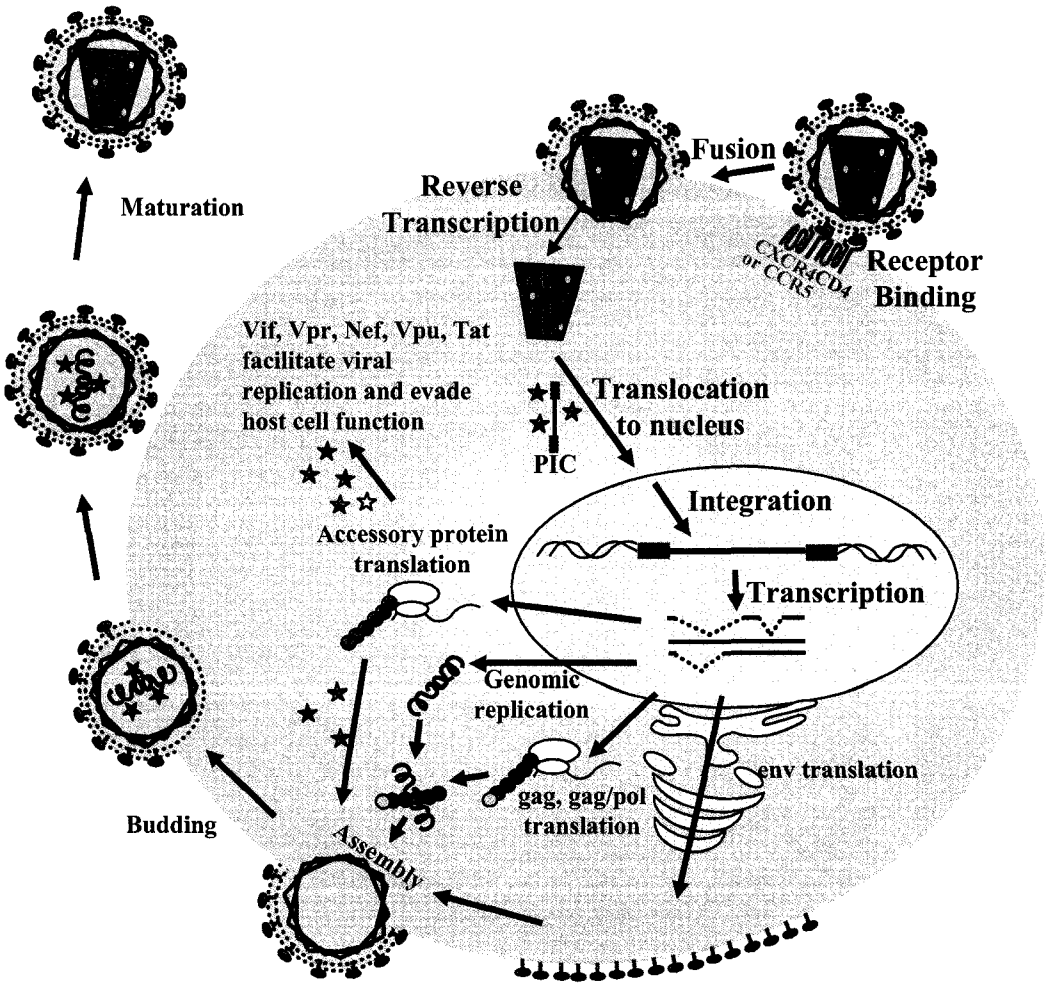


**FIGURE 1.4. HIV-1 REPLICATION CYCLE.** HIV-1 gp120 binds first to CD4, causing conformational changes to bind the coreceptor CXCR4 or CCR5; fusion between the plasma membrane and viral envelope occurs via gp41; viral capsid is released and reverse transcriptase reverse transcribes the genomic RNA to proviral DNA within the capsid; pre-integration complex (PIC) then translocates the provirus into the nucleus where integrase catalyzes integration into the host genomic DNA; the LTR is then transactivated, producing mRNA that is spliced or unspliced and transported to the cytosol for translation by host or viral (Rev) export proteins; translation of *env* occurs through the ER/golgi pathway resulting in Env translocation to the plasma membrane (or multivesicular body (MVB) not shown here); alternatively other viral proteins are translated by free ribosomes in the cytosol; the Gag/Pol polyprotein recruits HIV-1 genomic RNA along with other packaged viral and host proteins to the plasma membrane (or MVB not shown here); alternatively other viral protein products act on the host cell to facilitate viral replication or evade host defense mechanisms; once assembled, the virus buds from the host cell and full maturation through proteolytic cleavage occurs creating an infectious HIV-1 viral particle. Reviewed in (225)).



Other accessory proteins not mentioned in the life cycle here are important for the viral life cycle *in vivo*. Nef is a myristoylated protein that is important for the endocytosis of a number of proteins facilitating viral replication and evasion of the immune system. One specific effect of Nef is the downregulation of CD4 on the cell surface to reduce the amount of dual infection of a cell (6). Vif is another immune evasion protein that functions to remove an innate cellular antiviral protein, APOBEC3G, by targeting it for ubiquitin-mediated degradation (288). APOBEC3G induces mutations to the proviral DNA in the subsequent viral life cycle (121, 177, 196, 363). Vpu is unique to HIV-1, and functions to improve viral release through degradation of intracellular CD4, thus preventing intracellular gp120-CD4 interactions, facilitating viral progression (199). Lastly, Vpr is a conserved protein amongst HIV-1, -2, SIV and likely FIV, with multiple functions for facilitating HIV-1 infection including increasing accuracy of reverse transcription, integration of the provirus into the nucleus and transactivation of the LTR (reviewed in (365)). Vpr also mediates host cell interference and pathogenesis which will be discussed later.

### 1.1.7 DIVERSITY- GENES AND CLADES

Like many RNA viruses, HIV-1 is exceedingly heterogeneic at the nucleic acid and protein levels (26, 215). Since the HIV-1 reverse transcriptase is highly error-prone, an individual infected with HIV-1 can harbor a quasi-species of the virus, creating a more complex situation with regard to treatment and vaccine development (25). The mutations occur throughout the HIV-1 genome, but the region that varies the most is the gene encoding the envelope glycoprotein, gp120. Variable loops within this glycoprotein

change the tropism of the virus, while also evading the immune evasion with regard to neutralizing antibodies (134, 212, 218).

Though HIV-1 is highly diverse, the virus can be further subdivided into groups based on genetic sequence. These groups are: M for major, over 90% of the isolates originate from this group; O for outlier, these isolates contain less than 50% homology to M; and N for non-M or O, that have a completely different serology to groups M and O (267). Group M can be further subdivided into 8 clades: A, B, C, D, F, G, H and J (134). Within a clade, there can be up to 30% difference in sequence, depending on the gene (100). Clade A is the most divergent, while clade C is the most dominant virus (229). However, Clade B is the most studied, since this is the most prevalent virus in North America and Europe (234).

To increase the complexity of this virus, further variation can occur through recombination of the virus. During reverse transcription, strand displacement between the diploid RNA or dual infecting RNA can occur due to the nature of reverse transcription (358). The proviral DNA can therefore be distinct from the parent RNA, creating more diversity in HIV-1 and possibly a new clade (358).

### 1.1.8 PATHOGENESIS

Though both macrophages and $CD4^+$ T cells are the principal cells productively infected with HIV-1, the main pathogenic feature of HIV-1 infection is the depletion of $CD4^+$ T cells (23). The loss of $CD4^+$ T cells is partly due to direct infection of $CD4^+$ cells. Infection causes stress on the cell through loss of membrane due to excessive budding and causes syncytia formation between infected and uninfected $CD4^+$ T cells

resulting in apoptosis (65, 163). The type of virus infecting the organism also has an effect on disease progression, whereby X4 (CXCR4) viruses are more pathogenic than R5 (CCR5) viruses. Viruses isolated from early and late disease progression showed slow replication of a narrow group of cells *in vitro* and robust wide ranging cell type replication, respectively (15, 49). Typically, the disease process involves an R5-tropic infection followed by a highly pathogenic X4-virus tropic which indicates a significant decrease in $CD4^+$ T cells (62) as shown in **Figure 1.1**. It is not understood why this change in cell tropism causes a change in pathogenicity of the virus.

Although direct HIV infection of $CD4^+$ T cells causes loss of these cells, the actual number of infected $CD4^+$ T cells is 1/1000-1/100000 implying much of $CD4^+$ T cell loss is due to a high bystander effect (94). Moreover, analysis of infected lymph nodes from HIV-1 and SIV infected humans and primates, respectively, show that the predominant cell undergoing apoptosis are the uninfected cells (94). Theories for this bystander effect include HIV-1 mediated induction of inflammation and HIV-1 gene products. It has long been a mystery why SIV infection of non-human primates, such as rhesus macques causes AIDS-like disease (179) while the same virus produces minimal disease in other primates, such as Sooty mangabeys, despite high viremia (259). Research into SIV infection of Sooty mangabeys shows that there is no apparent immune activation with regard to cell-mediated immunity by $CD8^+$ T cells that occurs in pathogenic HIV-1 or SIV infection (294), suggesting the immune system exacerbates the disease. On the other hand, the basis of survival of the long term non-progressors (LTNP) of HIV-1 infection is their ability to decrease viral load with an effective cell-

mediated immune system (40). It is also thought that HIV-1 gene products, such as *nef* and *vpr* are important for causing pathogenesis and facilitating infection *in vivo*. Infection of rhesus macques with HIV-1 containing non-functional Nef caused a decrease in viral load and attenuation of disease (155). Nef could directly cause bystander pathogenicity through induction of FasL on infected $CD4^+$ T cells through interfering with TCR signaling causing apoptosis of uninfected cells expressing cell surface Fas (351, 352). Meanwhile, Nef protected infected cells from apoptosis through inhibition of pro-apoptotic ASK1 (104). Nef also indirectly mediated the bystander effect through induction of inflammation by causing chronic activation of CD40L on macrophages resulting in release of inflammatory cytokines (307). Vpr has also been shown to be involved in pathogenesis since SIV ΔVpr infection through mutation of the start codon caused a reduction of disease progression and low viral burden (169). While Nef suppresses apoptosis of infected cells, intracellular Vpr caused induction of apoptosis in infected cells while secreted Vpr mediates bystander killing (180, 301). The mechanism of Vpr-mediated cell death will be discussed later.

### 1.1.9 TREATMENT OF HIV-1 INFECTION

Since 1996, the life spans of HIV-1 infected patients have increased significantly with the introduction of combination antiretroviral therapy (cART). This therapy consists of three or more antiretroviral drugs, which target specific viral proteins, largely the reverse transcriptase and protease. Reverse transcriptase inhibitors (RTI) are classified into nucleoside analog RTI, such as lamivudine and non-nucleoside analog RTI, such as nevirapine (214, 300). Protease inhibitors are available, such as saquinavir to prevent

maturation and infectivity of the virus (141). Receptor interaction inhibition is currently being evaluated as a way to inhibit infection with drugs, such as TAK-779 (targeting CCR5) (19), while there are also fusion inhibitors, such as enfuvirtide (168). The viral integration within the host genome is also a potential target for drugs like MK-0518 directly targeting the integrase and currently in clinical trials (114). Through the use of cART, viral load can be suppressed for a decade while $CD4^+$ T cell counts remain stable for longer periods, opportunistic infections have decreased and transfer of HIV-1 from mother-to-child is markedly reduced (reviewed in (46)). Conversely, HIV-1 induced neurological disease has become more prevalent due to longer survival times and poor ART tissue penetration of the brain (56, 75).

## 1.2 HIV-1 ASSOCIATED DEMENTIA

Since HIV-1 infects macrophages, a highly motile and widespread cell type within the body, HIV-1 infects many organs. One such organ is the brain, and its infection by HIV-1 can lead to HIV-1 associated dementia (HAD). HAD is described as a subcortical disorder of the brain causing: motor abnormalities to the point of vegetative state; behavioural problems, such as personality changes and social withdrawal; and cognitive dysfunction, whereby short-term memory loss and reduction of concentration can occur (reviewed in (110)). This neurodegenerative disease occurs later on in the disease process and defined the presence of AIDS. Though this disorder does not occur in all HIV-1 infected persons, it is the major neurodegenerative disease occurring in humans aged forty years old and under (83). The course of dementia varies between cases, while some have a steep decline of function over a matter of weeks and others

show a steady decline in abilities (reviewed in (248)). One predicator of HAD is the manifestation of minor cognitive motor disorder (MCMD), a less severe form of encephalopathy; however a more common syndrome, occurring in 30% of symptomatic individuals (146, 275). Although MCMD is not as severe as HAD, it carries a prognosis for a worse AIDS disease course and a greater likelihood of the development of HAD (50, 276).

### 1.2.1 HAD STATISTICS

Prior to the introduction of cART the prevalence of HAD was between 20-30% for those patients with advanced stages of HIV-1 containing low $CD4^+$ T cell counts (210), while the prevalence post-cART has decreased significantly to 10% (274). However, there is an increase in the number of patients with $CD4^+$ T cell counts above 200 cells/μl who are showing clinical signs of HAD (275). There are reports that HAD prevalence is rising since the initial decrease post-cART, due to the increased life span of patients and the low efficiency of ARTs entering the brain (75, 208).

## 1.3 NEUROINVASION

### 1.3.1 BARRIERS

HIV-1 can infect the central nervous system (CNS) early in infection as the virus can be isolated from the cerebral spinal fluid (CSF) and brain early in the course of disease (9, 69, 209). Entry of virus into the brain is more complex than other organs because it is separated from the rest of the body by barriers to control potentially harmful substances from entering the brain (reviewed in (11)). There are two main barriers for the brain: the blood-brain barrier (BBB) that separates the peripheral blood from the

brain; and the choroid-plexus segregates the brain from the CSF. The vessels of the BBB contain microvascular endothelial cells that are linked together by tight junctions which is required for maintaining semi-permeability of the BBB (136). Inflammatory cytokines, such as TNF-α can structurally alter the BBB thus increasing the permeability to infiltrating cells (91, 157).

### 1.3.2 Mechanisms of Neuroinvasion

There are two proposed mechanisms for HIV-1 entry into the CNS. One mode of entry is through the Trojan horse mechanism. HIV-1 takes advantage of its tropism for macrophages with respect to causing a systemic and multi-organ infection. By studying a related *Lentivirus*, maedia visna virus (MVV), a solely macrophage-tropic virus, researchers revealed that this *Lentivirus* traffics through to the brain via hijacking the infiltrating macrophage (117, 237). Infected $CD4^+$ T cells can also enter the brain and X4 viruses were shown to be neurotoxic *in vitro* (231); however, this occurs less frequently and contributes less to infection of the brain since the principal virus type isolated from the brain is the R5-dependent isolate which is mainly macrophage-tropic (45, 230). The alternative mechanism is trancytosis by direct infection of free virus of the endothelial layer seen *in vitro* (60, 116). Direct infection of these $CD4^-$ cells was enhanced by inflammation, but does not induce a productive infection of these cells unless the cells are proliferating (60); instead, cytoplasmic vacuoles form for the virus to cross the endothelial cells (116). Trancytosis of free HIV-1 virus is not as well supported as the previous hypothesis and is not likely the dominant mechanism of HIV-1

entry into the brain. Once HIV-1 enters the brain it can then be released and infect other cells within the brain. The neuroinvasive mechanisms are displayed in **Figure 1.5.**

## 1.4 NEUROTROPISM AND CELLS IN THE BRAIN

The brain contains a number of different cell types in the brain that act in concert to form a complex system capable of controlling the body. Failure of one cell type to function properly severely disrupts whole system. Though the brain is a well recognized reservoir for HIV-1, there are few cell types that are productively infected with HIV-1 as shown in **Figure 1.5**.

### 1.4.1 PRODUCTIVE INFECTION

Cells that are productively infected by HIV-1 require a full life cycle of the virus including reverse transcription of the RNA to provirus, integration of the proviral genome, expression of viral proteins and release of mature infectious virions. Productive infection of a cell by HIV-1 entails having the correct receptor for the virus to enter and the necessary environment within the cell for completion of the life cycle, such as the activation state of the cell and the absence of innate cellular antiviral properties; however, HIV-1 has mechanisms to evade certain barriers (reviewed in (111)).

### 1.4.2 MICROGLIA AND PERIVASCULAR MACROPHAGES

Within the brain, the only cell types that are productively infected include microglia and perivascular macrophages (285, 344). Like most tissue-derived macrophages, such as Kupfer cells and Langerhans cells, microglia are permanent resident monocytoid cells that function to survey the surrounding tissue for evidence of intrusion or injury and are the first line of defense against pathogens (reviewed in (53)).

These glial cells are derived from the mesoderm and are detected within the neuroepithelial layer earlier in embryonic development than the development of monocytes, suggesting that microglia are not derived from monocytes infiltrating the brain (8, 309). Microglia are also morphologically distinct from other macrophages; resting microglia are ramified or highly branched while they are more round and ameboid shaped when activated (138). Like most macrophages, activation of microglia causes an increase in inflammation and release of cytokines to recruit monocyte reinforcement from the periphery and render the BBB permissible to monocyte entry (reviewed in (170)). Monocytes, originally derived from the bone marrow are recruited to the brain from the neighboring lymph nodes (130, 131, 133). Upon entry into the brain, these cells differentiate to perivascular macrophages, thus continuously replacing the existing perivascular macrophages (132, 133). HIV-1 and SIV exploit this high turnover rate of perivascular macrophages to gain entry into the CNS (347). Perivascular macrophages differ morphologically from microglia where perivascular macrophages exhibit a flat and elongated appearance (323, 324). Additionally, these cells closely interact with the microvascular endothelial cell layer of the BBB whereas microglia are located within the parenchyma of the brain but extend their protruding footpads to the BBB vasculature (132, 173, 174). Due to the close proximity to the periphery and the high turnover rate of infected infiltrating monocytes, the dominate cell infected by HIV-1 is the perivascular macrophage (347). Besides a productive infection, HIV-1 infection of macrophages presents many complications that affect the other cell types within the brain, including

induction of an inflammatory response and secretion of viral proteins, of which will be discussed later.

### 1.4.3 NEURONS

Neurons are the main driving force for transmitting information to, from and within the CNS. This highly structured and polarized cell type usually consists of a cell body containing the nucleus, the long axon and dendritic ends that form synapses with other neurons. Neurons are activated by stimuli, such as glutamate to open voltage-gated channels that facilitate depolarization across the neuronal membrane, causing release of transmitters at the synapse and transfer of information to neighboring or distant neurons and endocrine glands by saltatory conduction along the axon (reviewed in (316)). Neurons are not productively infected by HIV-1 because they do not express the appropriate receptor, CD4, though they do express the chemokine co-receptors (127, 287, 344). It has been shown that there might be proviral DNA present in neurons suggesting a non-productive infection during which HIV-1 enters the cell but does not replicate (318). However, this observation is controversial since it could not be confirmed by others (221, 262, 308, 359). The main pathogenic feature of dementia is the loss of neurons and their processes (synaptodendritic connections), suggesting other factors besides direct infection of neurons are the cause of this dysfunction (reviewed in (149)).

### 1.4.4 ASTROCYTES

Astrocytes are glial cells and are the most abundant cell type in the brain (181). These cells maintain homeostasis within the brain by reducing neurotoxins, such as reactive oxygen species while they also produce neurotrophins, such as nerve growth

factor (NGF) to neurons (278). An essential role of astrocytes is regulation of synaptic activity by the removal of extracellular excitatory amino acids, such as glutamate, cysteine and aspartate that may cause uncontrolled stimulation and possibly over-stimulation of neurons, resulting in excitotoxicity and ensuing apoptosis (122, 269, 345). Astrocytes take in extracellular glutamate and transform it into non-toxic glutamine, which neurons take up and use to make glutamate during excitation (119, 122, 298). In addition, astrocytes help sustain the BBB by release of soluble factors that regulate permeability via the microvascular endothelial cells (185). Thirdly, astrocytes function to maintain water and ion homeostasis within the brain by containing water and ion channels through to the BBB (reviewed in (295)). It has been shown that astrocytes can be infected with HIV-1 and even early genes are expressed and translated, such as Nef, Tat and Rev; however there is restricted productive infection of astrocytes (21, 254, 277, 317). It has been suggested that infection of astrocytes serve as a cell specific reservoir, where strains of HIV-1 isolated from astrocytes differ from the strains isolated from the rest of the brain (312). Along with neurons, there is also loss of astrocytes in HAD. Due to the important role astrocytes have on the survival of neurons, loss of astrocytes feedback on the destruction of neurons (38).

### 1.4.5 OLIGODENDROCYTES

The last type of cell in the brain is the oligodendrocytes. Also a glial cell, this cell intimately interacts with neurons to provide a protein and lipid rich myelin sheath around the neuronal axon. The myelin sheath acts to provide insulation to the neuron, as well, facilitates increased speed of nerve impulses along the axon during excitation. Damage

to myelin sheath causes damage to neurons as seen in multiple sclerosis, another neurodegenerative disease (reviewed in (78)). Along with neurons, oligodendrocytes are not productively infected by HIV-1 (344); however, soluble gp120 has been shown to bind to proteoglycans on oligodendrocytes causing an increase in intracellular $Ca^{2+}$ and a reduction of myelin production *in vitro* (57). It is not currently clear what are the effects of HIV-1 infection in the brain have on oligodendrocytes and their function with respect to the development of HAD. Therefore, these cells will not be discussed further.

## 1.5 NEUROPATHOLOGY

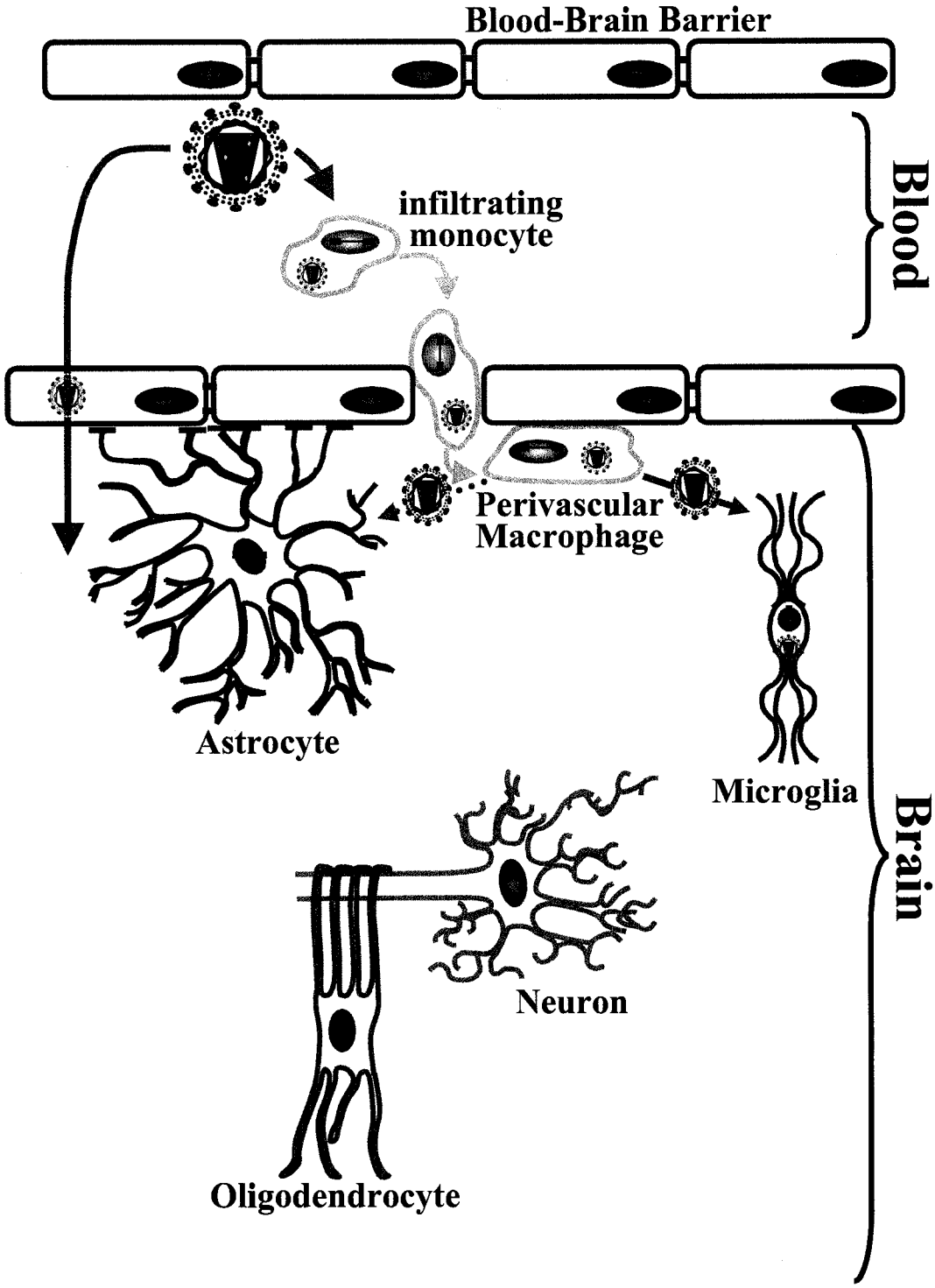
### 1.5.1 HALLMARKS OF NEUROINVASION AND DEVELOPMENT OF HAD

The hallmark of infection of the brain by HIV-1 is the presence of HIV-1 encephalopathy (HIVE). The neuropathological clinical features of HIVE are multinucleated giant cells, activation of astrocytes and microglia, macrophage recruitment to the brain, white and grey matter atrophy, decreased synaptic density and loss of neurons, particularly in the subcortical and cortical areas of the brain (16, 17, 35, 165, 226, 286, 303, 336, 343). The associated clinical symptoms and signs can therefore lead to the development of HAD.

### 1.5.2 REGIONAL DIFFERENCES WITHIN THE BRAIN

There are distinct regions within the brains of HAD patients that are affected by HIV-1 infection, resulting in loss of neurons within the brain. These regions include areas of the frontal cortex, cerebellum and basal ganglia (85, 87, 88, 113, 156, 260). Through MRI imaging, atrophic regions of the brains of HAD patients include both the cortical and subcortical areas (207, 297).

**FIGURE 1.5. HIV-1 NEUROINVASION, INFECTION AND CELLS IN THE BRAIN.** HIV-1 can enter the brain through two ways. The first way is by translocation through the microvascular endothelial blood brain barrier (BBB). The second way is through the Trojan horse mechanism, whereby HIV-1 infected monocytes infiltrate through the BBB and differentiate into a perivascular macrophage that closely associates with the BBB. Through this localization to the BBB, other cells that are located in this area, such as the astrocytes and microglia can become infected. Astrocytes are abundant cells that maintain homeostasis within the brain and can be non-productively infected, where provirus and some proteins are produced but no infectious particles are produced. Perivascular macrophages in the brain are associated with the BBB through their processes and are often productively infected by HIV-1. These cells along with the resident microglia regularly provide protection within the brain through surveillance of foreign pathogens and remove these foreign pathogens through inducing inflammation which can cause injury to the brain. Microglia and perivascular macrophages are the chief infected cells in the brain. In normal circumstances, microglia and astrocytes protect the neurons, which are the main driving force for transmitting information throughout the body. Though neurons are not infected, the infection of other cells in the brain creates a neurotoxic environment leading to neurodegeneration leading to dementia. Neurons are also protected by the oligodendrocytes, which produce the insulating myelin sheath around the neuronal axons. Like neurons, oligodendrocytes are not infected with HIV-1. Reviewed in (149)).



