Calcium Flux and Neuronal Cell Death Induced by the HIV-1 Proteins Tat and gp120

by

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A Thesis

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Calcium Flux and Neuronal Cell Death Induced by HIV-1 Proteins

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

of Manitoba in partial fulfillment of the requirements of the degree

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ABBREVIATIONS

AIDS	acquired immunodeficency syndrome
HIV	human immunodeficency virus
HTLV	human T-cell leukemia virus
$[Ca^{2+}]_i$	intracellular calcium
NMDA	N-methyl-D-aspartate
AMPA	amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
GluR	glutamate receptor
MK-801	(+)-5-methyl-10,11,dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine hydrogen maleate)
Dizoclipine	(+)-5-methyl-10,11,dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine hydrogen maleate)
AP5	($\pm$)-2-amino-5- phosphonopentanoic acid
CNQX	6-cyano-7nitroquinoxaline-2,3-dione
DNQX	6,7-dinitroquinoxaline-2,3-dione
TMB-8	([8-(diethylamino)octyl-3,4,5-trimethoxybenzoate, HCl])
DMSO	dimethyl sulfoxide
MIA	5-(N-methyl-N-isobutyl)-amiloride
LME	L-leucine methyl ester
BSA	bovine serum albumin
IP_3	inositol-1,4,5-trisphosphate
RyR	ryanodine receptors
TAR	*trans*-activation response element
CNS	central nervous system
TNF-α	tumor necrosis factor alpha
IL-1β	interleukin 1-beta
MIP-1α	macrophage inflammatory protein–1α
MCP-1	monocyte chemotactic peptide-1
SDF-1α	stromal derived factor 1-alpha

IFN-γ	interferon gamma
NO	nitric oxide
IP$_3$	inositol 1,4,5-trisphosphate
IP$_3$R	inositol 1,4,5-trisphosphate receptor
PKC	protein kinase C
PKA	protein kinase A
DAG	diacylglycerol
AMP	adenosine mono-phosphate
cAMP	cyclic adenosine mono-phosphate
ADP	adenosine di-phosphate
cADP	cyclic adenosine di-phosphate
ATP	adenosine tri-phosphate
SERCA	sacroplasmic/endoplasmic reticulum calcium pumps
S.E.M.	standard error of the mean
ANOVA	analysis of variance

This thesis is dedicated to my parents. Thank you for providing me with the love, strength and inspiration that made the completion of this work possible.

ABSTRACT

AIDS-related cognitive-motor complex is a dementing illness that is a

pathophysiological consequence of HIV-1 disease; it is characterized by deficits

in cognition, behavior and motor function. Although, neurons are not themselves

infected by HIV-1, the selective loss of neurons in brain almost certainly

contributes to AIDS-dementia. Viral proteins including gp120, Tat, Vpu and Nef

can be toxic to neurons. The HIV-1 coat glycoprotein, gp120, and the viral *trans-*

activator, Tat, are, at present, the two best characterized neurotoxic HIV-1

proteins. We showed that gp120 and Tat are both present in the brain of HIV-1

infected patients and are toxic to neurons *in-vivo* and *in-vitro*. Exogenously-

applied Tat depolarized neuronal membranes and caused biphasic increases of

cytosolic calcium; the first increase was from intracellular stores by IP_3-

dependant mechanisms and the second by glutamate-receptor mediated calcium

influx. Tat significantly potentiated glutamate and NMDA receptor-mediated

increases in cytosolic calcium by mechanisms dependant on IP_3-receptors and

potentiated glutamate-mediated neuronal cell death. Blockade of IP_3-receptors

protected neurons from the toxic effects of Tat. Applications of gp120 to mixed

neuron / astrocyte cultures resulted in increases of cytosolic calcium first in

astrocytes and second in neurons. Gp120-induced increases in cytosolic calcium

were blocked by inhibition of Na^+/H^+ exchange, and in neurons, by antagonists of

L-type voltage-sensitive calcium channels and glutamate receptors. Sub-toxic

amounts of Tat and gp120, when combined, produced synergistic increases of

cytosolic calcium and neuronal cell death. Antagonists of NMDA receptors but not L-type calcium channels or Na^+/H^+ exchange reversed the combined neurotoxic effects of gp120 and Tat. Together, these findings suggest that even very low levels of the HIV-1 proteins Tat and gp120 can combine to cause dysfunction and death of neurons in brain of HIV-1 infected individuals. Furthermore, our findings have identified mechanisms for and potential therapeutic strategies aganst HIV-1 dementia.

General Introduction

The Discovery of AIDS and HIV

In 1981, five cases of *Pneumocystis carinii* pneumonia (PCP) were discovered in Los Angeles among young homosexual men and reported to the Center for Disease Control [1]. These previously healthy men all showed signs of immunosupression without a discernable underlying cause. Shortly thereafter, 26 cases of Kaposi's sarcoma, a rare type of skin cancer, were reported in homosexual men from New York City and California [2] and seven of them presented with severe opportunistic infections, including four with PCP. Prior to this time, *Pneumocystis carinii* infection was rarely seen in young immunocompetent individuals and Kaposi's sarcoma was largely restricted to patients taking immunosupressive drugs. These discoveries suggested a severe disturbance of immune function. The occurrence of Kaposi's sarcoma and/or opportunistic infections in an individual with unexplained immune dysfunction became known as Acquired Immune Deficiency Syndrome (AIDS).

The first indication that a retrovirus may be responsible for AIDS came in 1983 when Barre'-Sinoussi and colleagues at the Pasteur Institute recovered from a patient with persistent lymphadenopathy syndrome a virus containing reverse transcriptase [3]. Their virus was later called lymphadenopathy-associated virus and in culture, it grew to a high titer in CD4$^+$ cells and killed them [4]. This finding suggested a possible link between this isolate and the immune suppression

observed in AIDS. In 1984, Gallo and his co-workers reported their discovery of a virus isolated from peripheral blood mononuclear cells of AIDS patients [5-8]. They believed the virus to be a new member of the human T-cell leukemia virus (HTLV) family and so named the isolate HTLV-III. At the same time, Levy and his co-workers were isolating retroviruses from individuals diagnosed with AIDS, with persistent lymphadenopathy syndrome, and from some 'healthy' people [9]. The viruses they isolated showed some cross-reactivity with lymphadenopathy-associated virus, however they felt it was sufficiently different to merit a new name, AIDS-associated retrovirus. Soon it was discovered that all three viruses, lymphadenopathy-associated virus, HTLV-III, and AIDS-associated retrovirus were members of the family lentivirinae and, in 1986, the International Committee on Taxonomy of Viruses recommended giving the AIDS virus the name Human Immunodeficency Virus (HIV) to distinguish it as a separate genus of the family Retroviridae. HIV-1 is now subdivided into clades A to H.

Genomic Organization of HIV-1

The genome of HIV-1 consists of a diploid (2 X 9.8 kb) plus sense RNA. The primary transcript of HIV-1 is a full length 9.2 kb mRNA that is translated into the Gag and Pol proteins (Fig 1). The synthesis rate of Gag vs Gag-Pol proteins is approximately 20:1. Singly spliced 4.5 kb transcripts are translated into the Env, Vif, Vpr and Vpu proteins and multiply spliced 2 kb transcripts are translated into the Tat, Rev, and Nef proteins. Primary transcripts and protein products are listed in Table 1.
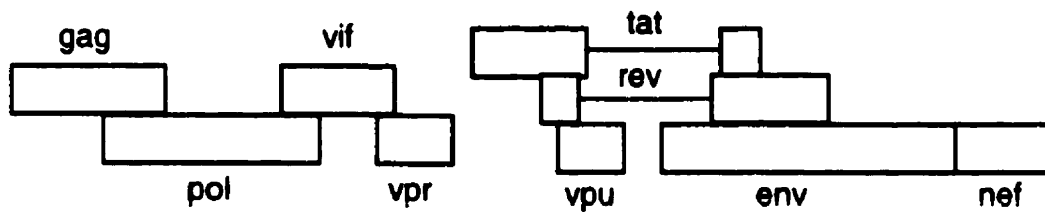
Figure 1: Genomic Organization of HIV-1. (Adapted from W. C. Greene, 1991 [10]).

Structure of HIV-1

HIV-1 is spherical in appearance with a diameter of 80-100 nm and contains a

unique three-layered structure (Fig 2). Innermost is the genome-nucleoprotein

complex that is closely associated with the viral reverse transcriptase (also

known as the viral polymerase), integrase, protease and the nucleocapsid

proteins p9 and p6. The protease is responsible for the processing of Gag and

Pol proteins while integrase is responsible for the integration of viral DNA into the

genome of host cells. This structure is enclosed within a icosahedral capsid that

appears rod-shaped in lentiviruses composed of p24 or Gag protein. The

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Table 1. HIV-1 Proteins and Known Functions.

Protein	Size (kd)	Function
Env	p120	Viral binding, determines tropism
	p41	Viral fusion and entry
Gag	p25(p24)	Capsid structural protein
	p17	Matrix protein-myristoylated
	p9	RNA binding protein
	p6	RNA binding protein; helps in viral budding
Polymerase	p66/p51	Reverse transcriptase; RNAse H activity (inside core)
	p10	Post-translation processing of viral proteins
Intergrase	p32	Viral cDNA integration
Tat	p14	Transactivation
Rev	p19	Regulation of viral mRNA expression
Nef	p27	Pleotropic, including virus suppression; myristoylated
Vif	p23	Increases virus infectivity and cell to cell transmission; helps in proviral DNA synthesis and/or in virion assembly
Vpr	p15	Helps in viral replication; *trans*-activation
Vpu	p16	Helps in virus release; disrupts gp160-CD4 complexes
Vpx	p15	Helps in infectivity
Tev	p26	Tat and Rev like activities

(Adapted from J. Levy., 1994 [11]).

integrity of the viral envelope is maintained by p17, a myristylated matrix protein that lines the inner surface of the viral membrane. The virus membrane is a lipid envelope derived from the host cell that can contain cellular proteins. There are 72 peplomers that project from the viral surface and are comprised of oligomers (trimers or tetramers) of the glycoprotein gp160 that is cleaved into two non-covalently linked peptides gp120 and gp41. Gp120 is responsible for the binding

Figure 2: The HIV-1 virion. A depiction of the location of structural proteins. (Adapted from W.C. Greene, 1991 [10])

of virus to host cells, while gp41 is responsible for viral entry by membrane fusion. Gp41 is a hydrophobic transmembrane protein that anchors gp120 to the viral envelope in a process stabilized by interactions between gp120 and envelope regions as well as by heavy glycosylation. The oligomeric arrangement and glycosylation patterns of gp120 are thought to protect the virus from neutralizing antibodies by sheltering the primary antigenic determinants.

HIV-1 Binding

The discovery that CD4$^+$ cells were preferentially depleted from blood of AIDS patients provided an initial clue for a cellular receptor for HIV-1. It was soon after the discovery of HIV, that the CD4 differentiation antigen was described as a receptor for HIV [12, 13]. Infection begins with the binding of gp120 to CD4 on helper T-lymphocytes and cells of macrophage lineage. The sequences of gp120 responsible for the binding involves a 159 amino acid region near the 3' region of gp120 (V3 region). Other noncontiguous regions of gp120 including regions of V1 and V2 likely participate in gp120 binding to CD4 (see [14] for a review) or may be involved with the determination of viral tropism [15, 16]. Nevertheless, the V3 loop appears to be the most important region for the determination of structure and binding characteristics of gp120 and thus viral tropism. Gp120 is the most heavily glycosylated protein known, with the N-linked glycans accounting for ~ 50% of it's weight. Glycosylation is important for the maintenance of the tertiary structure of gp120 and deglycosylated (expressed in bacteria) or non-glycosylated (enzymatic) gp120 do not bind to CD4 [17-19]. Although CD4$^+$ T-lymphocytes are the primary target for HIV-1, CD4-independant pathways of viral entry have been identified [20-23] and HIV can infect a variety of cell types (see Levy, 1994 [11] for a complete account of cells that can be infected by HIV).

After binding to CD4, the virus makes use of selected cellular proteases to cleave gp120 and the resulting conformational shift exposes a region of the V3 loop that can bind to a second receptor. This second receptor, initially termed fusin [24], is

now known as CXCR4 and is one of several members of the chemokine

superfamily of 7-transmembrane receptors that function as co-receptors for HIV-

1. These interactions pull the virus close to the cell surface allowing for insertion

of the now exposed gp41 and viral-cell membrane fusion [25-27]. Findings that

mutations in gp41 or the V3 region of gp120 affect viral fusion as well as viral

infectivity suggest that both proteins are important mediators of viral fusion and

infectivity [28, 29].

Postbinding Steps and Genomic Incorporation of HIV-1

Following binding and membrane fusion the viral capsid is released into the host

cell where partial uncoating takes place. The virus uses an RNA-dependent DNA

polymerase, reverse transcriptase, as well as RNAse H activity to produce an

RNA-DNA hybrid, from a plus sense diploid RNA genome, that is converted to

double-stranded cDNA. This ribonucleoprotein or preintegration complex

consisting of double-stranded DNA, matrix , nucleocapsid, integrase and Vpr and

Vif proteins is transported to the nuclear envelope of cells by an unknown

mechanism. Unlike other retroviruses, members of the lentivirus family, including

HIV-1, can infect non-dividing cells and thus are not dependent on breakdown of

the nuclear envelope for entry. Instead, the preintegration complex is

translocated to the nucleus with the aid of nuclear localization signals, importin-α

and importin-β, in co-operation with Vpr, matrix and intergrase proteins [30-32].

Integration into the host genome appears to be at random sites.

HIV-1 Transcription

Transcription of HIV occurs from a single promoter region located in the 5'-long

terminal repeat and involves a complex array of host factors (see [33] for a review).

The first HIV-1 mRNAs to appear in cytoplasm are the multiply-spliced transcripts

encoding for the major regulatory proteins Tat, Rev and Nef [34]. Unspliced and

partially spliced transcripts remain in the nucleus

until sufficient amounts of Rev have accumulated to

allow splicing and export of these remaining

transcripts by interaction with regions known as rev-

response elements (see [35] for a review of Rev

function).

Tat is a potent regulator of HIV-1 transcription and is

required for viral replication. Tat protein is

expressed early in infection from three different

multiply spliced viral mRNA's [36] and as a hybrid with

Rev, called Tev, that is formed by

splicing the first exon of Tat with the

env gene and the second exon of Rev

[37-39]. Alternate splicing can form Tat_{1-72}
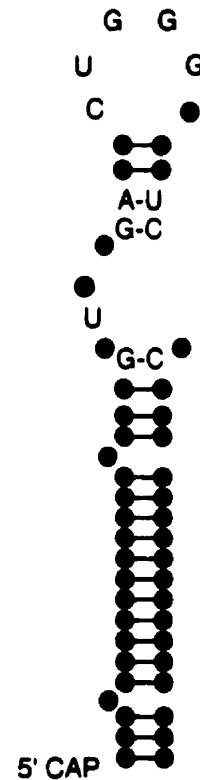
Figure 3. Structure of the TransActivation Response Element. Structure is found in the HIV-1 long terminal repeat.

and Tat_{1-86} in a process modulated by rev; expression of rev favors production of

the truncated form of Tat [40]. Tat is exported from cells by a leaderless secretatory

pathway [41] and is taken up by adjacent cells [42, 43]. Tat may enter cells following binding to membrane receptors at least one of which requires the amino acid sequence RGD in the second exon [44-46]. Alternatively, Tat may cross membranes by diffusion, using the exposed basic domain to insert into the lipid bilayer [47]. An unfolding step is required for the internalization of exogenous Tat [48]. Once inside the cell, Tat refolding is thought to occur with the help of chaperone proteins such as HSP90 [49]. Once in the cell, Tat targets to the nucleus [42, 50-52] and has been found to accumulate in the nuclear and peri-nuclear regions [46, 50, 51, 53].

Tat activates transcription through the transactivation response element (TAR) found in all viral transcipts at the 5' end of HIV-1 mRNAs. Tat binds to the UCU bulge region of TAR and enhances transcriptional initiation and elongation (Fig 3). Activation by Tat leads to increased phosphorylation of the carboxy terminus of RNA polymerase II via a Tat-associated kinase and additional elongation factors [54-56]. Tat derived from different HIV-1 isolates differ in terms of amino acid sequence and protein length; the length is known to vary from 82 to 101 amino acids (Fig 4). Expression of Tat species changes during the course of infection with full length Tat representing the dominant species early in infection and Tat$_{1-72}$ (exon 1) predominating late in infection [40].

The amino acid sequence of Tat can be divided into domains based on functional characteristics (Fig 5). The basic domain of Tat is an important determinant in the binding to TAR sequences and contains a functional nuclear localization

HIV1_BRU

MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFTTKAL

GISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQPRGDPTGPKE

Figure 4. Amino acid sequence of Tat$_{1-86}$. Sequence from HIV-1$_{BRU}$.
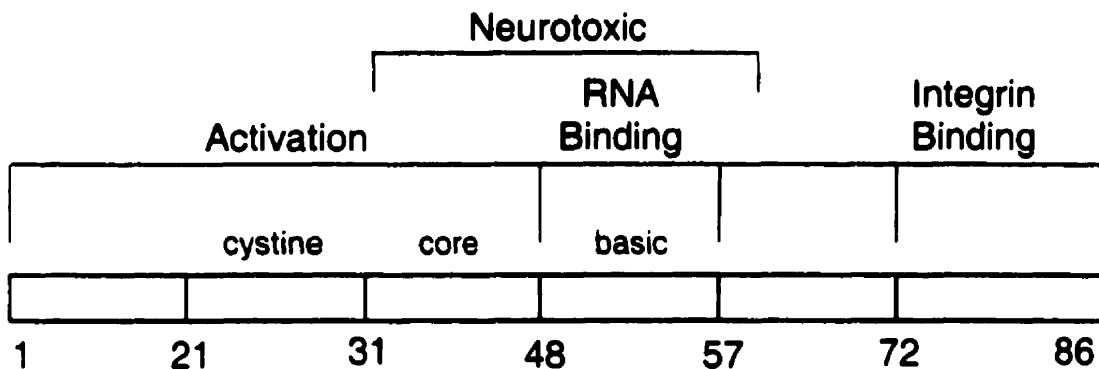
Figure 5. Functional Domains of HIV-1 Tat$_{1-86}$. Alignment of amino acid sequence with the known functions of Tat.

sequence [57, 58]. The core and flanking cystiene rich domains are the most highly conserved regions among Tat isolates and are critical for transactivation. Mutagenesis and domain -swapping experiments have indicated that the core, cystiene rich and amino-terminal regions constitute an independent *trans-*activation domain. Only 25 amino acids (10 core and 15 amino terminal) are

12

required for TAR binding and transactivation. The remainder of the protein stabilizes the interaction and may be required for the recruitment of co-factors that increase transcriptional efficiency[59, 60].

Pathogenesis of HIV-1 Infection to AIDS

HIV is transmitted by three principal means, sexual contact, blood or blood products, and maternal-child transfer[61]. Within 1-3 weeks following exposure to HIV-1, individuals present symptoms typical of most viral infections. Symptoms consist of headache, retro-orbital pain, muscle aches, sore throat, low or high grade fever, swollen lymph nodes and the appearance of a nonpruritic macular erythematous rash involving the trunk and latter extremities[62]. Primary HIV-1 infection is detectable at the time of seroconversion ~ 3 months after infection. An asymptomatic period follows that can last from months to years. Once termed viral latency, this period is now thought to represent a time of effective immune control during which time viral turnover is rapid[63-65]. As infection progresses and CD4$^+$ cell numbers decline, viral titer rises and opportunistic infections appear. Infection cycle of HIV-1 in a T-lymphocyte is depicted in Figure 6. Death most frequently occurs due to complications arising from *Pneumocystis carinii* infection.
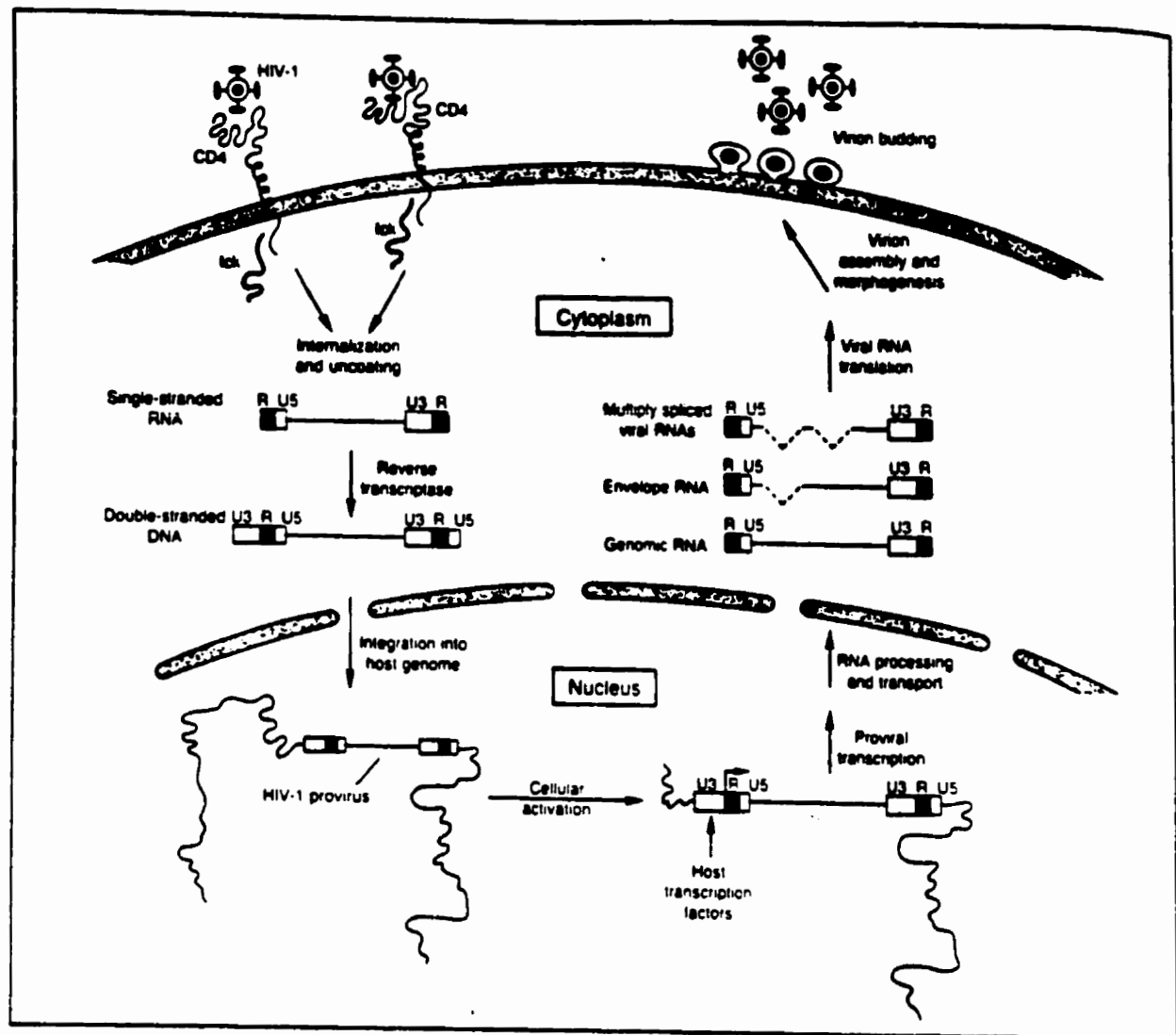
Figure 6: Overview of the HIV-1 replication cycle in a T-lymphocyte.
Infection begins with binding of gp120 to CD4 and co-receptors. Following fusion
of viral and host membranes, the genome of HIV-1 undergoes reverse
transcription to produce double stranded DNA with long terminal repeats (LTR)
derived from sequences in the 3' (U3) and 5' ends of the viral RNA.
Transcriptional activity is regulated by host factors (including Sp1 and TATA-
binding factors) and by inducible members of the Nf-κB family. An early HIV-1
gene product, Tat, binds to the TAR region in the LTR and amplifies transcription.
Following the synthesis of a full length RNA transcript, a complex array of
multiply spliced mRNAs are produced. The differential expression of multiply
spliced mRNAs and their export to cytoplasm for translation are controlled by
Rev. Encapsulation of the viral genome is followed by budding from the plasma
membrane. (From M.B. Feinberg and W.C. Greene, 1992 [66]).

HIV-1 Entrance to Brain – Macrophage Infiltration

HIV-1 enters the brain rapidly. HIV-1 and HIV-1-antibodies can be detected in the cerebral spinal fluid during acute infection [67, 68]. Rapid infection of brain was first demonstrated in an individual mistakenly injected with HIV-1 infected white blood cells. Several days later, HIV-1 was detected in post mortem tissue, including brain, from that individual [69]. Early infection of the brain is likely responsible for the appearance of self limiting aseptic meningitis during acute seroconversion reactions. Effective immune control during the asymptomatic period may restrict viral replication in the brain and limit further infiltration. Indeed, reduced immunologic control and reseeding of the brain during the period of CD4$^+$ cell depletion is thought to be responsible for the resurgence of neurologic symptoms [70]. Owing to it's relative isolation and immunoprivileged status, the brain functions as a protected reservoir for the virus.

A viral correlate for the severity or appearance of neurological symptoms is at present controversial. Although some studies emphasize the importance of CNS viral load or viral replication as determinants of the severity of HIV-1 induced neurological disease [71-73], other studies do not support such a conclusion [74, 75]. The best correlate of the severity of CNS disease appears to be the number of activated macrophages found in the CNS of infected individuals [76-78]. HIV-1 is most likely transported into brain in blood derived macrophages and microglia. During acute seroconversion, the primary HIV-1 isolates in blood are macrophage-tropic [79, 80] as are HIV-1 isolates from brain. [81, 82]. It has been

suggested that microvascular endothelial cells may be infected by HIV-1 and that the virus gains entry to brain by budding into the parenchymal side. Although direct infection of microvascular endothelial cells has been shown *in vitro* [83], *in vivo* findings do not support these results [81, 82, 84] and several other laboratories were unable to replicate HIV-1 infection of endothelial cells *in vitro* [85, 86]. The most likely route of HIV-1 entry into brain is thus infiltration of HIV-1 infected macrophages and may involve areas of vasculature with a less well defined blood brain barrier, such as choroid plexus and hypothalamus, although infected monocytes may transmit HIV to CNS tissues during normal trafficking.

The initial step of macrophage extravasation from vascular compartments occurs with macrophage binding to endothelial cells. HIV-1 infected macrophages show enhanced binding to endothelial cells possibly due to the overexpression of adhesion molecules in HIV-1 infected macrophages and the induced expression of E-selectin (a molecule that mediates the binding and extravasation of macrophages) in brain microvascular endothelial cells [86]. Infected macrophages secrete the vasoactive compound nitric oxide [87] and the cytokines tumor necrosis factor alpha (TNF-α) and interleukin 1-beta (IL-1β), that are expressed at higher levels during HIV-1 infection [88, 89], further enhance the production of nitric oxide. In addition, HIV-1 infected and gp120 stimulated macrophages secrete gelatinase B [90, 91] that can degrade basement membranes and increase the permeability of the blood brain barrier. Indeed, it has been demonstrated that HIV-1 infected macrophages increase the permeability of endothelial cells [90].

Messenger RNA levels for the chemokines macrophage inflammatory protein–1α (MIP-1α), MIP-1β and monocyte chemotactic peptide-1 (MCP-1), potent chemoattractants, are elevated in the brains of demented HIV-1 infected individuals compared with their non-demented counterparts and are found localized to areas of HIV-1 infected macrophage and microglia [92, 93]; effects that are augmented by cytokine stimulation [94].

Increased permeability of the blood brain barrier may be mediated by viral products, rather than viral infection *per se*. The viral protein Tat has been shown to induce the expression of TNF-α [95, 96], E-selectin [97], MIP-1α and MIP-1β [98], MCP-1 [99], increase the adhesion of monocytes to endothelial cells [100, 101], and increase macrophage migration [101-103]. Increased expression of adhesion molecules, cytokines and chemoattractants may thus lure HIV-1 infected macrophages to enter brain and further support their infiltration into brain parenchyma.

HIV-1 Infection of Brain – Involvement of Astrocytes

In addition to cells of macrophage/microglia lineage, astrocytes have been shown to be infected by HIV-1 *in vitro* [104, 105] and *in vivo* [106, 107]. Infection of astrocytes occurs by a CD4-independant mechanism [20, 108, 109] possibly gaining entrance by a partially characterized 260-kDa protein on astrocytes that binds gp120 [110]. Infection of astrocytes is not permissive and results in only low levels of viral

production [104, 111] that extinguish over time [112, 113, 111]. Restricted replication of

HIV-1 in astrocytes has been attributed to a poorly understood translational block

that prevents the formation of structural components of the virus but allows for

the complete assembly of Tat and Rev [114]. Active replication of HIV-1 can occur

in astrocytes stimulated by cytokines such as TNF-α, IL-1β, and IFN-γ [111, 115, 116]

by a mechanism involving TAR-independent transactivation of an HIV-1

enhancer domain [117, 118] that can be induced by Tat-facilitated upregulation of

NF-κB binding to the enhancer domain of HIV-1 long terminal repeat [119, 120].

Astrocytes can thus serve as reservoirs for HIV-1 in brain and active viral

production can be induced in these cells.

AIDS-Related Dementia Complex

HIV is the most common central nervous system (CNS) infection in the world and

commonest cause of dementia in North Americans < 60 years of age, affecting ~

30 % of the adult and 50 % of the pediatric population infected with HIV-1.

Patients with HIV-1 associated cognitive/motor complex (also termed AIDS

dementia complex) suffer from deficits in motor control, cognition and behavior

[121]. Neuropathological manifestations of HIV-1 infection include inflammation,

white matter pallor, wide spread gliosis, multinucleated giant cell formation,

dentritic pruning, synaptic simplification and loss of select populations of neurons

[122]. With the exception of isolated reports [107, 123] there is little evidence for direct

infection of neurons limiting the possibility that a lytic course of HIV-1 infection is

responsible for neuronal loss. One of the most intriguing hypotheses is that

neuronal dysfunction and death are the indirect consequence of HIV-1 infection

of macrophage/microglia cells, and astrocytes. This hypothesis suggests that the

release of HIV-1 proteins from infected cells serves as an amplification system

that induces the release of neurotoxic factors from infected and non-infected

cells. An amplification system mediated by viral proteins is able to account for the

widespread pathology seen in AIDS-related dementia despite limited infection.

Viral proteins have been implicated as the neurotoxic agents of infection and Tat,

gp120, Nef and Vpu have been found to be neurotoxic[124]. A complex array of

toxic factors released by interactions of these proteins with infected and non-

infected astrocytes, macrophage/microglia and direct interactions of these

proteins with neurons are thought to result in the dysfunction and degeneration of

neuronal populations. Tat and gp120 are the two HIV-1 proteins most frequently

linked with HIV-1 dementia.

Mechanisms of Gp120-Induced Neurotoxicity

The first indication that gp120 could at-least in part be responsible for the

neuronal degeneration seen in AIDS was in 1988 when Benneman and his

colleagues demonstrated that picomolar concentrations of gp120, in the absence

of virus, were toxic to hippocampal neurons in vitro[125]. In vivo studies further

implicated gp120 by showing that injections of gp120 into hippocampus[126] were

neurotoxic. Neuropathology found in gp120 transgenic mice was regionally

correlated with the expression of gp120 and was consistent with pathologies

seen in the brains of HIV-1 infected individuals[127]. Gp120-induced neurotoxicity

appears to involve a large and potentially lethal rise in intraneuronal Ca^{2+} that is mediated by the release of toxic factors from macrophage/microglia and astrocytes. When microglia cells were removed from neuronal cultures by L-leucine methyl ester treatment, neurons were protected from the toxic effects of gp120 [128, 129] suggesting an indirect mechanism of neurotoxicity involving macrophages/microglia. When activated by gp120, macrophages and microglia release arachidonic acid, that can facilitate neurotoxicity indirectly by inhibiting glutamate uptake in astrocytes [130, 131] and gp120 has been shown to inhibit the uptake of glutamate [132, 133] and stimulate glutamate release [134] through the actions of arachidonic acid. On neurons arachidonic acid can facilitate NMDA-evoked currents in neurons [135]. Inhibitors of phospholipase A_2, the major pathway affecting arachidonic acid release in macrophage/microglia, inhibited gp120-induced arachidonic acid release and restored the uptake of glutamate into astrocytes [132, 136, 137]. Increased extracellular glutamate and sensitization of NMDA receptors by arachidonic acid may thus be responsible for the potentiation of glutamate-induced toxicity by gp120 [136, 138]. Abnormal function of NMDA receptors may be responsible for neuronal demise because gp120-induced neurotoxicity was inhibited by dizocilpine (MK-801), an open channel blocker of NMDA receptors, and AP5, a competitive antagonist of the glutamate binding site, but not by antagonists of non-NMDA receptors [129, 138].

In addition to NMDA receptors, gp120 activates voltage sensitive calcium channels on neurons [129]. Activation may be due, in part, to membrane

depolarization provided by glutamate receptor-mediated ion flux and by a mechanism involving amiloride-sensitive Na^+/H^+ exchange and K^+ efflux in astrocytes [139-142]. In astrocytes, gp120 stimulates Na^+/H^+ exchange and increases intracellular pH [141]. Intracellular alkalinization can affect other transporters including K^+ channels [143, 144] thus increasing extracellular K^+ and depolarizing neuronal membranes. In astrocytes, increased extracellular K^+ inhibits Na^+-dependant glutamate uptake and can enhance glutamate release [143, 145]. Together these events remove the Mg^+ block from NMDA receptors on neurons thus priming them for activation [138, 143, 145, 146].

Nitric oxide is a well known pro-apoptotic agent in neurons, and free radical induced cellular damage is a central theme to many neurodegenerative processes, including AIDS-related dementia [147-149]. Gp120 has been shown to stimulate the inducible form of nitric oxide synthase in macrophages/microglia with consequent increases in nitric oxide [150, 151]. Numerous free radical scavengers reverse gp120-induced cell damage, suggesting that neuronal injury is mediated by free radical-induced injury [152, 153].

In the central nervous system, cytokines are differentially expressed during critical periods of growth and development. In the adult brain, overexpression of pro-inflammatory cytokines is associated with degenerative processes and has been shown to induce apoptosis in neurons. The inflammatory mediator platelet-

activating factor [154] and the cytokines TNF-α and IL-1β [151, 155, 156] are overexpressed in macrophage/microglia and astrocytes stimulated by gp120. Some of the effects that these cytokines can have on cellular function include stimulation of Na^+/H^+ exchange, increased arachidonic acid production and release, inhibition of glutamate uptake in astrocytes and facilitation of voltage dependant Ca^{2+} currents in neurons [140, 157-159]. The effects of cytokines can thus compliment or enhance the effects of gp120 and together potentiate neuronal toxicity. It should however be noted that in some model systems, TNF-α has been touted as a neuroprotective agent by mechanisms involving NF-κB and reductions in NMDA-receptor function [159, 160].

Neurons, astrocytes and macrophages/microglia are known to express a variety of chemokine receptors [161-163] and at-least one chemokine, SDF-1α is toxic to neurons [164-166]. Recently, apoptosis induced by gp120 was suggested to involve direct binding to, and activation of, the chemokine receptor CXCR4 in a neuronal cell line [164]. Another group did not reproduce this finding in primary mixed brain cultures and concluded that gp120-induced neuronal apoptosis depends predominantly on an indirect pathway via activation of chemokine receptors on macrophages/microglia [165]. The preponderance of evidence suggests that gp120-mediated neuronal cell death is indirect and involves a complex release of toxic factors from astrocytes, macrophages and microglia. Although gp120 undoubtably binds to chemokine receptors on neurons, the effects of this interaction on neuronal survival are not yet clear.

Mechanisms of Tat-Induced Neurotoxicity

The first evidence that Tat could be a neurotoxic agent appeared in 1991 when

Sabbatier and his co-workers reported that Tat reduced the viability of

neuroblastoma and glioma cells *in vitro* and *in vivo* (intracerebroventricular

injection was lethal to mice) [167]. These results have been substantiated in

separate reports by Philippon [168] and Jones [169] who demonstrated that a single

injection of Tat, or simply the basic domain of Tat, into the lateral ventricles or

gray matter (hippocampus or thalamus) resulted in the influx of inflammatory

cells, glial cell activation, ventricular enlargement, induction of inducible nitric

oxide synthase and neurotoxicity [168, 169]. It is now known that even sub-toxic

amounts of Tat can alter the normal organization of cells causing the aggregation

of astrocytes and neurons and the retraction of neuritic processes [52, 170]. Tat may

thus affect neuronal function following a single exposure and at concentrations

that do not result in frank cell loss.

Distinct from the effects of gp120, Tat can interact directly with neurons to

produce toxic results. Tat binds to rat brain synaptosomes with moderate affinity

(Kd of 2 µM) [167] and binds to membrane receptors with an apparent molecular

mass of 90-kDa on PC12 and NG108-15 cells [171]. Although a specific membrane

receptor for Tat has not been identified, downstream effects suggest a

metabotropic pathway linked to phosphoinositol hydrolysis. In PC-12 neural cells,

Tat activated phosphatidylinositol 3-kinase [172], increased levels of IP_3 and

increased activity of the protein kinase C isoforms-α-ε and ζ [173]. These effects

are likely coupled through MAP-kinase that is activated by Tat [174] since the

tyrosine kinase inhibitor genestein inhibited the upregulation of protein kinase C

by Tat [173]. The production of IP$_3$ and increased protein kinase C activity are

traditionally associated with the activation of trimeric G-proteins, thus suggesting

that the effects of Tat are mediated by a metabotropic receptor.

Additional evidence that Tat interacts directly with neurons was provided in

electrophysiological studies. Independent reports have shown that Tat

depolarizes neuronal membranes in a tetrodotoxin independent manner [175, 176]

and rapid, non-delimiting membrane currents were demonstrated in outside out

membrane patches obtained from human cortical and rat CA1 neuronal slices

when fmole amounts of Tat were applied to the outer but not to the inner surface

of the membrane [177]. The reversal potential for Tat-induced depolarization has

been independently determined to be ~ 0 mV, suggesting the involvement of a

non-selective cation channel [167, 177, 178]. Consistent with these reports, the

removal of extracellular Ca^{2+} resulted in a reduction of ~ 50 % in membrane

current. Calcium flux is thus an important component of Tat-induced membrane

current.

In astrocytes, Tat increased the activity of protein kinase C [120] and MAP kinase

[174] as well as increased binding of the transcription factors NF-κB and NF-IL6 [120,

24

[179]. Production of the cytokines TNF-α and IL-6 [96, 180, 181] are increased in astrocytes following Tat-stimulation, although the effect is more potent in Tat-stimulated macrophages [96, 180, 181]. A central role for TNF-α has been postulated in Tat –induced neuronal degeneration, by a mechanism involving glutamate accumulation and activation of non-NMDA receptors [182]. Indeed, blockade of TNF-α synthesis with pentoxyfilline, reduced the size of Tat-induced brain lesions thus suggesting a central role for this cytokine [168]. TNF-α was shown to potentiate Tat-induced neuronal cell death and the lethal effects of this combination were partially reversed by antioxidants thus suggesting a role for free radicals [183]. Indeed, inducible nitric oxide synthase, and the peroxynitrite scavenger uric acid protected neurons from Tat-induced neurotoxicity [184].

Calcium, The Common Denominator

Central to the mechanisms of gp120 and Tat-induced neuronal cell death discussed thus far, are the dysregulation of $[Ca^{2+}]_i$ homeostasis. Indeed, therapeutic strategies aimed to prevent or reduce the severity of AIDS-related dementia target Ca^{2+} flux [185-188]. The remainder of the chapter is devoted to a discussion of mechanisms by which cells control $[Ca^{2+}]_i$.

An Evolutionary Perspective of Calcium

Before considering the specific modes of Ca^{2+} flux, it is important to consider the evolutionary advantage for the development of a 10,000 fold Ca^{2+} gradient between extra- and intracellular compartments. When life first formed, the pH of

the sea was high, ~ 8, and the concentration of Ca^{2+} was low, ~10^{-7} M; under

these ideal conditions the basic building blocks of life processes, nucleic acids

and nucleotides could develop in thermal ducts under the ocean floor. The Ca^{2+}

salts of ATP and other phosphorylated organic anions necessary for the

synthesis of RNA, DNA and basic energy-transducing systems are only sparingly

soluble and would precipitate or form stable Ca^{2+} complexes in a high Ca^{2+}

environment. A gradual increase in the Ca^{2+} content of the sea [189, 190] thus

threatened the most fundamental of life's processes and necessitated a means of

environmental separation . By the time that multicellular life evolved, the

Precambrian sea contained a Ca^{2+} content that approached 1.0 mM and cells

developed a semipermeable membrane that isolated the Ca^{2+} rich extracellular

milieu and with the aid of calcium pumps, maintained an intracellular environment

with a low Ca^{2+} content. The Ca^{2+} concentration gradient then became exploited

as a means of signal transduction, transmitting information of cell surface events

to the intracellular environment. Brief, controlled movements of Ca^{2+} into the

cytosol transmit information in spatial and temporal frequencies that are

terminated by the removal of Ca^{2+}. Intracellular calcium levels are thus under

tight control (see [191, 192] for reviews of Ca^{2+} mediated signaling processes).

As organisms became more complex, it became necessary to eliminate certain

populations of cells during the developmental process. In the brain, during

development, only neurons that make appropriate synaptic connections survive.

Indeed, inhibition of this process is fatal. This process of "cell suicide", known as

apoptosis, exploits the Ca^{2+} gradient to trigger self destruction in a neat and orderly fashion (see [193] for a review of apoptosis in CNS development). Ironically, these same mechanisms that are necessary for life can be triggered by pathologic processes (see [194] for a recent review of apoptosis).

Calcium and Cell Death

Mechanisms of Ca^{2+} induced neurotoxicity can be classified into two stages (Fig. 7 A and B). The first stage results from pathologic mechanisms that cause initial and sustained increases of $[Ca^{2+}]_i$, while the second stage results from the effects of $[Ca^{2+}]_i$ excess. Energy failure is a central mechanism of Ca^{2+} induced neurodegeneration. Dramatic or prolonged increases of $[Ca^{2+}]_i$ result in the sequestration of Ca^{2+} into internal stores, including mitochondria where a disruption of the electron transport chain results in decreased cellular ATP. A general loss of ion homeostasis and membrane depolarization results due to the failure of energy dependent ion pumps. Voltage sensitive calcium channels are activated and the Mg^{2+} block that normally prevents NMDA receptor activation is removed [195]. In addition, Na^+ / Ca^{2+} exchange inverts and Ca^{2+} is actively transported into the cell rather than extruded [196, 197]. Membrane depolarization induces glutamate transmitter release and over activation of excitatory amino acid receptors [198] coupled with decreased energy dependant glutamate uptake by neurons and glia [199]. ATP depletion also leads to decreased macromolecular synthesis, loss of cytoskeletal integrity and altered membrane permeability.

Disruption of mitochondrial function results in the formation of damaging reactive

oxygen species and can lead to increased levels of prostaglandins and

leukotrienes, lipid peroxidation and inactivation of enzymes by nitrosalation[200]. A

sustained rise of $[Ca^{2+}]_i$ activates a family of Ca^{2+}-dependent cysteine proteases

known as calpains. Protease activation during pathological increases in $[Ca^{2+}]_i$

results in remodeling of membranes and cytoskeleton by cleavage events[201].

Calcium-dependant endonucleases are activated that degrade DNA in a specific

pattern of 200 bp cleavages typically associated with apoptosis[202]. Cytochrome c

that normally shuttles electrons between protein complexes in the inner

mitochondrial membrane is exported to the cytoplasm where it helps to activate a

family of killer proteases known as caspases[203]. Once activated, caspases act in

a manner similar to the calpains and help to kill the cell quickly and neatly by

cleaving other proteins[204, 205].

The following discussion details mechanisms by which Ca^{2+} enters the

cytoplasmic compartment. Events that result from Ca^{2+} excess are beyond the

scope of this work and the reader is directed to other sources for discussions of

these events[191, 194, 206].

Calcium Exchangers

Two classes of calcium exchanges have been characterized; an energy requiring

$Ca^{2+}/2H^+$ ATPase and the $3Na^+/Ca^{2+}$ exchanger that is driven by the Na^+

electrochemical gradient. When neural cells are transferred to sodium deficient

media, $[Ca^{2+}]_i$ rises, presumably reflecting an inverse mode of the exchanger[207].

The importance of this mechanism in neural cell function remains unresolved as reports suggest minor[208] to major[209] roles for the exchanger in the removal of Ca^{2+} from the cytoplasm.

Calcium Pumps

The role of calcium pumps in neural cells is to remove Ca^{2+} from the cytoplasm.

There are two known types of calcium pumps both of which belong to the P-class of high velocity ion-motive ATPases. Plasmalemmal calcium pumps extrude Ca^{2+} from the cytoplasm into the extracellular space. Pump activity is upregulated by increasing cytoplasmic Ca^{2+} concentrations and is efficient enough to remove as much as 40 % of Ca^{2+} entry induced by depolarization during the development of a Ca^{2+} transient[211].