### 1.5.3 CORRELATES OF DISEASE

Though the direct cause of HAD remains unclear, there are many clinical and pathological correlations with cognitive impairment seen in HAD, in terms of HIV-1 infection and its effects. It is generally accepted that there is a correlation between the viral load within the CSF and the degree of cognitive impairment though this notion is still controversial (34, 83, 211); however, the number of infected cells, viral antigens and multinucleated giant cells within the brain parenchyma do not correlate with the disease (108, 204). This suggests that the disease process is not dependent on the extent of infection in the brain, but rather on other pathogenic effects of HIV-1. On the other hand, inflammation within the brain caused by HIV-1 infection is correlated with cognitive impairment. For instance, increased microglial numbers, as well as inflammatory molecules, such as TNF-α, quinolinic acid, neurotoxic amines and excitotoxins are seen in HAD (107, 108, 129, 340). Lastly, neuronal dysfunction, such as loss of synaptic density as well as selective neuronal loss has been shown to occur in conjunction with cognitive impairment in HAD (86, 96, 203, 204). These specific types of neurons, such as large pyramidal neurons or excitatory neurons within the cortex and GABAergic or inhibitory neurons are most affected within the brain during HIV-1 infection (203). Another phenotype that occurs is synaptic injury to the neurons, whereby the dendrites that contain the synapses are damaged. Synaptic injury is thought to be the first sign of neurodegeneration in HAD and correlates largely with cognitive impairment (reviewed in (81)). Astrocyte apoptosis is also highly associated with impaired cognitive development

in HAD while neuronal loss through both apoptosis and necrosis is also a consistent feature of HAD (4, 103, 241, 289, 313).

### 1.5.4 VIRAL STRAINS AND DIVERSITY OF HIV-1 WITHIN THE BRAIN

HIV-1 infection within the brain somewhat differs from what occurs elsewhere within the body (reviewed in (328)). Though it is widely held that the brain acts as a reservoir for the virus, there is some evidence to suggest that the virus leaves the brain through the choroid plexus and thus the possibility of brain-derived HIV-1 on the periphery (43), although no further evidence of this has been shown. That said, however, proviral DNA persists in the brains of SIV-macaque animal models after suppression of virus by brain penetrating and non-penetrating ARTs suggesting the brain is a viral reservoir (55). Therefore, infection of cells within the brain may act as reservoirs for infection within the brain. For instance astrocytes harbor sequences that differ from the rest of the brain and although there is no productive infection in astrocytes, they still may secrete viral encoded proteins, which may be important for the neuropathogenicity of the virus (21, 254, 277, 312, 317).

Sequence homology between viruses isolated from different regions of the brain tends to cluster within regions of the brain but vary widely amongst different regions of the brain and from the periphery (47, 84, 161, 162, 257, 284, 349). The cause of viral genetic variation amongst the brain is not yet understood. Different viruses may be entering independently or specific types of virus may be adapting to the different regions of the brain in different ways. The idea of brain adaptation is supported by the observation that mutations in gp120 can occur to reduce the threshold of CD4 and CCR5

binding; therefore, allowing infection of cells that express lower CD4 and CCR5 (201, 239). Viral diversity may also be the reason why some patients acquire HAD and some do not, since certain strains of FIV and SIV are know to cause neurological diseases (197, 243, 251). In HIV-1 infection, it has been shown that mutations within the constant region C2 and variable regions V1 and V3 of gp120 differ between HAD and non-demented (ND) patients (250, 304). Moreover, certain variants of genes, such as Tat and gp120, that are known to be neurovirulent, have been shown to correlate with HAD and not ND patients (32, 250, 251, 329).

Another anomaly that occurs in the brain is that the principal virus infecting the brain is the R5 virus while X4 and dual tropic viruses are rarely found within the brain (45, 230, 231). This is particularly interesting since the natural disease progression consists of the conversion of the virus from R5 to the more pathogenic X4 virus, while the brain remains infected with the R5 virus. This may be due to a compensatory mechanism to reduce neurovirulence or R5 may survive in the brain better than X4, since most cells in the brain are R5-dependent since these cells express mainly CCR5 (344).

## 1.6 CELL DEATH

Cells throughout the body die by a number of ways, depending on the environment and the stimulus for cell death. The effects of each pathway differ widely and affect the surrounding environment.

### 1.6.1 NECROSIS

Cells that enter necrosis die accidentally, whereby entry into a necrotic phase occurs without order and without proper cell signaling. Cells often undergo necrosis

from detergents, oxidents or excessive treatment of a stimulus. Characterized by cell swelling, absence of zeiosis and inducers of inflammation, necrosis generally occurs under situations of tissue/cell injury, such as ischemia (reviewed in (224)). Often during apoptosis, phagocytosis cannot occur fast enough and secondary necrosis occurs (10, 29).

### 1.6.2 APOPTOSIS

Apoptosis, on the other hand, is a mechanism by which a cell programs itself to undergo cell death in an ordered fashion. The apoptotic pathway is a naturally occurring pathway throughout an organism's life, whereby it is required for tissue development and homeostasis, prevention of tumour growth through removal of damaged and redundant cells and removal of pathogens (reviewed in (178)). In an event where survival is futile or detrimental to the organism as a whole, such as viral infection, the cell will induce a series of cell signaling that will result in cell shrinkage, chromatin aggregation, DNA fragmentation and blebbing of the cellular membrane (reviewed in (154, 178)). The process of apoptosis will signal macrophages to phagocytose the remaining apoptotic blebs which reduce the risk of release of cellular components and thus induction of inflammation (82, 233, 315). If, for example there is extreme injury to neurons and macrophages are not fast enough in this process, secondary necrosis and thus inflammation will occur (10, 29, 77).

There are two general pathways that trigger apoptosis through activation of caspases, a family of cysteine proteases (reviewed in (223, 315)). Each pathway triggers the activation of different initiator caspases, such as caspase-8 and -9, though these

initiator caspases converge to activate the same effector caspases, such as caspase-3 and -7, which mediate apoptosis (reviewed in (315)).

One pathway for induction of apoptosis is through the extrinsic pathway or the death receptor pathway. Through this mode of cell death, communication between adjacent cells, such as cytotoxic T lymphocytes, occurs as a means of development or protection against pathogens, whereby extracellular signaling through soluble factors are released (reviewed in (24)). These soluble factors include FasL and TNF-α, which bind to their respective homotrimeric receptors Fas and TNF-α receptor. Recruitment, homo-oligomerization and auto-catalytic activation of an initiator caspase-8 occurs which can then proteolytically cleave and thus activate effector caspases (315, 354). Once activated, caspase-3 cleaves cellular substrates, such as nuclear lamin, inhibitor of caspase-activated DNase (ICAD), poly(ADP-1 ribose) polymerase-1 (PARP-1), which further lead the cell down the irreversible pathway of apoptosis (reviewed in (315)).

The second pathway is the intrinsic pathway, whereby intracellular signaling occurs within the cell under situations of stress, such as growth factor withdrawal, DNA damage or viral infection (reviewed in (315)). This pathway is initiated at the mitochondria level, where intracellular signals cause mitochondrial dysfunction and release of mitochondrial cytochrome *c* and apoptosis inducing factor (AIF) from the inner membrane which mediates the apoptotic pathway (188). Mitochondrial integrity is tightly regulated by the members of the Bcl-2 family which consists of both pro- and anti-apoptotic members, whereby pro- and anti-apoptotic proteins act against each other resulting in either cell survival or apoptosis depending on the balance between the

proteins (reviewed in (240)). Once released from the mitochondria, cytochrome *c* will interact with apoptotic protease-activating factor 1 (Apaf-1), initiator pro-caspase-9 and ATP causing the formation of a complex called the apoptosome (188, 367). This complex causes autocatalytic cleavage/activation of caspase-9, which can then activate caspase-3 and follow the pathway previously mentioned (188). In some cells, the extrinsic pathway is intertwined within the intrinsic pathway, whereby activated caspase-8 cleaves Bcl-2 family member, Bid to t-Bid, making it functionally active for facilitating mitochondrial dysfunction and thus amplifying the extrinsic apoptotic pathway (183, 190).

The cell cycle is tightly regulated by checks and balances to ensure proper replication of a cell. Unless there is a proper check in the cell cycle, tumors result from deregulated cell cycle and loss of apoptotic potential. An essential check or tumor suppressor is the transcription factor p53 (reviewed in (266)). If problems arise in the cell cycle, such as DNA damage, p53 will induce expression of proteins to arrest the cell cycle, such as p21, so DNA repair can be done (79). If the DNA can not be repaired, p53 induces the expression of pro-apoptotic proteins, such as Fas, Puma and Bax causing apoptosis (217, 265, 360). MDM2 negatively regulates the activity of p53 by inhibiting its transcriptional activity and mediating proteasomal degradation of p53 (reviewed in (216)). An important modulator of MDM2 is Akt, a serine/threonine kinase, which phosphorylates MDM2 causing nuclear localization and increased ubiquitin ligase activity (206). Thus Akt has anti-apoptotic activity by negatively regulating p53 through enhancing MDM2 activity. Akt also facilitates NFκB nuclear localization by advancing

the degradation of its inhibitor, IκB (152). Additionally, Akt kinase activity causes the sequestration of Bad from the mitochondria, caspase-9 inactivation and phosphorylation of ASK1 causing inhibition of downstream Bid cleavage and apoptosis (41, 158, 362).

## 1.7 MECHANISMS OF NEUROTOXICITY

The defining neuropathological feature of HAD is the loss and injury of neurons; however, these cells are not infected with HIV-1 (221, 262, 308, 359). There are therefore alternate mechanisms of neurodegeneration occurring during HIV-1 infection. The two proposed and most likely concurrent mechanisms of neurodegeneration are indirect and direct neurotoxicity. Indirect neurotoxicity occurs through the induction of pathogenic inflammation by HIV-1 infection and ensuing release of soluble host factors which cause neurotoxicity. Direct neurotoxicity occurs through release of viral proteins produced by HIV-1 that cause injury or death of neurons and astrocytes (reviewed in (149)). Another possible mechanism of neurotoxicity could be the effect of direct infection of macrophages on macrophage function, such as phagocytosis and inflammation; however, this idea has yet to be investigated.

### 1.7.1 INDIRECT NEUROTOXICITY

Like any viral infection, an inflammatory response is initiated to combat HIV-1 infection in the brain; however excessive inflammation causes damage to cells that can lead to HAD when terminal injury ensues. Inflammation can be induced by either direct infection of monocytoid and microglial cells (353) or by HIV-1 derived proteins released from infected macrophages and possibly astrocytes (33, 39, 180, 272). These factors can induce activation of adjacent macrophages and astrocytes resulting in release of

inflammatory mediators. Activated macrophages release such mediators as: TNF-α, IL-1β, iNOS, IL-6, arachidonic acid, matrix metalloproteinases, neurotoxic amino acids and metabolites, such as quinolinic acid (37, 105, 107). Astrocytes release MCP-1, IP-10, TNF-α, IL-8, IL-6 and IL-1β (14, 61, 64, 80, 167, 327). The effects of these mediators are recruitment of infected and uninfected peripheral monocytes, the breakdown of the BBB and apoptosis of neurons and astrocytes (91, 93, 327, 337).

### 1.7.2 DIRECT NEUROTOXICITY

Direct neurotoxicity occurs when the virus itself or the viral products induce apoptosis. HIV-1 infected cells have been shown to secrete proteins, such as gp120, gp41, Tat and Vpr, which have been detected within the CSF and are established neurotoxins (1, 33, 39, 128, 166, 180, 222, 236, 272). While some of these viral proteins induce an innate immune response that is indirectly neurotoxic, these proteins can induce neuronal apoptosis directly through different mechanisms. There are multiple ways in which these viral proteins can directly cause apoptosis. First, excitotoxicity can occur through directly binding the NMDA receptor causing excessive influx of $Ca^{2+}$, which together with release of $Ca^{2+}$ from intracellular compartments leads to the induction of apoptosis (93, 95, 187, 232, 357). Another means of neuronal apoptosis is through induction of reactive oxygen species within the neurons (1, 2, 37, 127, 128, 144). Lastly, gene products of HIV-1 can induce apoptosis through interference with the apoptotic pathway either by indirect induction of pro-apoptotic proteins are by directly inducing apoptosis (143, 153).

### 1.7.3 GP120

Soluble gp120 can bind CXCR4 independently of CD4 on neurons, causing the induction of the apoptotic pathway through the induction of superoxide resulting in ceramide production (127, 128, 144). However, the virus that is most commonly found in the brain is R5 tropic, not X4, so under physiological conditions, this observation may not occur. Neurotoxicity through gp120 is also thought to occur through disregulation of $Ca^{2+}$ homeostasis, causing apoptosis through excitotoxicity (95, 187, 357). Indirect neurotoxicity by gp120 also occurs through production of arachidonic acid, TNF-α, IL-1β by gp120-treated monocytes (332) and IP-10 by astrocytes (14).

### 1.7.4 GP41

The levels of the Env fusion protein, gp41 within the brain correlate with severity of disease in HAD (2). Moreover, the mechanism by which gp41 mediates neurotoxicity may be through the induction of inducible nitric oxide synthase (iNOS), an enzyme facilitating the production of neurotoxic reactive nitric oxide (1).

### 1.7.5 TAT

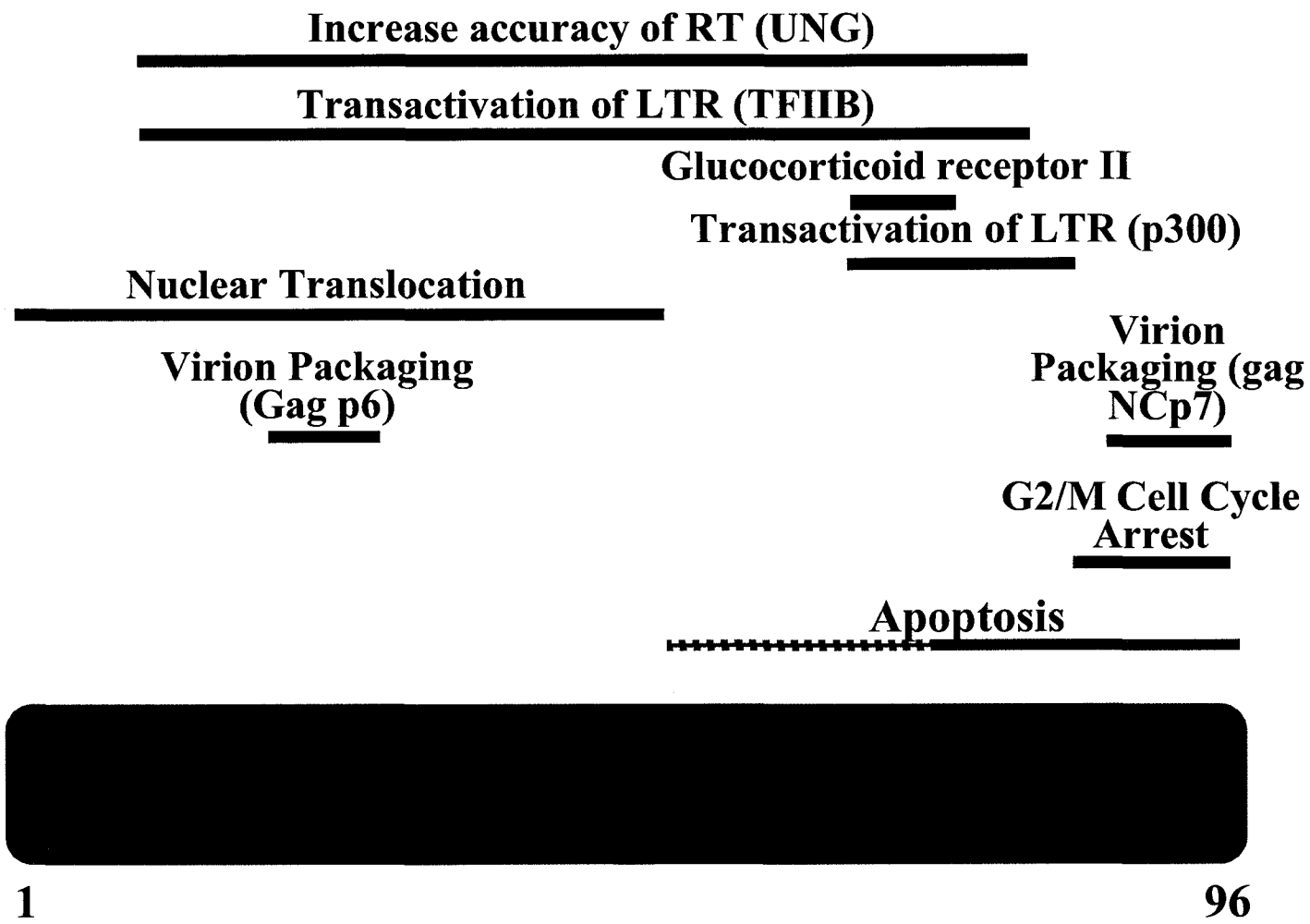
Tat has been shown to cause a direct neurotoxicity through multiple mechanisms, such as: excitotoxicity through the NMDA receptor, induction of reactive oxygen species and direct activation of caspases (166). Indirectly, Tat mediates increased production of TNF-α, IL-1β, iNOS, IL-6, MCP-1, IL-8, IP-10 and matrix metalloproteinases-7 and -2 in monocytes and astrocytes resulting in indirect neurotoxicity, influx of monocytes and BBB dysfunction (61, 93, 148, 167, 242).

## 1.8 VPR

The HIV-1 accessory protein, viral protein R (Vpr) is a small, basic protein that is highly conserved in HIV-1, -2 and SIV (319). The ORF of *vpr* overlaps the ORF of *vif* and *tat* encoding a 96-amino acid or 14kDa protein. Synthesized late in the viral life cycle, Vpr is packaged within the virion by Gag p6 and NCp7 (20, 70) and is essential for viral replication in macrophages, while it is not necessary for replication in $CD4^+$ T cells (306). Vpr has multiple functions with regard to viral replication including increasing the accuracy of reverse transcriptase (198), facilitating nuclear import of the provirus within the pre-integration complex (125, 247) while also inducing viral protein expression through transactivation of the LTR (59). Vpr also interferes with the host cell through halting the cell cycle progression at the G2/M phase through the blockage of $p34^{cdc3}$/cyclin B complex formation (123, 151, 256, 264) and causes apoptosis independently of cell cycle alterations (227, 291, 301, 302).

The structure of Vpr consists of three α helixes with the second α helix containing a amphipathic leucine zipper region between amino acids 60-81 which may account for dimer formation and other interactions (31). The domains for Vpr-mediated functions are shown in **Figure 1.6**. Vpr-mediated apoptosis in neurons has been localized to amino acids 70-96. Moreover, six arginine residues are located in this region with three residues at position 73, 77 and 80 have been implicated in apoptotic function (143, 253, 273).

**FIGURE 1.6. STRUCTURE OF VPR.** Vpr is 96 amino acids and has a molecular weight of 14kDa. The N-terminal region (1-52) contains the domains required for nuclear localization and virion packaging signals (194, 195). The C-terminal region (52-96) contains the domains for p300, glucocorticoid receptor II, a second virion packaging domain, G2/M cell cycle arrest and apoptosis while TFIIB and uracil DNA glycosylase (UNG) binding occurs through both the N and C terminal regions (5, 12, 70, 143, 159, 160, 194, 198). Apoptosis can occur with amino acids 52-96, however, amino acids 70-96 have been reported to be sufficient for inducing apoptosis(273).



### 1.8.1 VPR PATHOGENICITY

While Vpr is virtually dispensable for HIV-1 infection of T cells *in vitro,* its chief functions seems to be important for HIV-1 sustainability *in vivo*, suggesting Vpr's role in pathogenesis. Since Vpr can cross the plasma membrane (12, 143, 245) and induce apoptosis of $CD4^+$ T cells as well as monocytes and neurons (246, 301, 355), it is thought that this accessory protein may act as a mediator of bystander killing while also mediating cell death of infected cells.

A mechanism by which Vpr causes apoptosis in T cells was reported by the Kroemer group, who showed that Vpr interacted with a major component of the permeability transition pore complex (PTPC) called the adenine nucleotide translocator (ANT), located within the inner membrane of the mitochondria (143). This PTPC is essential for the maintenance of mitochondrial function, acting as the regulator of release of pro-apoptotic factors, such as cytochrome *c* and AIF-1 induced upon apoptotic stimuli like Vpr treatment (143). Vpr was also shown to pass through the outer membrane pore component, called the voltage dependent anion channel (VDAC) to interact with the apoptogenic region of ANT in the mitochondrial intermembrane space, which is the same region that anti-apoptotic Bcl-2 binds to inhibit cytochrome *c* release (142). Moreover, overexpression of Bcl-2 inhibits Vpr-mediated apoptosis (142). Thus, through directly acting on the mitochondria to induce the intrinsic pathway, Vpr causes the activation of caspase-9 and -3 (220, 302). Alternatively, it has also been shown that Vpr can mediate apoptosis that is dependent on the mitochondria but independent on the downstream apoptosome formation suggesting there is an alternate pathway occurring downstream of

the mitochondrial dysregulation, which does not utilize caspase-9 activation (270), but this pathway remains ill defined. The pro-apoptotic effects of Vpr are concentration dependent, whereby apoptosis generally occurs between 0.1μM and 1μM. However, at lower doses, Vpr has also been shown to have anti-apoptotic properties, whereby at lower concentrations of Vpr, anti-apoptotic proteins like Bcl-2 are upregulated and pro-apoptotic proteins, such as Bax are downregulated (63).

Vpr can also affect the apoptotic pathway through indirect mechanisms. Though the cell surface receptor(s) for Vpr is currently unknown, the cytoplasmic receptor or binding partner for Vpr is the glucocorticoid receptor II (258). This interaction was shown to be essential for forming a complex with PARP-1, an NFκB activator, thus sequestering PARP-1 from the nucleus and inhibiting NFκB expression (219). Therefore, anti-apoptotic factors as well as inflammatory factors could not be expressed. In addition to suppression of activation of NFκB, Vpr enhances repression of NFκB through induction of IκB, the negative regulator of NFκB (18).

Vpr may also indirectly induce apoptosis through mediation of DNA damage. Downstream effects of DNA damage have been observed through activation of DNA sensoring kinase, ATR downstream of p53, possibly through interference with damage-specific DNA replication (366). Recently, it has been shown that Vpr interacts with DNA-binding protein (DDB1) leading to proteasomal degradation of UNG2 and SMUG1, preventing DNA repair (279). On the other hand, it has been shown that p53 does not play a role in Vpr-mediated apoptosis (291).

It has long been studied why LTNP do not progress to AIDS. One observation is that some of these patients contain a mutation in Vpr. Sequence analysis of Vpr has shown that the arginine rich region located within the C terminal $(H(S/F)RIG)_2$ are mutated among LTNP individuals. When the amino acids R73, R77 and R80 were mutated, there was significant reduction in apoptosis (143, 189).

### 1.8.2 VPR IN THE CNS

The possibility that Vpr could contribute to the development of HAD was proposed when Vpr was detected within the CSF of HIV-1 patients and the concentration correlated with patients who had neurological disorders (180). Moreover, the level of Vpr within the CSF was comparable to its level in the serum and to the level of Gag p24 in the brain (180). Vpr was also detected within the basal ganglia and cortex of HIVE brains, localizing with macrophages and neurons, but not astrocytes (341), further confirming Vpr presence in HAD and suggesting a putative role in this neurological disorder.

Once it was known that Vpr was detected in the CSF, Vpr was further investigated for potential effects in HAD. Since the hydrophobic domain within the second α helix of Vpr made the protein a good candidate for creating a pore formation, Piller et al (1996) observed that Vpr was capable of creating ion channels, specific for $Na^+$ permeability in planar lipid bilayers (245) which also occurred in Vpr-exposed neurons (246). In fact, this selective cation ion channel in neurons was not formed by the hydrophobic second α helical domain, but sufficiently induced by the N-terminal region of Vpr (244) causing depolarization of the neuron and cell death (244, 246).

*In vitro* Vpr-mediated neurotoxicity was further confirmed through inducing cell death in neurons of the rat hippocampus (135, 246), striatum and cortex (273) and human cell lines extracellularly (236) and intracellularly (235), whereby through propidium iodide, TUNEL and Annexin V positive staining suggested that apoptosis may be occurring. Moreover, microarray of Vpr expressing neurons showed increased pro-apoptotic expression, such as Bad, Bik, caspase-3 and -8 and Fas L, suggesting potential for apoptosis (235).

An *in vivo* model for Vpr-mediated neurotoxicity was recently reported using cortical stereotactic injections of viral vectors containing Vpr into neonatal mice (48). Widespread neuronal loss and synapto-dendritic loss occurred with TUNEL and Annexin V positive staining occurred in the cortex, hippocampus, choroid plexus and cerebellum (48); however, the physiological relevance of this research must be examined since Vpr expression here is much greater than in the brains of HIV-1 infected patients. In addition, the authors used viral vectors that were targeting cell non-specifically causing Vpr intracellular expression of cell types, such as astrocytes and neurons that would not physiologically occur in an HIV-1 infection of the brain.

The pathway to apoptosis mediated by Vpr was further investigated showing activation of caspase-8, up-regulation of pro-apoptotic proteins (236) and activation of caspase-3 *in vitro* (273). Through addition of soluble peptides containing different regions of Vpr, the minimal apoptogenic region of Vpr was localized to amino acids 70-96; however, regions 52-96 were more toxic (273).

In addition to neuronal toxicity, Vpr was also toxic to astrocytes (48, 135). The exact mechanism of cell death is still controversial; however, through TUNEL, Annexin V and DNA fragmentation, it appears that Vpr may also be inducing apoptosis in astrocytes *in vitro* and *in vivo* (48, 135).

Though Vpr seems to induce apoptosis in neurons, the exact apoptotic pathway of soluble Vpr-treated neurons was not completely clear. Dr. Gareth Jones in the Power laboratory had previously shown through *in vitro* studies that low concentrations of soluble Vpr caused excitation of neurons through $Ca^{2+}$-activated $K^{+}$ channels or BK channels, which may suggest the occurrence of excitotoxicity and apoptosis of neurons at higher concentrations of Vpr. The exact Vpr-mediated apoptotic pathway of neurons was further elucidated to be through a caspase-9 pathway, whereby mitochondrial potential was lost, cytochrome *c* was released from the mitochondria and downstream caspase-9 could be activated to induce cleavage and activation of caspase-3. Moreover, Vpr treatment of neurons caused an increase in p53 expression, suggesting a role of DNA damage in Vpr-mediated neurotoxicity. The effects of Vpr on adjacent glial cells, monocytes and astrocytes showed that although no outstanding inflammation was occurring, supernatants of Vpr-treated glial cells was neurotoxic. Moreover, this effect was not due directly to Vpr presence within the supernatant, since blocking antibody to Vpr did not suppress this effect, suggesting that a neurotoxin independent of inflammation is produced by Vpr-treated monocytes and astrocytes. Therefore, soluble Vpr mediates neurotoxicity *in vitro* through both direct and indirect means (150).

## 1.9 HYPOTHESIS AND EXPERIMENTAL RATIONALE

I hypothesized that Vpr mediates neural cell death by way of apoptosis through direct interactions between Vpr and vulnerable neural cell types and indirectly through activation of glial cells, macrophages and astrocytes.

### 1.9.1 VPR MODULATES APOPTOTIC SIGNALLING

Vpr has been shown to induce the expression of pro-apoptotic proteins possibly leading to induction of apoptosis (235), while also suppressing the anti-apoptotic stimuli, such as NFκB (18, 219). Since Vpr seems to have wide ranging effects on apoptotic signaling, I investigated whether this accessory protein may have other modulating abilities.

### 1.9.2 WT AND R77Q VPR (70-96) INDUCTION OF NEURONAL OR ASTROCYTIC CELL DEATH

Previous literature described the 70-96 amino acid domain of Vpr was sufficient to induce neuronal cell death as well, the mutation of R73A caused a significant change in neuronal death, while R77A and R80A did not (273). Since this peptide is sufficient for causing neuronal death and the R77Q mutation correlates with sequences in LTNP (189), we investigated whether R77Q peptide affects Vpr-mediated neuronal and astrocytic cell death. In contrast to Lum *et al* (2003), previous work by Dr. Gareth Jones in the Power laboratory observed significantly more Vpr R77Q sequences occurring in HAD patients compared to ND patients.