Sacroplasmic/endoplasmic reticulum calcium pumps (SERCA) have an affinity for Ca^{2+} similar to the plasma membrane pump in the range of 0.3 to 0.9 μM and, like the plasma membrane calcium pump, remove Ca^{2+} from the cell cytosol. Pump activity increases with increasing concentrations of cytosolic Ca^{2+} and decreases with increasing concentrations of Ca^{2+} in the endoplasmic reticulum. SERCA transporters are formed by a tetrameric protein array containing two high affinity Ca^{2+} binding sites in the cytoplasmic domain. Calcium binding and subsequent ATP-dependent phosphorylation induce a conformational shift in the
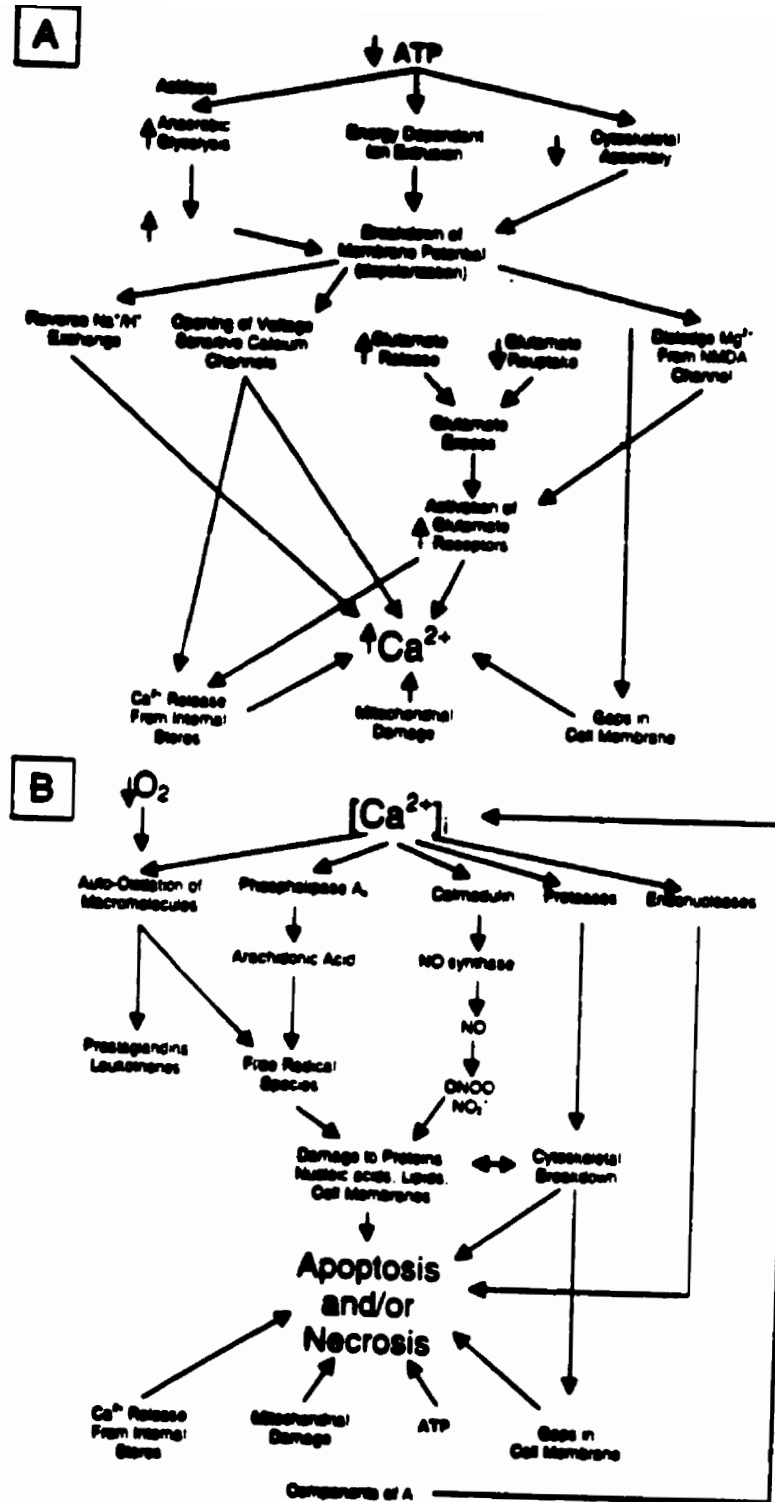
Figure 7. Calcium-triggered cell death. (A) A large increase in cytosolic Ca^{2+} can cause the dysfunction of mitochondria and decrease ATP production. Decreased energy charge results in further increases of cytosolic Ca^{2+} by the indicated mechanisms. (B) Mechanisms of secondary Ca^{2+} dependant phenomenon related to neurodegeneration (Adapted from Tymianski and Tator, Neurosurgery, 38(6), 1176-1195, 1996 [210])

receptor complex that moves Ca^{2+} into the sarco- or endoplasmic lumen and a subsequent reduction in the affinity of the binding sites for Ca^{2+} results in the release of Ca^{2+}. Calcium is sequestered within the organelle by binding to low affinity calcium binding proteins such as calsequesterin and calretinin (among others) and the size of the Ca^{2+} pool is determined by the area of the matrix defined by the sarco- endo-plasmic reticular membrane and by the quantity of calcium binding proteins in this compartment[212]. Thapsigargin and cyclopiazonic acid inhibit SERCA pumps and effectively deplete intracellular calcium stores. Release of Ca^{2+} from the sarco- endo-plasmic reticular matrix is governed by two classes of transmembrane protein complexes that form cation selective pores gated by inositol 1,4,5-trisphosphate (IP_3 receptors) or Ca^{2+} ions (ryanodine receptors).

Intracellular Calcium Release Channels

A membrane-limited subcellular domain, the endoplasmic (or sarcoplamic in muscle cells) reticulum is an important regulator of cytosolic Ca^{2+}. These $[Ca^{2+}]_i$ pools sequester Ca^{2+} by means of energy dependent pumps similar to those found in the plasma membrane. Within endoplasmic reticulum is sequestered concentrations of Ca^{2+} estimated to be in the low millimolar range. Calcium efflux from endoplasmic reticulum stores is controlled by two separate calcium release channels characterized by ligand binding as ryanodine-sensitive and IP_3-sensitive. It has been known for some time that these receptors co-exist within neurons[213] with differential distribution in thick dendritic spines (RyR's) and fine

processes (IP$_3$) [214] and recent evidence has demonstrated that these stores are not only spatially distinct but also are associated with functionally distinct calcium pools [215].

Ryanodine sensitive [Ca^{2+}]$_i$ pools were first discovered by binding of the plant alkaloid ryanodine to endoplasmic reticulum associated transmembrane proteins. Ryanodine receptors (RyR) are thought to consist of a hetero or homo-tetrameric structure arranged around a central pore with each subunit composed of 4 membrane spanning segments [216]. There are at-least three receptor subtypes designated RyR1, RyR2 and RyR3 and all types have been identified in murine [217], rat [218] and human [219] brain. (see [220] for a review). RNA editing and differential subunit inclusion are thought to produce several isoforms not yet fully characterized, although several known isoforms have been shown to exist within a single cell [221]. Individual channels preferentially conduct mono and divalent cations over anions with high conductance rates of 600-750 pS for monovalent (Na$^+$, K$^+$) and 100-150 pS for divalent cations (Ca^{2+}) and at-least 4 conductance states are apparent due to regulatory effects of divalent cations, pH, nucleotides, some lipid metabolites and calcium binding proteins (see [222] for a review). Calcium induced calcium release is the hallmark of ryanodine channel function although adenine nucleotides (including AMP-PCP, AMP, ADP, cAMP, cADP-ribose) can activate ion flux in the absence of Ca^{2+} and potentate channel function in the presence of activating concentrations of Ca^{2+}. Calcium release from ryanodine sensitive pools is thought to occur in an all or nothing manner [223].

Caffeine can act to release Ca^{2+} from RyR-regulated pools of $[Ca^{2+}]_i$. Ryanodine can lock RyR channels in an open state at low concentrations and in a closed state at high concentrations.

Inositol 1,4,5-triphosphate (IP_3)-sensitive calcium channels are activated by the second messenger IP_3 that is produced by the breakdown of phosphatidylinositol bisphosphate via phospholipase C. Diacylglycerol is also produced and affects the activity of protein kinase C. There are two major pathways of IP_3/diacylglycerol production. The first pathway is linked by trimeric G-proteins to the β-1 isoform of phospholipase C and is activated by metabotropic receptors including (but not inclusive of) metabotropic glutamate receptors, metabotropic purinoreceptors, muscarinic-cholinoreceptors and serotonin receptors. The second pathway is linked to tyrosine kinase receptors by the γ-1 isoform of phospholipase C and is activated by numerous growth factors. IP_3-receptors are thought to be arranged as a homotetramer with each individual subunit spanning the membrane four times [224]. At-least four isoforms of IP_3-receptors exist (IP_3R1, IP_3R2; IP_3R3 and IP_3R4) and alternate splicing is thought to account for several additional subtypes [225, 226]. As with ryanodine receptors several subtypes are thought to exist within a single cell and the relative distribution of the subtypes vary between brain regions [227]. Conductance rates are substantially less (10 - 26 pS) than those observed for ryanodine receptors and display multiple conductance states. In contrast to the all or none nature of Ca^{2+} release from ryanodine-sensitive stores, IP_3-sensitive Ca^{2+} release is quantal in nature [228].

Several regulatory sites have been identified on the transducing domain of the molecule that are sensitive to cAMP-dependant protein kinases, calmodulin and adenine nucleotides as well as phosphorylation sites that affect the functional properties of the channel. Cytosolic Ca^{2+} levels inversely affect channel function so that levels of Ca^{2+} above 1 µM decrease the open probability of the channel and concentrations below 300 nM increase channel openings [229]. The effect of Ca^{2+} on conductance states appears to be mediated by alterations in the affinity of IP_3 for it's binding site [230]. The filling state of the intracellular

Table 2. Pharmacological Modulation of Intracellular Calcium Stores

Intracellular Target	Compound	Action
SERCA pump	thapsigargin	Inhibits pump activity
	4-aminopyridine	Inhibits pump activity
Inositol Trisphosphate Receptors	heparin	Blocks receptor
	xestopongin	Blocks receptor
Ryanodine Receptors	ryanodine	Locks receptor in open or closed state [a]
	procaine	Antagonist
	ruthenium red	Antagonist
	caffeine	Agonist

[a] Action is concentration dependant.

store also affects the sensitivity of the IP_3 binding site so that increased luminal Ca^{2+} increases IP_3 binding and decreased luminal Ca^{2+} inhibits IP_3 binding thus allowing the store to re-fill [231, 232].

Voltage-Activated Calcium Channels

Calcium channels are heteromeric structures made up of 5 subunits designated α_1, α_2, β, γ, and δ. The α subunit is the largest structural component, forms the ion channel and contains binding sites for numerous modulators of ion channel conductivity [233-235]. Channel gating based on membrane potential is the defining feature of voltage sensitive calcium channels. Channel opening is accomplished by displacement of charged components of the channel molecule located in the transmembrane domains that results in a conformational shift and the opening of an ion selective pore. Amino acid residues lining the pore provide ion selectivity and stabilization energy as the ion sheds its water moiety during transport through the pore. Based on electrophysiological and pharmacological properties there are at-least 4 types of voltage-activated calcium channels present on neurons designated L, N, P and T (Table 1). L-type calcium channels are high voltage-activated, sensitive to dihydropyridine compounds and show single channel conductance of 22 - 27 pS. Channel activation is associated with neurotransmitter release and may promote neuronal survival [236] possibly by the induction of a phenomenon termed "delayed facilitation", where repetitive high frequency stimulation leads to a long lasting hyperpolarization [237]. N-type calcium channels are high-voltage activated, resistant to dihydropyridine compounds,

irreversibly blocked by ω-conotoxin and show single channel conductance of 12 -

20 pS. N-type channel activation is associated with neurotransmitter release [238]

and may play additional roles during development in the directed migration of

immature neurons [239]. P-type calcium channels (first identified in Purkinje cells)

are high voltage-activated, resistant to dihydropyridine compounds and ω-

Table 3. Pharmacological Antagonism of Voltage Sensitive Calcium Channels

Nomenclature	Antagonists	Conductance	Channel Kinetics
L	Calciseptine	25 pS	High-voltage activated, slow inactivation, PKA-modulated *
	Nicardipine		
	Nifedepine		
	Nimodipine		
	Nitreddipine		
	PN 200-110		
	TaiCatoxin		
N	ω-Conotoxin	12-20 pS	High-voltage activated, moderate rate of inactivation *
P	ω-Agatoxin	10-12 pS	Moderate voltage activated, non-inactivating
Q	ω-Conotoxin	8 pS	Low voltage-activated, slow inactivation

*Rate of inactivation may be accelerated by increased intracellular calcium.
(Adapted from Receptor and Ion Channel Nomenclature, Trends. Neurosci., supplement, 1996 [240]).

conotoxin but are selectively blocked by ω-agatoxin and funnel-web spider toxin. T-type calcium channels are low voltage-activated (activate at very negative membrane potentials; between −60 and −40) and are insensitive to the specific blockers of L, N and P-type channels but are sensitive to amiloride and octanol and show single channel conductance of ~ 8 pS. Both P- and T-type calcium channels have been associated with a form of synaptic plasticity known as long term depression [241].

Glutamate Receptors

Glutamate is the most abundant excitatory neurotransmitter in brain and affects pre- and post-synaptic cell function through actions on ionotropic and metabotropic excitatory amino acid receptors. Receptors for L-glutamate are among the best characterized group of plasma membrane receptors and are responsible for mediation of the majority of excitatory neurotransmission in the mammalian central nervous system. The discovery by Johnson in 1974 that L-glutamate exists in at-least two different conformations in solution provided the first indication that more than one receptor may be affected by this neurotransmitter [242]. Stable analogues of L-glutamate were produced that recapitulate the folded conformation, NMDA, and the extended conformation, AMPA, and were used to demonstrate that there were at-least two receptors for L-glutamate. Many more analogs later, glutamate receptors were classified into those sensitive to the selective agonists NMDA, AMPA or kainic acid and at-least 7 metabotropic receptors that are affected to differing degrees by the above

agonists. In total, twenty seven genes have been identified that code for specific receptor subunits with an additional five proteins that may function as receptor subunits or receptor associated proteins [243].

This discussion will focus on ionotropic glutamate receptors (AMPA and NMDA) because of their intimate association with neuron cell survival; those interested in metabotropic glutamate receptors are directed to Pin and Duvoisin for a review [244]. Ion channels of glutamate receptors are formed by a pentameric arrangement of subunits each of which spans the plasma membrane 4 times. Differences in subunit composition and post-translational RNA editing [245] result in functionally distinct ion conductance and agonist sensitivities, precluding the possibility of hundreds of receptor forms. NMDA receptor associated ion channels display the greatest degree of Ca^{2+} permeability (at-least 10 fold greater than sodium) although other divalent cations also permeate the channel. Specific receptor activation can be accomplished with NMDA although this compound is 100 times less potent an activator of the NMDA receptor than is the endogenous agonist glutamate. Activation of NMDA associated ion channel conductance requires the binding of co-agonists glutamate and glycine and membrane depolarization to remove a Mg^{2+} block that physically inhibits channel conductance. There are in addition several known modulator sites that serve to fine tune channel function and numerous competitive and non-competitive antagonists each of which targets a component of the channel complex necessary for optimal activation. AMPA-type glutamate receptors are 70 % less

permeable to Ca^{2+} than NMDA-type receptors but still allow for considerable Ca^{2+} influx. AMPA receptors display less Ca^{2+} permeability than NMDA channels and Ca^{2+} conductance is dependant on subunit composition such that the inclusion of the glutamate receptor subunit-2, (GluR-2), into the pentameric receptor array results in the formation of channels that are Ca^{2+} impermeable. Differential transcription rates of mRNA encoding distinct subunits varies during development [246], differs depending on brain region in adult [247] and may be altered during pathologic conditions [248]. This suggests both temporal and regional control of Ca^{2+} permeability.

Ion flux through NMDA receptors is an important determinant of post-synaptic cellular function including developmental morphogenesis, synaptic plasticity and cell survival. Estimates suggest that Ca^{2+} accounts for ~ 10% of the ion flux through NMDA receptor channels [249-252] with ion selectivity attributed to amino acid residues glutamine (Q) arginine (R) and asparagine (N) in the pore region located in the M2 segment of NMDA-receptor subunit 2 (NR2) [253, 254] and other ionotropic glutamate receptor subunits [253, 255-258]. Subunit inclusion into heteromeric channels therefore determines ion selectivity with additional modulation by a Ca^{2+} binding site at the external mouth of the pore that determines the fraction of Ca^{2+} current [259]. Modulation of ion current is accomplished by a voltage sensitive Mg^{2+} block deep within the pore, an external NO site and by phosphorylation of independent serine/threonine and/or tyrosine sites on the carboxyl terminus of NR1, NR2A and NR2B subunits [260, 261].

Table 4. Pharmacologic Modulators of Glutamate Receptor Function

Channel	Agonists	Antagonists
NMDA	*NMDA Site*	
	NMDA	DL-AP5
	(1R,3R)-ACPD	DL-AP7
	Homoquinolinic Acid	(R)-4-carboxy-3-hydroxyphenylglycine
	cis-ACBD	
	(±)-*cis*-2,4-Piperidinedicarboxylic acid	*cis*-1-Amino-3-(2-phosphonoacetly)-cyclobutane-1carboxylic acid
	(RS)-(Tetrazol-5-yl)glycine	
	Glycine Site	
	Glycine	ACBC
	ACPC	Clycloleucine
	(±)-HA-966	MNQX
	Ion Channel	
		Memantine
		(±)-MK801
	Polyamine Site	
	Spermidine	Arcaine
		Synthalin
AMPA/ Kainate		
	(RS)-AMPA	CNQX
	Kainic acid	DNQX
	Domoic acid	NBQX
	(RS)-Bromowillardiine	
	(S)-5-Fluorowillardiine	

Phosphorylation by serine/threonine and tyrosine kinases increase open

probability of NMDA-receptors while dephosphrolyation by phosphatase 1, 2A or

2B decrease open probability and thus control channel function by protein kinase

C (PKC) and protein kinase A (PKA) dependent mechanisms respectively[262-264].

Calcium Signaling in Neurons

Neurons have four main regions: A dendritric tree (each branch is referred to as

a spine) that is designed to receive incoming information, the cell body or soma

that contains the nucleus, a long axon arising from a specialized region of the

soma known as the axon hillock that relays outgoing information, and synaptic

terminals that are designed to transmit information to target cells. Dendritic

spines are highly specialized to receive chemical signals and typically contain

numerous types of membrane receptors many of which are Ca^{2+} permeable or

mobilize Ca^{2+} from internal stores as their primary mechanism of signal

transduction. Small cytosolic volumes of ~ 0.1 μm^3 in individual spines and the

subdivision of spines into regions that can function as autonomous

compartments result in a complex and highly sensitive signal receiver (see[265, 266]

for reviews of Ca^{2+} signaling in neurons). For instance, Ca^{2+} can modulate the

activity of membrane channels so that signals from separate neurotransmitters

can combine to result in super increases of cytosolic Ca^{2+}, Ca^{2+} waves or

oscillations of Ca^{2+} and can act as co-incidence detectors thereby expanding the

potential of signaling possibilities[266]. A similar dependence on the temporal and

spatial aspects of Ca^{2+} signaling is apparent at the synaptic terminal where

neurotransmitter release occurs. Subtle modifications in the temporal and spatial presentation of Ca^{2+} signals at the nerve terminal can stimulate or depress neurotransmitter release and may affect synaptic vesicle docking and alter the combination of neurotransmitters released. Neuronal excitability is modified by Ca^{2+} flux at the cell soma or axon hillock by activation of Ca^{2+}-dependent K^+ channels that allow for the efflux of K^+ ions thus depolarizing the plasma membrane. Depolarization increases the threshold for neuronal transmission by depressing the number or rate of impulses that enter the axon. Modulation of Ca^{2+} flux at the cell soma can also provide crucial information for modulation of gene transcription, sometimes referred to as the cellular basis of long term memory[267, 268]. Calcium ions are able to modulate transcriptional activity by encouraging the selective translocation of transcription factors from the cytosol to the nucleus and determine the duration of transctivation based on temporal and spatial aspects of Ca^{2+} flux. In addition, Ca^{2+} signaling within the nuclear compartment itself can directly modulate transcriptional events.

Calcium Signaling Between Astrocytes and Neurons

Astrocytes make up ~ 50 % of brain cells by number and there is increasing evidence that they play an active role in neuronal communication. Astrocytes respond to mechanical and selective agonist induced stimulation with increases of intracellular Ca^{2+} that propagate between astrocytes in complex wave patterns[269] at rates that vary from 10 to 20 μm s^{-1} for distances that can exceed 100 μm[270]. Cell to cell propagation of Ca^{2+} waves are thought to involve an extracellular

component mediated by the release of ATP and signaling through purinergic receptors as well as an intracellular component involving gap junctional pathways [271, 272]. This latter pathway is formed by a pentameric arrangement of proteins, known as connexins, that form a transcellular pore. Regulation of the distance that a Ca^{2+} wave propagates is in part regulated by alterations in gap junctional conductance and can be altered by neurotransmitters, neurohormones and Ca^{2+} itself. A Ca^{2+} signal is able to travel a distance that far exceeds it's diffusion capabilities by using IP_3 receptor-mediated Ca^{2+} release in adjacent cells to boost the Ca^{2+} signal in a relay fashion [273].

Astrocytes contain receptors for most neurotransmitters, neuropeptides, a number of hormones and neurotrophins [143, 274, 275]. The variety of stimuli to which an astrocyte is able to respond strongly suggests that they play an active role in brain function. Indeed, a two way communication exists between astrocytes and neurons. Calcium waves propagated by glia can elicit a Ca^{2+} signal from associated neurons presumably either directly by neuronal linked gap junctions or indirectly through soluble factors such a glutamate or ATP [276-278]. Communication also occurs in the opposite direction. The frequency of stimulus-induced Ca^{2+} events in neurons is paralleled by astrocytes *in vivo* [277, 279] and *in vitro* [280] at-least in part by glutamate and ATP-mediated mechanisms.

Thesis Objectives

This thesis addresses the mechanisms of Tat and gp120-mediated neurotoxicity. Our central hypothesis is that Tat and gp120-induced neurotoxicity are primarily dependent on increases of $[Ca^{2+}]_i$ that result in the dysregulation of intracellular Ca^{2+} homeostasis. It is this aberrant regulation of $[Ca^{2+}]_i$ that triggers processes leading to the dysfunction and death of neurons. The goals of the following studies were thus to determine the mechanisms by which Tat and gp120 increased levels of $[Ca^{2+}]_i$ and the relationship of specific modes of Ca^{2+} flux to neuron cell death.

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Chapter 1

Identification of a Human Immunodeficiency Virus Type-1 Tat Epitope that is Neuroexcitatory and Neurotoxic

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ABSTRACT

Tat is an 86-104 amino acid viral protein that activates Human Immunodeficiency Virus type-1 (HIV-1) expression, modifies several cellular functions and causes neurotoxicity. Here, we determined the extent to which peptide fragments of HIV-1$_{BRU}$ Tat$_{1-86}$ produced neurotoxicity, increased levels of intracellular calcium ($[Ca^{2+}]_i$) and affected neuronal excitability. Tat$_{31-61}$, but not Tat$_{48-85}$, dose-dependently increased cytotoxicity and levels of $[Ca^{2+}]_i$ in cultured human fetal brain cells. Similarly, Tat$_{31-61}$, but not Tat$_{48-85}$, depolarized rat hippocampal CA1 neurons in brain slices of rat. The neurotoxicity and increases in $[Ca^{2+}]_i$ could be significantly inhibited by non-N-methyl-D-aspartate (NMDA) excitatory amino acid receptor antagonists. Shorter 15-mer peptides which overlapped by 10 amino acids each and which represented the entire sequence of Tat $_{1-86}$ failed to produce any measurable neurotoxicity. Although it remains to be determined if Tat acts directly on neurons and/or indirectly via glial cells, these findings do suggest that Tat neurotoxicity is conformationally dependent, that the active site resides within the first exon of Tat between residues 31-61, and that these effects are mediated at least in part by excitatory amino acid receptors.

INTRODUCTION

It has been estimated that 15-17 million people world wide are infected with HIV-1 and that one-third of these individuals will develop a dementing illness. HIV-1 dementia is now the leading cause of dementia in people less than 60 years of age [1,2] and carries with it a very poor prognosis averaging 6 months from onset to death [3]. Clinical features of this complex include motor disabilities, as well as behavioral and cognitive changes that range in intensity from memory dysfunction to global dementia [4]. Neuropathological findings vary between patients and include microglial nodules, multinucleated giant cells, myelin pallor, astrocytosis, and neuronal loss [5,6]. Most commonly, microglial cells [7,8,9] and some astrocytes [10,11,12] are infected with HIV-1. A close relationship between neurons and infected glial cells has been shown *in vivo* [13]. As neurons are not infected by HIV-1, it has been hypothesized that HIV-1 viral proteins may produce neuronal dysfunction and/or loss.

One HIV-1 protein so implicated is the trans-acting nuclear regulatory protein, Tat. Tat is a non-structural viral protein composed of 86 to 104 amino acids that is formed from two exons. The first exon contributes to the initial 72 amino acids and the second exon forms the remaining 14 to 32 amino acids [14]. Tat is released extracellularly by infected lymphoid cells [15] and glial cells [16] *in vitro*. Evidence suggesting that Tat is cytotoxic includes findings that Tat, when injected intracerebroventricularly is lethal to mice and causes cytotoxicity to neuronal cell lines [17,18]. Although it remains to be determined if Tat acts directly on neurons or indirectly via glial cells it has been shown that Tat binds specifically to neuronal cell membranes with high affinity and depolarized interneurons [17], and that the neurotoxic properties of Tat in mammalian neurons

are due to activation of excitatory amino acid receptors [19]. Some of the same effects have been reproduced using peptides derived from Tat including microglial cell activation, astrocytosis and neuronal cell loss upon injection into rat striatum [20]. These pathological features resemble those observed in patients with HIV-1 dementia [5, 6]. Similar neuropathological changes have been shown to occur with homologous peptides derived from the Tat protein of another retrovirus, Visna virus [20]. Thus, HIV-1 Tat may be a causative factor of pathological features associated with HIV dementia.

In this study, we identify an epitope of Tat that is cytotoxic to cultured human fetal neurons, and show that mechanisms underlying the Tat-induced neurotoxicity may include Tat-activation of excitatory amino acid receptors and increases in intracellular calcium.

METHODS AND MATERIALS:

Cultures of human fetal neurons: Brain specimens of 12 to 15 weeks gestational age fetuses were obtained, with consent, from women undergoing elective termination of pregnancy. All aspects of these studies received approval from The University of Manitoba's Committee for Protection of Human Subjects. Blood vessels and meninges were removed, brain tissue was washed in Opti-MEM (GIBCO) and mechanically dissociated by repeated trituration through a 20 gauge needle. The cells were centrifuged at 270 x g for 10 min and resuspended in Opti-MEM with 5%-heat inactivated fetal bovine serum (FBS), 0.2% N2 supplement (GIBCO) and 1% antibiotic solution (10^4 units/ml of penicillin G, 10 mg/ml streptomycin, and 25 µg/ml amphotericin B in 0.9% NaCl). Cells (10^5 cells per well) were plated in 96-well microtiter plates and maintained in culture for a

minimum of 4 weeks before experimental use. Sample cells were immunostained for the neuronal marker, microtubule associated protein-2, and only wells where cells were >70% neurons were used for experiments. The remaining cells were predominantly astrocytes as determined by staining for glial fibrillary acidic protein with rare microglia (<1%) which stained for EBM-11 [21].

Neurotoxicity assay: Cell death in neuronal cultures treated with Tat peptides in the absence of FBS was determined by trypan blue exclusion three hours after addition of peptides. Neuronal cell counts were determined from five fields chosen randomly. Each field was photographed and coded, and then counted by an investigator blinded to its experimental identity. At least 200 cells were counted in each field. Individual experiments were conducted in triplicate wells. The mean percentage of dead cells ± S.E.M. were calculated from these data and statistical analyses were performed using a one way ANOVA with posthoc analysis using the Dunnett's test.

Tat peptides: Tat_{31-61}, Tat_{31-71}, and Tat_{48-85} were obtained as gifts from the AIDS Reagent Program of the Medical Research Council of U.K. and added to neuronal cultures at concentrations ranging from 7 to 17 µM concentrations. Fifteen-mer Tat peptides, each overlapping by 10 amino acids and completely spanning the 86 amino acid sequence of Tat from HIV_{BRU} were synthesized on a peptide synthesizer (Applied Biosystems) and were purified by reverse phase high pressure liquid chromatography. Stock solutions of these peptides were prepared in 0.9% (w/v) NaCl. Neuronal cultures were treated with these peptides at 100 µM concentrations and effects on cytotoxicity were determined in triplicate wells as described above. Neurotoxicity experiments with the 15-mer overlapping

peptides spanning the region 32-72 and the longer peptides 31-61, 31-71 and 48-85 were repeated at least three times.

To determine specificity of Tat$_{31-61}$ neurotoxicity, Tat$_{31-61}$, was incubated with 1:100 dilutions of rabbit anti-Tat serum or normal rabbit serum were bound to protein A-coated agarose beads (Pharmacia) for 90 min at room temperature followed by centrifugation. The supernatants were tested for neurotoxicity. Tat$_{31-61}$ (17 µM) solution was also treated with 0.05% trypsin (Life Tech Inc.) for 30 min at 37°C and following addition of trypsin inhibitor (Sigma) (final concentration of 0.1%) for 30 min at 37°C the effects on neurotoxicity were determined.

Intracellular calcium recordings: Human fetal neurons were cultured for 4-6 weeks as described above in T-75cm^2 flasks. The flasks were gently tapped manually and the cells released into the supernatant were replated on to 33 mm glass cover slips for 7-10 days. Intracellular calcium concentrations were measured using fura-2-acetoxymethyl ester (fura-2/AM). Cells were incubated with fura-2/AM for 40 min at 27°C in KREBS buffer containing in mM; 111 NaCl, 26.2 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, 4.7 KCl, 1.2 MgCl$_2$, 15 HEPES, 1.8 CaCl$_2$, 5 glucose and 1.5 µM bovine serum albumin. Cells were subsequently washed three times with KREBS to remove extracellular fura-2/AM and incubated at 37°C for 5 min. Fura-2 loaded cells were superfused at a rate of 2 ml/min in an open perfusion microincubator at 37°C. Cells were excited at 340 and 380 nm and emission was recorded at 510 nm using a video-based imaging system (EMPIX, Missassauga, Ontario). Rmax/Rmin ratios were converted to nM [Ca^{2+}]$_i$ according to the method of Grynkiewich [22].

Tat$_{31-61}$ (100 µM) and Tat$_{48-85}$ (700 µM) were dissolved in KREBS solution and loaded into glass micropipettes and administered by pressure injection (3 x 15 ms pulses at 8 psi). The cells nearest the micropipette were monitored for 20 min. Superfusion of cells was stopped during application of peptides and was continued after $[Ca^{2+}]_i$ returned to baseline.

Preparation of brain slices: Young Sprague-Dawley rats (14-21 days old) were anaesthetized with halothane and decapitated. Brains were placed in gassed (95% O_2, 5% CO_2) normal artificial cerebrospinal fluid (ACSF) (mM: NaCl 118, KCl 3.0, NaH_2PO_4 1.0, $MgSO_4$ 0.81, $CaCl_2$ 2.5, glucose 10 and $NaHCO_3$ 24) at 4°C, for 1-2 min and blocked by hand. Slices, 200-250 µm thick were prepared and incubated at 28-30°C in gassed ACSF for 1-4 hours. Single slices were then transferred to a continuously perfused (2-3 ml/min, 30°C) glass bottomed recording chamber (Warner Instruments) and held in place by a nylon grid. Neurons were visualized (Hoffman modulation optics) and impaled with sharp glass microelectrodes containing 2.0 M potassium acetate and 1% biocytin (100-150 MΩ resistance).

Intracellular recording from brain slices: Tat peptides were applied extracellularly to neurons by pressure ejection (General Valve), using pressures of 1-20 psi and ejection times of 5-5000 ms. Initially, five short applications were made at 1 Hz and doses were increased by increasing the pulse duration; a 5 sec continuous application represented a maximum dose. The minimum time for complete evacuation of the pressure electrodes containing 2 µl of solution was 20 sec. The maximum dose of Tat$_{31-61}$ and Tat$_{48-85}$ was 670 µM, each applied at 1-20 psi for 5 sec.

To determine the morphology of recorded neurons, biocytin was included in each recording microelectrode, and impalements of 20 min or longer resulted in neurons being filled adequately for histological examination. Slices were fixed overnight at 4°C in 4% paraformaldehyde, and transferred to 10% sucrose for 48 hours. Fifty-μm thick frozen sections were exposed to streptavidin CY3 for 3 to 5 hours in phosphate buffered saline (pH=7.4), and viewed with a microscope equipped for epiflorescence microscopy using a rhodamine filter cube to visualize the label CY3. All of the recorded neurons were CA1 hippocampal neurons.

RESULTS

Neurotoxicity of Tat peptides to human fetal neurons: Tat_{31-61} was toxic to human fetal neurons. Maximal toxicity was seen at 0.5-2 hrs as determined by trypan blue exclusion with a drop in the number of trypan blue staining cells at 24 hrs (Fig. 1) due to either rupturing or dislodging of the injured cells. Tat_{31-61} produced significant cytotoxicity; cell loss expressed as mean percentage of total neurons counted was 7.7 ± 1.0 (Fig. 2). Tat_{31-71} produced 75% less neurotoxicity than did Tat_{31-61} and this level of toxicity was not statistically significant. The percent neuronal loss of 0.5 ± 0.1 following application of Tat_{48-85} was indistinguishable from control values of 0.5 ± 0.1. None of the sixteen peptides, each 15 amino acids in length and overlapping by ten amino acids, spanning the entire molecule of Tat produced significant neurotoxicity even when used at concentrations of 100 μM; a concentration 100-fold higher than what was used for full-length Tat_{1-86} (Table 1). Toxicity to Tat_{31-61} that was heat treated at 60°C for 30 min (7.7 ± 2.5) was not statistically different from untreated Tat_{31-61} (7.7 ± 1.0) (Fig. 2).
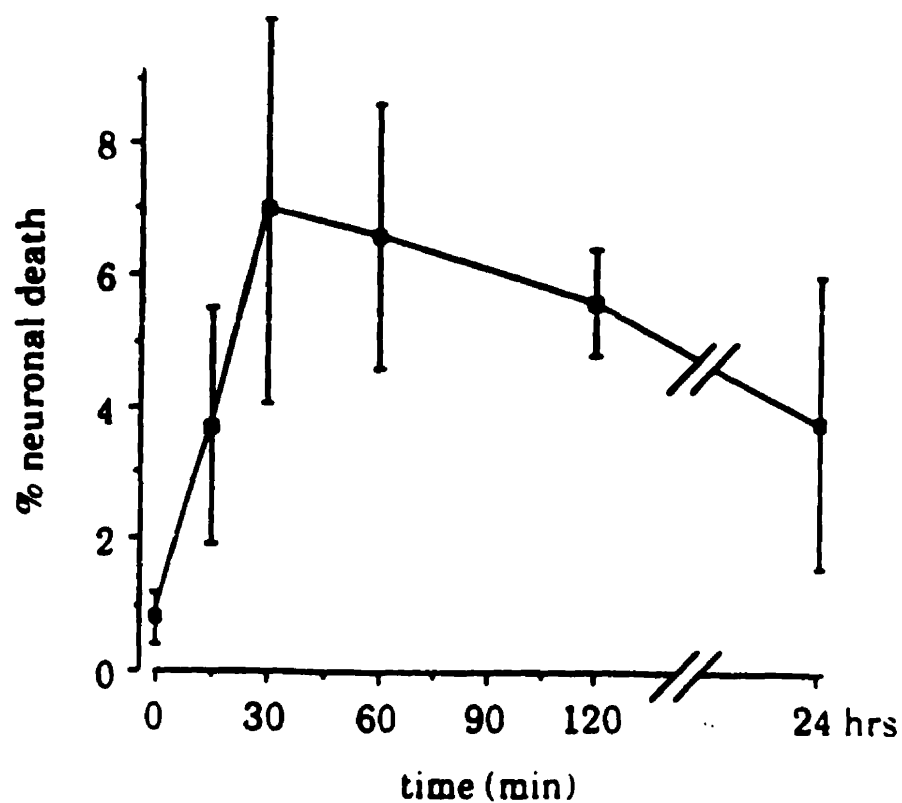
Figure 1: Tat$_{31-61}$ induced neurotoxicity. Cultures of human fetal neurons were treated with Tat$_{31-61}$ (17 µM) and the amount of neuronal cell death was analyzed by trypan blue exclusion at different time intervals. Maximal neurotoxicity was seen by 0.5-2 hrs. Error bars represent standard deviations.
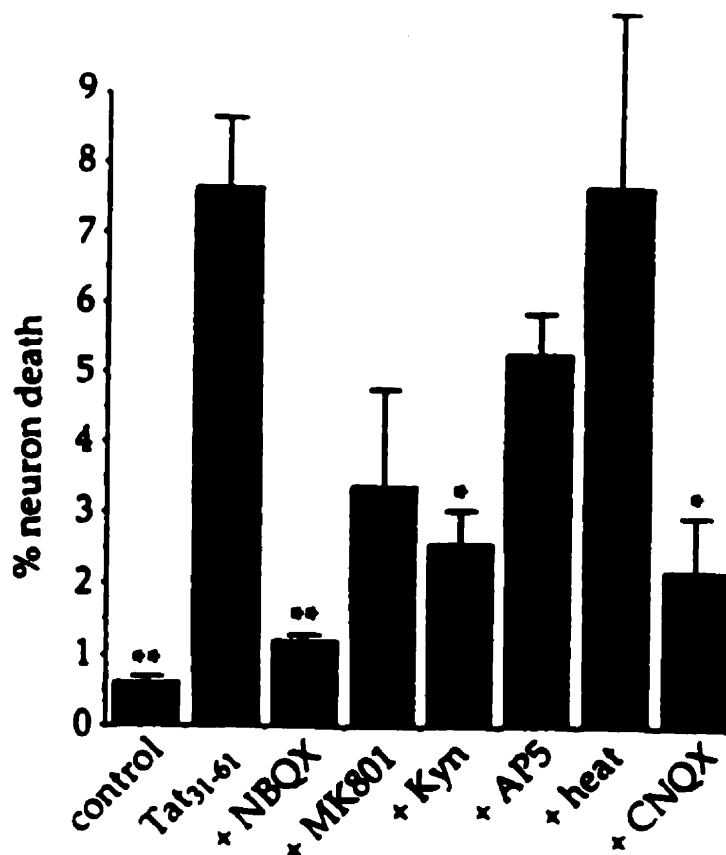
Figure 2. Excitatory amino acid antagonists attenuate Tat31-61 toxicity: Each value represents the mean±S.E.M.. Values marked with an asterix show significant cell sparing compared to Tat31-61 (17µM) alone (*P<0.05; **P<0.01). Kynurenate (Kyn; 1mM), CNQX (1µM) and NBQX (10µM) significantly decreased Tat31-61 toxicity. Some attenuation by MK801 (20µM) and AP5 (100µM) was also observed, however, it was not statistically significant. Heat treatment (60°C for 30min) of the peptide did not effect its toxic properties.

TABLE 1. Treatment of human fetal neurons with by 15-mer Tat peptides[a].

Peptide	Neuron death[b]	Peptide	Neuron death[b]
1-15	-1.15 ± 0.47^1	38-52	$+0.05 \pm 0.15^3$
3-17	-1.20 ± 0.26^1	43-57	-0.28 ± 0.19^3
8-22	-0.68 ± 0.38^1	48-62	-0.34 ± 2.64^4
13-27	$+0.06 \pm 0.27^1$	53-67	$+0.16 \pm 0.88^5$
18-32	-0.04 ± 0.03^1	58-72	$+1.63 \pm 2.66^4$
23-37	-0.33 ± 1.20^1	63-77	-4.30 ± 0.80^2
28-42	-2.71 ± 0.30	68-82	-1.12 ± 0.37^1
33-47	$+1.80 \pm 0.80^3$	72-86	-1.37 ± 0.47^2

Table 1: [a] Sixteen Tat peptides each 15 amino acids in length and overlapping by 10 amino acids each, spanning the entire molecule of Tat from HIVBRU did not produce toxicity. [b] Values represent the mean of the difference between control wells and treated wells ± S.E.M. Each experiment was done in triplicate wells. Superscripts beside values indicate the number of times the peptide was tested.

Role of excitatory amino acid receptors in Tat31-61 mediated toxicity:

Previously, we found that Tat1-86 induced neurotoxicity of human fetal neurons was blocked at least in part by NMDA and non-NMDA excitatory amino acid receptor antagonists [8]. To determine the mechanism of toxicity of Tat31-61, the following pharmacological agents were tested: kynurenate (1 mM), a non-selective excitatory amino acid (EAA) receptor antagonist; D,L-2-amino-5-phosphovaleric acid (AP5, 100 µM) and dizocilpine (MK801, 20 µM), selective NMDA-receptor antagonists; and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 1 µM) and 2,3-dihydro-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX, 10 µM), selective non-NMDA receptor antagonists. Tat31-61-induced neurotoxicity was blocked significantly (P<0.05) by kynurenate, CNQX and NBQX. MK801, and to a

lesser degree AP5, blocked neurotoxicity, although in neither case was the blockade found to be statistically significant (Fig. 2).

Specificity of Tat₃₁-₄₁ induced neurotoxicity: The possibility that the neurotoxicity of Tat_{31-61} was due to contaminants was excluded by using only highly purified peptides. Further, the immunabsorption of Tat_{31-61} with rabbit anti-Tat serum coupled to protein A-conjugated agarose beads (Fig. 3A) and, treatment of Tat_{31-61} with trypsin (Fig. 3B) resulted in loss of Tat neurotoxicity. The neurotoxicity was unaffected by Tat_{31-61} solutions treated with normal rabbit serum coupled to protein A-conjugated agarose beads (Fig. 3A).