### 1.9.3 TRANSGENIC VPR EXPRESSION CAUSES IMMUNE DYSREGULATION

Research on Vpr-mediated neurotoxicity has been primarily done *in vitro*, through tissue culture of neuronal cell lines and of primary rat neurons. Though it has previously been shown that Vpr is present in the CSF (180) and brain (341), the effects of Vpr *in vivo* have not been properly investigated. Though a model has recently been used to observe Vpr-mediated neurotoxicity *in vivo* (48), the biological relevance of this latter model is problematic. During HIV-1 infection within the brain, the cells that are productively infected causing production and secretion of Vpr are perivascular macrophages and microglia. An *in vivo* Vpr transgenic mouse model was available through our collaborator, Dr. Peter Dickie, in which Vpr was expressed under the *c-fms* promoter and hence only in monocytoid cells (73). Since Vpr can interfere with immune modulation, namely through NFκB, this transgenic mouse model was used to observe induction or suppression of inflammation. In addition, through *in vitro* treatment immune activators to bone marrow derived macrophages from the transgenic mouse, we were able to define Vpr-mediated inflammatory affects.

### 1.9.4 VPR MEDIATES NEURODEGENERATION AND NEURONAL APOPTOSIS *IN VIVO*

Vpr has been shown to be neurotoxic causing apoptosis in neurons, *in vitro*; therefore, this effect required confirmation through a biologically relevant *in vivo* Vpr transgenic model. Additionally, using the transgenic model, we determined if Vpr caused abnormal behaviour in the mouse model.

### 1.9.5 VPR MEDIATES ASTROCYTE DEATH AND APOPTOSIS *IN VITRO*

Previously it has been shown that Vpr might cause death of astrocytes, although the authors were uncertain if death was due to apoptosis (135). Confirmation of Vpr-mediated astrocyte apoptosis necessitated further investigation. In addition, since previous work in the Power laboratory has shown an inflammation-independent neurotoxic substance released from Vpr-treated astrocytes, more insight into the possible indirect neurotoxicity should also be investigated.

### 1.9.6 VPR MEDIATES ASTROCYTE LOSS *IN VIVO*

In view of the observation that Vpr is cytotoxic to astrocytes, this effect was examined in the transgenic mouse model. Possible connections between astrocytic loss and neuronal loss were investigated.

### 1.9.6 EXPERIMENTAL DESIGN

To pursue the previously mentioned objectives, two models were used to analyze Vpr's actions. For *in vitro* analyses for objectives **1.9.1** and **1.9.2**, I used soluble recombinant Vpr (NL4-3 derived) expressed by a baculovirus in insect cells. For *in vivo* analysis for objectives **1.9.3, 1.9.4** and **1.9.6**, I used a transgenic mouse model expressing Vpr (NL4-3 derived) (Tg-Vpr) in monocytoid, cells under the *c-fms* promoter, which allowed me to observe Vpr effects under HIV-1 infection conditions.

# CHAPTER 2- METHODS

## 2.1 METHODS FOR CHAPTER 3

### 2.1.1 VPR PREPARATION

The procedure for producing full-length recombinant HIV-1 Vpr protein derived from pNL4-3 has been previously described (180). Briefly, Vpr was produced following infection of high five insect cells ($2x10^9$ cells per liter of culture infected at a multiplicity of infection of 5 to 10) with recombinant baculovirus encoding the HIV-1 NL4-3 *vpr* open reading frame fused to a His-tag at the N-terminus. The Vpr baculovirus vector was kindly provided by Dr Ned Landau (the Salk Institute for Biological Studies, La Jolla, CA, USA). Vpr preparations that were over 90% pure were routinely obtained. Fractions containing Vpr were dialyzed against three changes of 100 volumes of PBS, aliquoted and store at -80°C. The yield of recombinant Vpr obtained by this method was 1mg/L of high five cell culture. Recombinant Vpr preparations were gratefully done by Dr. Eric Cohen's laboratory (Institut de Recherches Cliniques de Montréal, Montreal, PQ, Canada). Of note, there were variations of neuronal and astrocyte toxicity occurring with different fractions of Vpr. Depending on the fraction used, Vpr was added at concentrations that were toxic to neurons and astrocytes, whereby the fraction caused at least 50% decrease in β-tubulin expression through LI-COR assays (discussed later).

### 2.1.2 *IN VIVO* MOUSE MODEL

Vpr transgenic mice were generated as previously described (73) in which *vpr* (NL4-3 derived) was under the control of the *c-fms* (macrophage-colony stimulating factor, M-CSF receptor) promoter, permitting expression chiefly in monocytoid cells. 4

month old Tg-Vpr and littermate wildtype (Wt) mice were subjected to three behavioural tests: the inverted screen test at 20cm and at 40cm, and the horizontal bar test as previously described (115). For the inverted screen test, the mice were measured for the length of time it took them to climb across a mesh screen that measured 20cm x 20cm and 40cm x 40cm. The horizontal bar test consisted of timing how long a mouse could hold onto a horizontal bar (1m long lengthened hanger). The maximum time taken was 300 seconds. The Z-score and mean deficit score was measured for each animal and averaged using previous described calculations (126) described below. Brains from these animals were then harvested and stored for immunohistochemistry, RT-PCR and Western blot studies.

Z score = (time taken - AVE time for Wt mice)/ STDEV of Wt mice

| Z score | Deficit score |
|---|---|
| $\geq 1$ or $\leq -1$ | 0 |
| -1.1-1.5 or 1.1-1.5 | 1 |
| -1.6-2.0 or 1.6-2.0 | 2 |
| -2.1-2.5 or 2.1-2.5 | 3 |
| -2.6-3.0 or 2.6-3.0 | 4 |
| $<-3$ or $>3$ | 5 |

### 2.1.3 VPR PEPTIDES

The sequences of 70-96 Vpr Wt and R77Q were analyzed using PredictProtein (268). Once we saw no aberrant differences between the peptides and the full-length Vpr with respect to predictions, such as secondary structure and solubility differences due to

change in polarity, these peptides were made (Cedarlane). Peptides were then solubilized in PBS at stock concentration of 20mM. Peptides were then exposed to neurons and astrocytes at 1, 5, 10, 20, 30, 40, 50, 60 and 70μM and analyzed for cell viability through LI-COR (described later).

### 2.1.4 CELL CULTURES

LAN-2 (human cholinergic neuroblastoma) and SK-N-SH (human neuroblastoma) cell lines were cultured in MEM (Gibco) and U373 (human astrocytic) cell line was cultured in DMEM (Gibco) containing 10% fetal bovine serum (FBS, Gibco), 1% penicillin (100U/ml)/ streptomycin (100μg/ml, P/S, Gibco). LAN-2 cells were also supplemented with 1% sodium pyruvate (1mM, Gibco) and 1% N2 (Invitrogen) while SK-N-SH cells were supplemented with 1% sodium pyruvate. Prior to use, LAN-2 and SK-N-SH cells were differentiated for 3 or 5 days in L-15 medium (Sigma-Aldrich) containing 10% FBS, 1% P/S and 1mM or 2mM dibutyryl-cAMP (Sigma-Aldrich), respectively.

Mouse bone-marrow derived macrophages (BMDM) were isolated from the pelvis and femurs of mice using culture methods previously described (321). Briefly, remaining muscle tissue from pelvis and femurs were removed and the bone marrow was washed out using a 25 gauge needle. Cells were cultured at $8x10^5$ cells per well of a 24-well plate in DMEM containing 10% FBS, 10% L929 supernatant and 1% P/S. Cultures were left undisturbed for 5 days at 37°C and 10% $CO_2$. Half the media was changed on day 5 and all of the media was changed on day 6. Cells were treated on day 7 or 14. If cells went longer than 7 days, media was changed every other day.

Rat fetal neurons (RFN) were isolated from the septum of day 17 and 18 old Sprague-Daley rat fetuses (Charles River, Canada) by methods previously described (147) following approved protocols set out by the local Health Sciences Animal Policy and Welfare Committee. Briefly, basal forebrain neurons from the septal regions were dissected into Hanks balanced salt solution (HBSS, Invitrogen) containing 15mM HEPES (Sigma-Aldrich), 10U/ml penicillin (Sigma-Aldrich), and 10mg/ml streptomycin (Sigma-Aldrich); using 0.05% trypsin (Sigma-Aldrich), dissections were then digested; triturated; and plated at $8x10^5$ per well on a 96-well plate. The cultures were left to grow for 24 hours in Neurobasal medium (Sigma-Aldrich) containing with 1% N2 at 37°C and 5% $CO_2$ to remove access glial cells. Medium was changed 1 day later and neurons were treated 7 days later. The isolation of RFN was appreciatively done by David MacTavish of the Jhamandas Laboratory (University of Alberta, Edmonton, AB, Canada).

Human fetal neurons (HFN) were isolated from 15-19 week old aborted fetuses and cultured by methods previously described (251) supported by University of Alberta Ethics Committees. Brain tissue was isolated from the vasculature and digested with 0.25% trypsin (Gibco) and 0.2mg/ml of DNase I (Roche) for 30 minutes at 37°C. Once the tissue had reached a viscous texture the digested tissue was then passed through a 125μm mesh using the end of a plunger to push individual cells through. After centrifugation and a PBS wash, cells were seeded at around $6x10^7$ cells per flask into poly-orthinine (Sigma-Aldrich) coated T75 flasks. HFN growth media contained DMEM, 10% FBS, 1% P/S, 0.2% amphotericin B (0.5μg/ml, Gibco), 0.04% gentomycin (20ug/ml, Gibco), 1% minimal essential amino acids (0.1μM, Gibco), 1% L-glutamine

(2mM, Gibco), 1% sodium pyruvate. Cytosine arabinoside (Ara-C, 25μM, Sigma-Aldrich) was added to HFN to purify the neurons from the mixed culture. Cells were cultured for 3-4 weeks with change of media containing Ara-C every 5 days. About 10-14 days after culture in T75 flask, cells were trypsinzed with 0.25% trypsin, 0.4mg/ml DNase I and 2mM EDTA/PBS and reseeded into respective plates for experiments. Neurons were continued to culture and purify in the respective plates for 2-3 weeks before experimentation.

### 2.1.5 IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

Paraffin-embedded 5μm brain sections from HIV-1 seropositive and seronegative patients and mice after neurobehavioral studies were immunostained with antibodies to HIV-1 Vpr (1/200; rabbit polyclonal raised against bacterially expressed recombinant Vpr (175)), CD45 (1/25; Zymed), Iba-1 (1/100; Wako) and F4/80 (1/100; Serotec) for macrophage and microglia detection, NeuN (1/200; Chemicon) for neuron detection and cleaved caspase-3 for detection of activated caspase-3 (C-Casp-3, 1/100; Cell Signaling Technology). Immunostaining protocols for single and double labelling were performed, as previously reported (321).

For immunofluorescent staining, dehydrated sections were baked at 60°C for 1 hour; rehydrated through sequential wash steps from xylene (Anachemia) to decreasing 100%, 95% and 70% ethanol to water; to retrieve antigen, slides were boiled for 10 minutes in 0.01M sodium citrate buffer (pH 6, OmniPur); slides were blocked in solution containing PBS, 0.2% triton X-100 (Sigma-Aldrich), 10% normal goat serum (NGS, Sigma-Aldrich) and 2% bovine serum albumin (BSA, Sigma-Aldrich) for 2 hours at

room temperature; primary antibody was diluted in PBS, 0.2% triton X-100, 5% NGS and 1% BSA overnight at 4°C using antibody dilutions according to the respective antibody protocol; after 3 washes in PBS, secondary antibody was added in the same block solution as primary antibody and incubated for 2 hours at room temperature; then washed 3 times in PBS; and mounted in gelvatol (PBS/ 2 glycerol (Anachemia): 1 polyvinyl alcohol (Sigma-Aldrich)). Immunofluorescent secondary antibodies were goat anti-mouse and goat anti-rabbit Alexa Fluor 488 (1/500, Molecular Probes), goat anti-rat FITC (1/500, Serotec) and goat anti-rabbit Cy3 (1/500, Jackson). Sections were then analyzed on a LSM Zeiss confocal microscope at 63x magnification.

For immunohistochemical staining, sections were baked and rehydrated as in immunofluorescent staining. For BCIP (purple) staining, sections were blocked with levamiscle (Vector) in 100mM Tris-HCl (pH 9.5, Sigma-Aldrich) for 30 minutes; then blocked in serum (as mentioned above); after staining with the first primary antibody overnight (as mentioned above), sections were incubated in goat anti-mouse alkaline phosphatase secondary antibody (1/500, Jackson) for 2 hours and then stained with BCIP (Vector) for 30 minutes. For DAB (brown) staining, sections were blocked in 0.3% $H_2O_2$ (Caledon) for 20 minutes; blocked in the above mentioned serum; incubated in the second primary antibody (as above); after washing, incubated in goat anti-rabbit biotinylated secondary antibody (1/500, Vector); then after washing, sections were incubated in vecastain (Vector) for 2 hours; and then stained with DAB (Vector) for 3-10 minutes; after washes, sections were then dehydrated in 70%, 95% and 100% ethanol and

mounted with acrytol (Surgipath). Sections were then analyzed on a Zeiss Fluorescent microscope at 40x and 100x magnification.

### 2.1.6 DIRECT AND INDIRECT NEURONAL INJURY ASSAYS

For the direct neuronal injury assay, differentiated LAN-2 cells, RFN and HFN were cultured in 96-well flat bottom plates in serum free AIM-V media (Gibco) with and without different concentrations of HIV-1 full-length Vpr and Vpr peptides (70-96) for 48 hours. After the culture period, cells were fixed with 4% formalin, washed in PBS containing 0.1% Triton-X 100 and blocked for 90 minutes at 4°C with LI-COR Odyssey Blocking Buffer (LI-COR, Lincoln, USA). After removal of the blocking reagent the cells were incubated overnight at 4°C with mouse anti-β tubulin (1/600, Sigma-Aldrich). Following primary antibody application, the cells were washed in PBS containing 0.1% Tween 20 and incubated with goat anti-mouse Alexa Flour 680 (1/200, Molecular Probes) secondary antibodies. All antibody dilutions were made with LI-COR Odyssey Blocking Buffer. After removal of the secondary antibodies, the cells were washed in PBS/0.1% Tween 20 and left to dry in the dark prior to quantification of β-tubulin using the Odyssey Infrared Imaging System (LI-COR). Abundance of β-tubulin in each treatment was analyzed relative to the AIM V alone control.

For the indirect neuronal injury assay, supernatants were also removed from Wt and Tg-Vpr BMDM cultures. These supernatants (S/N) were then applied in the presence or absence of HIV-1 Vpr specific antibodies (1/100) to RFN. As a positive control, soluble Vpr (1000nM) was added to Wt S/N just before applying to RFN. After 48 hours

of exposure, neuronal cell death was quantified by β-tubulin immunoreactivity (as described above).

### 2.1.7 HUMAN BRAIN SAMPLES

Autopsied frozen and fixed brain tissue (frontal lobe) from HIV-1 B clade seropositive (all were AIDS-defined) and seronegative individuals, collected with consent and stored in the Laboratory for Neurological Infection and Immunity Brain Bank (University of Alberta) were used for immunohistochemistry.

### 2.1.8 WESTERN BLOTTING

Differentiated LAN-2 cells were cultured with or without HIV-1 Vpr for up to 6 hours, following which cells were lysed in lysis buffer (20mM Tris, 1% NP-40, 50mM NaCl, protease inhibitors (1/1000; Calbiochem Protease Inhibitor cocktail set III) serine phosphatase inhibitor, 1μM Okadaic acid (Chemicon) and 0.5mM EDTA). Protein lysates were also prepared from the brains of Tg-Vpr and Wt animals. Protein lysates were separated by 10% SDS-polyacrylamide, and the membrane blotted for phosphorylated Akt (1/1000; Cell Signaling), total Akt (1/000; Cell Signaling), $PI_3K$ (1/500; Cell Signaling), GAD65 (1/1000; Chemicon), synaptophysin (Synap, 1/1000; Santa Cruz), vesicular acetylcholine transferase (VAChT, 1/1000; Sigma-Aldrich), cleaved caspase-3 (C-Casp-3, 1/250; Cell Signaling Technology) and β-actin (1/1000; Santa Cruz). Ratios of Western blot immunoreactivity in Wt and Tg-Vpr mice relative to β-actin were quantified by densitometry using Scion imaging software.

### 2.1.9 REAL-TIME PCR

RNA from Wt and Tg-Vpr brains were isolated using TRIzol (Life Technologies, Gaithersburg, MD) in accordance with the manufacturer's guidelines. Briefly, 200µl of chloroform (24:1 chloroform:isoamyl alcohol, Sigma-Aldrich) was added to 1ml of TRIzol and the aqueous layer containing RNA was purified and precipitated using isopropanol and 70% ethanol. Genomic RNA was dissolved in diethylpyrocarbonate (DEPC) treated water and used for the synthesis of cDNA where 2µg of RNA was treated with final concentration 0.17U DNase (initial concentration 1U/µl, Promega), DNase buffer (10x, Promega) and 1.73U RNase out (40U/µl, Roche). RNA was then reverse transcribed to cDNA using 2.43mM dNTP (10mM, Invitrogen), 9.75$A_{260}$U random primers (50$A_{260}$U, Roche), 7.92mM DTT (0.1M, Invitrogen), reverse transcription buffer (5x, Invitrogen), 0.40U RNase out, and 3.96U superscript (200U/µl, Invitrogen). Refer to Table 1 for the list of primers used for Chapter 3 to observe gene expression. Set up of real-time PCR includes making a 25µl mix of 5µl cDNA made previously; supermix containing PCR buffer (10x, New England Biosciences), 1.08mM dNTP (1.6mM, Invitrogen), 0.675% Tween-20, 6.75% glycerol (Omnipur); 1/16000 Syber-green (1/6000, Molecular Probes); 1/200000 Fluorescene (1/10000, Biorad); 0.5µM Primer (except Nestin at 0.75µM); and 0.05U *Taq* polymerase (5U/µl, New England Biosciences). *Semi*-quantitative analysis was performed by monitoring in real time the increase in the fluorescence of the SYBR-green dye on a Bio-Rad i-Cycler (Bio-Rad Laboratories, Hercules, CA). Threshold cycle value was taken from the point at which fluorescent measurements were at an increasing slope. All data were normalized against

the GAPDH mRNA level and expressed as fold increases relative to the average un-stimulated control. Confirmation for all real-time PCR was done by melt-curve analysis through loss of fluorescence of Fluorescene when the dye is released from the double stranded DNA helix at the corresponding melting temperature.

### 2.1.10 TREATMENT OF BMDM

To induce *vpr* expression and observe effects of Vpr on immune system isolated BMDM were treated on day 7 and/or 14 with TNF-α (10 and 50ng/ml, R&D Systems), retinoic acid (RA, 10 and 50nM, Sigma-Aldrich), M-CSF (50ng/ml, Cedarlane), IL-3 (100ng/ml), Lipopolysaccharide (LPS, 100ng/ml, Sigma-Aldrich), pertusis toxin (PTX, 1 and 0.1 μg/ml, Sigma-Aldrich), phorbol 12-myristate 13 acetate (PMA, 50ng/ml, Sigma-Aldrich). In addition, to induce *vpr,* variations on L929 supernatant supplementation was done at 0%, 10% and 20% while the total volume of media containing 10% L929 supernatant was reduced by half. After a 24 hour treatment, supernatants were taken for indirect neurotoxicity assays and RNA was isolated from the cells, cDNA was made and *vpr* induction and inflammatory genes were analyzed through real-time PCR using protocols described above.

## 2.2 METHODS FOR CHAPTER 4

### 2.2.1 VPR PREPARATION

Recombinant Vpr preparations were prepared as previously described in 2.1.1.

### 2.2.2 *IN VIVO* TRANSGENIC MOUSE MODEL

The *in vivo* Tg-Vpr mouse model was produced as previously described in 2.1.2.

### 2.2.3 CELL CULTURE

Human fetal astrocytes (HFA) were isolated from the same aborted fetuses as HFN. Tissue was digested the same as above. However, cells were not passed through a wire mesh, therefore, after digestion cells were centrifuged and washed, counted and seeded at about $6x10^7$ cells per T75 flask in media previously described for HFN, but without AraC. HFA were passaged 4-6 times to remove HFN. To remove cells from the flasks cells were trypsinzed with 0.25% trypsin and 2mM EDTA/PBS followed by FBS wash. HFA may have also been frozen down to remove HFN as well.

### 2.2.4 IMMUNOFLUORESCENCE

Using the protocol described above, tissue sections from the brains of Wt and Tg-Vpr and HFA were immunostained with rabbit anti-GFAP (1/1000, Dako) for detection of astrocytes. Wt and Tg-Vpr sections and Vpr treated HFA were double labeled for astrocytes with mouse anti-GFAP (1/200, BD Pharmagen) and rabbit anti-C-Casp-3 (1/100). Immunofluorescent secondary antibodies were with goat anti-rabbit Alexa Flour 488 (1/500), goat anti-mouse Alexa Flour 488 (1/500) and goat anti-rabbit Cy3 (1/500), respectively. Sections and HFA were analyzed using an LSM Zeiss confocal microscopy at 63x magnification.

### 2.2.5 ASTROCYTIC VIABILITY ASSAY

In a 96-well plate, HFA and U373 astrocytes were treated with Vpr at 0, 1, 10, 100 and 1000nM in replicates of 5 wells or more for 48 hours. Cells were then stained with mouse anti-β-tubulin (1/600) using the LICOR protocol previously mentioned above. The percentage of β-tubulin expression for each treatment was relative to the average intensity for control wells containing no Vpr.

### 2.2.6 MITOCHONDRIAL MEMBRANE POTENTIAL

HFA were analyzed for cell death and loss of mitochondrial membrane potential. One day before treatment, HFA were seeded in 6-well plates at $5x10^5$ cells well. Cells were then treated with cytotoxic concentration of Vpr at 1000nM for 8, 12 and 16 hours. As a positive control, HFA were treated with UV-C ($200mJ/cm^2$). Cells were first stained with tetramethylrhodamine ethyl ester (TMRE, 0.2μM, Molecular Probes) for 30 minutes at 37°C and 5% $CO_2$. After one wash with PBS, cells were then trypsinzed with 0.25% trypsin and 2mM EDTA/PBS and washed with FBS containing media. Cells were washed once with PBS containing 1% FBS and then stained with 2μg 7-amino-actinomycin D (7AAD, 1μg/μl, Sigma-Aldrich). Cells were then analyzed by flow cytometry using FACSCanto (BD Bioscience) with the following voltage settings: FSC-A (250V, lin), SSC-A (311V, lin), FL-2 TMRE (352V, log), FL-3 7AAD (440V, log). Compensation was set for TMRE 8.05% of 7AAD and 7AAD 28.02% of TMRE. Data was collected from 10 000 cells per sample and analyzed using BD FACSDiva software.

### 2.2.7 CASPASE-3 ACTIVATION ASSAY

HFA were pre-treated with zVAD-fmk (50μM, Calbiochem) for 1 hour, followed by treatment with and without Vpr (1000nM) for 10 hours. Lysates were taken and 100μg were loaded onto a 15% SDS-PAGE agarose gel.

### 2.2.8 WESTERN BLOTTING

Western blotting protocol for Wt and Tg-Vpr brain protein lysates were the same as above until blocking. Once transferred, blots were blocked in LI-COR Odyssey Blocking Buffer for 1 hour and then incubated overnight in primary antibody at 4°C; blots were then washed 5 times in PBS/ 0.1% Tween 20 and secondary antibody was added for 1 hour each; after 5 washes in PBS/ 0.1% Tween 20 blots were scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR). Antibodies used were astrocyte marker, mouse anti-GFAP (1/1000), rabbit anti-C-Casp-3 (1/100) and β-actin (1/1000) as a loading control. Secondary antibodies used here were donkey anti-goat Alexa Flour 680 (1/5000, Molecular Probes) and then goat anti-mouse IRDye 800CW (1/10000, Rockland Immunochemicals) or goat anti-rabbit IRDye 800CW (1/10000, Rockland Immunochemicals). Densitometry was calculated as follows:

$$\text{Densitometry} = \frac{(\text{AI protein of interest}/\ \text{AI } \beta\text{-actin})}{\text{wildtype AVE } (\text{AI protein of interest}/\ \text{AI } \beta\text{-actin})}$$

(AI= average intensity-background)

### 2.2.9 REAL-TIME PCR

RNA from Wt and Tg-Vpr brains and treated HFA were isolated using the previous protocol. Of which, cDNA was made and gene expression was *semi*-quantified

using primers listed in **Table 1** for Chapter 4. HFA were treated for 6 hours with 0, 10, 100, 1000nM of Vpr for 6 hours before isolation of RNA.

| Gene | Species | | Sequence (5'-3') | Annealing Temp (°C) | Chapter |
|---|---|---|---|---|---|
| GAPDH | Hm/Ms | Fwd | AGC CTT CTC CAT GGT GGT GAA GAC | 50-60 | 3 and 4 |
| | | Rev | CGG AGT CAA CGG ATT TGG TCG | | |
| HIV-Vpr NL43 outer flanking | HIV | Fwd | AGA GGA CAG ATG GAA CAA GCC | 60 | 3 |
| | | Rev | CTA GTC TAG GAT CTA CTG GCT CC | | |
| HIV-Vpr NL43 inner flanking | HIV | Fwd | GAC ACT AGA GCT TTT AGA GG | 57 | 3 |
| | | Rev | GGA TAA ACA GCA GTT GTT GCA G | | |
| IL-1β | Hm | Fwd | CCA AAG AAG AAG ATG GAA AAG CG | 58 | 3 and 4 |
| | | Rev | GGT GCT GAT GTA CCA GTT GGG | | |
| IL-1β | Ms | Fwd | CAA CCA ACA AGT GAT ATT CTC CAT G | 60 | 3 |
| | | Rev | GAT CCA CAC TCT CCA GCT GCA | | |
| IL-6 | Hm | Fwd | ACC CCT GAC CCA ACC ACA AAT | 58 | 3 and 4 |
| | | Rev | AGC TGC GCA GAATGA GAT GAG | | |
| IL-6 | Ms | Fwd | CAA CCA CGG CCT TCC CTA CT | 54 | 3 |
| | | Rev | TCA TTT CCA CGA TTT CCC AGA G | | |
| TNF-α | Hm | Fwd | ACC TCA TCT ACT CCC AGG TCC | 58 | 3 and 4 |
| | | Rev | CTC TTG ATG GCA GAG AGG AGG | | |
| TNF-α | Ms | Fwd | ATG CTG GGA CAG TGA CCT GG | 60 | 3 |
| | | Rev | CCT TGA TGG TGG TGC ATG AG | | |
| IL-10 | Ms | Fwd | GGT TGC CAA GCC TTA TCG GA | 60 | 3 |
| | | Rev | ACC TGC TCC ACT GCC TTG CT | | |
| F4/80 | Ms | Fwd | GCC ACC TGC ACT GAC ACC | 54 | 3 |
| | | Rev | GCT GCA CTT GGC TCT CC | | |
| GFAP | Ms | Fwd | GGA CAT CGA GAT CGC CAC CTA CAG | 58 | 4 |
| | | Rev | CTC ACC ATC CCG CAT CTC CAC AGT | | |
| Nestin | Ms | Fwd | GAC ACC TGG AGG AAG TTC | 53 | 4 |
| | | Rev | GTG TCT TCA GAA AGG CTG | | |
| S100β | Ms | Fwd | GAG CTG GAG AAG GCC ATG GTT | 59 | 4 |
| | | Rev | GTC CAG CGT CTC CAT CAC TTT | | |
| AQP4 | Ms | Fwd | GCC ACG CTT ATC TTT GTT TTG | 57 | 4 |
| | | Rev | CCT AGC GAT GCT GAT CTT TCG | | |
| GLAST-1 | Ms | Fwd | ACG GTC ACT GCT GTC ATT G | 58 | 4 |
| | | Rev | TGT GAC GAG ACT GGA GAT GA | | |
| GLT-1 | Ms | Fwd | GGA AGA TGG GTG AAC AGG C | 58 | 4 |
| | | Rev | TTC CCA CAA ATC AAG CAG G | | |
| NGF | Hm/Ms | Fwd | GGC AGT GTC AAG GGA ATG CGA AGT T | 56 | 4 |
| | | Rev | CCA AGG GAG CAG CTT TCT ATC CTG G | | |
| IGF | Ms | Fwd | CCC CGG GTC CCA CTT AGA | 58 | 4 |
| | | Rev | TGA GGC TGC CAT AGA AAA ATA G | | |
| BDNF | Ms | Fwd | GAA AGT CCC GGT ATC CAA AG | 54 | 4 |
| | | Rev | CCA GCC AAT TCT CTT TTT | | |
| VEGF | Ms | Fwd | GGA CCC TGG CTT TAC TGC T | 56 | 4 |
| | | Rev | TGG CTT TGG TGA GGT TTG AT | | |

**TABLE 1. LIST OF PRIMERS USED FOR REAL-TIME PCR**

# CHAPTER 3 – HIV-1 VPR CAUSES NEURONAL APOPTOSIS AND *IN VIVO* NEURODEGENERATION

## PREFACE

The preceding data was published in the Journal of Neuroscience and formatted to comply with the traditional thesis format. The data presented was acquired from G. Jones, K. Harris and myself with contributions from J. Jhamandas and C. Power. Brain samples, soluble Vpr and the transgenic mouse model were acquired from J. Holden, É. Cohen and P. Dickie, respectively. The manuscript was jointly written by G. Jones and C. Power with contributions from myself. Figures 3.4, 3.8 and 3.9 were not part of the published data.