Effect of Tat peptides on intracellular calcium: Human fetal brain cells were treated with Tat_{31-61} (100μM) or Tat_{48-85} (700μM) and analyzed by video imaging to determine the effect of Tat peptides on intracellular calcium. For Tat_{31-61}, peak increases in $[Ca^{2+}]_i$ of 955 ± 280 nM were reached within 0.21 ± 0.06 min (n=19) (Fig. 4). NBQX (10 μM) did not itself affect $[Ca^{2+}]_i$, but almost completely blocked responses to Tat_{31-61}; time to peak was delayed to 1.0 ± 0.14 min and increases in $[Ca^{2+}]_i$ were significantly (P<0.01) reduced to 69 ± 26 nM (n=13), i.e. 7% of original response. After NBQX washout, Tat_{31-61}-induced peak increases in $[Ca^{2+}]_i$ were 770 ± 256 nM (81% of the original response). These responses to Tat_{31-61} after NBQX washout were not significantly different from values obtained for Tat_{31-61} prior to NBQX treatment (n=13) (Fig. 4). Treatment of neurons with Tat_{48-85} even at concentrations of 7 fold greater than Tat_{31-61} resulted in a negligible change in $[Ca^{2+}]_i$ of 7 ± 3nM (n=13).
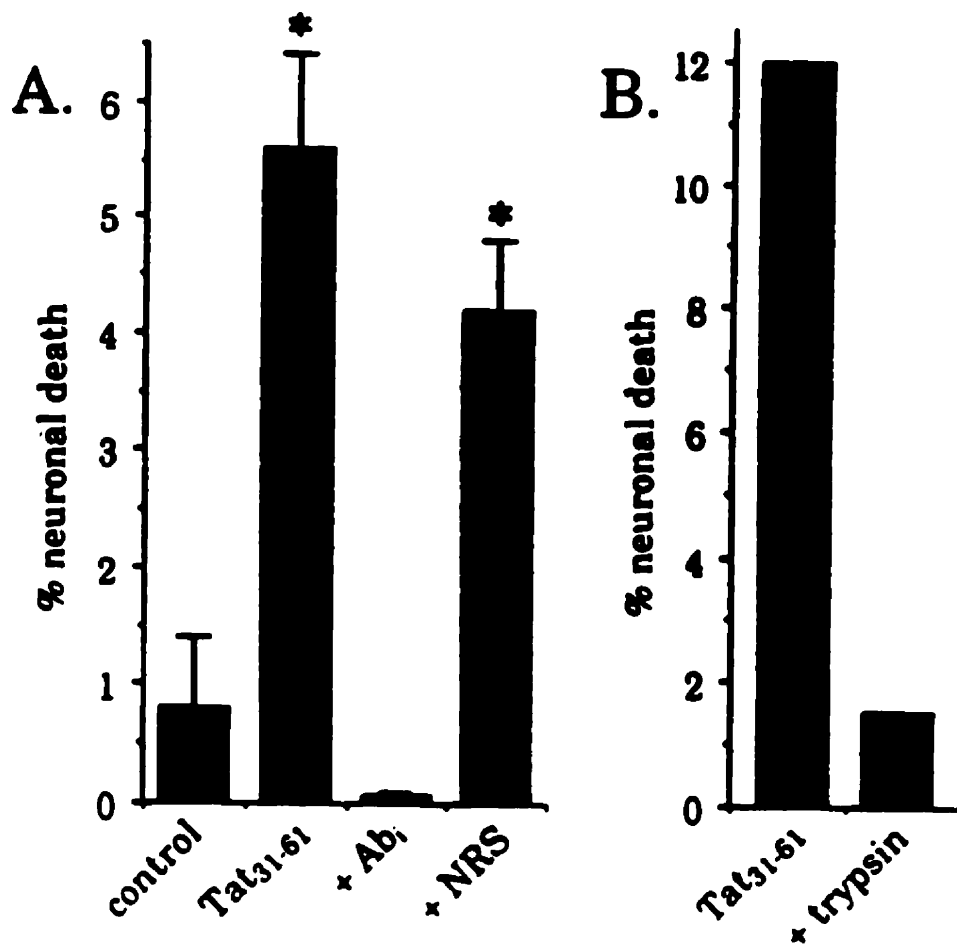
Figure 3: Specificity of Tat$_{31-61}$ neurotoxicity. (A) This graph represents three individual experiments using triplicate wells. Each value represent the mean ± S.E.M. Values marked with an asterix show significant cell death compared to control (*p < 0.01). The neurotoxicity of Tat$_{31-61}$ (17 µM) was completely abolished following immunoabsorption with antisera to Tat (Abi). Similar treatment with normal rabbit serum (NRS) did not affect the neurotoxicity. (B) Each value represents the mean percentage cell death above control from a single representative experiment done in triplicate wells. Treatment of Tat$_{31-61}$ (17 µM) with trypsin completely abolished neurotoxicity.
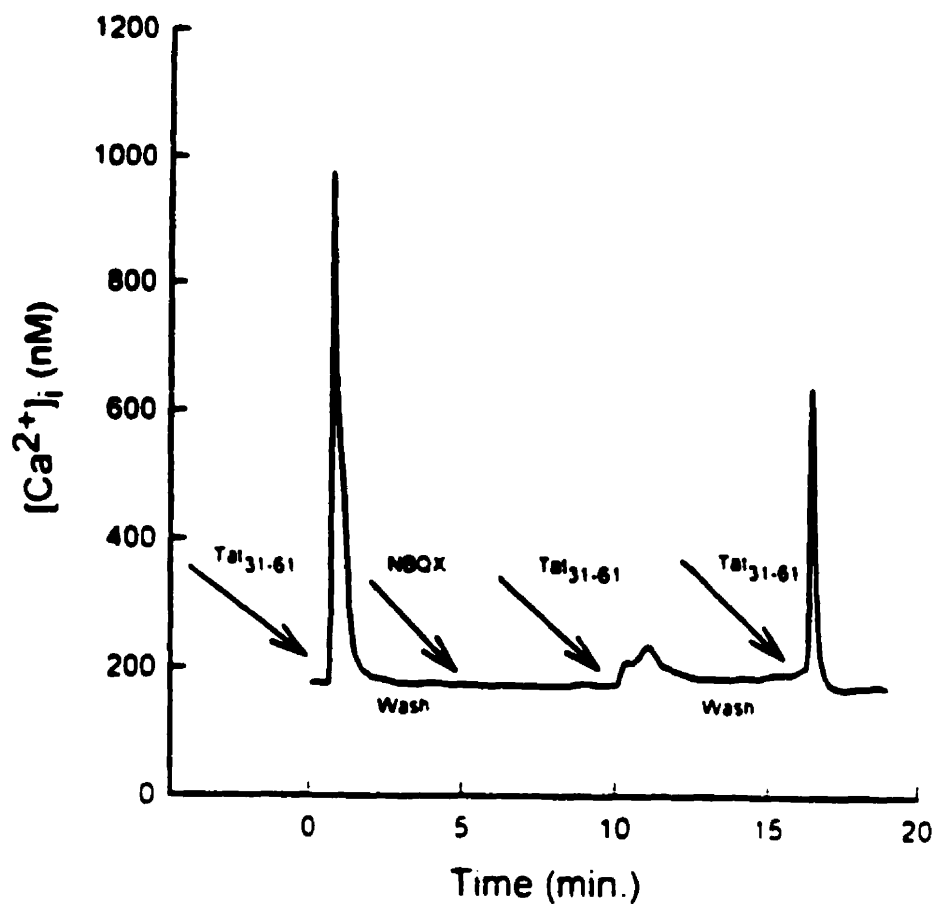
Figure 4: A representative trace depicting the response of a single neural cell showing blockade of Tat_{31-61} response with NBQX. Baseline $[Ca^{2+}]_i$ was 177 nM. The application of Tat_{31-61} (100 µM) resulted in an increase in $[Ca^{2+}]_i$ of 799 nM. In the presence of NBQX (10 µM) the Tat_{31-61} response was diminished to 59 nM (applied 4 min after application of NBQX) and following a washout, reapplication of Tat_{31-61} resulted in an increase in $[Ca^{2+}]_i$ of 461 nM.
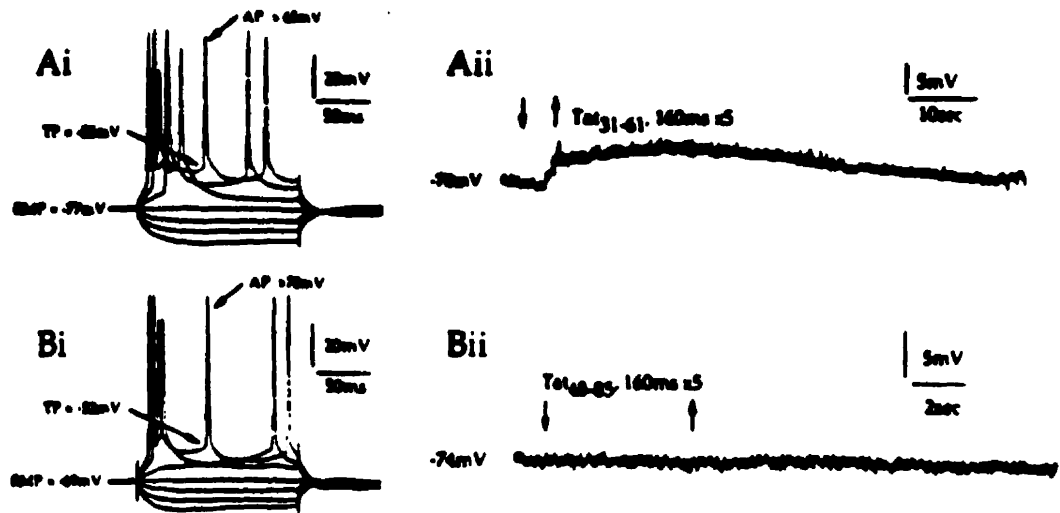
Figure 5: Electrophysiologic properties of Tat$_{31-61}$. This figure shows representative intracellular recordings of two rat hippocampal neurons during intracellular current injection (-0.6 to 0.6 nA in 0.2 nA steps) and exposure to the Tat peptides 31-61 and 48-85 applied extracellularly be pressure injection. Ai and Bi demonstrate that these hippocampal CA1 pyrimidal neurons have normal current-voltage responses, resting membrane potentials (RMP), membrane input resistances (Rin), thresholds for action potential generation (TP) and action potential (AP). (Aii). The neuron demonstrated in Fig. 4Ai when exposed to Tat$_{31-61}$ shows a change in membrane potential (depolarization). (Bii). The neuron demonstrated in Fig. 4Bi when exposed to Tat$_{48-85}$ showed no change in the membrane potential.

DISCUSSION

HIV-1 proteins including Tat, gp120, Nef, and Rev have been shown to be cytotoxic and have been implicated in the pathogenesis of HIV-1 dementia [23, 24, 25, 26]. Previously, we showed that full length Tat (Tat$_{1-86}$) caused neuronal cell death and increased neuronal excitability through interactions with NMDA and non-NMDA type excitatory amino acid receptors [19]. Further, we showed that Tat$_{1-86}$ significantly increased levels of intracellular calcium in mixed cultures of human fetal brain [27, 19]. In this paper we report that, in terms of neurotoxicity, neuronal depolarization and calcium mobilization, an active region of Tat resides in the 31-61 amino acid sequence.

HIV-Tat protein influences a large number of viral and host functions. For some of these the functional regions have been defined. The basic region which contains an arginine rich region (positions 49-57) has been reported to be important for nuclear localization of Tat [28], cell attachment [29, 30], and for cytotoxicity [31, 17]. The cysteine rich region (positions 22-37) was found to be responsible for the metal binding properties of Tat [32, 28] and Tat (positions 1-58) was shown to be required for inhibition of antigen-induced lymphocyte proliferation [33]. The RGD (arginine-glycine-aspartic acid) sequence in the second exon of Tat (positions 72-74) is a well known integrin receptor recognition sequence [34] and has been shown to be important in mediating adhesion and cell aggregation [35, 36]. Our description here that the distinct, conformationally dependent region of Tat within the first exon contained within positions 31-61 that causes excitation of neurons, increases in intracellular calcium, and neurotoxicity suggests potentially an important role of Tat in mediating CNS effects in HIV-1 infected individuals.

It appears clear that neuro-excitatory and -toxic properties of full length Tat as well as Tat$_{31-61}$ are mediated predominantly through interactions non-NMDA and to a lesser degree via NMDA excitatory amino acid receptors. Possible explanations for this include specific interactions of Tat with the non-NMDA receptors or a differential expression of non-NMDA receptors in fetal neurons. It has been shown that non-NMDA receptors are expressed earlier and are more abundant than NMDA receptors in fetal brain [37]. Thus if Tat were to cause a release of a glutamate-like substance from glial cells that activates both types of excitatory amino acid receptors, it would appear as though the toxicity is mainly mediated via non-NMDA receptors. Further studies are needed to determine if Tat acts directly on excitatory amino acid receptors or indirectly via action on glial cells.

At present, the cause and effect relationships between neuronal depolarization, toxicity and build up of intracellular calcium remains uncertain. It is known that excessively high levels of intracellular calcium ultimately leads to cell death [7]. Indeed, in some of our first experiments, we found that microinjection of high concentrations of Tat$_{31-61}$ led to dramatic increases in levels of intracellular calcium and cell demise [27]. Given that excitation of NMDA and non-NMDA receptors results in increased levels of intracellular calcium and at sufficiently high levels can lead to cell death [38], and that the time course of neuronal depolarization by Tat precedes the Tat-induced increases in intracellular calcium, it is possible that the calcium response is secondary to increased neuronal excitability. If so then extracellular calcium may enter the cell through calcium permeable excitatory amino acid receptor coupled channels or alternatively through depolarization-induced opening of voltage sensitive calcium channels [38].

Results of our video imaging of calcium in brain cells treated with Tat or Tat$_{31-61}$ suggests that calcium levels adjacent to the plasma membrane are highest initially as well as during periods of maximal increases in intracellular calcium; findings consistent with Tat-induced influx of extracellular calcium [39]. However, these data do not exclude the possibility that calcium released from intracellular pools contributes to the Tat-induced rises in intracellular calcium.

The tertiary structure of the Tat molecule, as governed by its length, appears to be important in mediating neurotoxicity. Tat peptides, 15 amino acids in length, do not produce significant increases in cell death. A 31-mer Tat peptide (Tat$_{31-61}$), produced significant levels of neurotoxicity. At 10-20 fold higher concentration of the peptide as compared to our previous study using full length Tat, the amount of toxicity was small, however, the pharmacological properties of the neurotoxicity were similar to that of full length Tat [19]. Increasing the length of the Tat peptide to a 41-mer (Tat$_{31-71}$) reduced the degree of neurotoxicity. Further, heat treatment of Tat$_{31-61}$ did not change its toxicity. However, we and others have previously shown that similar heat treatment of Tat$_{1-86}$ resulted in loss of toxicity [19, 18] suggesting that Tat$_{31-61}$ has a stable tertiary configuration. Since Tat$_{31-61}$ consists of the core region (32-47) and the basic region (48-57), it suggests that both regions are essential for causing neurotoxicity. These core and basic domains of Tat are highly conserved and have exposed hydrophobic and helical regions available for membrane interaction (3) which may mediate neurotoxicity. Sabatier et al., [17] reported neurotoxicity with the basic region alone. In their studies, death of mice following intracerebroventricular injections was used as a measure of toxicity but pathological confirmation of neurotoxicity was not included. We, however, did not observe any toxicity with two 15-mer peptides (Tat$_{43-57}$ and Tat$_{48-62}$) and another 38-mer peptide (Tat$_{48-85}$) all of which

contained the basic region of Tat. These results are consistent with those of Weeks et al., [18] who were also unable to demonstrate any toxicity with the basic peptide Tat49-58 in neuronal cell lines. The basic region however, does play a role in cell surface binding [18].

Circulating or intracerebral levels of Tat have not yet been determined. Localization of Tat by immunohistochemistry in the brain has been difficult due to cross reactivity of anti-Tat antibodies with normal brain antigens [40]. Our studies and those of others show that micromolar concentrations of Tat are required to produce neurotoxicity [19, 40, 17]. Although it might be unlikely for such high levels to be present in the circulation, it is conceivable that these levels may be achieved in close vicinity to HIV infected cells. Alternatively, Tat may act synergistically with other neuroexcitatory/toxic molecules released from HIV infected cells since Tat has been shown to be released extracellularly from HIV infected cells *in vitro* and neurons are frequently observed in close proximity to HIV infected glial cells [10, 13]. Clearly, further studies are needed to determine the biological relevance of Tat neurotoxicity.

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Chapter 2

Role of Na^+/H^+ Exchangers, Excitatory Amino Acid Receptors and

Voltage Operated Ca^{2+} Channels in HIV-1 gp120-Mediated Increases

of Intracellular Ca^{2+} in Human Fetal Neurons and Astrocytes.

C.P. Holden, N.J. Haughey, A. Nath, and J.D. Geiger

INTRODUCTION

A subacute encephalopathy known as AIDS dementia complex in patients infected with human immunodeficiency virus type-1 (HIV-1) is the commonest form of dementia in North Americans ≤ 60 years old. AIDS dementia complex is characterized clinically by progressive cognitive, motor and behavioral dysfunction[1,2,3] and its onset is associated with a poor prognosis for survival. In brain, macrophages, microglia and astrocytes are infected directly with HIV-1 and neuropathological findings include multinucleated giant cells, myelin pallor, astrogliosis and neuronal cell loss[4,5,6,7,2,8,9]. Direct infection of neurons with HIV-1 is rare[10,11]. Therefore, research to determine cause(s) of neuronal dysfunction and/or loss has focused on viral products of HIV-1 as well as potentially toxic non-viral factors.

HIV-1 viral products implicated in neurotoxicity include accessory proteins involved in viral replication, Tat, Nef and Rev, as well as envelope glycoproteins, gp120 and gp41[12]. HIV-1 Tat is neuroexcitatory through direct actions on neurons, Tat peptides increase levels of intracellular calcium ($[Ca^{2+}]_i$) via activation of excitatory amino acid receptors, and Tat and Tat peptides cause neurotoxicity[13,14,15]. In contrast, gp120 appears to cause neurotoxicity indirectly following interactions with bystander cells such as microglia and astrocytes[16,17].

Gp120 induces the release of soluble products from brain cells that may contribute to gp120-induced neurotoxicity[18,19,20,17]. In neurons, gp120 increased

$[Ca^{2+}]_i$ by causing an influx of calcium through voltage- and receptor-operated calcium channels, and releasing calcium from intracellular pools[21]. In addition, nitric oxide synthase was induced, and the production of nitric oxide and other reactive oxygen intermediates were increased by gp120[22,23,24,20]. Many of the neurotoxic actions of gp120 appear to be mediated through interactions with glial cells because, in glial cells, gp120 dysregulated the production and release of endogenous neurotrophic substances and cytokines[23,25,26], inhibited glutamate reuptake[27,28], and increased Na^+/H^+ exchange, K^+ conductance, and tyrosine kinase activity[29,30,31].

Gp120-induced increases of $[Ca^{2+}]_i$ in neurons and astrocytes may be an important determinant of neuronal dysfunction in patients infected by HIV-1. Using single cell calcium imaging, recombinant gp120 (gp120) and cultured human neurons and astrocytes, we compared responses of neurons and astrocytes to gp120. Here, we report that gp120-induced calcium influx occurs first in astrocytes followed by neurons. Mechanistically, gp120 increased calcium influx following activation of Na^+/H^+ exchangers and (±)-2-amino-5-phosphonopentanoic acid (AP5)-sensitive NMDA receptors in cultured human fetal neurons and astrocytes, and via voltage-operated Ca^{2+} channels and dizocilpine-sensitive NMDA receptors in neurons, but not astrocytes.

EXPERIMENTAL PROCEDURES

Materials: Fura-2-acetoxymethyl ester (Fura-2/AM) and Fura-2 were obtained from Molecular Probes Inc. (Eugene, OR). 5-(N-methyl-N-isobutyl)-Amiloride (MIA), dizocilpine maleate (MK-801), ($\pm$)-2-amino-5-phosphonopentanoic acid (AP5), memantine and diltiazem hydrochloride (diltiazem) were purchased from Research Biochemicals International (Natick, MA). N-Methyl-D-glucamine, EGTA, L-leucine methyl ester, thapsigargin and chloride salts of cadmium and nickel were purchased from Sigma Chem. Co. (St. Louis, MO). Nimodipine, ω-conotoxin GVIA, ω-agatoxin IVA, and ω-conotoxin MVIIC were purchased from Alomone Labs (Jerusalem, Israel). Recombinant gp120 of HIV-1$_{IIIB}$ was purchased from Intracel (Cambridge, MA) and gp120 of HIV-1$_{SF2}$ was obtained as a gift from Chiron Corporation. Recombinant non-glycosylated gp120 of HIV-1$_{SF2}$ and rabbit sera containing polyclonal antibody to gp120 were obtained through the AIDS Research and Reference Reagent Program of the NIH. All other reagents were of analytical grade or the highest purity available. All drugs were dissolved to the desired stock concentrations in Krebs buffer on the day of experimentation except for MIA, nimodipine and thapsigargin that were dissolved in DMSO and diluted with Krebs buffer (final DMSO concentration was < 0.1 %).

Mixed cultures of human fetal astrocytes and neurons: Brain specimens from fetuses at gestational ages of 12 to 15 weeks were obtained, with consent, from women undergoing elective termination of pregnancy. All aspects of these studies received approval from the University of Manitoba's Committee for

Protection of Human Subjects. Blood vessels and meninges were removed and brain tissue was washed in Opti-MEM (Gibco BRL, Burlington, ON) and brains were mechanically dissociated by repeated trituration through a 20 gauge needle. The cells were centrifuged at 270 g for 10 min and resuspended in Opti-MEM with 5 % heat inactivated fetal bovine serum, 0.2 % N2 supplement (Gibco BRL, Burlington, ON) and 1 % antibiotic solution (10^4 U of penicillin G/ml, 10 mg streptomycin/ml and 25 µg amphotericin B/ml in 0.9 % NaCl). Approximately 10^6 cells were plated in 75 cm^2-T flasks and incubated for 4-6 weeks in a humidified environment at 37°C (95% O_2/5% CO_2). Flasks were gently tapped manually, and cells released into supernatant were collected and plated onto 33-mm diameter poly-D-lysine-coated glass coverslips. After replating, cells were incubated for 7-10 days in a humidified environment at 37°C (95% O_2/5% CO_2) before being taken for assay. Cells were routinely treated with 7.5 mM L-leucine methyl ester (LME) for 12 h to remove microglia; prior to LME treatment microglia that stained positively for EBM-11 (Dako, Denmark) were in-frequent (<1%) and following treatment were not observed. Astrocytes and neurons were identified morphologically, and in sample coverslips from each fetus, cells were immunostained with either neuronal marker microtubule-associated protein 2 (Boehringer Mannheim, Laval, P.Q.) or with astrocyte marker glial fibrillary acidic protein (Chemicon, Temecula, CA). Neuron-enriched cultures used for experimentation were > 70% pure and astrocyte cultures were > 98% pure.

Measurements of intracellular calcium ([Ca²⁺]ᵢ): Free $[Ca^{2+}]_i$ was determined

using the calcium-specific fluorescent probe Fura-2/AM. Sparsely plated cells

(approximately 250,000 per plate) were incubated for 1 h at 25°C in Krebs-BSA

buffer consisting of (in mM): 111 NaCl, 26.2 $NaHCO_3$, 1.2 NaH_2PO_4, 4.7 KCl, 1.2

$MgCl_2$, 15 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1.8

$CaCl_2$, 5 glucose, 1.5 µM bovine serum albumin (BSA) and 2 µM Fura-2/AM.

Cells were washed 3-times with Krebs (no BSA) to remove extracellular Fura-2

and were incubated at 37°C for 5 min. The coverslip containing Fura-2 loaded

cells was placed in a LU-CSD Leiden coverslip dish (Medical Microsystems

Corp., Greenvale, NY) and a PDMI-2 open perfusion micro-incubator (Medical

Microsystems Corp., Greenvale, NY) and cells were superfused at a rate of 2

ml/min with Krebs buffer warmed to 37°C. Cells were excited at 340 and 380

nm, and emission was recorded at 510 nm with a video-based Universal imaging

system (EMPIX, Missassauga, ON). All images were acquired by real time

averaging of 16 frames of each wavelength that included a background reference

subtraction from each of the acquired images. R_{max}/R_{min} ratios were converted to

nanomolar $[Ca^{2+}]_i$ according to the method of Grynkiewicz et al.[32]. From our

calibration studies using calcium standards obtained from Molecular Probes

(Eugene, OR), it was determined that Fura-2 saturated calcium concentrations of

approximately 10 µM. Typically, in the fields analyzed, 3 to 5 cells including their

cell bodies and most processes were imaged.

Gp120 was loaded into glass micropipettes (1.0 mm OD, 0.78 mM ID) positioned approximately 3 cell body widths away from target cells, and gp120 was pressure-applied (3 x 100 ms pulses at 5 psi) using a Picospritzer (General Valve Corp, Fairfield, NJ). Cells within 5 cell body widths of the micropipette were monitored for time periods up to 1 h. Typical experiments were conducted as follows. Cells were superfused with Krebs buffer at a rate of 2 ml/min for ~5 min and after basal $[Ca^{2+}]_i$ were determined, superfusion was stopped, and gp120 was pressure-applied to obtain control responses. Upon return to basal $[Ca^{2+}]_i$, with superfusion still stopped, either MIA, diltiazem, nimodipine, $CdCl_2/NiCl_2$, $MgCl_2$, ω-conotoxin GVIA, ω-agatoxin IVA, ω-conotoxin MVIIC, thapsigargin, dizocilpine, memantine or AP5 were manually pipetted into the culture dish. When $[Ca^{2+}]_i$ were stable, gp120 was pressure-applied a second time. Following this, cells were superfused with Krebs buffer for at least 10 min to wash away any remaining drug and/or gp120. Once basal $[Ca^{2+}]_i$ were obtained gp120 was pressure-applied a third, and final, time.

Controls for gp120 specificity: For immunoabsorption experiments, a 1:100 dilution of a rabbit polyclonal anti-gp120 antisera or normal rabbit serum was bound to protein A-coated agarose beads (Pharmacia Biotech Inc., Baie d'Urfé, P.Q.), and incubated with gp120 for 90 min at 25°C. Following centrifugation at 500 g for 3 min at 4°C, supernatants were loaded into glass micropipettes, pressure-applied onto the cultured cells and $[Ca^{2+}]_i$ responses were monitored. The degree to which freeze/thaw cycles affected gp120-induced increases of

$[Ca^{2+}]_i$ was determined by freezing and thawing, 3-times, a 1 nM aliquot of gp120. To control for the effects of gp120 on cellular membrane integrity, cells were incubated for 30 min in Krebs buffer containing cell impermeable Fura-2 and exposed for 20 min to concentrations of bath-applied gp120 ranging from 1 to 25 nM; calcium ionophore A23187 (40 µM) was added at the end of each of these experiments as a positive control.

Data Collection: Data were reported as mean ± SEM values. Statistical significance was determined using a two-way analysis of variance (ANOVA) with a Student-Neuman-Kuels test.

RESULTS

[Ca^{2+}]$_i$ response profiles induced by recombinant HIV-1 gp120: Positive

responses to gp120 were defined as increases in [Ca^{2+}]$_i$ at least two standard

deviations above basal levels; 83 ± 1% of neurons and 85 ± 1% of astrocytes

responded positively to gp120. Basal [Ca^{2+}]$_i$ of 154 ± 1 nM in 1619 astrocytes

analyzed (n = 1619 from 21 separate fetuses) were not significantly different from

those of 155 ± 1 nM in neurons (n = 1749 from 25 separate fetuses). Pressure

application of 25 nM gp120 (HIV-1$_{IIIB}$ or HIV-1$_{SF2}$) near neurons resulted in a 10-

to 15-fold increase of [Ca^{2+}]$_i$ (Table 1). However, responses to gp120 (HIV-1$_{IIIB}$)

Table 1. Effect of HIV-1 strain difference and glycosylation on gp120-induced increases of [Ca^{2+}]$_i$

Treatment	[Ca^{2+}]$_i$ Increase (nM)
gp120 (HIV-1$_{IIIB}$)	2003 ± 215[a]
gp120 (HIV-1$_{SF2}$)	1432 ± 145[a,b]
Non-glycosylated gp120 (HIV-1$_{SF2}$)	941 ± 177[a,b,c]

Table 1: HIV-1 recombinant gp120 proteins at 25 nM were pressure-applied (3 x 100 ms, 5 psi) onto human fetal neurons. Data are mean ± SEM values (above background [Ca^{2+}]$_i$) for at least 40 different cells from 2 separate fetuses. [a]p<0.05 compared to background [Ca^{2+}]$_i$. [b]p<0.05 compared to HIV-1$_{IIIB}$ gp120. [c]p<0.05 compared to glycosylated HIV-1$_{SF2}$ gp120.

were significantly (p<0.05) greater than those to gp120 (HIV-1$_{SF2}$). Non-

glycosylated gp120 (HIV-1$_{SF2}$) also produced significant increases in [Ca^{2+}]$_i$,

however the responses were nearly two-thirds those of glycosylated gp120

(HIV-1$_{SF2}$) (Table 1).

Similar results were obtained for astrocytes. When astrocytes and neurons were in close proximity in a single field, astrocyte responses preceded neuronal responses by seconds to minutes; a example of this was illustrated in Figure 1. All subsequent experiments were conducted with glycosylated gp120 (HIV-1$_{IIIB}$).

Source of gp120-induced increases of [Ca^{2+}]$_i$: Basal levels of [Ca^{2+}]$_i$ did not vary significantly between cells incubated for 20 to 60 min in either normal Krebs buffer containing 1.8 mM Ca^{2+} or in Ca^{2+}-free Krebs buffer that contained 2 mM EGTA. In the presence of Ca^{2+}, pressure-ejected gp120 (25 nM) significantly ($p<0.01$) increased [Ca^{2+}]$_i$ by 2076 ± 147 nM in neurons (see Fig. 2A for a typical response). In the absence of Ca^{2+}, no statistically significant changes in [Ca^{2+}]$_i$ were observed (Fig. 2B). However, following reperfusion with Ca^{2+}-containing buffer, gp120 significantly ($p<0.01$) increased [Ca^{2+}]$_i$ by 1700 ± 236 nM (Fig. 2B); these latter increases were not significantly different from gp120-induced rises of [Ca^{2+}]$_i$ observed prior to superfusion of cells with calcium-free buffer. These results suggested that gp120 caused an influx of extracellular calcium into these cells. However, to determine if additionally, gp120 might release calcium from intracellular stores we used the endoplasmic reticulum Ca^{2+}-ATPase pump inhibitor thapsigargin. Thapsigargin (5 µM) typically increased neuronal [Ca^{2+}]$_i$ starting 2 min after its application - [Ca^{2+}]$_i$ increased significantly ($p<0.01$) to peak levels of 1958 ± 111 nM and remained elevated for ~5 min before returning to basal levels. In the continued presence of thapsigargin (but after [Ca^{2+}]$_i$ returned
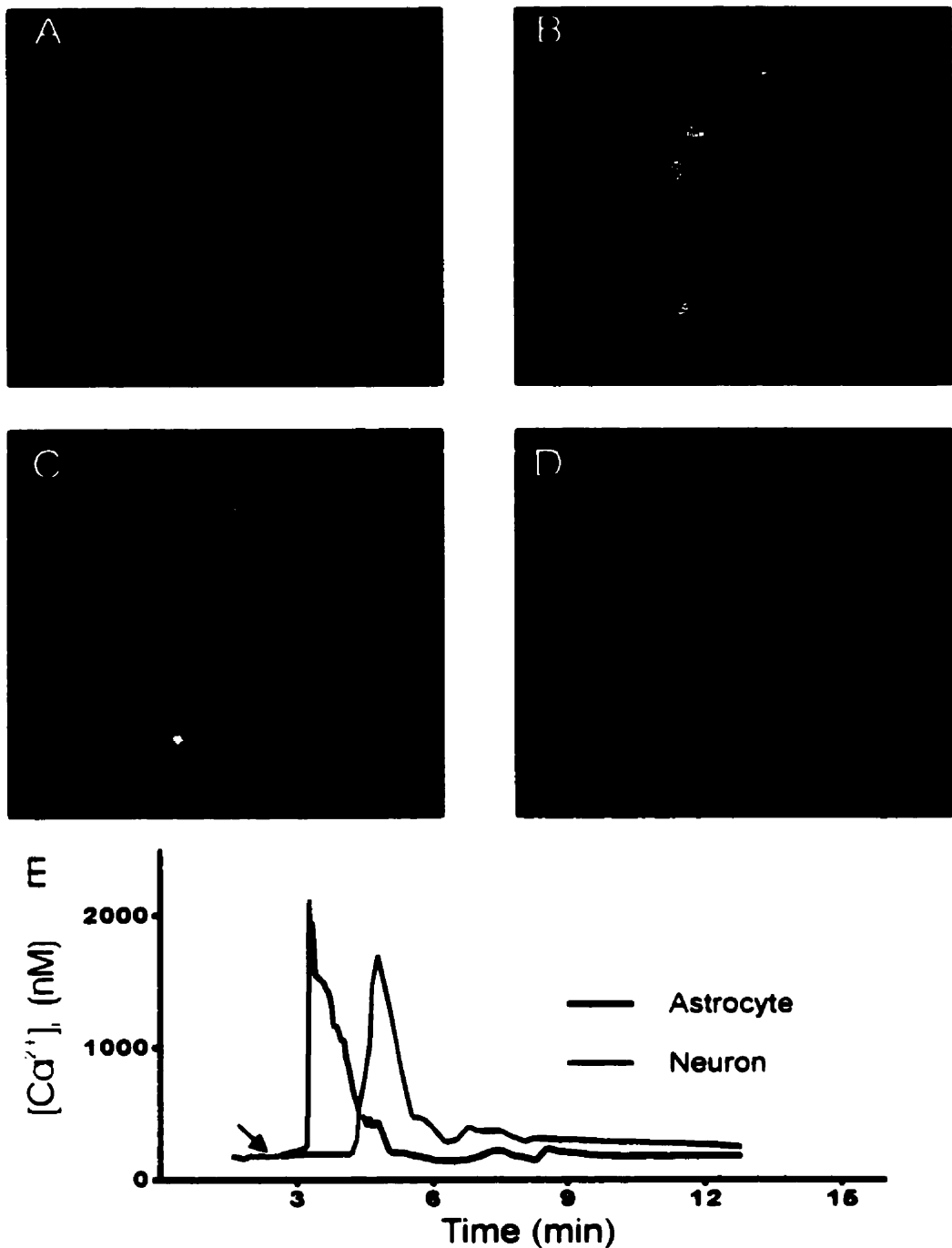
Figure 1. $[Ca^{2+}]_i$ increase in astrocytes prior to increases in neurons. gp120 (25 nM) was pressure-applied (3x 100 ms, 5 psi; arrow) 3 cell body widths away from the targeted neuron and astrocyte. gp120 produced an increase of $[Ca^{2+}]_i$ in astrocytes first followed seconds to minutes later by an increase in neurons. **A.** Control response in the absence of gp120 showing baseline levels of $[Ca^{2+}]_i$ in a human fetal astrocyte (top cell) and neuron (bottom cell). Pressure-applied gp120 induced a significant increase of $[Ca^{2+}]_i$ in the astrocyte (**B**) prior to an increase in the neuron (**C**). **D.** $[Ca^{2+}]_i$ returned to levels not significantly different from baseline. **E.** Graphical representation of the $[Ca^{2+}]_i$; maximum $[Ca^{2+}]_i$ was 2119 nM in the astrocyte and was 1700 nM in the neuron.
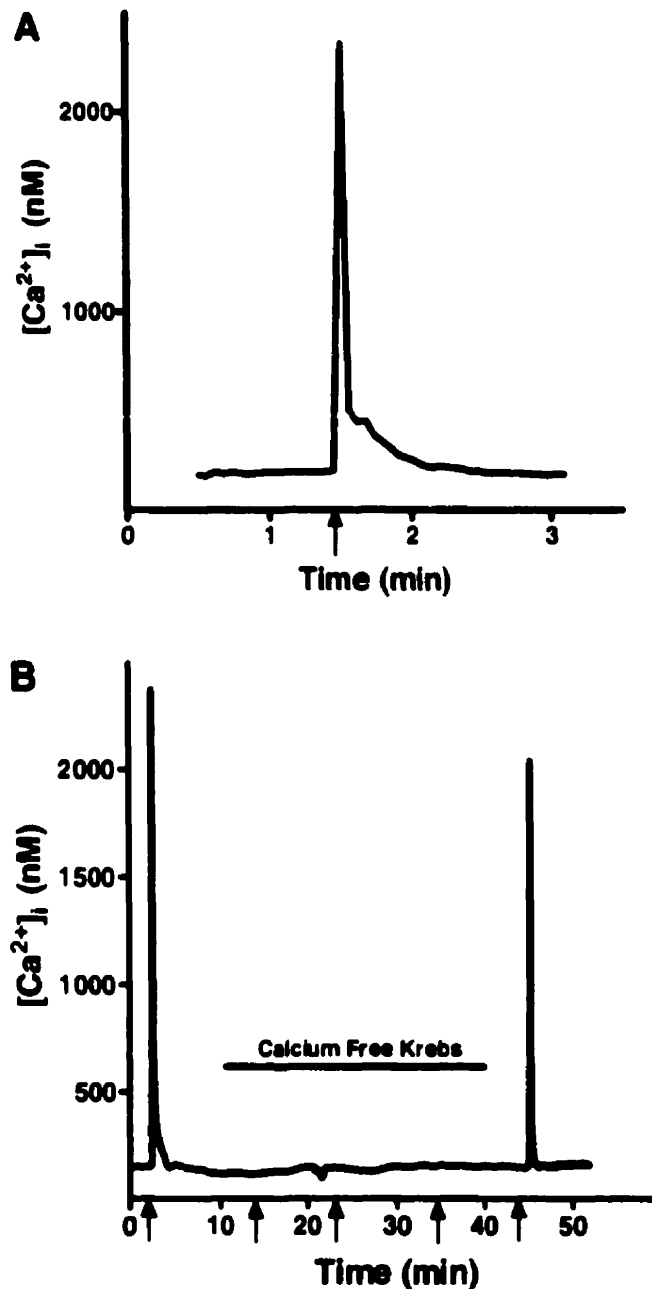
Figure 2: HIV-1 gp120 induced increases of $[Ca^{2+}]_i$ in human fetal brain cells. (A) Typical increases of $[Ca^{2+}]_i$ (2342 nM) from a cultured human fetal neuron following pressure-application (arrow) of HIV-1 recombinant gp120 (25 nM, 3 X 100 ms, 5 psi) onto the cell. (B) In the presence of 1.8 mM extracellular Ca^{2+} ($[Ca^{2+}]_e$), gp120 significantly (p < 0.01) increased $[Ca^{2+}]_i$ by 2373 nM. Pressure application of gp120 following the removal of $[Ca^{2+}]_e$ did not significantly affect $[Ca^{2+}]_i$. Pressure application of gp120 following the removal of $[Ca^{2+}]_e$ did not significantly affect $[Ca^{2+}]_i$. Gp120 significantly (p < 0.01) increased $[Ca^{2+}]_i$ by 2038 nM following reperfusion with normal $[Ca^{2+}]_e$.
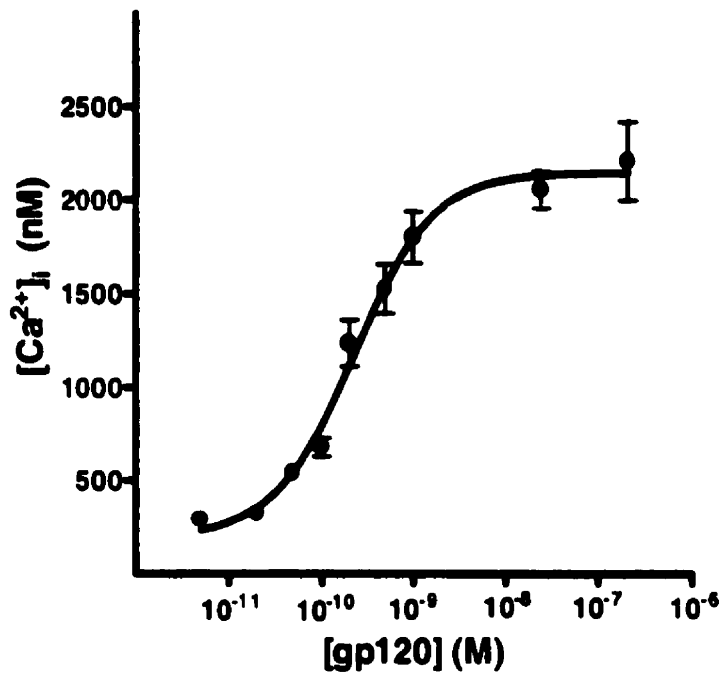
Figure 3: Gp120 dose-dependently increased $[Ca^{2+}]_i$ in human fetal brain cells. Recobinant gp120 pressure-applied at concentrations of gp120 from 120 pM to 209 nM (3 X 100 ms, 5 psi) onto human fetal neurons induced significant ($p < 0.005$) increases of $[Ca^{2+}]_i$ ranging from 209 ± 13 to 2210 ± 211 nM. The apparent EC50 value for gp120-induced increases of $[Ca^{2+}]_i$ in neurons was 223 ± 40 pM (95% confidence, n = 20). Similar results were observed with astrocytes (data not shown). Data are means ± S.E.M. values (bars) for at least 20 different cells from 2 different fetuses.

to baseline levels), gp120-induced increases in $[Ca^{2+}]_i$ of 2241 ± 129 nM were

not significantly different from those of gp120 alone (2021 ± 408 nM). Similar

responses to thapsigargin were found in neurons.

Dose dependency of gp120-induced $[Ca^{2+}]_i$ responses: Gp120 dose-dependently

increased $[Ca^{2+}]_i$ in human fetal neurons (Fig. 3). Statistically significant

(p<0.005) increases of 209 ± 13 nM were observed beginning at 5 pM

pressure-applied gp120 and maximal increases were observed at about 10 nM

gp120. At the highest concentration of gp120 tested, 209 nM, increases of $[Ca^{2+}]_i$

were 2210 ± 211 nM. The apparent EC_{50} value for gp120-induced increases of

$[Ca^{2+}]_i$ in neurons was 223 ± 40 pM (n = 20). Maximal increases of $[Ca^{2+}]_i$ in

astrocytes were not significantly different from those found in neurons (Table 2).

Specificity of responses: No significant increase of $[Ca^{2+}]_i$ was observed after

pressure-application of either Krebs buffer or BSA onto neurons or astrocytes.

Solutions from which gp120 had been immunoabsorbed, and gp120 subjected to

repeated freeze/thaw cycles failed to significantly increase $[Ca^{2+}]_i$ above

background levels (Figs. 4A and 4B). Gp120 did not appear to compromise cell

integrity because bath-applied gp120 at concentrations up to 25 nM did not

cause cell-impermeable Fura-2 to enter neurons (Fig. 4C) or astrocytes. In
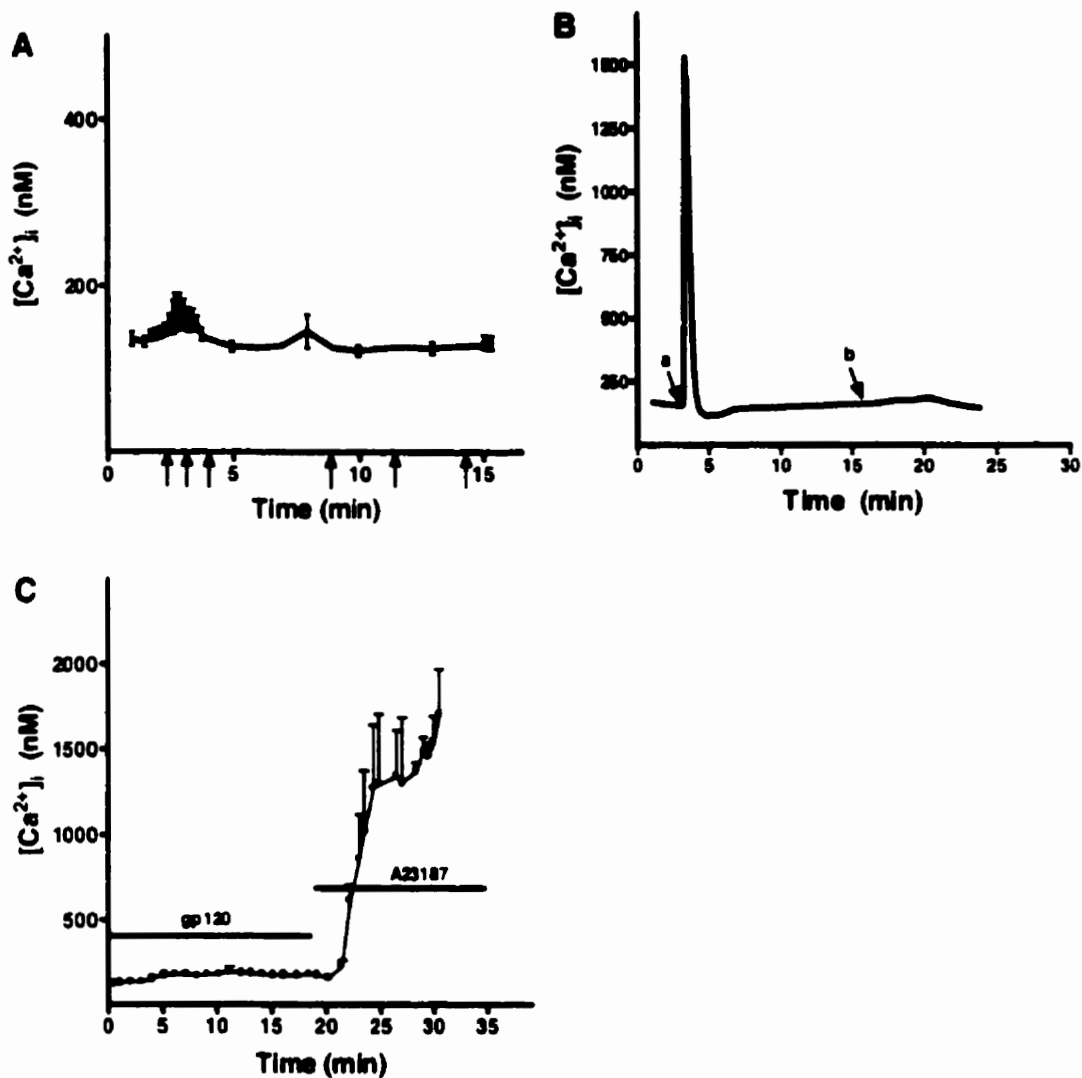
Figure 4: Experiments testing the specificity of gp120 action in human fetal brain cells. (A) Supernatant from immunoabsorbed recombinant HIV-1 gp120 was pressure-applied (arrow) (3 X 100 ms, 10 psi) onto cultured human fetal neurons and astrocytes. Immunoabsorbed gp120 supernatants failed to significantly increase $[Ca^{2+}]_i$ above background levels. Data are means ± S.E.M. values (bars) from 4 separate immunoelution experiments performed with at least 40 different cells from 2 different fetuses. (B) Recombinant HIV-1 gp120 at 1 nM (a), but not freeze/thawed gp120 (b) significantly increased $[Ca^{2+}]_i$ when pressure-applied (3 X 100 ms, 5 psi) onto human fetal neurons. Similar results were observed with astrocytes. Data shown are representative of experiments that were preformed 3-times using 2 batches of gp120. (C) Recombinant gp120 at concentrations ranging from 1-25 nM did not disrupt neuronal plasma membrane integrity as shown by lack of Fura-2 entry. Addition of Ca^{2+} ionophore A23187 (40 µM) significantly ($p < 0.01$) increased $[Ca^{2+}]_i$ by at least 10-fold over background levels. Data are means ± S.E.M. values (bars) for at least 30 different cells from 2 separate fetuses.

Table 2. Effects of the Na^+/H^+ antiporter Inhibitor 5-(N-methyl-N-isobutyl)-amiloride (MIA) and Na^+-free Krebs on gp120-induced increases of $[Ca^{2+}]_i$

Treatment	Cell Type	$[Ca^{2+}]_i$ Increase (nM)
gp120	neurons	2357 ± 290[a]
gp120 + MIA	neurons	89 ± 20[b]
gp120	neurons	1864 ± 474[a]
gp120 + Na^+-free Krebs	neurons	137 ±14[b]
gp120	astrocytes	1992 ± 312[a]
gp120 + MIA	astrocytes	87 ± 41[b]
gp120	astrocytes	1758 ± 228[a]
gp120 + Na^+-free Krebs	astrocytes	104 ±13[b]

Table 2: Recombinant gp120 (25 nM) was pressure-applied (3 x 100 ms, 5 psi) onto cells treated with either Krebs buffer (gp120), Krebs buffer plus 10 µM 5-(N-methyl-N-isobutyl)-amiloride (gp120 + MIA) or Na^+-free Krebs buffer (gp120 + Na^+-free Krebs). Data are mean ± SEM values for at least 30 different cells from 2 separate fetuses. MIA and Na^+-free Krebs did not affect basal $[Ca^{2+}]_i$. [a]$p<0.005$ compared to background $[Ca^{2+}]_i$. MIA and Na^+-free Krebs significantly ([b]$p<0.005$) inhibited responses to gp120.

contrast, the calcium ionophore A23187 (40 µM) significantly ($p<0.005$) increased Fura-2 entry and resulted in at-least 10-fold increase of $[Ca^{2+}]_i$ over background in neurons (Fig. 4C) and astrocytes.