# HIV-1 VPR CAUSES NEURONAL APOPTOSIS AND *IN VIVO* NEURODEGENERATION

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* GJJ AND NLB CONTRIBUTED EQUALLY TO THIS PAPER

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## 3.1 HIV-1 VPR *IN VIVO* ABUNDANCE AND NEUROTOXICITY

Although Vpr is known to exert neuropathogenic actions and has been observed in the brain (135, 235, 236, 246, 273, 341), the extent of Vpr detection in brain is unknown. Hence, we examined Vpr expression in brains from HIV-1 infected and uninfected persons using a polyclonal antibody, which specifically recognized HIV-1 Vpr (**Figure 3.1A (i, inset)**). Vpr immunoreactivity was not present in sections from HIV-1 uninfected brains (**Figure 3.1A (i)**). However, in brain sections from HIV-1 infected patients, Vpr immunoreactivity was detectable in perivascular and parenchymal cells that resembled monocytoid cells (**Figure 3.1A (ii)**). Furthermore, we observed that Vpr immunoreactivity was co-localized with CD45 immunoreactivity in brain sections from HIV-1 infected brains (**Figure 3.1A (ii), inset**). Hence, Vpr was expressed in the brains of patients with HIV-1 infection, chiefly in monocytoid cells.

Given that Vpr is a secreted protein, detected in both serum and cerebrospinal fluid (180), we next investigated its effects on a human cholinergic neuronal cell line (LAN-2) as well as primary human and rat fetal neurons (HFN and RFN, respectively). Soluble Vpr was neurotoxic in a concentration-dependent manner, as indicated by loss of β-tubulin immunoreactivity (**Figure 3.1B**) with the extent of neurotoxicity hinging on the individual cell type. Human neuronal cells (LAN-2 and HFN) exhibited greater vulnerability to Vpr-mediated neurotoxicity at 100nM compared to RFN where there was evident loss of neuronal viability occurring at 500nM (**Figure 3.1B**). Importantly, the neurotoxic effects of Vpr were reduced by immunoabsorption of Vpr with an anti-Vpr polyclonal antibody in both Vpr-treated (100nM) and untreated (**Figure 3.1C**). To

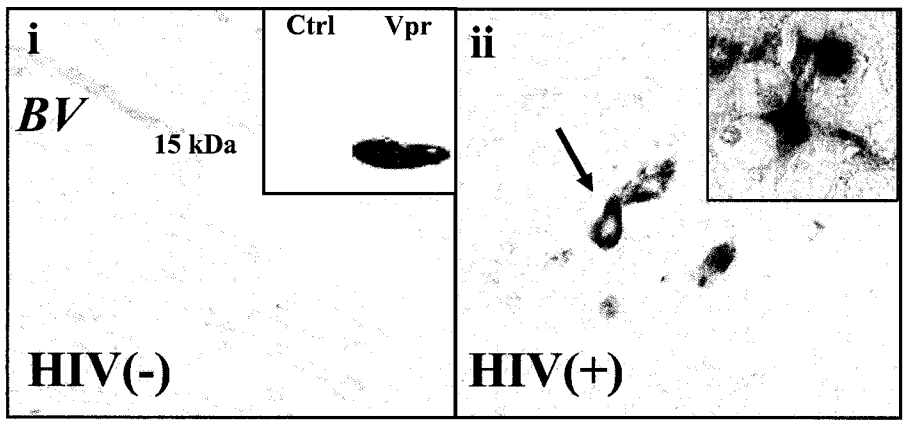
pursue the mechanism by which neurons were injured by Vpr, we assayed activated or cleaved caspase-3 (C-Casp-3) abundance (normalized to β-tubulin abundance), revealing an increase in caspase-3 activation in Vpr-treated neurons (**Figure 3.1D**). Complementing these latter findings, neuronal p53 level was also elevated in neurons following Vpr treatment, suggesting that apoptosis was the underlying process that caused neuronal damage (**Figure 3.1D**). **Figure 3.1 A** (not insets), **C** and **D** were done by G. Jones.

### 3.2 HIV-1 VPR MODULATES NEURONAL MEMBRANE RESPONSES

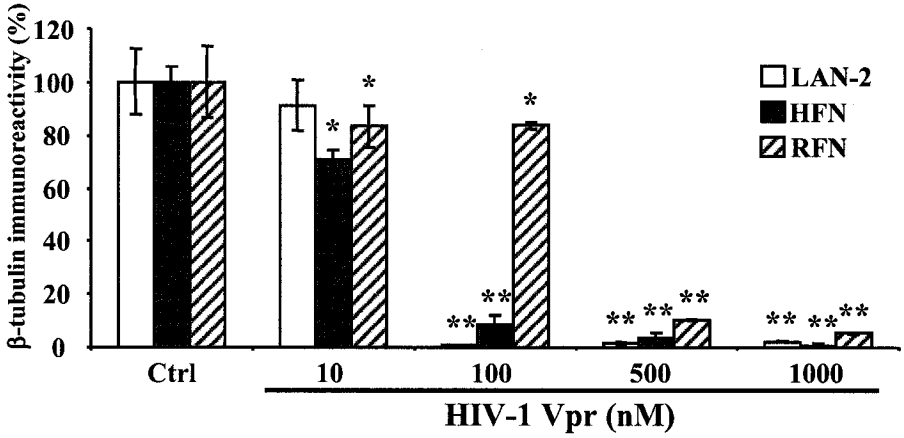
Earlier studies showed that high concentrations of Vpr exerted variable electrophysiological effects on neurons (246). To define the precise pathophysiological actions of Vpr on neuronal membrane activity, we examined the current-voltage relationship in dissociated rat septal neurons, indicating that Vpr (10nM) caused a reduction in outward whole-cell currents in the voltage range from -30 mV to +3 mV (**Figure 3.2A**). Indeed, a Vpr concentration-dependent effect was observed in terms of a reduction in whole cell currents in dissociated neurons (**Figure 3.2B**). When the high conductance $Ca^{2+}$-activated $K^+$ channels (BK channels) were inhibited with iberiotoxin (IBTX, 25nM), the Vpr-induced reduction in current was significantly blocked (**Figure 3.2C**; inset represent results from 5 cells). Vpr (10nM) also caused a reduction in the delayed rectifier ($I_K$) potassium current (**Figure 3.2D, top panel**) but not the transient outward ($I_A$) potassium current (**Figure 3.2D, bottom panel**). Thus, Vpr was found to induce rapid changes in neuronal membrane currents involving an inhibition of voltage-dependent potassium channels. **Figure 3.2** was done by K. Harris.

**FIGURE 3.1. HIV-1 VPR IS EXPRESSED IN BRAINS OF HIV-1 INFECTED PATIENTS AND INDUCES NEURONAL CELL DEATH.** (A) Detection of recombinant HIV-1 Vpr using an anti-Vpr polyclonal antibody on Western blot (Ai, inset). HIV-1 Vpr immunoreactivity was absent in brain sections from HIV-1 uninfected controls (Ai). Conversely, Vpr was detectable in brain sections from HIV-1 infected patients in perivascular and parenchymal cells (Aii, arrow) (magnification=40x). These cells resembled monocytoid cells and co-expressed CD45, whereby Vpr was stained brown (DAB) and CD45 was stained purple (BCIP) (Aii, inset) (magnification=100x). (BV; blood vessel). (B) β-tubulin expression in human neurons: LAN-2 cholinergic cell line and human fetal neurons (HFN) and rat fetal neurons (RFN) treated with increasing concentrations of HIV-1 Vpr for 48 hours, showing a concentration-dependent reduction in neuronal viability as evidenced by reduced β-tubulin immunoreactivity. (C) β-tubulin immunoreactivity in LAN-2 cells with or without HIV-1 Vpr (100nM) treatment, with and without anti-HIV-1 Vpr antibodies for 48 hours, showing that pre-treatment with the antibody rescued neurons from Vpr-mediated neurotoxicity. (D) Active caspase-3 and total p53 abundance was increased in LAN-2 cells treated with 100nM Vpr for 24 hours. Values were normalized to the β-tubulin immunoreactivity, and expressed as the percentage increase relative to control cells. For methods not described, refer to (150). Statistical analysis of B and C were done by Dunnet multiple comparisons test while D was analyzed through students' t-test. Error bars represent the SEM (*$p<0.05$, **$p<0.01$).

A




B

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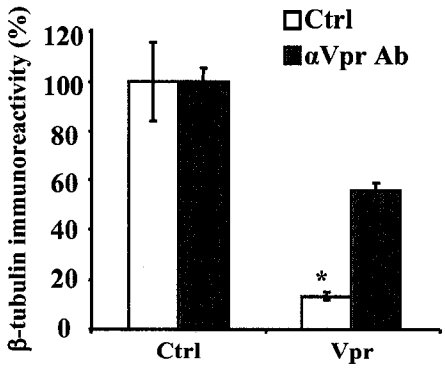



D

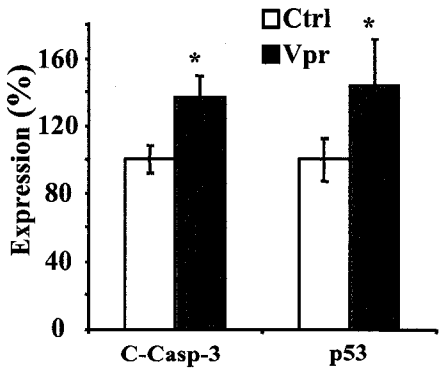
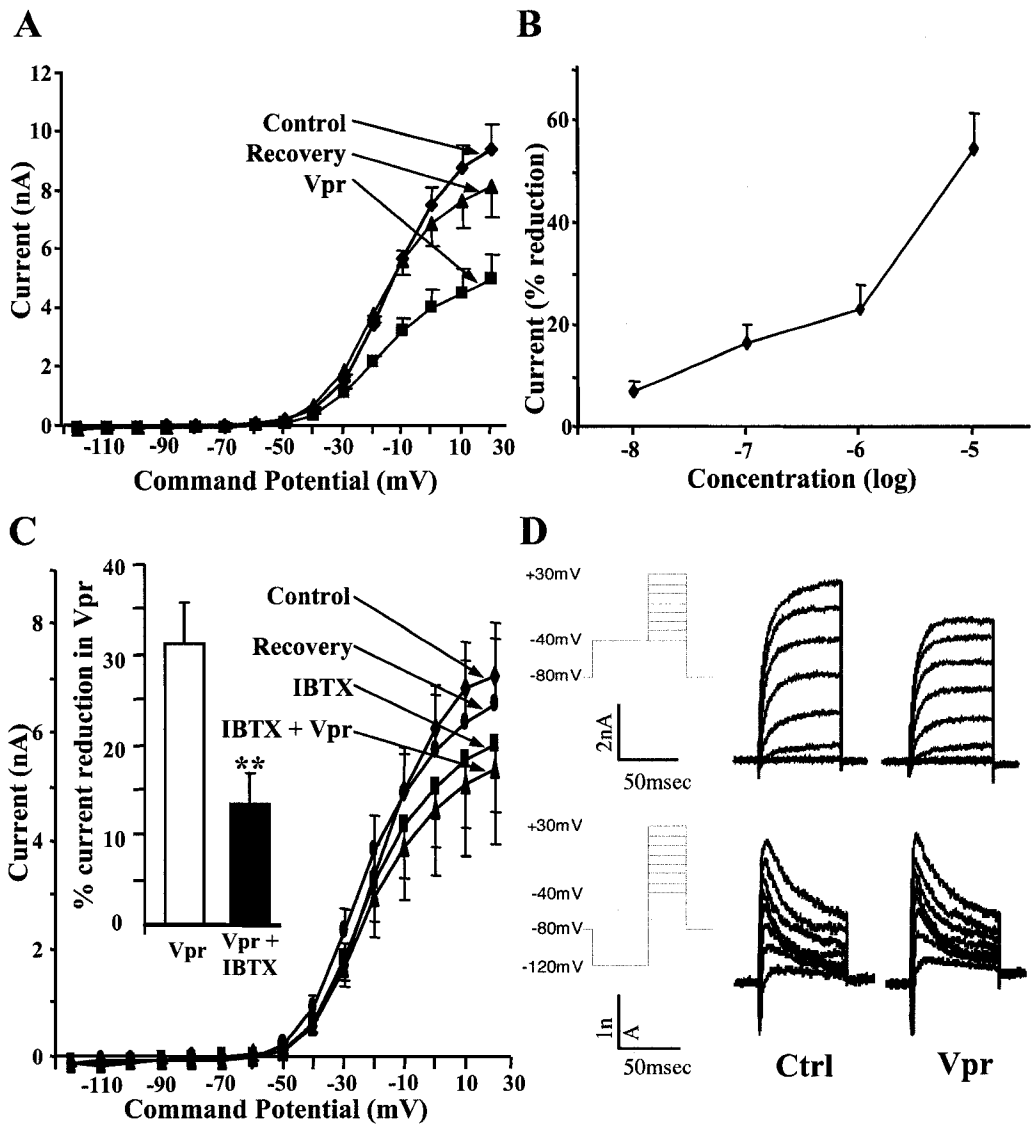


**FIGURE 3.2. HIV-1 VPR MODULATES NEURONAL MEMBRANE RESPONSES.** (A) Whole-cell currents were reduced in acutely dissociated rat basal forebrain neurons (n=9 cells) following treatment with soluble HIV-1 Vpr (10nM). (B) Concentration-response relationship for HIV-1 Vpr showing a reduction of whole-cell currents (n= 4-9 cells at each concentration). (C) HIV-1 Vpr-mediated reduction in whole-cell current was blocked in the presence of 25nM iberiotoxin (IBTX) (histograms in inset represents 5 cells). (D) HIV-1 Vpr (10nM) reduced the delayed rectifier ($I_K$) potassium current (top panel) but not the transient outward ($I_A$) potassium current (bottom panel). Insets show voltage protocols used to identify $I_K$ and $I_A$. $I_A$ obtained from subtracting currents derived with the two voltage protocols shown. Statistical analysis of the inset in C was done by students' test. Error bars represent the SEM. For methods, refer to (150). (** $p<0.01$).



### 3.3 HIV-1 VPR MEDIATES NEURONAL APOPTOSIS THROUGH CASPASE-9 ACTIVATION

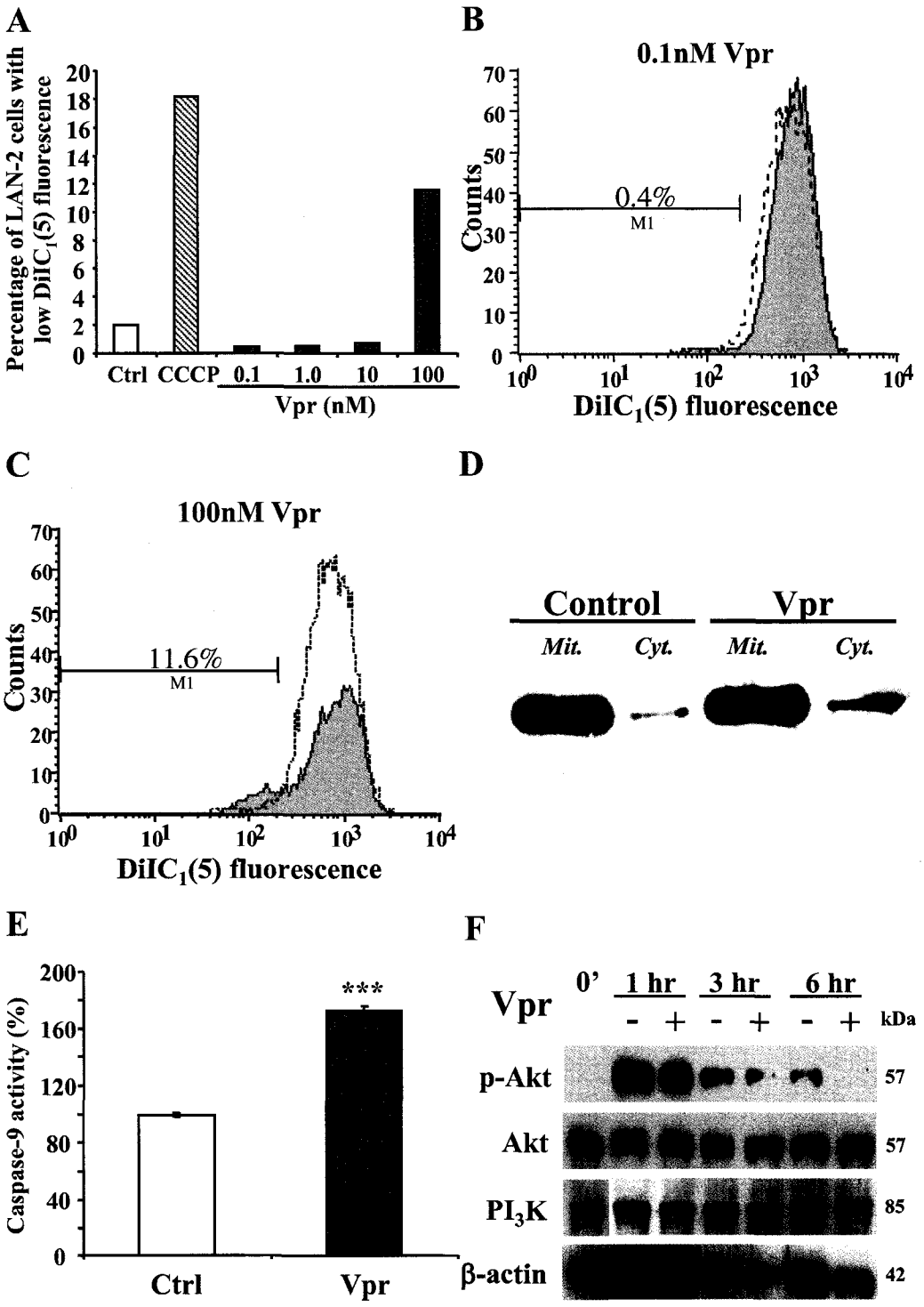
Since activation of BK channels and caspase-3 are involved in the apoptosis signalling pathway (184), we next investigated the effects of HIV-1 Vpr on the 'up-stream' events, eventually leading to caspase-3 activation. As increased cleaved caspase-3 level was only observed in neuronal cells treated with 100nM HIV-1 Vpr (**Figure 3.1D**), we used this 'neurotoxic' concentration in the next series of experiments. It is well established that p53 can mediate the 'intrinsic' apoptosis pathway, and thus, we examined whether Vpr-induced neuronal cell death was mediated through this pathway. Mitochondrial membrane permeability was assessed with the cyanine dye $DiIC_1(5)$, which accumulates in mitochondria with active membrane potentials (283). In contrast to the controls, $DiIC_1(5)$ staining intensity diminished when neuronal cells were treated with cyanide 3-chlorophenylhydrazone (CCCP), a reagent that disrupts the mitochondrial membrane potential, resulting in an increased percentage of LAN-2 cells with low $DiIC_1(5)$ staining (**Figure 3.3A**). Exposure of neuronal cells to low concentrations of Vpr (0.1 to 10nM) had no effect on the mitochondrial membrane potential (**Figure 3.3A and B**). However, after four hours of exposure to a neurotoxic concentration of Vpr (100nM), there was an approximate six fold increase in the percentage of neuronal cells showing diminished $DiIC_1(5)$ staining compared to untreated cells (**Figure 3.3A and C**). Indeed, the proportion of neuronal cells showing diminished $DiIC_1(5)$ staining upon Vpr exposure approached that of the positive control CCCP. In addition to increased mitochondria membrane permeability, neuronal cells cultured in the presence of Vpr demonstrated greater cytochrome *c* immunoreactivity in cytosolic fractions compared to

untreated controls (**Figure 3.3D**). Moreover, caspase-9 activity was significantly higher in lysates prepared from Vpr-treated neuronal cells, compared to untreated controls (**Figure 3.3E**). These latter studies were supported by reduced phosphorylated Akt abundance in neurons following Vpr exposure compared to induction of phosphorylated Akt in untreated cells due to culture medium change, while total Akt and $PI_3K$ were unaffected by Vpr application (**Figure 3.3F**). Taken together, these results suggested that induction of the intrinsic apoptosis pathway, including increased mitochondria membrane permeability, cytochrome *c* release, caspase-9 activation and reduced phosphorylated Akt, participated in Vpr-mediated neurotoxicity. **Figure 3A-E** were done by G. Jones.

## 3.4 R77Q MUTATION IS ASSOCIATED WITH HAD BUT HIV-1 VPR (70-96) FAILED TO CAUSE LOSS OF NEURONAL VIABILITY AT PHYSIOLOGICAL CONCENTRATIONS

Since Vpr seems to contribute to the development of HAD, we investigated whether there were differences in *vpr* sequences in the brains of HAD and non-demented (ND) patients. Sequences were taken from 9 patients with HAD and 5 patients that were ND (**Figure 3.4A**). While some sequences were obtained by direct sequencing of the *vpr* amplicon from nested PCR (denoted patient ID#-HAD-Br), others were sequenced after insertion of the nested PCR amplicon into the coloning vector, pSL1180 (denoted patient ID#-clone #-HAD-Br). Sequences were then aligned through Bioedit software. At position 77 of Vpr, 94% of sequences isolated from HAD brains presented the R77Q mutation while there was significantly less ($p<0.01$) R77Q mutations in ND isolated brain sequences (22%) (**Figure 3.4A**). Since this mutation occurred significantly more

**FIGURE 3.3. HIV-1 VPR INDUCES THE INTRINSIC APOPTOSIS PATHWAY IN NEURONAL CELLS.** (A-C) $DiIC_1(5)$ staining in LAN-2 cells pre-treated with Vpr. (A) Percent of LAN-2 with reduced $DiIC_1(5)$ staining. Individual $DiIC_1(5)$ fluorescence plots shown for LAN-2 cells treated with (B) 0.1nM Vpr and (C) 100nM Vpr (solid line represents Vpr-treated cells, dotted line represents control treated cells). M1 region represents the percentage of LAN-2 cells with reduced $DiIC_1(5)$ abundance (data summarized in panel A). (D) Increased cytochrome c immunoreactivity was present in the cytosolic fraction from LAN-2 cells pre-treated with Vpr compared to control (*Mit* denotes mitochondrial fraction, *Cyt* denotes cytosolic fraction). (E) Enhanced caspase-9 activity was evident in lysates from LAN-2 cells treated with HIV-1 Vpr. (F) Phosphorylated Akt (p-Akt), total Akt, $PI_3K$ and β-actin immunoreactivity in lysates prepared from LAN-2 cells cultured with or without Vpr for up to 6 hours, showing downregulation of p-Akt. For methods not described, refer to (150). Error bars represent the SEM. Statistical analysis for E was done by a students' test. (***$p<0.001$).



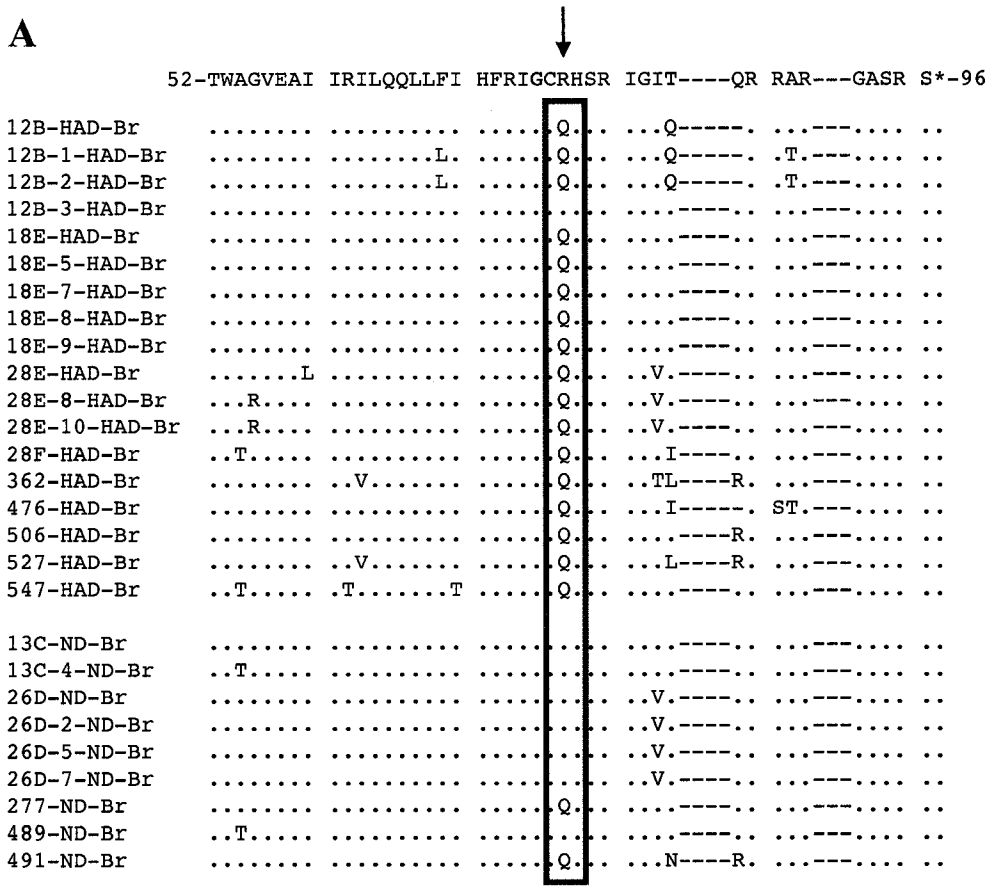
frequently in HAD brain sequences, we investigated whether this mutation had an affect on neurotoxicity. Previous literature revealed that Vpr (70-96) could induce neuronal cell death (273), therefore, we used Vpr (70-96) peptides containing the Wt arginine or mutant glutamine at position 77 of Vpr. When applied to the neuroblastoma cell line, SK-N-SH cells at increasing concentrations, significant ($p<0.05$) loss of neuronal viability occurred in the Wt peptide at 30μM while the R77Q peptide caused loss of viability at 20μM; however at 30 and 40μM, the R77Q peptide did not cause significant loss of viability (**Figure 3.4B**). Therefore, significant and consistent loss of viability occurred at 50μM in the R77Q peptide (**Figure 3.4B**). A general trend occurred at increasing concentration of R77Q peptide between 30 and 60μM where there was comparatively higher β-tubulin immunoreactivity than the Wt peptide, which was significant ($p<0.05$) at 30 and 40μM. Also of note, when compared to full-length Vpr at 1μM, both peptides do not cause neurotoxicity until much greater concentrations (**Figure 3.4B**). Hence, Vpr (70-96) peptides do not cause toxicity to neurons at physiological concentrations, while at higher concentrations, the R77Q mutant peptide is less neurotoxic. Sequences from HAD and ND brains were obtained from G. Jones.