Involvement of Na^+/H^+ exchangers in blocking $[Ca^{2+}]_i$ responses to gp120: MIA (10 µM), a selective blocker of Na^+/H^+ exchangers at a concentration that itself did not affect $[Ca^{2+}]_i$, significantly ($p<0.005$) reduced by 96 % gp120-induced increases of $[Ca^{2+}]_i$ in astrocytes and neurons (Table 2) to levels not significantly different from background (see Fig. 5 for a typical response). When extracellular

Na^+ was replaced with equimolar N-methyl-D-glucamine, responses to pressure-applied gp120 were significantly ($p<0.005$) reduced in neurons and astrocytes (Table 2).

Involvement of excitatory amino acid receptors: AP5, a competitive blocker of NMDA receptors significantly ($p<0.01$) reduced by 94 % at 100 μM and by 90 % at 10 μM, gp120-induced increases of $[Ca^{2+}]_i$ in neurons. Similar results were observed for astrocytes; AP5 significantly ($p<0.01$) inhibited gp120-induced increases of $[Ca^{2+}]_i$ by 96 % at 100 μM and 86 % at 10 μM (Table 3). At 100 μM concentrations, memantine and dizocilpine, both non-competitive blockers of NMDA receptors, significantly ($p<0.01$) inhibited by 95 % and 44 %, respectively, gp120-induced increases of $[Ca^{2+}]_i$ in neurons. Memantine, significantly ($p<0.01$) inhibited gp120-induced increases of $[Ca^{2+}]_i$ by 89 % in astrocytes, however, dizocilpine was ineffective (Table 3).

Involvement of voltage-operated calcium channels: The non-selective voltage-operated calcium channel antagonists $CdCl_2$ (50 μM) plus $NiCl_2$ (100 μM) significantly ($p<0.005$) reduced gp120-induced increases of $[Ca^{2+}]_i$ in neurons by 94 % (Table 4) to values not significantly different from background. Both the dihydropyridine nimodipine (100 nM) and the benzothiazepine diltiazem (100 nM) significantly ($p<0.005$) reduced gp120-induced increases of $[Ca^{2+}]_i$ in neurons by about 96 % to values not significantly different from background. Neither
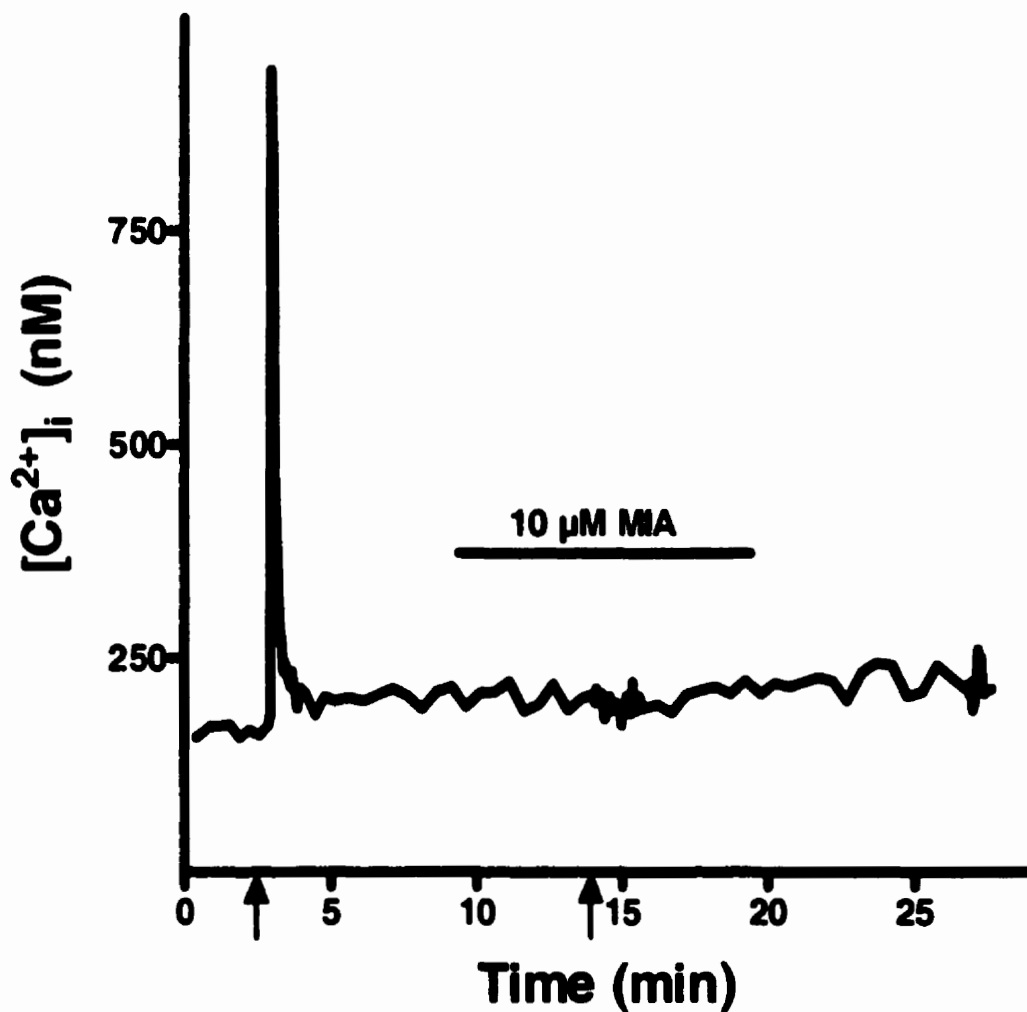
Figure 5: The Na⁺/H⁺ antiporter inhibitor MIA blocked gp120-induced increases in [Ca²⁺]ᵢ in human fetal brain cells. A typical increase in $[Ca^{2+}]_i$ in a neuron to gp120 applied at 25 nM (3 X 100 ms, 5 psi; arrow) was 2565 nM, and MIA (10 µM) significantly ($p < 0.001$) reduced gp120-induced increases in $[Ca^{2+}]_i$ to 48 nM.

Table 3. Effects of NMDA receptor antagonists on gp120-induced increases of [Ca^{2+}]$_i$

Treatment	Cell Type	[Ca^{2+}]$_i$ Increase (nM)
gp120	Neurons	1996 ± 169[a]
gp120 + 10 µM AP5	Neurons	181 ± 33[b]
gp120 + 100 µM AP5	Neurons	106 ± 28[b]
gp120 + 100 µM memantine	Neurons	96 ± 8[b]
gp120 + 100 µM dizocilpine	Neurons	1056 ± 159[a,b]
gp120	Astrocytes	1938 ± 179[a]
gp120 + 10 µM AP5	Astrocytes	266 ± 71[b]
gp120 + 100 µM AP5	Astrocytes	79 ± 7[b]
gp120 + 100 µM memantine	Astrocytes	215 ± 16[b]
gp120 + 100 µM dizocilpine	Astrocytes	1633 ± 178[a]
gp120 following washout of NMDA antagonists	Neurons + Astrocytes	2078 ± 143[a]

Table 3: Recombinant gp120 (25 nM) was pressure-applied (3 x 100 ms, 5 psi) onto cells in the absence or presence of the NMDA receptor antagonists AP5 (10 or 100 µM), memantine (100 µM) or dizocilpine (100 µM). Data are mean ± SEM values for at least 35 different cells from 2 separate fetuses. NMDA antagonist in the absence of gp120 did not significantly affect basal [Ca^{2+}]$_i$ (data not shown). [a]p<0.01 compared to background [Ca^{2+}]$_i$. [b]p<0.01 significantly different from control response to gp120.

non-selective (CdCl$_2$ and NiCl$_2$) nor selective (nimodipine and diltiazem) L-type Ca^{2+} channel antagonists significantly blocked Ca^{2+} responses to gp120 in

astrocytes (Table 4). Selective antagonists to P-(ω-agatoxin IVA at 200 nM),

N-(ω-conotoxin GVIA at 5 μM) and Q-(ω-conotoxin MVIIC at 1 μM) type Ca^{2+}

channels did not significantly affect responses to gp120 in neurons or

astrocytes.

Table 4. Effects of calcium channel blockers on gp120-induced increases of $[Ca^{2+}]_i$

Treatment	*Cell Type*	$[Ca^{2+}]_i$ Increase (nM)
gp120	Neurons	1804 ± 167[a]
gp120 + $CdCl_2$ + $NiCl_2$	Neurons	109 ± 11[b]
gp120 + nimodipine	Neurons	78 ± 9[b]
gp120 + diltiazem	Neurons	64 ± 7[b]
gp120	Astrocytes	1528 ± 148[a]
gp120 + $CdCl_2$ + $NiCl_2$	Astrocytes	1440 ± 113[a]
gp120 + nimodipine	Astrocytes	1469 ± 101[a]
gp120 + diltiazem	Astrocytes	1420 ± 130[a]

Table 4: Recombinant gp120 (25 nM) was pressure-applied (3 x 100 ms, 5 psi) onto cells in the absence or presence of the voltage-operated calcium channel blockers $CdCl_2$ (50 μM) + $NiCl_2$ (100 μM), nimodipine (100 nM) or diltiazem (100 nM). Data are mean $\pm$ SEM values (above background $[Ca^{2+}]_i$) for at least 31 different cells from 2 separate fetuses. $[Ca^{2+}]_i$ of cells exposed to $CdCl_2$ + $NiCl_2$, nimodipine or diltiazem in the absence of gp120 were not significantly different from background (data not shown). [a]$p<0.005$ compared to background $[Ca^{2+}]_i$. [b]$p<0.005$ compared to responses to gp120 in the absence of calcium channel blockers.

DISCUSSION

HIV-1 gp120 causes neuronal cell death via apoptosis and/or necrosis, but appears to do so secondary to interacting with glial cells[16]. Proposed mechanisms for gp120-induced neuronal injury/death include increases of $[Ca^{2+}]_i$, increased levels of extracellular glutamate that then activate excitatory amino acid receptors, and increased generation and accumulation of levels of reactive oxygen intermediates[23,33,34,35,36]. Gp120-induced increases of $[Ca^{2+}]_i$ is an issue of importance because increased $[Ca^{2+}]_i$ can activate several Ca^{2+}-dependent processes and lead to neurotoxicity[36,34,37]. Here, we report that increases of $[Ca^{2+}]_i$ observed in neurons appear to be due to gp120-activation of astrocytes and a vicious cycle that includes Na^+/H^+ exchanger, NMDA-type excitatory amino acid receptor, and L-type calcium channel activation.

In these studies, we tested gp120 from two different lymphotropic strains of HIV-1 and found that both were able to induce significant increases in $[Ca^{2+}]_i$ although some differences in the magnitude of responses between the two strains were noted. Because these proteins were obtained from different commercial sources, the possibility that the differences were, in part, due to variations in preparation and storage can not be excluded. Although most studies have used gp120 from lymphotropic strains[12] a recent study showed that, at comparable doses, gp120 from macrophage-tropic strains of HIV was neurotoxic[38]. Hence it remains to be determined if HIV strain differences and in particular gp120 derived from brain-specific strains may differ in their neurotoxic properties. It was clear, however,

that the three-dimensional configuration of this protein was important because repeated freeze/thaw cycles attenuated the activity of gp120, and non-glycosylated gp120 (HIV-1$_{SF2}$) produced a much smaller increase in [Ca^{2+}]$_i$ than did the glycosylated form. These results further indicate that it is the protein framework and not the carbohydrate moities of gp120 that interact with astrocyte cell membranes.

A number of experimental approaches were used to ensure that the observed actions of gp120 were specific and the concentrations used were appropriate. Specificity was confirmed and artifactual increases of [Ca^{2+}]$_i$ were discounted because no statistically significant changes in [Ca^{2+}]$_i$ were observed with pressure-applied Krebs buffer, BSA, supernatant from immunoabsorbed gp120, or freeze/thawed gp120. Furthermore, gp120-induced increases of [Ca^{2+}]$_i$ did not result from changes in cell membrane integrity because gp120 did not allow cell impermeable Fura-2 to enter cells, bind free [Ca^{2+}]$_i$, and increase fluorescence signals. Picomolar concentrations of gp120 initiate neuropathological processes in vivo[39] and we found that concentrations of gp120 as low as 5 pM were able to induce significant increases of [Ca^{2+}]$_i$ in neurons and astrocytes; a dose lower than that necessary to elevate [Ca^{2+}]$_i$ and cause neurotoxicity in vitro in other studies[12]. In terms of cellular specificity, responses of astrocytes and neurons to gp120 were not subsequent to activation of microglia because only cultured cells treated with L-leucine methyl ester were used. However, it was clear that neuronal responses to gp120 occurred after [Ca^{2+}]$_i$ increased in astrocytes. This

later finding is consistent with findings that astrocytes, but not neurons, have binding sites for gp120[25,40,41].

Gp120 was shown previously to stimulate Na^+/H^+ exchange, cause cellular alkalization, increase K^+ conductance through interactions with apamin-sensitive K^+ channels, increase excitatory amino acid release, and all of these effects were blocked by the Na^+/H^+ exchange inhibitor amiloride[29,31,28]. These actions may form a vicious cycle not unlike one previously proposed[42] whereby stimulation of Na^+/H^+ exchange precedes, and is an important regulator of, gp120-induced increases of $[Ca^{2+}]_i$. Stimulation of Na^+/H^+ exchangers on astrocytes would release glutamate and K^+, cause neuronal depolarization, activate neuronal voltage-operated calcium channels and excitatory amino acid receptors, and participate in gp120-induced neurotoxicity. Although we have no data to support this, it is likely that subtype 1 of Na^+/H^+ exchangers (NHE1) was responsible for these effects because of the 4 subtypes identified only NHE1 and NHE4 have been found in brain and NHE4 is not inhibited by MIA[43]. Our results are consistent with, and three lines of evidence in the current study support, this model. First, we found that gp120-induced increases of $[Ca^{2+}]_i$ in astrocytes and neurons were blocked by the amiloride analog MIA as well as by the absence of Na^+ ions. These findings help explain previous results that tetrodotoxin, a Na^+ channel blocker, protected against gp120 neurotoxicity[44]. Second, we found that AP5, a competitive NMDA receptor antagonist and memantine, a non-competitive NMDA receptor antagonist, blocked gp120-induced increases of

$[Ca^{2+}]_i$ in neurons as well as astrocytes. These findings suggest that subsequent to gp120 application, NMDA receptor-channel complexes are activated, extracellular calcium enters, and glutamate release may be enhanced. Neurons and astrocytes express NMDA- and non-NMDA-type ionotropic glutamate receptors[45,46] and stimulation of neuronal NMDA receptors by gp120 is secondary to the production of arachidonic acid, inhibition of glutamate re-uptake, and increased accumulation of extracellular glutamate[27]. These events may lead to excitation of and injury to neighboring neurons. In our human cell model system, this cycle was overridden by the open-channel blocker memantine, and our results lend support to the use of memantine as an effective therapeutic agent against AIDS dementia complex. Memantine is currently in clinical trials for this purpose (NIAID ACTG 301). Third, we found that a broad spectrum of L-type voltage-operated calcium channel blockers, but not N-, P-, or Q-type blockers significantly reduced responses to gp120 in neurons, but not in human fetal astrocytes. These findings are consistent with recent findings that astrocytes do not express functional L-type voltage-operated calcium channels[47] and may help explain why voltage-operated calcium channel blockers (U.S. Patent # 5,614,560) failed in clinical trials against HIV-1 dementia[48]. Because Na^+/H^+ exchangers are present on neurons and astrocytes, we interpret our results that gp120 stimulates increases of $[Ca^{2+}]_i$ in astrocytes prior to responses in neurons as further evidence that gp120 binds to and activates binding sites on astrocytes[40] that once activated cause receptor-mediated changes in neurons and astrocytes.

Although $[Ca^{2+}]_i$ can change as a result of influx of extracellular calcium or release from intracellular pools, we found that gp120-induced increases of $[Ca^{2+}]_i$ in human fetal brain cells were due solely to influx of extracellular calcium, despite suggestions in the literature that gp120 may release Ca^{2+} from intracellular pools[36,49,50,21,34]. Our results showing that thapsigargin did not alter gp120-induced increases of $[Ca^{2+}]_i$ and that gp120 was unable to elicit a response in the absence of extracellular Ca^{2+} suggest strongly a lack of significant involvement of intracellular pools of Ca^{2+} in the observed responses.

Thus, Na^+/H^+-exchange and NMDA receptor antagonists, may, through their ability to block gp120-induced increases of $[Ca^{2+}]_i$ in neurons as well as astrocytes, help attenuate neuronal dysfunction and cell loss associated with HIV-1 dementia. Further, the ability of drugs to inhibit gp120-induced increases of $[Ca^{2+}]_i$ in neurons and astrocytes may be an additional screen[51] for drugs to nter pre-clinical trials.

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Chapter 3

Involvement of IP$_3$-Regulated Stores of Intracellular Calcium in Calcium Dysregulation and Neuron Cell Death Caused by HIV-1 Protein Tat

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ABSTRACT

HIV-1 infection commonly leads to neuronal cell death and a debilitating syndrome known as AIDS-related dementia complex. The HIV-1 protein Tat is neurotoxic and because cell survival is affected by levels of intracellular calcium ($[Ca^{2+}]_i$), we determined mechanisms by which Tat increased $[Ca^{2+}]_i$ and the involvement of these mechanisms in Tat-induced neurotoxicity. Tat increased $[Ca^{2+}]_i$ dose-dependently in cultured human fetal neurons and astrocytes. In neurons, but not astrocytes, we observed biphasic increases of $[Ca^{2+}]_i$. Initial transient increases were larger in astrocytes than in neurons and in both cell types were significantly attenuated by antagonists of IP$_3$-mediated $[Ca^{2+}]_i$ release - TMB-8 and xestospongin, an inhibitor of receptor-G$_i$ protein coupling - pertussis toxin, and a phospholipase C inhibitor -neomycin. Tat significantly increased levels of IP$_3$ three-fold. Secondary increases of neuronal $[Ca^{2+}]_i$ in neurons were delayed and progressive as a result of excessive calcium influx, and were inhibited by glutamate receptor antagonists ketamine, MK801, AP5, and DNQX. Secondary increases of $[Ca^{2+}]_i$ did not occur when initial increases of $[Ca^{2+}]_i$ were prevented with TMB-8, xestospongin, pertussis toxin, or neomycin, and these inhibitors as well as thapsigargin inhibited Tat-induced neurotoxicity. These results suggest that Tat, via pertussis toxin-sensitive phospholipase C activity induces calcium release from IP$_3$-sensitive intracellular stores which leads to glutamate receptor-mediated calcium influx, dysregulation of $[Ca^{2+}]_i$, and Tat-induced neurotoxicity.

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HIV-1 infection is the most common CNS infection in the world and the leading

cause of dementia in North Americans < 60 years of age. Patients with HIV-1

associated dementia (AIDS-related dementia complex) suffer from deficits in

motor control, cognition and behavior [1] and typically survive only a few months

after onset. Morphological changes including inflammation and loss of select

populations of neurons have been described in HIV-1 positive patients and these

changes have been implicated in the pathogenesis of HIV-1 dementia [2]. In brain,

macrophage/microglial cells and to a lesser degree astrocytes are productively

infected with HIV-1 [3-6]. With the exception of isolated reports [4,7] there is little

evidence for direct infection of neurons with HIV-1. Neurotoxicity is therefore

attributed to indirect mechanisms, such as the release from infected cells of

potentially toxic viral encoded proteins and cellular factors [8]. HIV-1 proteins that

show varying degrees of neurotoxicity include the envelope glycoproteins gp120,

and gp41, and non-structural regulatory proteins Nef, Rev, and Vpr and Tat [8].

Increasingly, over the past few years, attention has focused on the neurotoxic

properties of Tat, an HIV-1 viral protein whose first exon contributes to the initial

72 amino acids and the second exon forms the remaining 14 to 32 amino acids.

Tat mRNA levels are elevated in brains of patients with HIV-1 dementia [9] and

HIV encephalitis [10]. Tat protein is present in the blood of HIV-infected individuals

[11], can be detected in brains of patients with HIV encephalitis and in macaque

monkeys infected with a chimeric simian-human immunodeficiency virus [12], and

is released from HIV-infected and *tat*-transfected cells [13] via a leaderless

secretory pathway [14]. Extracellularly, full length Tat$_{1-86}$ and to a much lesser extent Tat$_{1-72}$, both of which transactivate the long terminal repeat of HIV-1, are taken up into neural cells and accumulate in the nucleus [15]. Tat has been shown to bind to cell membranes [16] and specifically to a 90-kDa cell surface protein on neural tissue-derived PC12 and NG108-15 cells [17].

Functionally, Tat is a promiscuous effector of cellular function. Tat has been found to decrease expression of superoxide dismutase [18], increase NF-κB binding, increase protein kinase C activity [19, 20], increase production of cytokines [21-23] and chemokines [24], activate phosphatidylinositol 3–kinase [25], and increase chemotaxis [26]. Interestingly, Tat has been reported to have both protective [27, 28] and toxic effects [29-35] *in vitro* and *in vivo*. However, in contradistinction to other neurotoxic HIV-1 proteins, Tat directly activated neurons and depolarized neuronal cell membranes [36, 37]. Furthermore, only a transient exposure of Tat may be sufficient to produce neuronal cell death or glial cell activation [35]. Tat and Tat fragment-induced membrane depolarizations, neurotoxicity and apoptosis, and increases in levels of intracellular calcium ($[Ca^{2+}]_i$) were blocked, to varying degrees by inhibitors of caspase activation and calcium mobilization, anti-oxidants, and glutamate receptor antagonists [34, 37].

Increased $[Ca^{2+}]_i$ as a result of either influx of extracellular calcium through plasma membrane voltage- and receptor-operated calcium channels or by

release from calcium stored in intracellular pools is an important cause of cell death [38] and has been ascribed a central role in HIV-1 protein-induced neuronal cell death [8, 39]. The two intracellular pools defined to date appear to be distinct entities both in terms of the receptors that regulate them and the pools themselves - one is regulated by IP_3 receptors and the other by ryanodine receptors [38]. Defining the different pools of calcium that are mobilized and determining the mechanism of calcium influx into cells in response to Tat may be important to the development of strategies for therapeutic intervention. In this study, initial transient Tat-induced increases of $[Ca^{2+}]_i$ were observed in neurons and astrocytes, but secondary prolonged increases of $[Ca^{2+}]_i$ were observed only in neurons. The initial spikes of $[Ca^{2+}]_i$ were due to pertussis toxin-sensitive release of calcium from IP_3-sensitive intracellular pools while the secondary increases of $[Ca^{2+}]_i$ in neurons were blocked by antagonists of glutamate receptors. Pharmacological blockade of initial $[Ca^{2+}]_i$ spikes in neurons was sufficient to inhibit Tat-induced calcium dysregulation and neurotoxicity.