## 3.5 HIV-1 VPR EXERTS CELL TYPE-DEPENDENT IMMUNOGENIC AND NEUROTOXIC EFFECTS

In addition to the direct neurotoxic effects of HIV-1 viral proteins, neuronal apoptosis may also result from neurotoxic host factors secreted by brain resident non-neuronal cells in response to stimulation by viral proteins. To investigate potential indirect mechanisms of Vpr-mediated neurotoxicity, supernatants from HIV-1 Vpr-

**FIGURE 3.4. R77Q MUTATION IS ASSOCIATED WITH HAD AND HIV-1 VPR (70-96) FAILED TO CAUSE LOSS OF NEURONAL VIABILITY AT PHYSIOLOGICAL CONCENTRATIONS.** (A) Vpr sequences of 9 HAD patients and 5 ND patients were taken by direct sequencing through nested PCR with Vpr-specific primers (denoted patient ID#-HAD/ND-Br) or sequenced from a cloning vector, pSL1180, through which Vpr was cloned into (denoted patient ID#-clone #-HAD/ND-Br). Sequences were then aligned and analyzed using Bioedit software. Mutation at position 77 is highlighted showing a significant increase ($p<0.01$) in R77Q mutation in HAD sequences (94%) than ND sequences (22%). (B) β-tubulin immunoreactivity in SK-N-SH cells treated for 48 hours with of Vpr (70-96) Wt and R77Q peptides at increasing concentration (μM) compared to untreated and full-length Vpr (1μM) showing a high concentration dependent loss of β-tubulin immunoreactivity for both Wt and R77Q Vpr (70-96) while R77Q had less neurotoxic effects than the Wt peptide. Data is a representation of three experiments including the mean (± SEM) of 4 technical replicates per treatment. Statistical analysis for B was done using Tukey-Kramer multiple comparisons test. (*$p<0.05$, ***$p<0.001$)

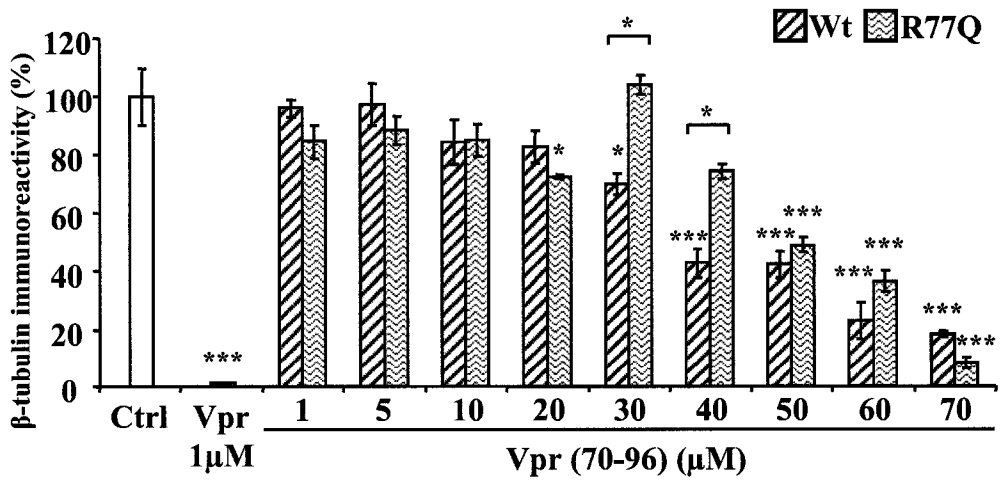
A




**94 % HAD-Br sequences R77Q substitution.**

**22% ND-Br sequences R77Q substitution. ($p<0.01$)**

B



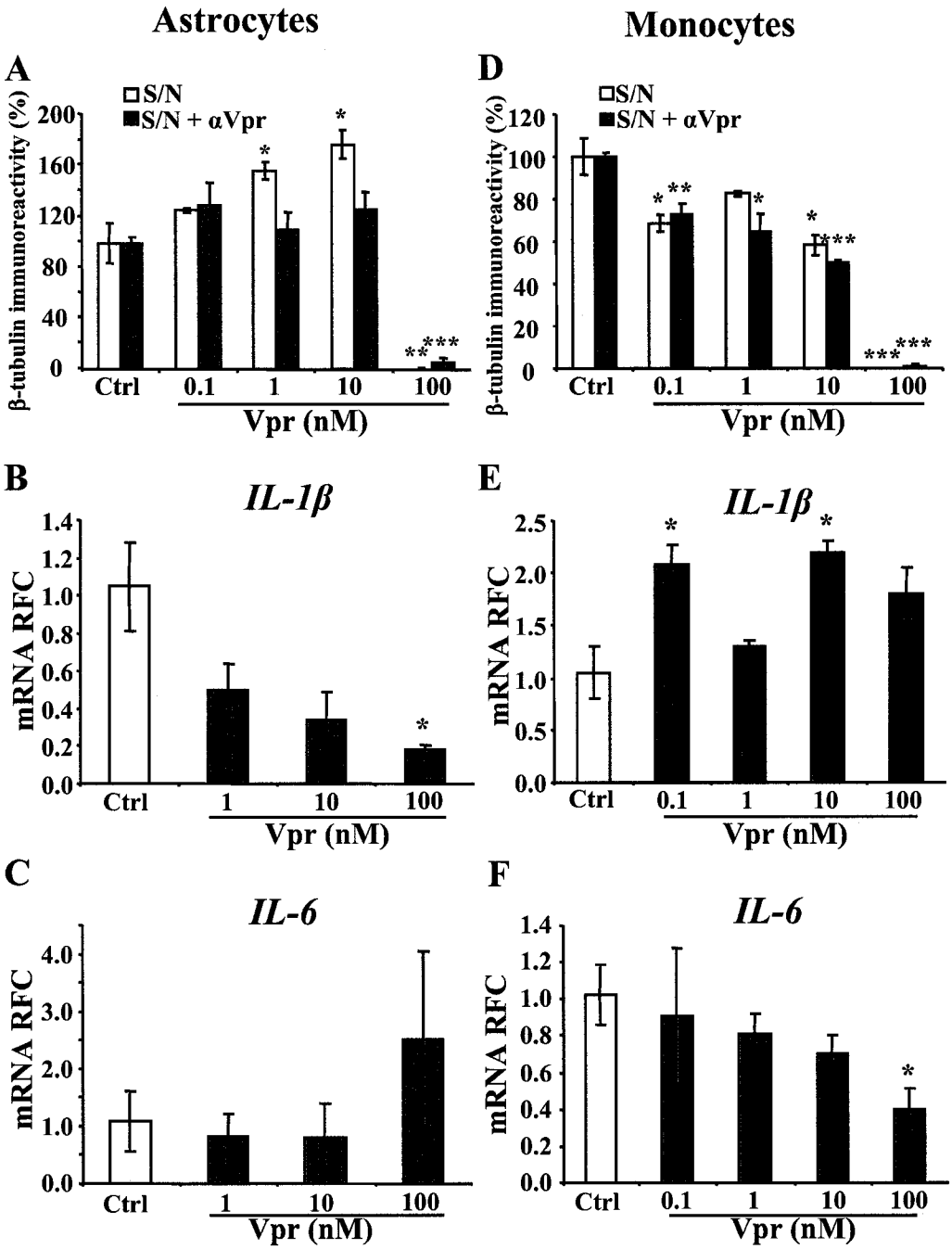
stimulated astrocytic cells were applied to neuronal cells. As determined by β-tubulin expression, no neurotoxicity was observed with supernatants from astrocytic (U373) cells stimulated with 10nM Vpr or less (**Figure 3.5A**). Indeed, at these Vpr concentrations there appeared to be a modest concentration-dependent increase in β-tubulin immunoreactivity, which was abrogated in the presence of Vpr-specific antibodies. However, conditioned media from astrocytic cells stimulated with 100nM Vpr induced marked neuronal cell death, which was not reversed by Vpr-specific antibodies. Immune dysregulation within the central nervous system is a cardinal feature of HIV-1 infection and is assumed to contribute to reduced neuronal viability through the secretion of neurotoxic molecules. Compared to unstimulated controls, *IL-1β* transcript levels were reduced in Vpr-stimulated astrocytic cells in a concentration-dependent manner (**Figure 3.5B**). In contrast, *IL-6* was increased in Vpr-treated astrocytic cells, although this finding was apparent at 100nM concentration only (**Figure 3.5C**). Indirect neurotoxicity also was evident when monocytoid cells (U-937) were stimulated with as low as 0.1nM of Vpr although the maximal effect was seen when monocytoid cells were stimulated with 100nM Vpr. Irrespective of the Vpr concentration used, prior treatment of neuronal cells with Vpr-specific antibodies failed to abrogate the neurotoxicity of Vpr-activated monocytoid cell-derived supernatants (**Figure 3.5D**). In monocytoid cells, soluble Vpr induced *IL-1β* transcript abundance (**Figure 3.5E**) while *IL-6* expression was markedly reduced in a concentration-dependent manner (**Figure 3.5F**). These studies suggested that soluble Vpr influenced glial cell immune responses, which was accompanied by the

secretion of cellular neurotoxins depending on the individual cell type that was stimulated by Vpr. **Figure 3.5** was done by G. Jones.

## 3.6 *IN VIVO* HIV-1 VPR EXPRESSION RESULTS IN NEUROTOXIC AND NEUROIMMUNE EFFECTS

Although soluble Vpr exerted immunogenic and neurotoxic properties in different assays of neural function, the *in vivo* neurotoxic effects of Vpr expression in monocytoid cells are unknown. To investigate this question, we examined transgenic mice expressing HIV-1 (NL4-3) *vpr* under the control of the *c-fms* promoter (Tg-Vpr), which drives transgene expression in monocytoid cells, together with littermate wild type controls (Wt) (**Figure 3.6A**). Macrophages from Tg-Vpr and Wt mice showed that *vpr* transcripts were detected in Tg but not Wt animals (**Figure 3.6B, inset**). In fact, supernatants from Tg-Vpr-derived macrophages were highly neurotoxic to RFN, which was prevented by pre-treatment with the anti-Vpr antibody (**Figure 3.6B**). Likewise, soluble Vpr (1000nM) was neurotoxic to RFN when added to supernatants from Wt-derived macrophages but again was reversed by pre-treatment with the anti-Vpr antibody (**Figure 3.6B**). To further investigate the effects of Vpr in the brain, we initially examined Vpr transcript levels, disclosing that Vpr was present in the basal ganglia (BG) of Tg-Vpr animals, but not in Wt littermates (**Figure 3.6C, inset**). Vpr transcripts were also detected in the cortex (CTX) and hindbrain (HB) of these animals, although higher Vpr transcript levels were found in the BG and CTX compared to the HB (**Figure 3.6C**). Of note, Vpr immunoreactivity was not present on Western blot using brain tissue from both

**FIGURE 3.5. HIV-1 VPR EXERTS CELL TYPE-DEPENDENT IMMUNOGENIC AND NEUROTOXIC EFFECTS.** LAN-2 cells were cultured in the presence or absence of anti-HIV-1 Vpr antibodies for 48 hours with supernatants (S/N) from HIV-1 Vpr stimulated U373 cells (A) or U-937 cells (D). (A and D) Controls represent LAN-2 cells cultured with supernatant from un-stimulated U373 or U-937 cells. Relative fold change (RFC) in mRNA expression for *IL-1β* (B and E) and *IL-6* (C and F) in U373 cells (B and C) and U-937 cells (E and F), respectively, 6 hours post-stimulation with soluble Vpr. Data represent mean (± SEM) from a minimum of 3 replicates, and are expressed relative to controls. Statistical analysis for all data was done by Dunnet multiple comparisons test. For methods, refer to (150). (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

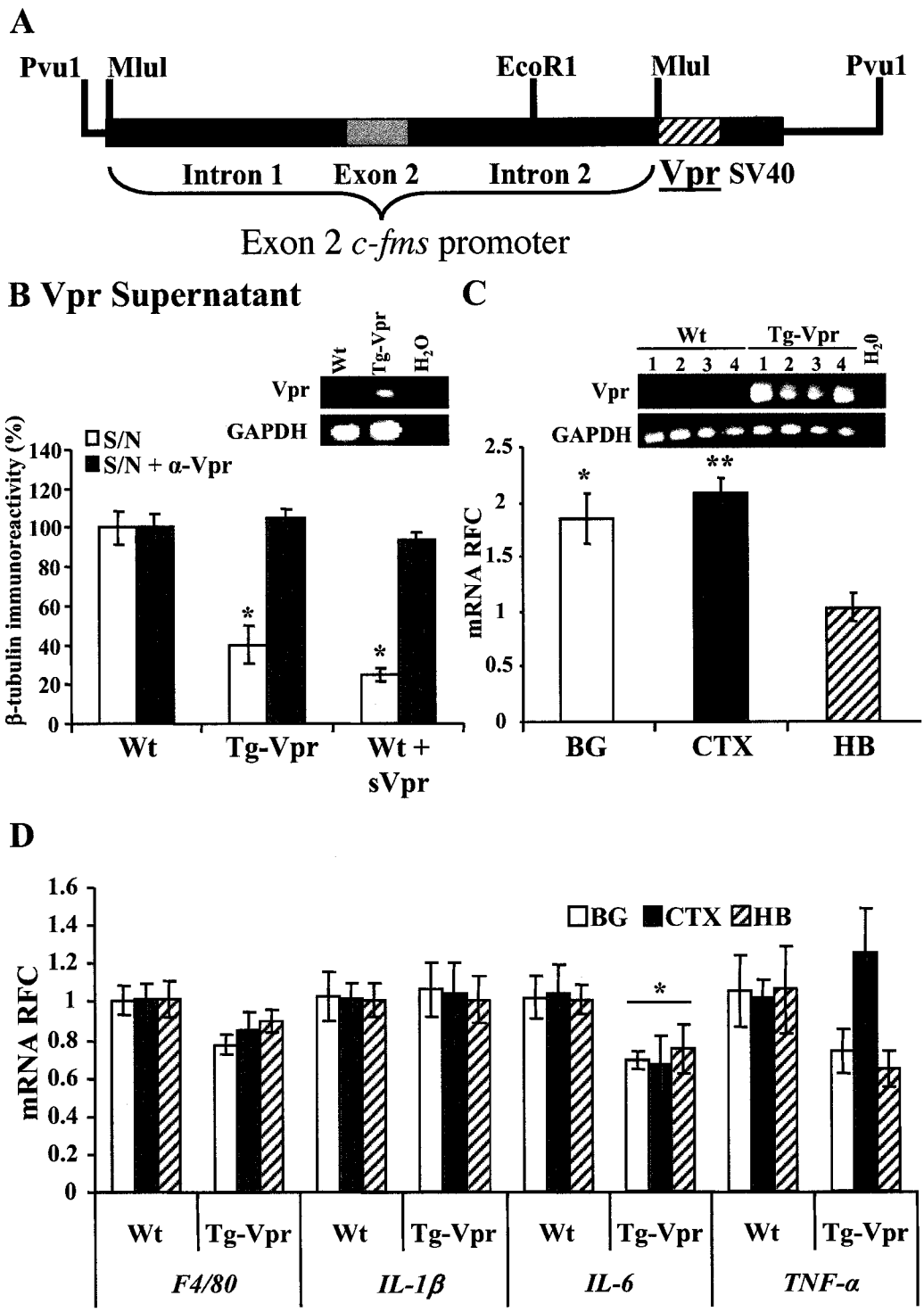


Tg-Vpr and Wt animals. Analysis of brains from Tg-Vpr and Wt animals revealed that there were no differences in F4/80 (a marker for activated macrophages), IL-1β or TNF-α transcript levels (**Figure 3.6D**). In contrast, IL-6 transcript levels were significantly ($p<0.05$) lower in the BG, CTX and HB of Tg-Vpr animals compared to Wt controls. These studies indicated that Vpr was expressed and secreted by monocytoid cells in Tg-Vpr animals. Moreover, Vpr was expressed in the brains of Tg-Vpr animals, which was accompanied by a marked reduction in IL-6 transcript levels, thereby recapitulating the effects of soluble Vpr in monocytoid cells (**Figure 3.5E**).

### 3.7 VPR CAUSES *IN VIVO* NEURODEGENERATION AND NEUROBEHAVIOURAL ABNORMALITIES

Although Vpr was expressed at the transcript level, it was critical to determine if Vpr protein was present in the brains of the Tg-Vpr mice. Hence, we investigated Vpr's presence in the brains of Tg-Vpr animals and Wt littermates, disclosing that Vpr immunoreactivity was absent in the brains of Wt littermate controls (**Figure 3.7A**) but present in the brains of Tg-Vpr animals (**Figure 3.7B**). Furthermore, Vpr co-localized with F4/80 immunoreactivity, suggesting it was expressed principally in activated monocytoid cells in both perivascular (**Figure 3.7B**) and parenchymal regions (**Figure 3.7B, inset**), whereas only F4/80 immunoreactivity was observed in the Wt sections. Analysis of Iba-1 immunoreactivity on monocytoid cells did not show substantial differences in microglia/macrophage morphology and numbers between Wt (**Figure 3.7C**) and Tg-Vpr animals (**Figure 3.7D**). Conversely, the neuronal nuclear protein,

**FIGURE 3.6. HIV-1 VPR EXPRESSION AND SECRETION BY MONOCYTOID CELLS IN TG-VPR ANIMALS.** (A) Schematic figure of the HIV-1 Vpr transgene expressed under the control of exon 2 of the *c-fms* promotor. (B) Vpr transcripts were detected in macrophages from Tg-Vpr animals but not in Wt littermate controls (inset). RFN were cultured with supernatants from either Tg-Vpr macrophages, Wt macrophages or Wt macrophages supplemented with soluble HIV-1 Vpr and pretreated with or without anti-Vpr antibodies and β-tubulin abundance was evaluated after 48 hours. (C) Greater levels of Vpr transcripts were detected in the basal ganglia (BG) and cortex (CTX) compared to the hind brain (HB) in Tg-Vpr animals. Inset depicts the detection of HIV-1 Vpr specific transcripts in the BG of 4 Tg-Vpr animals. (D) *F4/80*, *IL-β*, *IL-6* and *TNF-α* transcript levels in the BG, CTX and HB did not change between Wt and Tg-Vpr animals except for *IL-6* levels. Data for C and D represents the mean (± SEM) of 4 Wt and Tg-Vpr animals. Statistical analysis for B and C were done by Tukey-Kramer multiple comparisons test while D was analyzed by students' t-test (*$p<0.05$, **$p<0.01$).



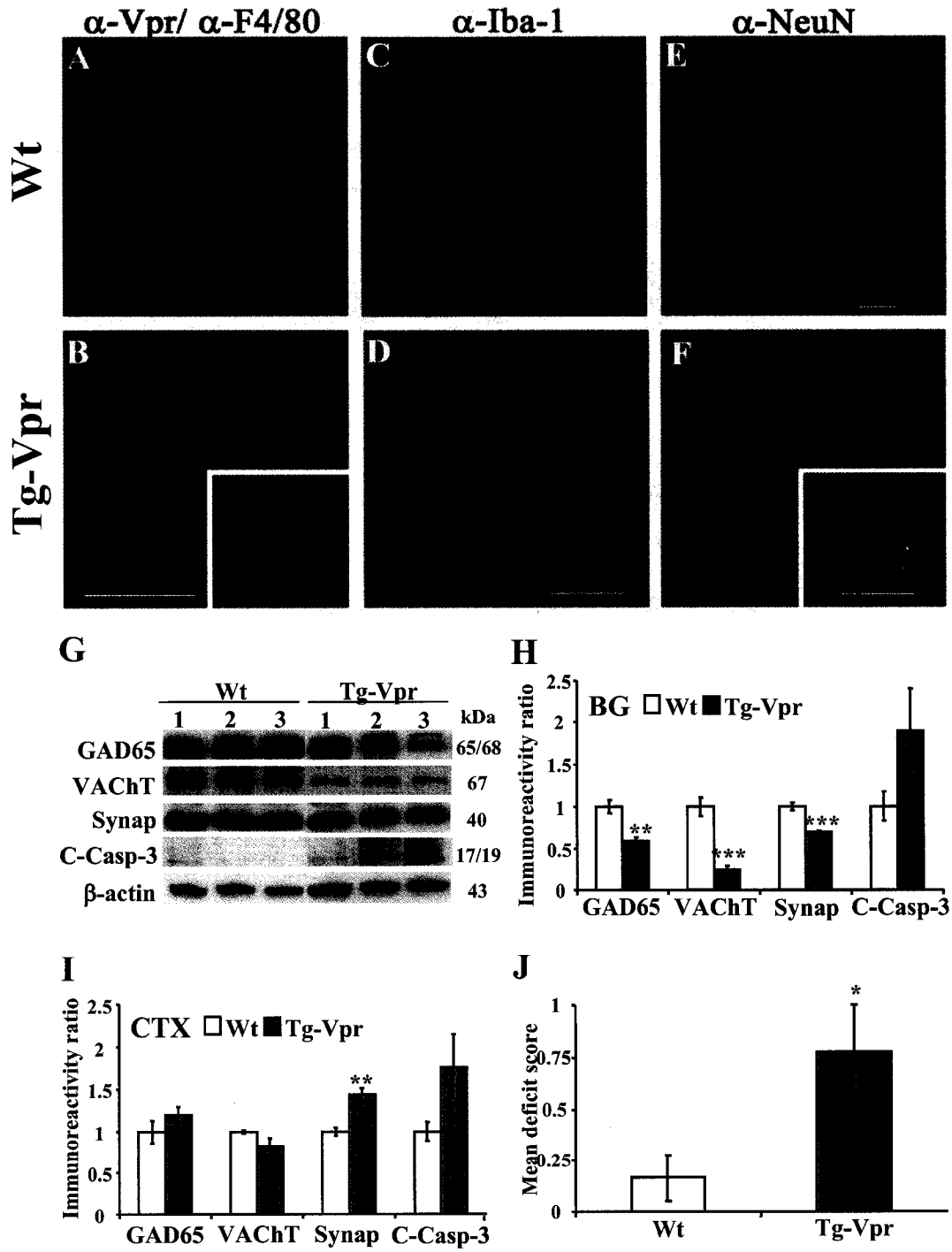
NeuN, exhibited reduced immunoreactivity in the Tg-Vpr animals (**Figure 3.7F**), compared to Wt controls (**Figure 3.7E**), particularly in subcortical regions. Moreover, activated caspase-3 immunoreactivity was detected within neurons by NeuN and cleaved caspase-3 double immunolabeling (**Figure 3.7F, inset**). Given that we observed reduced NeuN immunoreactivity with increased activation of caspase-3 in Tg-Vpr animals, we next investigated the expression of three representative neuronal proteins, glutamic acid decarboxylase 65 (GAD65) (GABA-ergic neurons), vesicular acetylcholine transferase (VAChT) (cholinergic neurons) and synaptophysin (synap) (most pre-synaptic terminals) in the brains of Tg-Vpr and Wt animals by Western blotting, revealing that all three neuronal proteins were reduced in the BG of Tg-Vpr animals along with increased immunoreactivity of cleaved caspase-3 relative to β-actin, which was slightly higher in Tg-Vpr BG (**Figure 3.7G and H**). However, the relative expression of GAD65 and VAChT were similar in the CTX of Tg-Vpr animals compared to Wt controls (**Figure 3.7I and Supplementary Figure 7.1**). Conversely, relative synaptophysin levels were significantly higher in the Tg-Vpr animal CTX (**Figure 3.7I and Supplementary Figure 7.1**). Similar to the BG, the CTX also showed an increase in immunoreactivity of cleaved caspase-3 (**Figure 3.7I and Supplementary Figure 7.1**). Since Vpr expression was associated with neuronal injury *in vivo* and *in vitro*, we also examined the neurobehavioral effects of Vpr expression in the present transgenic line. These studies revealed that Tg-Vpr animals exhibited abnormalities in neurobehavioral performance evidenced by the Tg-Vpr animals being significantly more hyperexcitable compared to

the Wt mice (**Figure 3.7J**). Thus, these studies indicated that Vpr was cytotoxic to subcortical neurons, together with exhibiting aberrant motor activity.

### 3.8 INDUCTION OF *VPR* TRANSCRIPT IN BONE MARROW DERIVED MACROPHAGES

Since the Tg-Vpr mouse model contained the *c-fms* promoter driving *vpr* expression, different approaches were used to induce expression of *vpr* through the promoter. The *c-fms* gene encodes the M-CSF receptor which, upon binding M-CSF results in macrophage maturation leading to release of inflammatory cytokines and nitric oxide (reviewed in (51)). TNF-α and retinoic acid (RA) were used to induce the *c-fms* promoter since this promoter has been shown to be upregulated in mature dendritic cells and these two compounds previously increased expression of the CSF receptor (192, 348). After 7 days of maturation, BMDM were treated with 10 and 50nM of RA and 10 and 50ng/ml of TNF-α as well as combinations of these cytokines at both concentrations. BMDM were also treated with L929 media or PMA (50ng/ml). Neither of these inflammatory molecules alone or in combination induced a significant increase in the expression of *vpr* relative to L929 treated Tg-Vpr macrophages (**Figure 3.8A**). While low levels of TNF-α (10nM) suppressed transcript levels, higher levels of TNF-α (50ng/ml) increased transcript levels, albeit inconsistently (**Figure 3.8A**). Treatment with RA and combinations of RA and TNF-α also suppressed the expression of *vpr* (**Figure 3.8A**). The conditioned medium of mouse L929 fibroblasts contains haematopoietic growth factors for growth and differentiation. The growth factors that are thought to be required for macrophage survival are M-CSF, GM-CSF and IL-3 (292). To investigate whether variations of these growth factors affect induction of the *vpr*

**FIGURE 3.7. HIV-1 VPR ABUNDANCE IN THE BRAIN OF TG-VPR ANIMALS IS ACCOMPANIED BY NEURONAL CELL LOSS.** HIV-1 Vpr immunoreactivity (red) was not detected in the brains of (A) Wt littermate controls but was observed in Tg-Vpr animals that co-localized with F4/80 immunoreactivity (green) in the perivascular cells (B) and parenchymal cells (B, inset). Iba-1 immunoreactivity in monocytoid cells in Wt (C) and Tg (D) animals did not differ. However, NeuN immunoreactivity in neurons of the basal ganglia of Wt littermate controls (E) was substantially greater than NeuN immunoreactivity Tg-Vpr animals' basal ganglia (BG) (F) with an increase in cleaved caspase-3 colocalizing, as shown in yellow, with NeuN (green) (Fig. 6F, inset). (G) GAD65, VAChT and synaptophysin (synap) immunoreactivity was reduced while there was an increase in cleaved caspase-3 in the BG of Tg-Vpr compared to Wt animals. (H) Graphic analysis of GAD65, VAChT and synaptophysin immunoreactivity in the BG disclosed all proteins were suppressed in Tg animals compared to Wt littermates, while cleaved caspase-3 was increased. (I) Conversely, GAD65 and VAChT immunoreactivity relative to β-actin immunoreactivity did not differ between Wt and Tg-Vpr animals while both synaptophysin and cleaved caspase-3 immunoreactivity in the cerebral cortex (CTX) was higher in Wt animals. (J) Tg-Vpr animals exhibited marked deficits in neurobehavioral performance compared to Wt littermates at 14 weeks of age. Data represents the mean (± SEM) of 3 Wt and 4 Tg-Vpr animals tested. Statistical analysis for H-J were done by students' t-test. (*$p<0.05$, **$p<0.01$) (Scale bars= 15μm)

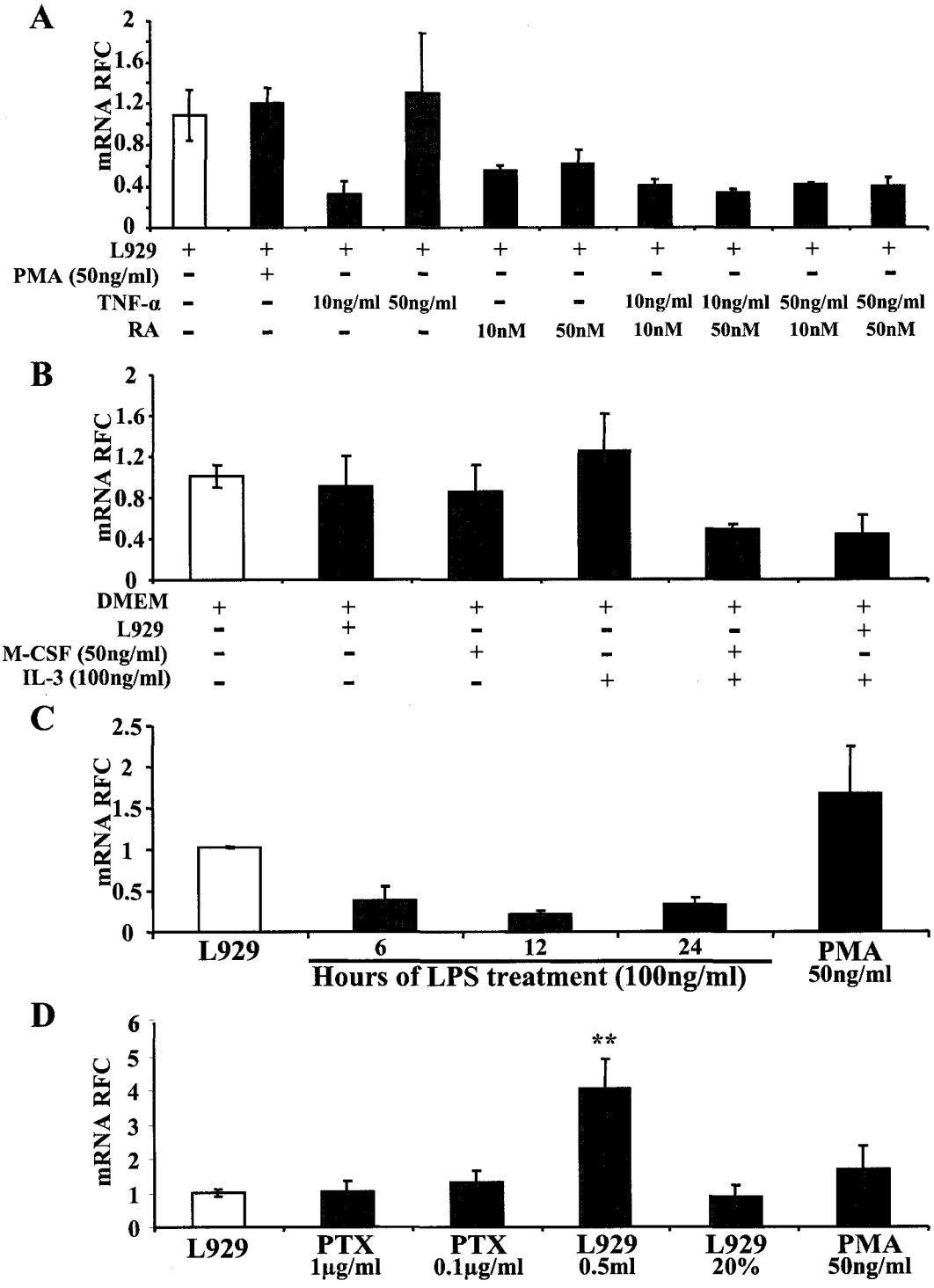


transcript levels, L929, M-CSF, IL-3 and combinations of these factors were cultured with Tg-Vpr BMDM (**Figure. 3.8B**). While IL-3 alone increased *vpr* transcript non-significantly, combinations with L929 and M-CSF reduced *vpr* expression while L929 and M-CSF alone did not change expression levels (**Figure 3.8B**). Lastly, just as M-CSF induces maturation of macrophages, so does pro-inflammatory lipopolysaccharide (LPS) and pertusis toxin (PTX) and thus these molecules were evaluated for their ability to induce *vpr* through the *c-fms* promoter (**Figure 3.8C and D**). PTX had no effect on expression while LPS, in fact caused a suppression of *vpr* transcript level. Attempts were also made to increase *vpr* expression through manipulation of medium volume and L929 concentration (**Figure 3.8D**). While reducing the total volume by half to 0.5ml of DMEM containing 10% L929 resulted in increased *vpr,* though not significantly, increasing the concentration of L929 conditioned medium to 20% in 0.5ml total volume of media had no effect. Therefore, modulation of expression from the *c-fms* promoter reveals a potentially complex system that requires more research into the regulation of M-CSF receptor expression.

### 3.9 *IN VITRO* TG-VPR BONE MARROW DERIVED MACROPHAGES DO NOT INCREASE INFLAMMATION

Though *vpr* expression did not significantly increase with any of the treatments, there was still *vpr* transcript present in all Tg-Vpr BMDM isolated. Since the treatment of macrophages with soluble Vpr did not cause a marked inflammatory reaction, we investigated whether expression of *vpr* within macrophages caused inflammation. Treatments with LPS and PMA (**Figure 3.9A-C**) and treatments with

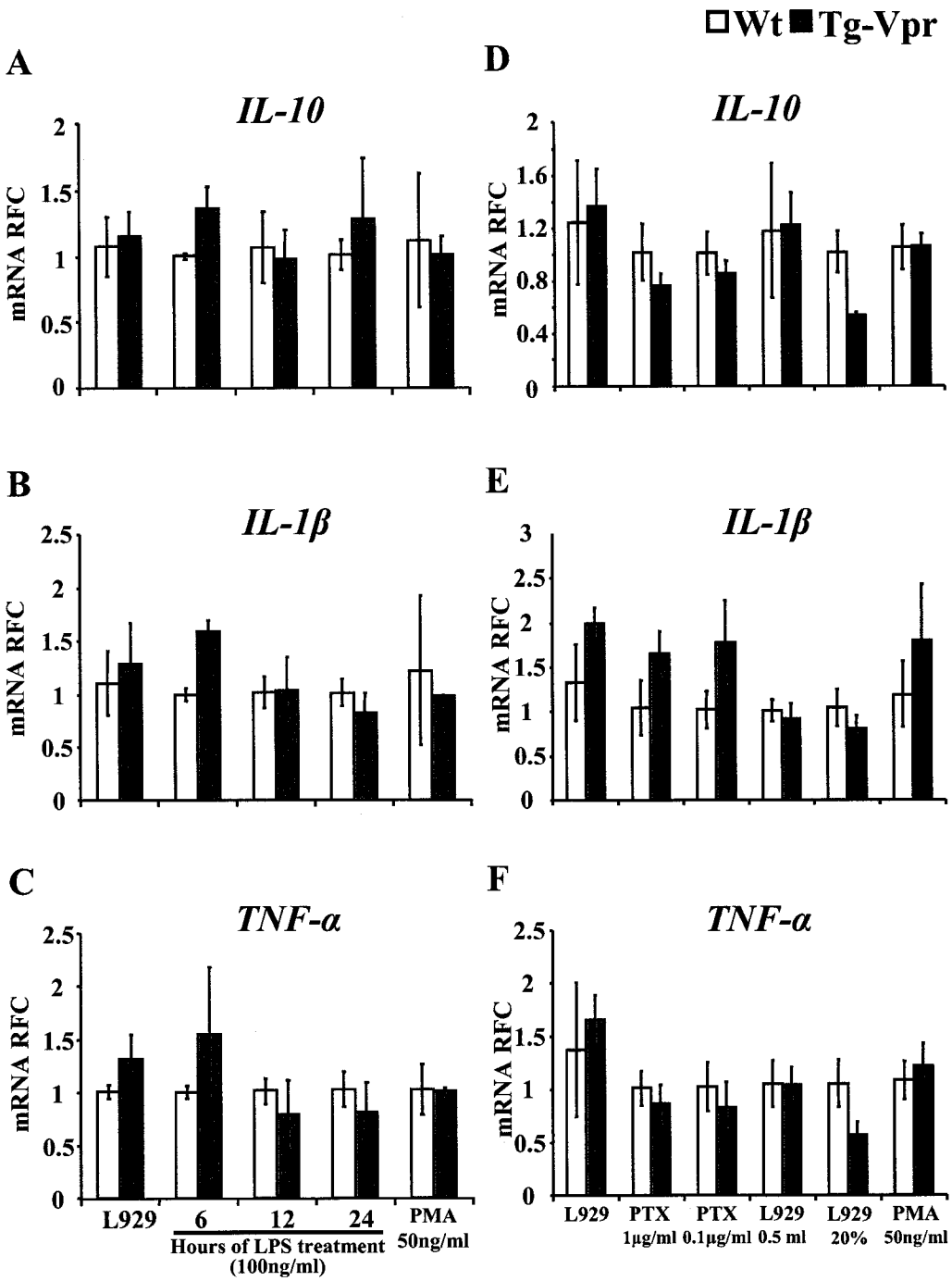
**FIGURE 3.8. INDUCTION OF *VPR* TRANSCRIPT IN BONE MARROW DERIVED MACROPHAGES.** (A) BMDM treated on day 7 with DMEM/10% L929 alone, PMA (50ng/ml) TNF-α (10 and 50ng/ml) and RA (10 and 50nM) and combinations of TNF-α and RA with the previous concentrations resulting in either no change or a decrease in *vpr* expression relative to L929 alone treatments. (B) Day 7 treated BMDM with DMEM medium alone, 10% L929, M-CSF (50ng/ml), IL-3 (100ng/ml) and combinations of IL-3 with M-CSF and L929 where IL-3 alone had a slight increase while combinations with L929 and M-CSF showed slight suppression of transcript. (C) BMDM treated on day 7 with 10% L929 alone, LPS (100ng/ml) at 6, 12 and 24 hours and PMA (50ng/ml) showing suppression of *vpr* with LPS treatment compared to L929 alone. (D) Day 14 treated BMDM with 10% L929, PTX (1 and 0.1μg/ml), 0.5ml of 10% L929, 0.5ml of 20% L929 and PMA (50ng/ml) showing a significant increase in *vpr* transcript with 0.5ml 10% L929 treatment. A and B are the mean (± SEM and range) of technical triplicate and duplicate wells and C and D were the mean (± SEM) of 3 Tg-Vpr animals. Statistical analysis for C and D were done by Dunnet multiple comparisons test. (**$p<0.01$).



PTX, PMA and manipulations of the BMDM maturation media (**Figure 3.9D-F**) did not induce any significant changes in *IL-10*, *IL-1β* or *TNF-α* expression (**Figure 3.9**).

Moreover, there were no associations between changes in *vpr* transcript levels (**Figure 3.8**) and changes in any of the inflammatory transcripts for any treatment (**Figure 3.9**). There were, however, decreases in *IL-10* and *TNF-α* for Tg-Vpr BMDM treated with 20% L929, though not significant (**Figure 3.9D-F**), which did correlate with a slight suppression of *vpr* transcript (**Figure 3.8D**). On the other hand, the modest increase in *vpr* expression with decreased media volume (**Figure 3.8D**) did not induce a change in inflammatory transcript levels (**Figure 3.9D-F**). Also of note, there was an increase in *IL-1β* transcript for L929, PTX and PMA treated Tg-Vpr macrophages relative to Wt, although not significant (**Figure 3.9E**). Thus, expression of *vpr* in BMDM does not seem to induce or suppress inflammation relative to Wt BMDMs.

**FIGURE 3.9.** ***IN VITRO*** **TG-VPR BONE MARROW DERIVED MACROPHAGES DO NOT INCREASE INFLAMMATION.** Treated BMDM were analyzed for *IL-10* (A and D), *IL-1β* (B and E) and *TNF-α* (C and F) transcripts and compared to Wt mice. (A-C) BMDM treated on day 7 with 10% L929 alone, LPS (100ng/ml) at 6, 12 and 24 hours and PMA (50ng/ml) revealing no significant changes in inflammatory transcript levels, though there were slight increases in *IL-1β* and *TNF-α* expression in Tg-Vpr compared to Wt BMDM at 6 hours of LPS treatment. (D-F) BMDM treated on day 14 with 10% L929, PTX (1 and 0.1μg/ml), 0.5ml of 10% L929, 0.5ml of 20% L929 and PMA (50ng/ml) showing no significant changes in transcript levels, though there was a slight suppression in *IL-10* and *TNF-α* with treatment with 0.5mL of 20% L929 and a slight increase in *IL-1β* in L929, PTX and PMA treatments. The data represents the mean (± SEM) of 3 Tg-Vpr and Wt animals.



# CHAPTER 4- HIV-1 VPR MEDIATES ASTROCYTE TOXICITY

## 4.1 HIV-1 VPR MEDIATES ASTROCYTE TOXICITY BUT VPR (70-96) IS NOT SUFFICIENT TO INDUCE ASTROCYTE DEATH

Previous research has shown that soluble Vpr might cause cell death in astrocytes (135). When soluble Vpr was added at increasing concentrations to cultured primary astrocytes, human fetal astrocytes (HFA) and astrocyte cell line, U373, there was a significant decrease in β-tubulin abundance that was concentration dependent (**Figure 4.1A**). While U373 lost significant ($p<0.01$) β-tubulin immunoreactivity relative to the control at 100nM of Vpr, HFA had a significant decrease ($p<0.01$) in expression at 1000nM (**Figure 4.1A**). Moreover, at the concentrations of 10 and 100nM Vpr exerts a trophic effect in HFA occurring where there was a significant increase ($p<0.01$) in β-tubulin abundance. Hence, treatment of astrocytes with Vpr caused loss of cell viability at higher concentrations; however, at lower concentrations, Vpr was trophic to astrocytes.

Since HFA were subject to toxicity due to Vpr treatment, some preliminary analysis on the inflammatory expression profile of HFA treated with Vpr was done to see if indirect toxicity may be a factor. HFA were treated with 0, 10, 100 and 1000nM of Vpr for 6 hours and the RNA was extracted and cDNA quantified by real-time PCR (**Figure 4.1B-D**). At the lower concentrations (1-100nM), there was no induction of *IL-1β, IL-6* or *TNF-α;* however at 1000nM, all transcripts were induced by Vpr treatment. Thus, Vpr is inducing an inflammatory effect in astrocytes at toxic concentrations.

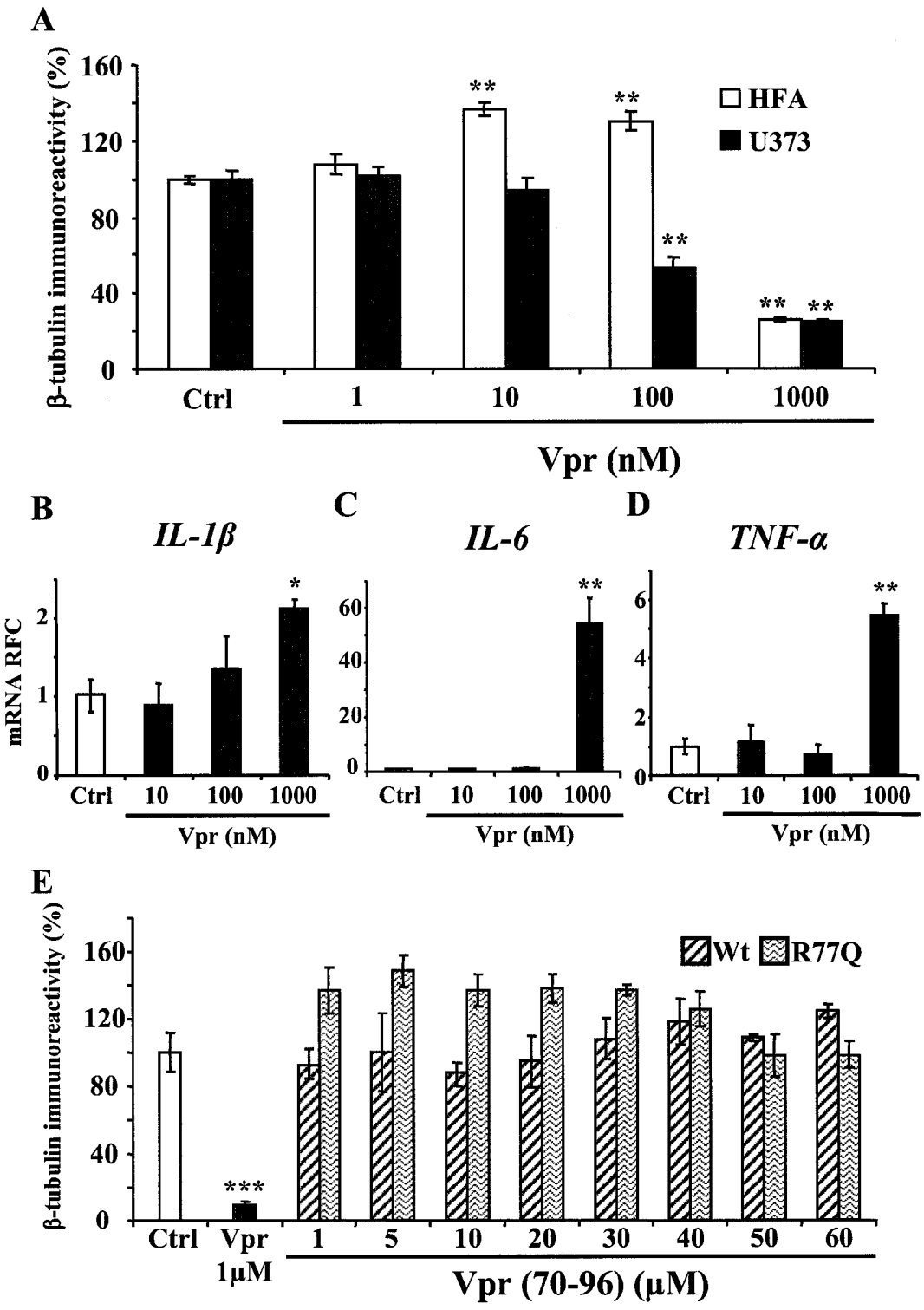
Since there was no effect of Vpr Wt and R77Q peptides on neurons at physiological concentrations, it was hypothesized that since Vpr can cause astrocytic cell

death, this mutation may be important for astrocyte loss. However, when applied to U373 there was no astrocyte toxicity, while toxicity did occur with full-length Vpr treatment (1μM) (**Figure 4.1E**). In fact, there was a trophic effect occurring with R77Q mutant peptide treatment between 1 and 40μM concentrations and between 30 and 60μM for the Wt peptide (**Figure 4.1E**). Of note, this assay was done only once in U373 and must be further defined in replicates and in HFA. However, analysis of this preliminary data show that while there is no astrocyte toxicity occurring in the astrocyte cell line, there seems to be a trophic effect occurring at different ranges of concentrations in Wt and R77Q Vpr (70-96) treatments.

## 4.2 VPR CAUSES LOSS OF MITOCHONDRIAL POTENTIAL IN ASTROCYTES

One major hallmark of Vpr-mediated apoptosis is the interaction with the adenine nucleotide translocator (ANT) of the permeability transition pore complex (PTPC), causing loss of mitochondrial potential and cytochrome *c* release (143). Thus, through the use of tetramethylrhodamine ethyl ester (TMRE) we could observe whether Vpr caused loss of astrocytic mitochondrial membrane potential through loss of TMRE staining. HFA were treated for 8, 12 and 16 hours with cytotoxic concentration of Vpr (1000nM) as well as UV treatment (200mJ/cm$^2$) as a positive control. Cells were then stained with TMRE and 7-amino-actinomycin D (7AAD) and analyzed by two color flow cytometry. While mock HFA were mostly all TMRE$^+$/ 7AAD$^-$, Vpr-treated HFA exhibited increasing loss of TMRE staining correlating with duration of the treatment of HFA (**Figure 4.2A**). As well, there were more cells that were 7AAD$^+$ as treatment time increased (**Figure 4.2A**).

**FIGURE 4.1. HIV-1 VPR MEDIATES ASTROCYTE TOXICITY BUT VPR (70-96) IS NOT SUFFICIENT TO INDUCE ASTROCYTE DEATH.** (A) Loss of β- tubulin immunoreactivity in HFA and U373 cells treated with increasing concentrations of Vpr for 48 hours. Data is representative of three experiments showing the mean (SEM ±) of 4 technical replicates. (B-D) *IL-1β, IL-6* and *TNF-α* expression was analyzed from HFA treated with Vpr for 6 hours resulting in a significant increase in the respective transcript at the highest Vpr concentration compared to untreated control. Data represents the mean (SEM ±) of 3 replicates. (E) Three was no loss of β-tubulin immunoreactivity in U373 cells treated with Vpr (70-96) for 48 hours compared to untreated control, while full-length Vpr (1μM) caused significant loss of β-tubulin immunoreactivity. R77Q Vpr (70-96) reveals a slight trophic effect though not significant. Data represents one experiment showing the mean (SEM ±) of 3 technical replicates. Statistical analysis for all data was done by Dunnet multiple comparisons test. (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

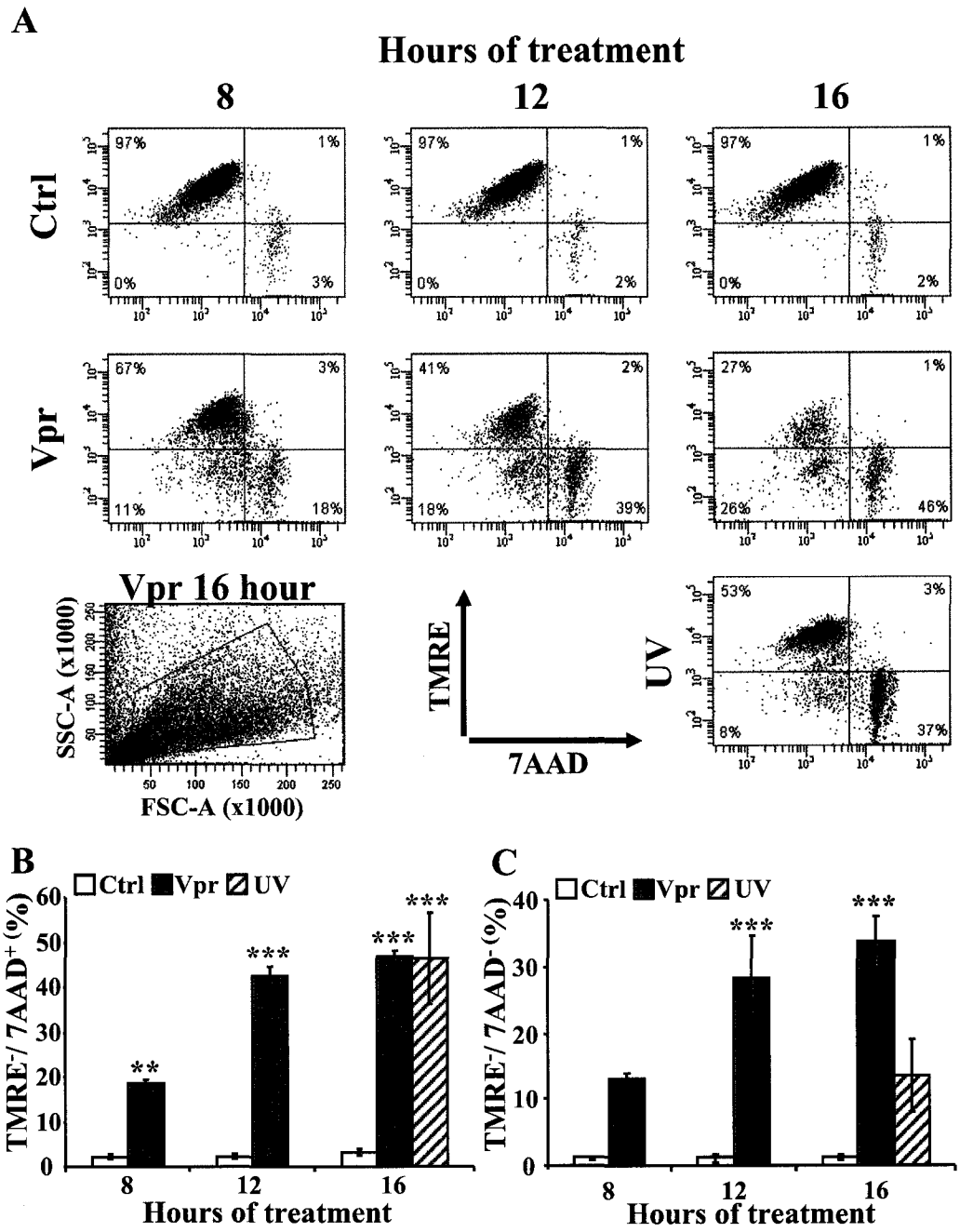


These results were displayed graphically by the percentage of cells that have undergone the full pathway of apoptosis and are now dead with $TMRE^-$/ $7AAD^+$ staining showing a significant increase in cells that died by apoptosis at 8 ($p<0.01$), 12 and 16 hours ($p<0.001$), which was comparable to the positive control of UV treatment at 16 hours ($p<0.001$) (**Figure 4.2B**). The percentage of cells that were undergoing apoptosis that were $TMRE^-$/$7AAD^-$ was also shown graphically showing a significant increase at 12 and 16 hours ($p<0.001$) (**Figure 4.2C**). Thus, Vpr causes loss of mitochondrial potential in primary astrocytes, strongly suggesting that these cells undergo apoptosis with Vpr treatment.

### 4.3 HIV-1 VPR CAUSES ACTIVATION OF CASPASE-3 IN ASTROCYTES

Given that we observed loss of mitochondrial potential in HFA treated with Vpr, suggesting apoptosis might be occurring; further confirmation of the induction of apoptosis through activation of caspase-3 was investigated. HFA were treated with and without Vpr (1000nM) for 10 hours and analyzed for activation of caspase-3 through immunofluorescence and Western blotting (**Figure 4.3**). Activated caspase-3 co-localized with GFAP in Vpr-treated HFA while there was no centralized immunofluorescence in the untreated HFA (**Figure 4.3A**). Through Western blotting, the Vpr-treated protein lysates were immunopositive for cleaved caspase-3 showing bands at 17 and 19kDa, corresponding to the cleavage products of activated caspase-3 while cleaveage was inhibited with treatment of pan-caspase inhibitor, zVAD-fmk (**Figure 4.3B**). Through analysis of the cleaved bands, there is almost 100 fold increase in fluorescence (**Figure 4.3C**).

**FIGURE 4.2. HIV-1 VPR CAUSES LOSS OF MITOCHONDRIAL POTENTIAL IN ASTROCYTES.** HFA were treated with Vpr (1000nM) for 8, 12 and 16 hours followed by TMRE and 7AAD staining and analysis through flow cytometry. (A) Scatter plots showing TMRE fluorescence on the y-axis and 7AAD fluorescence on the x-axis. The bottom scatter plot shows necrotic cells were removed in the forward and side scatter. As treatment time increased, the amount of $TMRE^-/ 7AAD^+$ cells increased in the Vpr-treated groups. (B and C) Graphical presentation of percentage of untreated, Vpr and UV cells that were (B) $TMRE^-/ 7AAD^+$ or dead apoptotic cells and (C) $TMRE^-/ 7AAD^-$ or cells that are undergoing apoptosis. Scatter plots are representative of three replicated experiments while bar graphs are the mean (SEM ±) of three experiments. Statistical analysis for B and C were done by Dunnet multiple comparisons test. (*$p<0.05$, **$p<0.01$, ***$p<0.001$)



However, these are preliminary results and require further replication with positive and negative controls. Thus, activation of caspase-3 further confirms that apoptosis might be occurring in astrocytes treated with Vpr.

### 4.4 HIV-1 VPR CAUSES LOSS OF CORTICAL *GFAP* TRANSCRIPT *IN VIVO*

Since treatment with Vpr *in vitro* caused loss of astrocytes through apoptosis, it was hypothesized that these effects occurred *in vivo*. Therefore, by using dissected brain samples from 4-month-old Wt and Tg-Vpr mice, we analyzed the cDNA made from the brain RNA for astrocytic markers. Through real-time PCR we investigated *GFAP, S100β, nestin, AQP4, GLT-1* and *GLAST-1* in the BG, CTX and HB (**Figure 4.4**). Expression levels of *GFAP* were significantly suppressed ($p<0.01$) in the CTX of Tg-Vpr mice compared to Wt while levels in the BG and HB were slightly lower as well, though not significant (**Figure 4.4A**). With respect to the CTX, there was a slight decrease in *AQP4,* though not significant (**Figure 4.4D**), while there was no change in other astrocyte markers (**Figure 4.4B, C, E, F**). There were no significant changes in astrocyte markers within the BG; however, there was an increase in *nestin* transcript (**Figure 4.4C**) as well as a slight increase in *S100β* (**Figure 4.4B**) compared to Wt BG. Besides *GFAP*, there were also decreases in *S100β* and *AQP4* in the HB, though none were significantly lower (**Figure 4.4B and D**) while no changes in *nestin, GLAST-1* and *GLT-1* (**Figure 4.4C, E and F**). Thus, though there was a general trend of decreased astrocytic markers with the exception of some markers in the BG, there is a significant decrease in *GFAP* transcript in the CTX.

**FIGURE 4.3. HIV-1 VPR CAUSES ACTIVATION OF CASPASE-3 IN ASTROCYTES.** HFA were treated with Vpr (1000nM) for 10 hours and either (A) immunostained for GFAP (green) and cleaved caspase-3 (C-Casp-3) (red) or (B) immunoblotted for C-Casp-3 where the cleaved bands represent activated caspase-3 (17 and 19kDa). HFA were treated with and without Vpr and zVAD-fmk (50μM). Treatment with Vpr and zVAD-fmk inhibited activation of C-Casp-3. (C) The graphical representation of immunoreactivity relative to β-actin and untreated control show an increase in C-Casp-3 in Vpr treated lysates. Data represents one preliminary experiment. (Scale bar = 15μm).

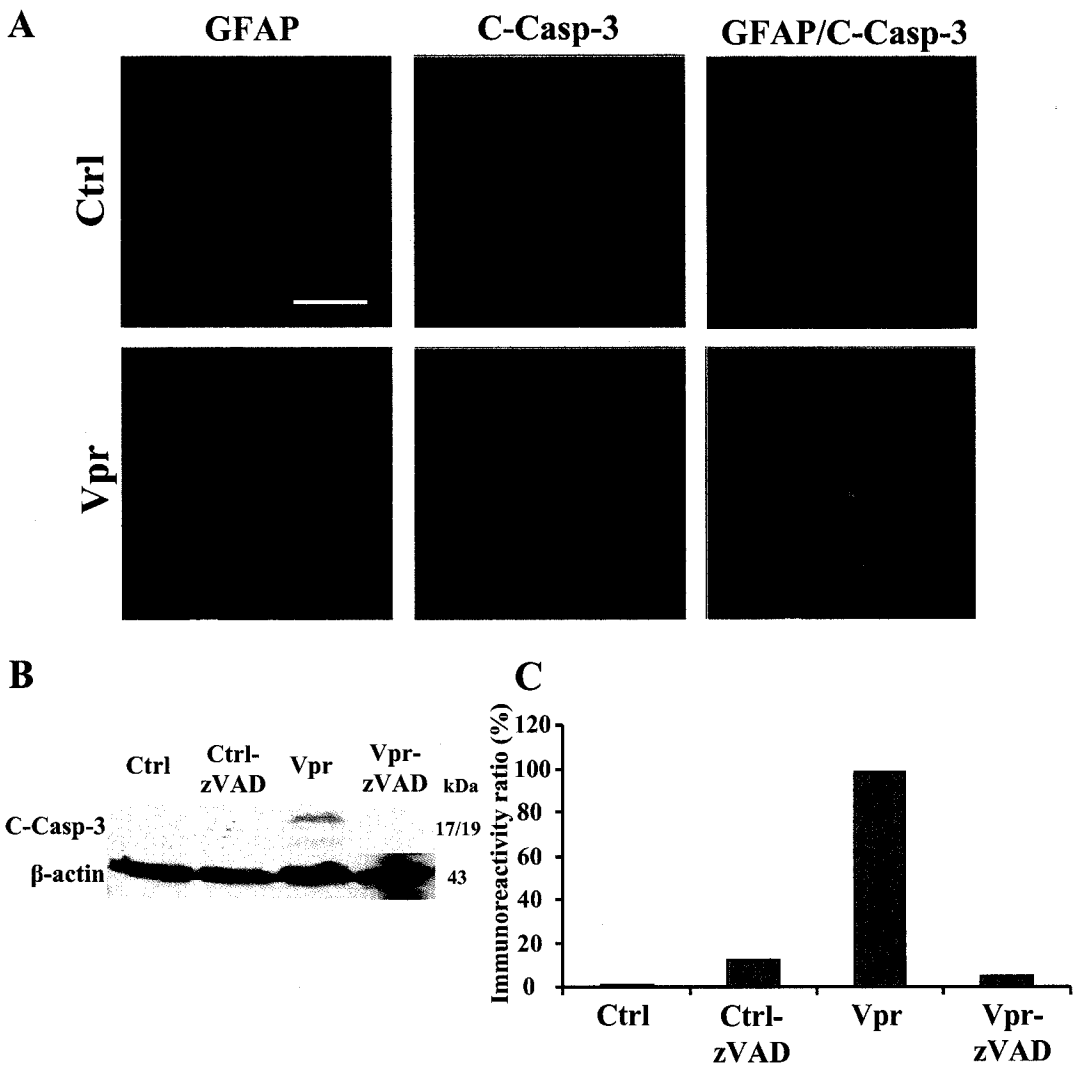
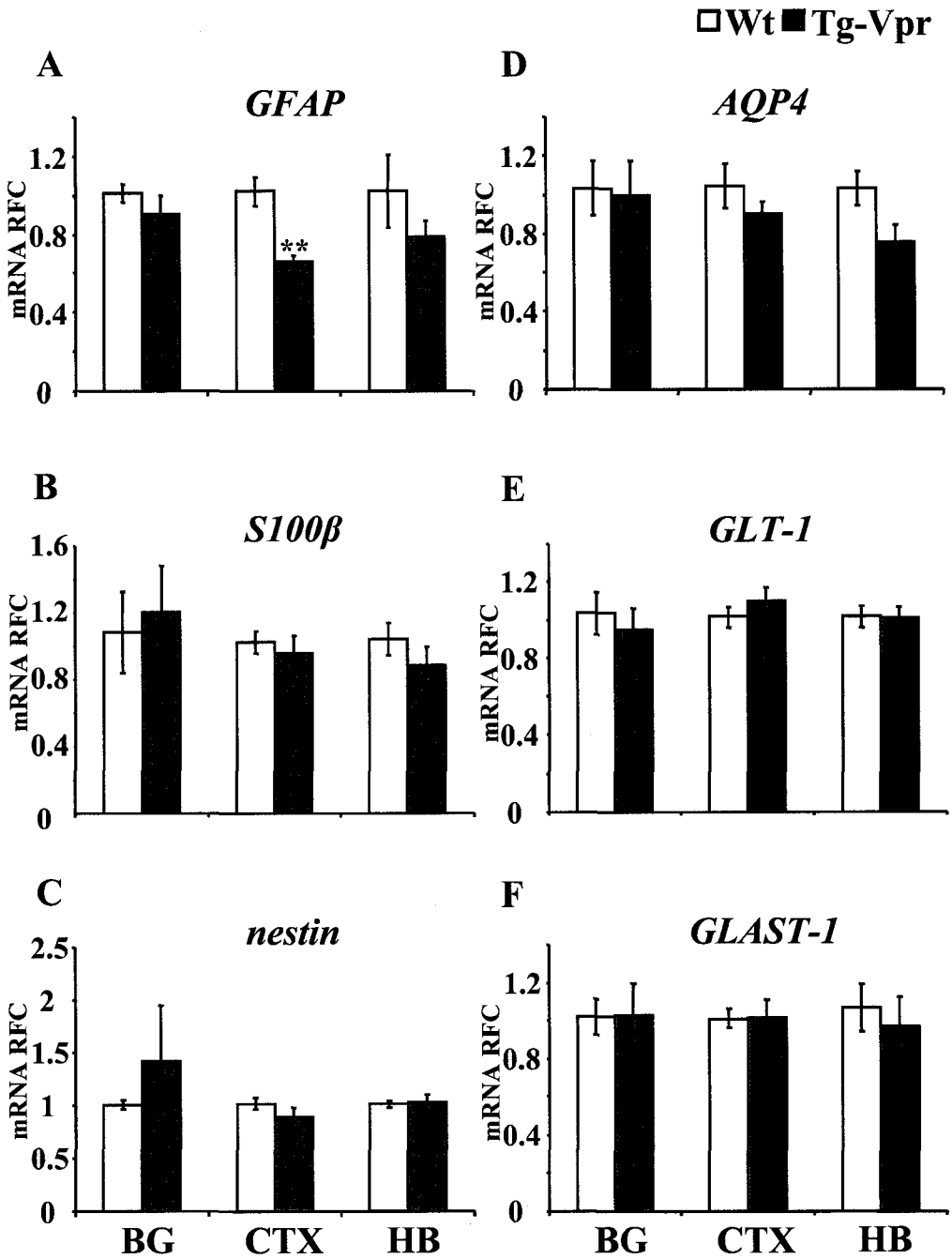


**FIGURE 4.4. HIV-1 VPR CAUSES LOSS OF CORTICAL *GFAP* TRANSCRIPT *IN VIVO.*** Wt and Tg-Vpr dissected brains were analyzed for (A) *GFAP* (B) *S100β* (C) *nestin* (D) *AQP4* (E) *GLT-1* and (F) *GLAST-1* expression. A significant decrease if *GFAP* transcript occurred in the CTX of the Tg-Vpr mice compared to Wt mice, while there was a general decreased trend of astrocyte transcripts in the BG, CTX and HB Tg-Vpr mice, with the exception of an increase in *nestin* transcript in the Tg-Vpr BG. The data represents the mean (SEM ±) of 6 Wt and 3 Tg-Vpr mice for the BG and the mean (SEM ±) of 10 Wt and 7 Tg-Vpr mice were used for the CTX and HB. Statistical analysis for all data was done by students' t-test. (**$p<0.01$)



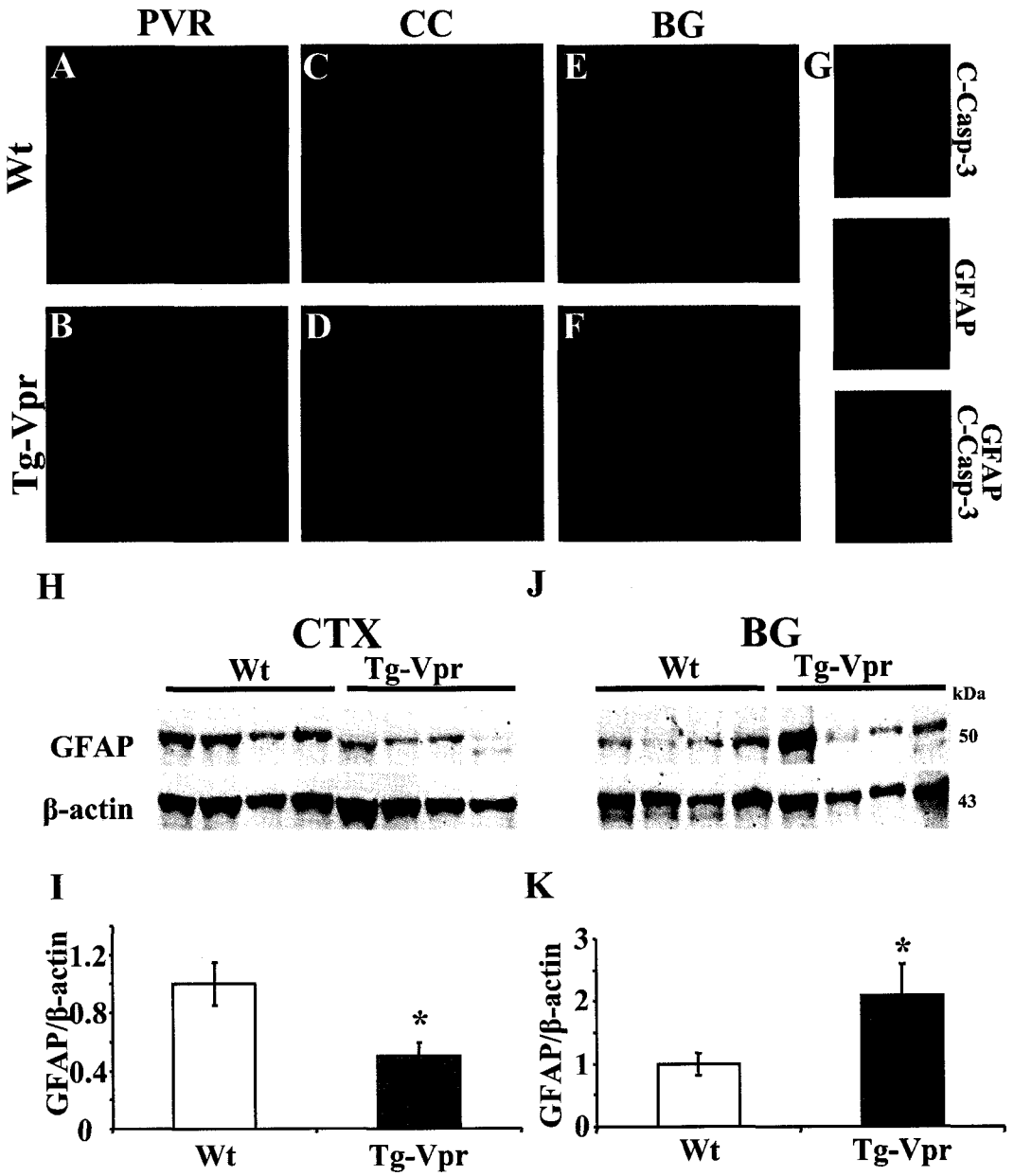
## 4.5 HIV-1 VPR MEDIATED REDUCTION OF CORTICAL GFAP$^+$ ASTROCYTES *IN VIVO*

Upon observing the significant decrease in *GFAP* transcript levels in the Tg-Vpr mouse model, we then examined the *in vivo* model for GFAP at the protein level through immunofluorescence (**Figure 4.5A-F**) and Western blotting (**Figure 4.5H-K**). While there was wide spread immunofluorescence for GFAP especially in the periventricular regions (PVR) in the Wt, there was noticeably less immunoreactivity in the Tg-Vpr mice (**Figure 4.5A and B**). The same was also true for the corpus collosum (CC) (**Figure 4.5C and D**) and BG (**Figure 4.5E and F).** When sections were double stained for C-Casp-3 (green) and GFAP (red), there were double positive cells in the Tg-Vpr mice (**Figure 4.5G**). GFAP protein was also shown to be significantly reduced in the CTX of Tg-Vpr mice by Western blotting (**Figure 4.5H**). The GFAP immunoblot shown in **Figure 4.5H** as well as another GFAP immunoblot from a second group of 4-month-old mice were quantified showing a significant decrease ($p<0.05$) in the ratio between GFAP and β-actin immunoreactivity in Tg-Vpr compared to Wt mice (**Figure 4.5I**). The BG was also immunoblotted for GFAP (**Figure 4.5J**); however, due to a few highly immunopositive animals in both litters analyzed, there was a significant increase ($p<0.05$) when GFAP fluorescence intensity was quantified (**Figure 4.5K**). Therefore, while there is a significant decrease in GFAP immunoreactivity within the CTX of Tg-Vpr mice, there is a significant increase in GFAP in the BG.

## 4.6 TG-VPR MICE EXHIBIT DECREASED EXPRESSION OF NEUROTROPHIC FACTORS

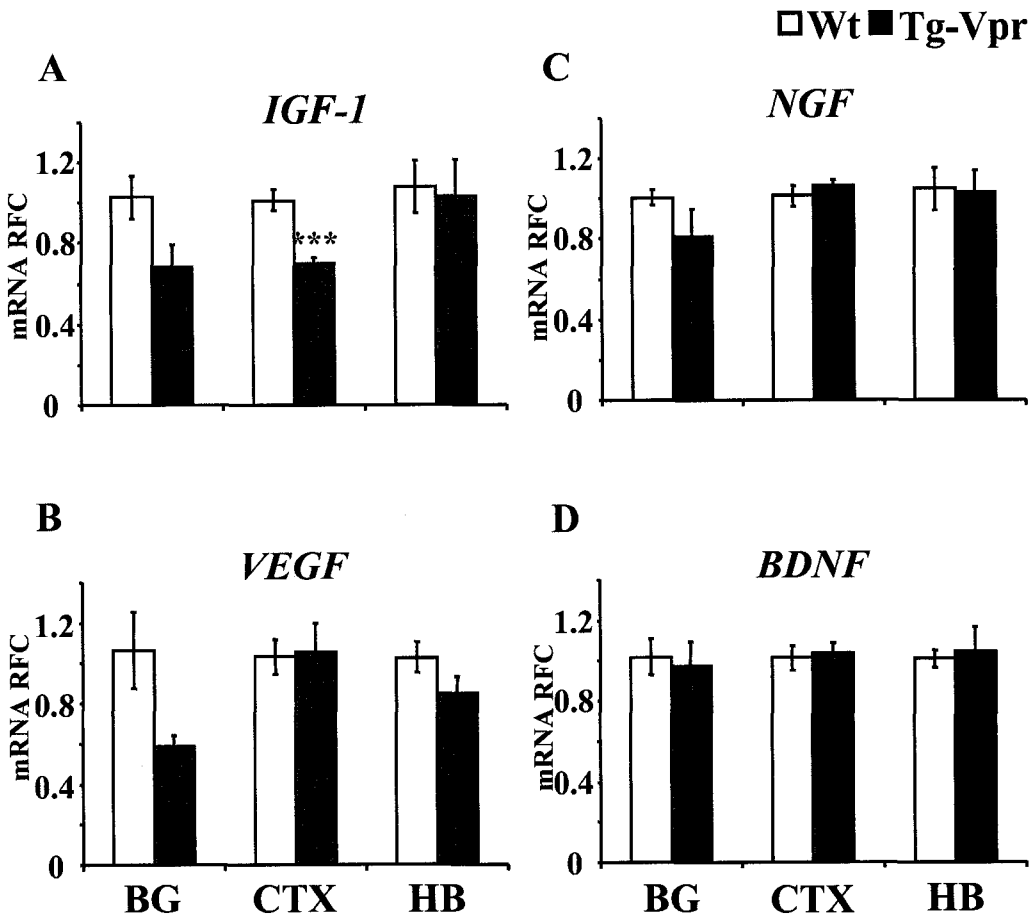
Since the brain works as a very complex network, tightly interconnected between different types of cells, the effect of reduced astrocyte abundance in the CTX may have

**FIGURE 4.5. HIV-1 VPR MEDIATED REDUCTION OF CORTICAL GFAP+ ASTROCYTES *IN VIVO*.** Immunoreactivity of GFAP in Wt and Tg-Vpr brain within the (A and B) periventricular region (PVR), (C and D) corpus collosum (CC) and (E and F) BG reveal a reduced immunoreactivity in the Tg-Vpr brains. (G) Co-localization of GFAP and C-Casp-3 occurred in Tg-Vpr brain. (H and I) GFAP immunoreactivity was significantly reduced ($p<0.05$) in the CTX (J and K) while it was significantly increased ($p<0.05$) in the BG. Western blots are representative of 1 litter of mice while the graphs represent the mean (SEM ±) of 2 litters containing 10 Wt and 7 Tg-Vpr mice. Statistical analysis for I and K were done by students' t-test. (*$p<0.05$).



indirect effects on neurons, causing the loss of neurons. Astrocytes are essential for neuronal survival through the removal of toxins and providing trophic factors to neurons. Therefore, we investigated whether the Tg-Vpr mice had significant changes in trophic factors within the brain. The transcript levels of *IGF-1* (insulin-like growth factor-1), *VEGF* (vascular endothelial growth factor), *NGF* (nerve growth factor) and *BDNF* (brain derived neurotrophic factor) were evaluated in the BG, CTX and HB of Wt and Tg-Vpr mice (**Figure 4.6**). There were decreases *IGF-1* in both the BG and CTX with a significant decrease in the CTX ($p<0.001$) while there was no change in expression in the HB (**Figure 4.6A**). The expression of *VEGF* had decreased in the BG and slightly in the HB while there was no change in the CTX (**Figure 4.6B**). The BG showed a slight decrease in *NGF* transcript, though not significant (**Figure 4.6C**). Finally, there were no changes between Wt and Tg-Vpr with regard to *BDNF* expression in any part of the brain (**Figure 4.6D**). Therefore, there were select decreases in neurotrophic factors that do not seem to be specific to one trophic factor or one area of the brain.

**FIGURE 4.6. TG-VPR MICE EXHIBIT DECREASED EXPRESSION OF NEUROTROPHIC FACTORS.** Wt and Tg-Vpr dissected brains were analyzed for (A) *IGF-1* (B) *VEGF* (C) *NGF* (D) *BDNF* expression through semi-quantitative RT-PCR, which revealed a significant decrease ($p<0.001$) in IGF-1 in the CTX of Tg-Vpr mice compared to Wt mice. Additionally there are decreases in the BG of Tg-Vpr with respect to *IGF-1* and *VEGF*, though not significant. The data represents the mean (SEM ±) of 6 Wt and 3 Tg-Vpr mice for the BG and the mean (SEM ±) of 10 Wt and 7 Tg-Vpr mice were used for the CTX and HB. Statistical analysis for all data was done by students' t-test. (***$p<0.001$)



# CHAPTER 5- DISCUSSION

## 5.1 VPR PRESENCE IN HIV$^+$ BRAINS

Localization of Vpr in HIV$^+$ brains is essential for further studies on Vpr-mediated neurodegeneration. Indeed Vpr was detected in HIV$^+$ brains in activated macrophages **(Figure 3.1A ii and inset)**. These observations confirm and extend Vpr detection in the CSF (180) and in HIV$^+$ brain tissue (341). The detection of Vpr within the brain of HIV$^+$ patients was a prerequisite to our study to assess its physiological relevance to HIV infection and to further investigate its potential role in HAD development.

## 5.2 VPR DETECTION IN *IN VIVO* MOUSE MODELS

Detection of Vpr in the Tg-Vpr mouse model was also required to validate this model for *in vivo* studies of Vpr and its effect in the brain. In the Tg-Vpr mice, we detected v*pr* transcript in the BMDMs **(Figure 3.6B)** as well as in the basal ganglia (BG), cortex (CTX), and hindbrain (HB), with two fold higher transcript levels within the BG and CTX than in the HB **(Figure 3.6C)**. Moreover, Vpr protein was detected in both perivascular and perenchymal macrophages of Tg-Vpr mice brains by immunofluorescence **(Figure 3.7A, B and inset,** respectively) while the absence of immunoreactivity in Wt mice brains confirmed the specificity of the antibody. The result confirmed the validity of our model of Tg mice expressing Vpr in brain macrophages as detected in the human HIV$^+$ brain tissue **(Figure 3.1A)** and previous literature (180, 341).

Other Tg-Vpr models have been used in which Vpr was expressed in adipose tissue (22), myocytes (182) and podocytes (368). However, only one other Tg-Vpr

mouse model was used to look at Vpr in the correct type of HIV-1 infected cell, whereby Vpr was expressed off the *CD4* promotor causing Vpr expression in T cells and macrophages (356). This model was sufficient for the groups' objective to look at Vpr-mediated apoptosis in T cells (356); however, for our studies, we were specifically investigating Vpr effect in the brain, where chiefly macrophages are productively infected. Thus the Tg-Vpr mouse model we used was most pertinent to our objectives. Other models have also been used to show Vpr-mediated effects *in vivo*. Recently, stereotactic injection of viral vectors containing Vpr were used to express Vpr (48); however, this method allowed infection of the vectors into non-specific cells in the brain or neuronal specific cells, which are not productively infected with HIV and do not express Vpr in a physiological infection. Therefore, our Tg-Vpr mouse model is more relevant for investigating Vpr-mediated neurodegeneration than previously used *in vivo* mouse models (48, 356).

The level of *vpr* transcript in different regions of the brain reflects HIV-1 infection of the brain, whereby there is more reported HIV-1 mediated neurodegeneration in the BG and the CTX, while less so in the HB (85, 87, 88, 113, 156). Therefore, the use of this model to investigate Vpr-mediated neurodegeneration was further justified due to its similarities to regional differences in HAD. Additionally, there are more macrophages in the BG and CTX relative to the HB, which correlates with *vpr* transcript level regionalization (176).

## 5.3 NEURONAL MEMBRANE DISTURBANCES

$Ca^{2+}$ plays an important role for homeostasis within a cell with regards to differentiation, growth, exocytosis, and for neurons, excitation and synaptic ability (reviewed in (13)). If not carefully regulated, an increase in $Ca^{2+}$ could cause neuronal apoptosis due to excitotoxicity. Since neurons are even more dependent on $Ca^{2+}$ homeostasis than other cells, regulation of this ion is essential for neuronal survival. Through electrophysiology studies, we showed neuronal membrane disturbances within the $Ca^{2+}$ dependent BK channels, resulting in a reduction of current due to reduced efflux of potassium ($K^+$) from these channels (**Figure 3.2**). Efflux of $K^+$ from BK channels leads to hyperpolarization and decreased excitation. Both membrane potentials and intracellular $Ca^{2+}$ levels activate BK channels and thus act as a negative feedback regulator of excitation (reviewed in (106)). Therefore, if neuronal excitation occurs, causing release of intracellular stores of $Ca^{2+}$ into the cytosol, the resulting increase in cytosolic $Ca^{2+}$ can lead to the induction of apoptosis via excitotoxicity or other cell stresses. Moreover, our results complement the changes in membrane potentials previously described (246).

## 5.4 *IN VITRO* AND *IN VIVO* VPR NEUROTOXICITY

The mechanisms underlying neuronal injury and death in HIV-mediated neurodegeneration remain controversial although apoptosis is a recognized component (reviewed in (149)). Previous results by Dr. Gareth Jones showed that soluble Vpr was specifically neurotoxic resulting in the induction of apoptosis through the intrinsic pathway. The activation of this pathway resulted in the loss of mitochondrial potential,

cytochrome *c* release, activation of caspase-9 and-3 occurred associating with increased expression of p53 (**Figure 3.1C and D, Figure 3.3A-E**). Additionally, I showed the anti-apoptotic factor, Akt had suppressed activation with Vpr treatment, suggesting Vpr-mediated imbalance towards an apoptotic pathway (**Figure 3.3F**). Investigating Vpr effects in the brain *in vivo*, we observed loss of neuronal markers through immunofluorescence (**Figure 3.7E and F**) and Western blotting in the BG, but not in the CTX of Tg-Vpr mice, compared to the Wt animals, suggesting that Vpr mediates loss of a wide range of neuronal subtypes in specific regions (**Figure 3.7G-I and supplementary Figure 7.1**). There was also an increase in activation of caspase-3 seen in the BG and the CTX, as well as co-localization of activated caspase-3 within neurons (**Figure 3.7F inset, G-I and Supplementary Figure 7.1**). Analysis of the fine motor skill behaviour of the Tg-Vpr mice showed that our neuropathological observations were associated with a significant decrease in fine motor skills, whereby the Tg-Vpr showed increased ataxic behaviour compared to Wt littermate controls (**Figure 3.7J**).

The results we observed *in vitro* with activation of intrinsic apoptotic pathway fall in line with the changes in neuronal membrane currents with Vpr treatment. These results also complement previous work showing Vpr–mediated activation of intrinsic apoptotic pathway in the periphery, where cytochrome *c* was released (143), mitochondrial potential was lost (143) and caspase-9 and -3 were activated (220, 302). Vpr has been shown to induce DNA damage (279, 366) and thus induction of p53 is not surprising; however, it has been suggested that p53 is not essential for Vpr-mediated apoptosis (291). Recent results also show that Vpr suppresses Akt activation through

downregulation of NHE1, a sodium/hydrogen exchanger at the plasma membrane important for maintaining proper pH balance and homeostatic cell volume (145). NHE1 acts as a scaffolding protein to recruit ezrin/radixin/moesin (ERM) to the plasma membrane, facilitating recruitment and interaction of Akt with phosphoinositide 3-kinase ($PI_3K$), leading to activation of Akt through phosphorylation (350).

The *in vivo* results we reported recapitulate the *in vitro* experiments done with BMDM supernatant application to neurons in which we showed that not only is Vpr being secreted from the BMDM, but also that Vpr is neurotoxic by itself (**Figure 3.6B**). Vpr-mediated neurotoxic effects of the Tg-Vpr model were observed from *in vitro* macrophages prepared from the mice as well as *in vivo* neurotoxicity through loss of neurons in the brain. Furthermore, since we observed secretion of Vpr *in vitro,* it is highly possible that this same event might be occurring *in vivo* within the brain. Indeed soluble Vpr was also described in the CSF of patients with neurological disorders (180), assessing our Tg-Vpr as a relevant model for the study of the Vpr role in HAD, we showed here that secreted Vpr can be neurotoxic.

The loss of neurons observed in the BG correlates with observations of neuronal loss and apoptosis in the BG of HAD patients (3, 260). However, we did not see this occurring in the CTX, an area in the brain that also has increased apoptosis in neurons (3). Previous experiments also did not show Vpr-mediated cell death of cortical neurons *in vitro* (135), suggesting a higher threshold of tolerance to Vpr or possibly resistance to Vpr-mediated neuronal cell death. It is possible that even though there was *vpr* transcript in the CTX, Vpr protein may not have been produced in the macrophage or not secreted

enough to cause neuronal death. On the other hand, we did observe an increase, though not significant, in cleaved caspase-3 in both the BG and CTX suggesting that while induction of apoptosis may be occurring in the neurons in the BG (**Figure 3.7F inset**), apoptotic pathways may be activated in another cell type within the CTX.

Synaptophysin is a marker of neuronal pre-synaptic dendrites. Its function has not been fully elucidated, but it has been suggested that synaptophysin plays a role in vesicle formation in the pre-synaptic neurons (reviewed in (325)). It has been previously shown that neuronal injury through synaptodendritic degeneration strongly correlates with the presence and severity of cognitive impairment observed in HAD (86, 204). It is thought that the initial stages of neurodegeneration in HAD start with neurons presenting dendritic pruning and beading (86, 101, 204). This type of injury causes synaptic dysfunction through decreased capacity for long term potentiation (LTP) important for memory (102). We also observed loss of synaptophysin in the BG of the Tg-Vpr mice, suggesting neuronal injury thorough reduced presynaptic processes. On the other hand, this did not occur in the CTX. In fact a significant increase in synaptophysin immunoreactivity was detected within the CTX. This may highlight a compensatory reaction to the BG where we did see loss of neurons and neuronal injury. These results are similar to the chronically treated animals with neurotoxic cocaine, where the synaptic boutons also increased (263). In studies of brain injury, an increase in synaptophysin was also observed, but was later followed by neuronal cell body degeneration. It was also noted that though there was an increase in synaptophysin immunoreactivity, the neurons were dysfunctional in synaptic transmission (290).

Since Dr. Gareth Jones showed a significant increase in the frequency of the R77Q Vpr mutation in HAD brains compared to ND brains, we investigated the effect of Vpr (70-96), the Vpr domain described as possessing the neurotoxic properties, representing the Wt or R77Q mutation on a neuronal cell line. Neurotoxicity did not occur at physiologically relevant concentrations. Moreover, these results did not confirm the previously reported observation showing that Vpr (70-96) was sufficient to induce neuronal death, at 5μM (273). The difference with our observations was that they dissolved their peptides in water containing DTT, a reducing agent to prevent oligomerization. Initially, we followed this protocol, but our control with DTT alone was neurotoxic, therefore, the experiments were repeated with peptides dissolved without DTT, resulting in negligible neurotoxicity at lower concentrations. Therefore, it might be more revealing to compare Wt and R77Q Vpr using the full-length protein, which could be made through a baculovirus system.

If we ignore the high concentrations of Vpr-derived peptide required to induce neurotoxicity, there was a discrepancy in toxicity, whereby the R77Q mutation was slightly less toxic than the Wt peptide at certain concentrations. This might suggest that the R77Q mutant causes less neurotoxicity than Wt. This could suggest that in the brain, the virus is mutating toward a less toxic strain, possibly to prevent the loss of the host. It has long been accepted that a strategic mechanism for viruses in general is to be able to replicate within the host without killing the host, allowing for viral survival. This observation confirms the observation that long term non-progressors of HIV-1 contain the R77Q mutation (189).

## 5.5 CORRELATING NEURONAL LOSS WITH BEHAVIOURAL ABNORMALITIES

In light of the behavioural abnormalities observed in the Tg-Vpr mice, showing hyperactivity and ataxic in their behaviour, we can infer from our Vpr effects regarding the BG and CTX with how this behavioural phenotype could occur. The BG is the core region of the brain, important for fine tuning of motor skills. Information from the CTX, cerebellum and spinal cord converge within the BG, where the information is integrated so that there is an appropriate reaction to a stimulus. For example, information generated by the activation of pyramidal excitatory neurons in the motor CTX will travel down to the striatum of the BG and from there, two pathways can be taken resulting in either inhibition or excitation. The direct pathway connects the striatum to the internal globus pallidus and substantia nigra pars reticulata through inhibitory neurons resulting in inhibition or dampening of signal. The indirect pathway connects the striatum to the external globus pallidus and subthalamic nucleus resulting in amplification of excitation. Therefore the BG determines which pathway to take depending on what the appropriate response should be (reviewed in (27, 261)). Since we did not observe a change in excitatory (VAChT) and inhibitory (GAD65) neuronal abundance in the CTX and there was a significant decrease in both excitatory and inhibitory neurons in the BG, we assume that excitation could be occurring from the motor CTX; however, there is no amplification of stimulus or more importantly no dampening of stimulus due to loss of these neurons in the BG. Therefore, if the mouse is reacting to a movement on the screen or the horizontal bar, there is no regulation of the movement which may account for the hyperactive, ataxic and tremulous behaviours, which can characterize HAD.

## 5.6 INDIRECT NEUROTOXICITY

There is an indirect neurotoxic mechanism occurring concurrently with the direct effect of Vpr on neurons, whereby application of conditioned media of Vpr-treated glial cells, macrophages and astrocytes causes loss of neuronal viability that does not seem to be due to inflammation induced by these adjacent glial cells (**Figure 3.5**). These glial cells may be secreting some other form of neurotoxin, such as excitatory amino acids or proteases which could cause excitotoxicity in neurons. Further research must be done to understand what is being released from these cells. Of note, there was a trophic effect occurring in the supernatants from astrocytes treated with lower concentrations that did seem to be due to Vpr, since the blocking antibody inhibited this trophic effect (**Figure 3.5C**). Vpr has been previously shown to be anti-apoptotic at lower concentrations (18, 63, 98).

## 5.7 SUPPRESSION OF INFLAMMATION *IN VIVO*

Surprisingly, *in vivo* Vpr-mediated neuronal cell death occurred in the absence of microglia activation, which is usually a feature of HIV-1 brain infection (reviewed in (149)); nonetheless, associated inflammation is not usually a feature of apoptosis. The *in vivo* expression of Vpr in the brains of mice failed to enhance (1) pro-inflammatory gene expression and (2) F4/80 expression (a marker of activated microglia) (3) morphological changes in microglia marked by Iba-1 in Tg-Vpr mice (**Figure 3.6D and 3.7C and D**). These results parallel our previous data showing the absence of inflammation associated with Vpr-treated astrocytes and macrophages (**Figure 3.5A, B, D, E**). Vpr has also been shown to repress cytokine expression in a glucocorticoid-like manner, whereby the

effects of NFκB mediated induction of inflammation are suppressed causing reduced IL-4, IL-2, IL-10, IL-12 and TNF-α (18), while others have shown that there is an increase in some cytokines, such as IL-8 (271). The similarities between Vpr and glucocorticoid suppression of inflammation were connected when it was revealed that Vpr might co-activate the glucocorticoid receptor II (GR) (159). This complex formation between Vpr and GR causes the sequestration with PARP-1 from the nucleus, resulting in inhibition of NFκB transcription and thus reduced expression of inflammatory mediators (219). Suppression of inflammation through inhibition of NFκB may also occur through induction of its inhibitor, IκB by Vpr (18).

These results are paradoxal to the current model of HAD, where increased microglial reactivity including inflammation within the brain are among the properties associated with HAD (107, 108, 129, 340). On the other hand, we did observe some inflammation occurring in HFA with increased concentration of Vpr, suggesting a threshold effect (**Figure 4.1**); however, the amount of Vpr expressed in the brains of HAD patients may not be as high as the concentration required to induce an inflammatory effect. Although we did not observe the characteristic inflammation occurring in these models, we did observe neuronal injury and apoptosis, astrocyte loss as well as behavioural abnormalities characteristic of HAD (3, 4, 86, 96, 103, 203, 204, 241, 289, 313). Thus, Vpr might cause the apoptosis observed in the brains of individuals with HAD while other viral proteins (e.g. gp120, Tat, gp41) dictate the extent of immune activation, as suggested by previous studies (reviewed in (149)).

## 5.8 INDUCTION OF *VPR* EXPRESSION IN TG-VPR MICE AND INFLAMMATION

The *c-fms* gene encodes the M-CSF receptor, important for growth and maturation of macrophages. Attempts to induce the expression of *vpr* through the *c-fms* promoter in the BMDM were not successful. Treatment of macrophages with macrophage maturation factors, such as inflammatory cytokines (TNF-α), inflammatory inducers, such as LPS and through manipulation of media, there did not seem to be an increase in expression of *vpr* off the *c-fms* promoter (**Figure 3.8**). In addition, there was no aberrant change in inflammation over the Wt controls (**Figure 3.9**). Induction of *vpr* through PMA, LPS and M-CSF actually downregulates the *c-fms* promoter (361). Therefore, it is possible that we were inducing the macrophages to differentiate resulting in the absence of induction or even a downregulation of the *c-fms* promoter. Macrophages may need to be partially differentiated or harvested at an earlier timepoint of differentiation to observe an increase in *vpr* transcript. Alternatively, we may also need to decrease the amount of M-CSF in the media, but M-CSF is required for survival. However, if we reduce the concentration, the macrophages may induce the expression of the M-CSF receptor to bind as much M-CSF in the media as possible. When we treated the BMDM with 0.5ml of media containing 10% of L929 supernatant, there was a slight increase in *vpr* expression. Though there was no change in concentration of L929, there was a reduced amount of growth factors per cell in culture, which may suggest that the macrophages were increasing expression of M-CSF, or *vpr* in this case, to bind as much M-CSF as possible.

The absence in inflammation was not surprising, since Vpr seems to suppress inflammation; however, since Vpr is immunosuppressive, we thought we would observe

more decreases in *IL-10, IL-1β* and *TNF-α* relative to the amount of *vpr* expression for each treatment. Instead, we did not observe any association between *vpr* expression and suppression of these genes. This might be because the threshold value for Vpr-mediated suppression of inflammation was reached and thus we did not see the suppressive effects previously described (18, 219). These results, however, reflect our *in vivo* results seen in the brain, in which there was no change in inflammation except a suppression of *IL-6* **(Figure 3.6D)**. These results also complement our previous data showing Vpr-mediated toxicity in BMDM due specifically to Vpr **(Figure 3.6B)**.

Of note however, we did see a discrepancy between our results, in that we observed a neurotoxic effect in supernatants from activated macrophages treated with Vpr **(Figure 3.5F)**, suggesting an indirect neurotoxin was released while we did not observe an indirect effect in the Tg-Vpr BMDM **(Figure 3.6B)**. The difference may be due to the activation state of the macrophages, where the U-937 monocytes were activated with PMA before Vpr treatment while the BMDM were only treated with media containing L929 for maturation of the macrophages. As well, the BMDM were producing the Vpr intracellularly, rather than being treated with soluble Vpr. These differences may have resulted in different signalling between the two groups resulting in the release of a non-inflammatory neurotoxin versus a direct Vpr-mediated neurotoxicity. Thus, this data further confirms that Vpr does not seem to be mediating its neurotoxic mechanism through induction of inflammation.

## 5.9 ASTROCYTE DEATH AND APOPTOSIS *IN VITRO* AND *IN VIVO*

Astrocytes are essential for providing neuronal support in the brain through prevention of infection and inflammation by maintaining the BBB, reducing neuronal death through removal of neurotoxins, such as glutamate and nitric oxide as well, providing neurotrophic factors for neuronal growth and survival (reviewed in (200)). While it has been shown that Vpr causes the loss of neurons, it was also cytotoxic to astrocytes, whereas the Vpr peptides, Wt and R77Q, were not (**Figure 4.1**). Furthermore, these peptides were slightly trophic to the astrocytes in preliminary studies as indicated by increased β-tubulin immunoreactivity (**Figure 4.1E**). Through analysis of full-length Vpr on astrocytes, we confirmed astrocyte death through 7AAD positive staining in flow cytometry while also showing that there was loss of mitochondrial potential in the Vpr-treated astrocytes through loss of TMRE staining suggesting apoptosis was occurring in the Vpr-treated astrocytes (**Figure 4.2**). To further confirm the induction of apoptosis, we observed activation of caspase-3 in astrocytes through immunofluorescence and Western blotting of astrocytes which was abrogated by zVAD-fmk treatment (**Figure 4.3**). Previous reports have also shown that Vpr causes astrocyte death through apoptosis *in vitro* and *in vivo* (48, 135). There were some doubts by Huang *et al* (2000) about Vpr causing apoptosis in astrocytes due to patterns in DNA fragmentation; however, our data and those of Cheng *et al* (2007) further confirm apoptosis may be occurring. Loss of astrocytes is a characteristic feature of HAD in which there was an increased number of apoptotic astrocytes in HAD brains (241, 289, 313).

GFAP or glial fibrillary acidic protein is an intermediate filament protein specific to astrocytes and an important protein for astrocyte structure (140). Using the *in vivo* Tg-Vpr mouse model, we also saw the loss of astrocyte markers including a significant reduction of *GFAP* transcript and GFAP protein in the CTX, though this did not occur in the BG (**Figure 4.4 and 4.5**). In fact, there was a significant increase in GFAP within the BG (**Figure 4.5I and K**). Additionally, we noted a general trend of reduced expression of astrocyte markers through real-time PCR (**Figure 4.4**). Loss of astrocytes in the CTX may be due to apoptosis as we did show evidence of co-localized activated caspase-3 with astrocytes (**Figure 4.5G**). Previous observations of postmortem brains show that compared to $HIV^-$ brains, rapidly progressive HAD brains had significantly higher apoptotic astrocytes in both the BG and the mid-frontal gyrus, while slow progressive HAD brains had significantly higher apoptotic astrocytes in just the mid-frontal gyrus (313). This suggests that astrocytes in the CTX are more sensitive to HIV-1 infection and Vpr toxicity than the BG. If such is occurring, we are probably observing neurotoxicity similar to slow progressing or initial stages of HAD, since the Tg-Vpr does not contain all of the viral and host neurotoxins occurring in HIV-1 infection and Vpr is not highly expressed in the Tg-Vpr mice. However, this does not explain the increase in GFAP in the BG in the Tg-Vpr mice. Even with the removal of the 2 outlying Tg-Vpr mice within the 2 litters tested, there is still an increase (though not significant) in relative GFAP abundance. Moreover, there was an increase in *nestin* expression in the BG, though not quite significant, suggesting that there is proliferation of stem cells that may result in increased astrocytes (**Figure 4.4C**). It is possible that reactive astrogliosis may be

occurring here causing an increase in GFAP expression. While there are some cases reporting loss of astrocytes in HAD, there is also evidence suggesting astrogliosis is occurring with an increase in astrocytes (171, 207). Moreover, during neuronal injury, astrocytes become reactive and significantly increase GFAP expression (330).

Since GFAP was the only astrocyte marker significantly decreased in the CTX of Tg-Vpr mice Vpr mediated effect may be specific $GFAP^+$ astrocytes. There are different subsets of astrocytes, moreover there are $GFAP^-$ astrocytes within the brain which are mainly radial glia cells and astrocyte progenitors which both eventually become $GFAP^+$ astrocytes (334). Perhaps the increase in GFAP protein and *nestin* expression within the BG reflects an increase in turnover of astrocytes from stem cell precursor stage ($Nestin^+/GFAP^-$) to mature astrocytes ($Nestin^{+/-}/GFAP^+$).

### 5.10 CORRELATING NEURONAL LOSS WITH ASTROCYTE LOSS *IN VIVO*

Astrocytes are important producers of growth factors and neurotrophins to maintain survival of cells dependent on astrocytes, such as the microvascular endothelia and neurons. Since there was a decrease in astrocytes we also examined whether there was loss of growth and neurotrophic factors in effort to connect the loss of astrocytes with the loss of neurons. There was a significant loss of *IGF-1* in the CTX as well as a general trend of reduced expression of growth and neurotrophic factors in the Tg-Vpr mice compared to the Wt mice (**Figure 4.6**). Similar observations have been reported with other viral proteins, such as gp120 and Tat, which have been shown to reduce BDNF and NGF, respectively (228, 238). Reduction of *IGF-1* is a significant factor that may be contributing to the reduced amount of neurons in the BG and astrocytes in the

CTX. Previous studies show that when IGF-1 is added to supernatants from HIV-1 infected cultures, this neurotrophic factor prevents the loss of neurons (335). The decrease in *IGF-1* may be linked to the decreased activation of Akt, where administration of IGF-1 causes activation of anti-apoptotic Akt (137). Since we have previously observed *in vitro* that Vpr inhibits activation of Akt, activation of this anti-apoptotic protein is probably further inhibited with the reduced *IGF-1 in vivo*, which may push the neurons further to apoptosis mediated by pro-apoptotic signals induced by Vpr. IGF-1 is induced by growth hormone in neurons and astrocytes. Previously, it had been reported that treatment of FIV-infected cats with growth hormone reduced neurotoxicity most likely through the induction of IGF-1 in neurons (326).

There was also a general trend of reduced *VEGF* and neurotrophins in the BG, suggesting that the astrocytes in the BG are not providing the neurons with these important supplements. It is possible that these astrocytes may either be non-functional or non-functional with regard to release of neurotrophic factors. Even though inflammatory cytokines are known for inducing tissue damage when excessively produced, their main function is to remove dangerous substances and facilitate tissue repair. Thus, cytokines are important for the induction of neurotrophins and growth factors. Astrocytes are important for the production of IL-6, a trophic factor important for neuroplasticity and synaptic scaling (139, 339). IL-6 causes the release of neurotrophins, such as NGF and BDNF, factors that mediate neuroplasticity (202). In our model, we observed a slight suppression of inflammation including a significant reduction of *IL-6,* which may be responsible for the decrease in these neurotrophins and

growth factors. However, in an HIV-1 infection of the brain, IL-6 is upregulated in astrocytes due to HIV-1 mediated inflammation induced by infection and other viral mediators (92). Thus, it is difficult to conclude that these neurotrophic factors may be downregulated in a full HIV-1 infection.

Though there was a significant decrease of *IGF-1* in the CTX, there were no decreases in the other neurotrophins and growth factor expression. Microglia and perivascular macrophages can also secrete neurotrophic factors that may be compensating for the reduced astrocytes in the cortical region (reviewed in (322)).

From this data, we can not conclude that the decrease in neurotrophic factors results in the loss of neurons; however, the possibility of Vpr-mediated direct neurotoxicity with the addition of lost support from astrocyte-mediated release of neurotrophic factors is plausible in this context.

While VEGF has neurotrophic properties (reviewed in (42)), we investigated the expression of this growth factor for vascular cells, to determine if there might be an astrocyte-mediated effect on the BBB. We did in fact see a decrease in *VEGF* expression in the BG, though not quite significant, suggesting that there may be a possibility of a compromised BBB which recapitulates previous reports (249). If so, the loss of astrocytes due to Vpr may be having a direct effect on the permeability of the BBB, which may cause increased infiltration of infected cells into the brain. However, this suggestion is very speculative, since it is only being based on reduced expression of a *VEGF*. Thus, further research into this area must be done to confirm whether this is occurring. Previous research has shown that there is actually an increase in VEGF in the

serum of HIVE patients compared to non-encephalitic and HIV-1$^-$ individuals suggesting its role in HIVE (299). Therefore, it may be that the decrease in VEGF is a Vpr specific effect and dominated by the effects we see in HIV-1 infection.

## 5.11 CONCLUSIONS

Taking everything we have observed regarding Vpr-mediated effects in neurons and astrocytes we can conclude that Vpr induces an intrinsic apoptotic pathway in neurons, whereby neuronal death can stem from direct Vpr treatment or indirect Vpr-treated adjacent glial cells. *In vivo* Vpr is secreted from macrophages leading to neurotoxicity through apoptosis that could lead to downstream behavioural abnormalities similar to HAD. Concurrently, Vpr is also toxic to astrocytes, possibly causing astrocyte apoptosis seen *in vitro* and *in vivo*. The downstream effect of reduced astrocyte abundance is evident through the decreased expression of neurotrophic and growth factors, which contributes to neuronal loss. A schematic diagram is shown in **Figure 5.1**.

## 5.12 FUTURE DIRECTIONS

Throughout our studies, we observed Vpr-mediated effects occurring at a threshold, where inflammation or toxicity occurred at a certain concentration rather than a gradual effect. Although there was Vpr expression through RNA and protein, there was not a great abundance of Vpr in the Tg-Vpr model, since we could not get immunoreactivity through Western blotting. Therefore, it would be interesting to define the effects of increased *vpr* expression. The treatments we had previously used are known activators of macrophages, which seemed to either downregulate the *c-fms*

**FIGURE 5.1. SCHEMATIC DIAGRAM OF RESULTS.** HIV-1 infects perivascular macrophages and microglia. These cells secrete Vpr which act on themselves or neurons and astrocytes. Direct Vpr treatment on neurons results in activation of caspase-9 and -3 and increased p53. Indirect treatment of Vpr on adjacent glial cells results in neurotoxicity through an unknown neurotoxin. Astrocytes treated with Vpr undergo apoptosis. The effects of Vpr *in vivo* results in loss of neurons and astrocytes possibly connected through decreased neurotrophic factors. The compounding effects we observe may lead to the neurobehavioural deficits associated with HAD.

promoter or not change its activity. However, when we treated with 0.5ml of 10% L929 we in effect decreased the total amount of L929 containing M-CSF, which caused an increase in *vpr* transcript level. Therefore, in order to increase expression of the *c-fms* promoter, we must not activate the macrophages to full maturity. This could be done by blocking the M-CSF receptor with blocking antibodies which will inhibit M-CSF signaling causing maturation and eventual negative feedback to reduction of expression off the *c-fms* receptor. If this works, we may be able to observe more effects of Vpr on the brain and perhaps see if the neurons in the CTX and the astrocytes in the BG become more vulnerable to Vpr. If we can manipulate the expression of this Tg model, we could observe other genes in HIV-1 alone and together.

Though we are observing Vpr-mediated effects in our *in vivo* mouse model, the model is not truly physiological in terms of the degree of immunosuppression. The present animals are healthy immunocompetent mice whereas in an HIV-1 infection, particularly later on, HAD patients are immunocompromised. Thus, it is necessary to observe the effects of Vpr in immunocompromised mice. To do this, we should produce a Tg-Vpr mouse in a Rag-1 knock out background which would not have functional T lymphocytes. There is some evidence to suggest that T cells infiltrating the brain have neurotrophic functions, however, with depletion of $CD4^+$ T cells in HIV-1 infection, this would not occur physiologically (120).

Previously we showed that both activated macrophages and astrocytes treated with Vpr secrete some form of neurotoxin while Vpr is also toxic to astrocytes. It would therefore be interesting to investigate what is being secreted from these macrophages and

astrocytes. First we could do the same experiment as we did previously with Vpr-treated astrocyte supernatants except apply the supernatants to astrocytes with and without blocking antibody to observe whether Vpr-mediated astrocyte toxicity is direct or indirect. For instance, the death of astrocytes may be releasing excitatory amino acids, which might also explain the neurotoxin released by macrophages, since these amino acids can be produced and released from these cells (357). Additionally, we currently have a microarray chip containing 100 genes known to the Power Laboratory. Preliminary experiments using this technique on HFA treated with ranging concentrations of Vpr have shown some interesting possibilities to investigate, including the induction of known neurotoxin, IP10; however, more analysis into this technique must be done as well as confirmation by real-time PCR and Western blotting to make any conclusions. Another way to test the contents released is take the supernatants from Vpr-treated astrocytes and macrophages and analyze the contents using mass spectrometry and NMR with immunoblotting for confirmation.

Although we did observe apoptotic markers occurring in astrocytes treated with Vpr, it would be beneficial to further confirm these results with other apoptotic assays. While caspase-3 activation must be repeated with a positive control, apoptosis can also be additionally confirmed through DNA fragmentation, cytochrome *c* release assays, Bax activation and activation of caspase-9 could be done.

The observation of reduced *IGF-1* expression may have implications on the loss of neurons and is worth investigating the pathway to reduced IGF-1. IGF-1 is produced by stem cells when stimulated by growth hormone (326) while IGF-1 may contribute as a

neurotrophic factor by reconstituting neurons from HIV-1 infection (335). It is possible that the loss of neurons resulted in the down-regulation of IGF-1 in the BG and significantly in the CTX or the loss of neurons resulted from the loss of IGF-1. Therefore, it would be interesting to investigate the pathway of IGF-1 loss and neuronal loss while also looking into the effects of Vpr on growth hormone production.

Throughout our studies, one constant observation was the occurrence of Vpr-mediated trophic effect on both neurons and astrocytes at the lower concentrations. Vpr has been shown to be anti-apoptotic at lower doses (18, 63, 98). It is therefore possible that either Vpr is signaling through the glucocorticoid receptor (GR) pathway at a threshold level, where at low doses Vpr is anti-apoptotic and at high doses, apoptotic. Alternatively, Vpr may be binding two or more different receptors. At lower doses, Vpr could be binding one receptor with more avidity resulting in an anti-apoptotic result, whereas at higher doses, Vpr could be binding the GR leading to a dominant apoptotic pathway. To define this possibility, we must do blocking experiments using antagonists to the GR, RU486, along with other antagonists for potential second receptors to see changes in cell loss.

Since our sequence data show a significant difference in Vpr in HAD brains compared to ND brains, with respect to an arginine at position 77 mutating to glutamine in HAD brains, it is necessary that we define the mechanism of this mutation. Since the peptides were not resulting in a physiologically relevant toxicity, we must use full-length Wt and R77Q Vpr to solve the question. I would predict, based on the data extracted

from the peptide experiments that R77Q is less neurotoxic and fits the results of Lum *et al* (2003), resulting from HIV-1 selection towards a less neurotoxic virulent Vpr.

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## SUPPLEMENTARY DATA

**FIGURE 7.1. *IN VIVO* TG-VPR CORTICAL IMMUNOREACTIVITY ASSOCIATED WITH APOPTOSIS BUT NOT NEURONAL LOSS.** GAD65 and VAChT immunoreactivity showed no change while there was an increase in synaptophysin and cleaved caspase-3 immunoreactivity in the CTX of Tg-Vpr compared to Wt animals. Graphical depiction of this is represented in **Figure 3.7I**.