MATERIALS AND METHODS

Chemicals and Recombinant Tat$_{1-72}$: Fura-2-acetoxymethyl ester (Fura-2/AM) was obtained from Molecular Probes Inc. (Eugene, OR). Dizocilpine maleate (MK-801), (+)-5-methyl-10,11,dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine hydrogen maleate), (±)-2-amino-5-phosphonopentanoic acid (AP5), and ryanodine were purchased from Research Biochemicals International (Natick, MA). EGTA, L-leucine methyl ester and neomycin were purchased from Sigma Chem. Co. (St. Louis, MO). TMB-8 ([8-(diethylamino)octyl-3,4,5-trimethoxybenzoate, HCl]), pertussis toxin, and xestospongin were purchased from Calbiochem (San Diego, CA). DNQX (6,7-dinitroquinoxaline-2,3-dione) was purchased from Tocris Cookson (Ballwin, MO). Antibodies against the astrocyte marker protein glial fibrillary acidic protein (GFAP), the neuron marker protein microtubule-associated protein 2 (MAP-2), and the microglia marker protein EBM-11 were purchased from Chemicon (Temecula, CA.), Boehringer Mannheim (Laval, P.Q.), and Dako (Denmark), respectively. All other reagents were of analytical grade or the highest purity available. All drugs were dissolved in Krebs buffer except for ryanodine that was dissolved in ethanol and diluted with Krebs (final ethanol concentration was 0.05 %). Polyclonal Tat antiserum against the recombinant Tat protein was raised in rabbits and purified on a protein A sephadex column. The antisera recognized both monomeric and dimeric Tat, but did not cross-react with any bacterial proteins or LPS as determined by western blot analysis.

Tat$_{1-72}$ was prepared as described previously [15]. Tat protein was determined to be at least 95% pure by SDS-PAGE and silver staining. By western blot analysis > 90% of the Tat was determined to be monomeric (14 KDa); the remaining was dimeric Tat (28 KDa). Tat's biological activity was confirmed by activation of β-galactosidase in transfected HeLa cells (AIDS Repository, NIH). Tat protein was lyophilized and stored at - 80°C until used. All studies were performed using Tat$_{1-72}$ because the neurotoxic domain is contained in this protein [34]. We had previously shown that Tat$_{31-61}$ could increase [Ca^{2+}]$_i$ in neurons, however the magnitude of the responses were much smaller than those induced by Tat$_{1-72}$ and much higher concentrations of Tat$_{31-61}$ were required. Amounts of Tat were reported in pmoles to reflect the doses of Tat that were pressure-applied onto the cells. Results from our previous experiments demonstrated that the concentration of Tat that reached cells following pressure-application was at-least 10-fold less than the concentration of Tat in the micropipettes [36]. Tat specificity was determined by incubating Tat with 1:100 dilutions of rabbit anti-tat serum or normal rabbit serum bound to protein A-coated agarose beads (Pharmacia) for 90 min at room temperature and this was followed by centrifugation. The supernatants were tested for effects on [Ca^{2+}]$_i$. Tat was also treated with 0.05% trypsin (Life Tech Inc.) for 30 min at 37°C and following the addition of trypsin inhibitor (Sigma, final concentration 0.1%) for 30 min at 37°C, the effects on [Ca^{2+}]$_i$ were determined.

Cultured fetal human brain cells: Brain specimens from fetuses at gestational ages of 12 to 15 weeks were obtained, with consent, from women undergoing elective termination of pregnancy. All aspects of these studies received approval from the University of Manitoba's Committee for Protection of Human Subjects. Blood vessels and meninges were removed and brain tissue was washed in Opti-MEM (GIBCO BRL, Burlington, ON) and mechanically dissociated by repeated trituration through a 20 gauge needle. Cells were centrifuged at 270 g for 10 min and resuspended in Opti-MEM with 5 % heat-inactivated fetal bovine serum, 0.2 % N2 supplement (GIBCO BRL, Burlington, ON) and 1 % antibiotic solution (10^4 U of penicillin G/ml, 10 mg streptomycin/ml and 25 µg amphotericin B/ml in 0.9 % NaCl). Approximately 10^6 cells were plated in 75 cm^2-T flasks and incubated for 4-6 weeks in a humidified environment at 37°C (95% O_2/5% CO_2). Flasks were gently tapped manually, and cells released into the supernatant were collected and plated onto 33-mm diameter poly-D-lysine-coated glass coverslips for an additional 5 – 10 days prior to use. These re-plated cells were enriched in neuronal populations, developed neuritic processes, and had normal resting membrane potentials [36]. Sample cells were immunostained for MAP-2 and only wells with >70% neurons were used for experimentation. The remaining cells were predominantly astrocytes as determined by GFAP immunostaining. Cells were treated with 7.5 mM L-leucine methyl ester for 12 h to remove microglia; prior to L-leucine methyl ester treatment microglia that stained positively for EBM-11 (Dako, Denmark) were in-frequently (<1%) observed and after treatment were not observed.

Intracellular Calcium Determinations: [Ca^{2+}]$_i$ were determined using the

Ca^{2+}-specific fluorescent probe Fura-2/AM as described previously [40]. Cells were

incubated for 1 h at 25°C in Krebs-BSA buffer consisting of (in mM): 111 NaCl,

26.2 $NaHCO_3$, 1.2 NaH_2PO_4, 4.7 KCl, 1.2 $MgCl_2$, 15 HEPES, 1.8 $CaCl_2$, 5

glucose, 1.5 μM bovine serum albumin (BSA) and 2 μM Fura-2/AM. Cells were

washed 3-times with Krebs (no BSA) to remove extracellular Fura-2 and were

incubated at 37°C for 5 min. Coverslips containing Fura-2 loaded cells were

placed in a LU-CSD Leiden coverslip dish situated in a PDMI-2 open perfusion

micro-incubator (Medical Microsystems Corp., Greenvale, NY) and the cells were

superfused at the rate of 2 ml/min with KREBS buffer pre-warmed to 37°C. Cells

were excited at 340 and 380 nm, and emission was recorded at 510 nm with a

video-based Universal imaging system (EMPIX, Missassauga, ON). R_{max}/R_{min}

ratios were converted to nM [Ca^{2+}]$_i$ as described previously [41]. Calibrations for

[Ca^{2+}]$_i$ were conducted using calcium standards obtained from Molecular Probes

(Eugene, OR) and [Ca^{2+}]$_i$ in cultured cells were obtained by comparing ratio

fluorescence values with generated standard curves. Images were acquired

every 30 seconds during baseline, every 5 sec for the first 5 min after

experimental treatments and approximately every 15 sec thereafter for the

remaining 40 min. At this acquisition rate we were able to maintain stable

baseline [Ca^{2+}]$_i$ levels in control experiments. An eight wheel rotary valve allowed

switching between normal KREBS, and buffers containing nominal calcium (no

added Ca^{2+}) or zero Ca^{2+} (no added Ca^{2+} plus 2 mM EGTA). TMB-8 (100 μM)

and neomycin (100 µM) were added during Fura-2/AM loading. Ketamine (100 µM), dizocilpine (10 and 100 µM), AP5 (100 µM) and DNQX (100 µM) were bath-applied 5 min prior to Tat. Tat (100 nM to 100 µM) was loaded into glass micropipettes (1.0 mm OD, 0.78 mM ID) pulled to a final outer tip diameter of < 1.0 µm. Micropipettes were positioned approximately 3 cell bodies away from target cells and Tat was pressure-applied to cells (3 X 100 msec, 8 psi) using a Picospritzer (General Valve Corp, Fairfield, NJ). Cells within 5 cell body widths of the micropipette were monitored for time periods up to 1 h. At these concentrations of Tat in the micropipettes, and having calculated that under our experimental conditions (micropipette diameter, tip diameter, and pressure and duration of applications) 0.04 µl were pressure-ejected from the micropipettes, it was determined that 4 fmoles (100 nM in pipette) to 4 pmoles (100 µM in pipette) of Tat were applied to the neural cells.". Peak increases of $[Ca^{2+}]_i$ were determined by subtracting the maximum $[Ca^{2+}]_i$ achieved during a 5 min period following Tat applications from baseline $[Ca^{2+}]_i$. Increases of $[Ca^{2+}]_i$ greater than two standard deviations above those produced from vehicle applications (> 39 nM increase over basal levels in neurons and astrocytes) were considered positive responses and were included for data analyses. $[Ca^{2+}]_i$ were averaged over the cell body and large processes. Significant differences between groups were determined by one-way ANOVA with Tukey's post-hoc comparisons.

Neurotoxicity Assay: Tat (125 nM) was applied to cultured human fetal brain cultures in Locke's buffer consisting of (in mM) 154 NaCl, 5.6 KCl, 2.3 $CaCl_2$, 1.0

MgCl$_2$, 3.6 NaHCO$_3$, 5.0 glucose and 5.0 HEPES (pH 7.2). Following incubation

for 12 h, cell death was determined using a trypan blue exclusion technique;

approximately 250 cells were examined in each of 5 pre-determined fields per

experiment and each experiment was conducted three-times and used cells from

two separate fetuses; over 4000 cells were counted for each experimental

condition. Neomycin (100 µM), thapsigargin (100 nM), or xestospongin (10 µM)

was applied to cultures 30 min prior to Tat. At the time of counting, the

investigator was unaware of the identity of the treatment groups.

Immunohistochemistry: Human fetal brain cells grown on glass coverslips were

rinsed 3-times in phosphate-buffered saline (PBS), fixed in acetone/methanol

(1:1) for 30 min at ˉ20°C, washed 3-times with PBS, and incubated for 30 min at

room temperature with a PBS solution containing 10% horse serum and 1%

BSA. The neuron specific marker MAP-2 (1:1000; anti-mouse) and the astrocyte

specific marker GFAP (1:100; anti-rabbit) were applied to coverslips for 90 min at

room temperature. Cells were washed 3-times with PBS and secondary

antibodies conjugated to fluorescein (anti-mouse; 1:50) and CY3 (anti-rabbit;

1:50) were added to coverslips for 30 min at room temperature. Coverslips were

inverted onto glass slides with adhesive mount and stored at 4°C for no longer

than 5 days before viewing. Cells were examined and photographed with a Nikon

Optiphot fluorescent light microscope using 40x magnification with excitation

filters 546 ± 10 nm for CY3 and 485 ± 22 nm for fluorescein with respective

excitations of 580 ± 30 nm and 530 ± 30 nm (Omega Optical). A bravado frame grabber board (True Vision) was used for video digitization.

D-Myo-Inositol-1,4,5-Trisphosphate (IP₃) Measurements: Culture flasks especially enriched (> 85%) in neurons (the remaining cells were astrocytes) were shaken lightly, and cells were centrifuged for 5 min at 500 x g, and suspended in buffer containing in mM; 25 $NaHCO_3$, 118 NaCl, 4.7 KCl, 1.2 mM KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 10 D-glucose, and 10 HEPES. Neurons were incubated in the absence or presence of 700 nM HIV-1 Tat protein or 100 µM ATP (positive control) for times ranging from 5 to 60 sec. Reactions were stopped by adding an equal volume of 1 M trichloroacetic acid and following placement on ice for 15 min, samples were centrifuged for 15 min at 5000 x g. Supernatants (160 µl) were removed and 40 µl of 10 mM EDTA and 200 µl of freon/octylamine (1:1) mix was added. Samples were shaken vigorously for 30 sec, placed on ice for 15 min, centrifuged at 5000 g for 15 min, and 100 µl of upper phase was added to vials containing 50 µl of 25 mM $NaHCO_3$. IP_3 was measured by radioreceptor assay performed on ice in a final volume of 200 µl. Samples (50 µl) or 10 µM IP_3 (to define non-specific binding) were added to 50 µl of 100 mM Tris-base/ 4 mM EDTA, pH 8.0, 50 µl of [^{3}H]IP_3 (≈ 5500 dpm/tube), and 50 µl of bovine adrenal-cortical binding protein. Samples were incubated for 2 h. Bound and unbound [^{3}H]IP_3 were separated by rapid filtration through presoaked Whatman GF/B glass-fiber filters that were then washed twice with 5 ml of ice cold 20 mM Tris-base/1 mM EDTA buffer, pH 8.0. Radioactivity was

determined by liquid scintillation spectroscopy. Protein content in pellets was

determined using bovine serum albumin as the reference standard.

RESULTS

Specificity and dose-related effects of Tat$_{1-72}$ on [Ca^{2+}]$_i$: Baseline levels of [Ca^{2+}]$_i$ were 149 ± 6 nM (n=45) in neurons and 151 ± 4 nM (n=42) in astrocytes. Pressure-application of 0.5 pmoles of Tat, but not buffer, significantly (p < 0.001) increased [Ca^{2+}]$_i$ over baseline levels by about three-fold in neurons and about eight-fold in astrocytes (Table 1). These results demonstrated that the effects of

Table 1. Tat Specificity.

Treatment	Neurons	Astrocytes
Tat (2 pmoles)	276 ± 52[a]	733 ± 63[a]
Rabbit serum + Tat	275 ± 75[a]	541 ± 96[a]
Anti-Tat + Tat	40 ± 4[b]	28 ± 5[b]
Trypsin + Tat	36 ± 5[b]	134 ± 5[b]

Table 1: Tat specificity. Conjugation of Tat with polyclonal anti-Tat and trypsinization of Tat both inhibited Tat-induced increases of [Ca^{2+}]$_i$ in neurons and astrocytes. Buffer pressure-applied to neurons and astrocytes did not significantly affect levels of [Ca^{2+}]$_i$ (data not shown). Levels of [Ca^{2+}]$_i$ were determined in 14 to 99 cells from 3 to 4 experiments and two separate fetuses per condition. [a]Indicates p < 0.001 compared with baseline [Ca^{2+}]$_i$. [b]Indicates p < 0.001 compared with Tat or rabbit serum plus Tat.

Tat on [Ca^{2+}]$_i$ could be observed at very low doses and were not artifacts caused by mechanical stimulation of cells following pressure application. Tat protein was specifically responsible for increasing [Ca^{2+}]$_i$ because overnight digestion of Tat with trypsin significantly (p < 0.001) reduced peak increases in [Ca^{2+}]$_i$ by 87 % in neurons and by 82 % in astrocytes (Table 1). Furthermore, conjugation of Tat
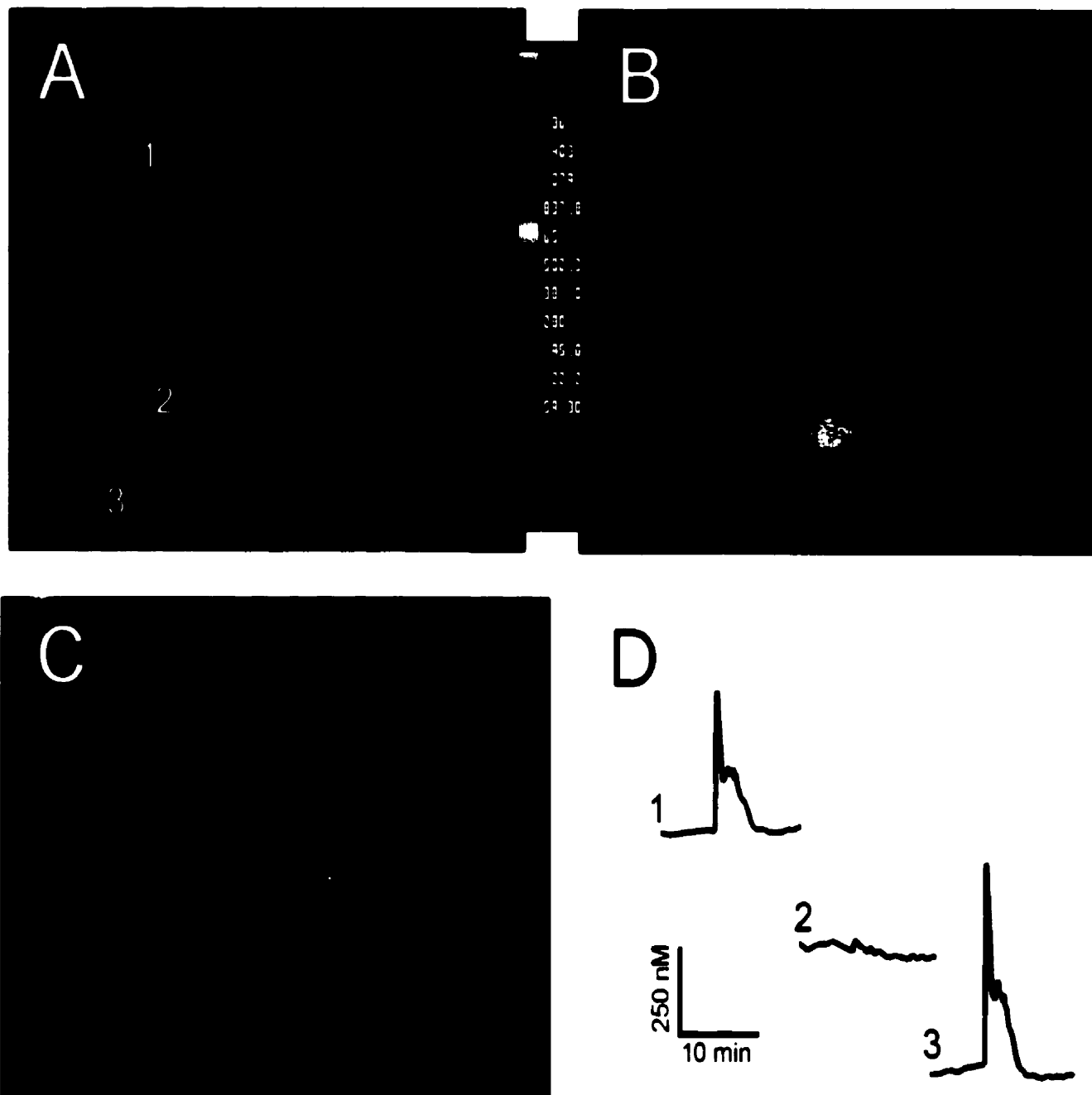
Figure 1: Tat (2 pmoles) increased $[Ca^{2+}]_i$ in immunohistochemically-identified human fetal neurons. In panel A, baseline levels of $[Ca^{2+}]_i$ were measured in neurons numbered 1, 2 and 3. In panel B, Tat applied to these three neurons increased $[Ca^{2+}]_i$ in neurons 1 and 3, but not 2. In panel C, all three neurons stained positive for MAP-2 and in panel D, $[Ca^{2+}]_i$ was graphically represented.

with polyclonal anti-Tat significantly ($p < 0.001$) reduced peak increases of $[Ca^{2+}]_i$ by 86 % in neurons and 96 % in astrocytes (Table 1).

Doses of Tat pressure-applied to cells were expressed as the amount (pmoles) of Tat calculated to have been ejected from micropipettes. In preliminary studies we found that proportionately greater increases of $[Ca^{2+}]_i$ were observed with up to 9 pressure applications (100 msec, 8 psi) of Tat; maximal responses were observed with greater than 9 pulses (data not shown). This was the first indication that responses to Tat were dose-related. Further experimentation demonstrated dose-dependent increases of $[Ca^{2+}]_i$ in cultured human fetal neurons and astrocytes (Table 2). The apparent ED_{50} value for neurons was 2.5 pmoles and for astrocytes, was 1.6 pmoles (data not shown). Responses to Tat were greater in astrocytes than neurons at all concentrations tested. In all experiments, cells were identified morphologically and in select experiments were identified post-imaging by immunostaining with MAP-2 or GFAP. Illustrated in Figure 1 is a typical experiment where Tat-induced increases of $[Ca^{2+}]_i$ were determined in neurons that stained positive for the neuronal marker protein MAP-2.

$[Ca^{2+}]_i$ response profiles: Two basic profiles of Tat-induced increases of $[Ca^{2+}]_i$ were observed – initial transient increases and secondary prolonged increases. Three patterns for Tat-induced initial transient increases of $[Ca^{2+}]_i$ were observed;

Table 2: Amplitude of increase in [Ca^{2+}]$_i$ (nM) and occurrence (percentage) of three patterns (single, sustained and multiple spikes) of Tat-induced initial transient increases of [Ca^{2+}]$_i$ in neurons and astrocytes following pressure application of 0.5 to 4.0 pmoles Tat.
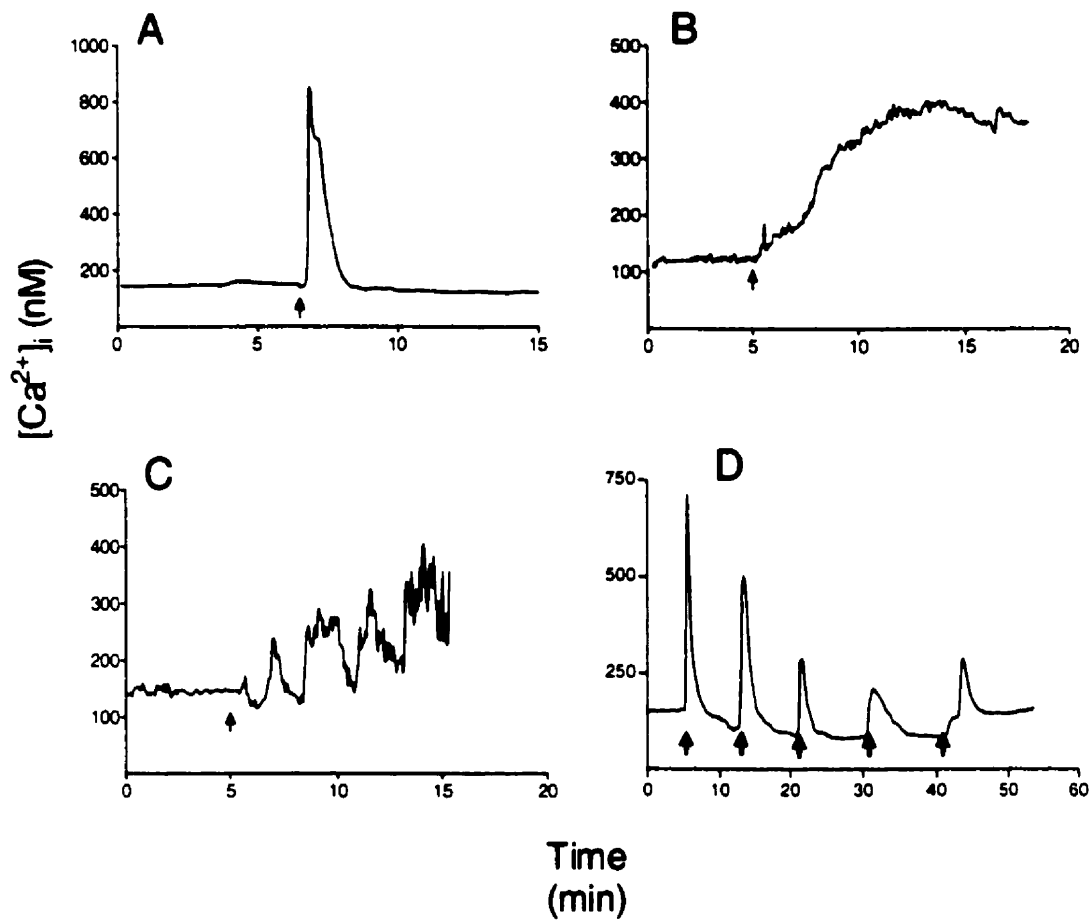
Tat (pmoles)	Response	[Ca^{2+}]$_i$ Increase	
		Neurons	Astrocytes
0.5	Single spike	257 ± 52 (32%)	320 ± 55 (36%)
	Sustained	70 ± 4 (41%)	83 ± 8 (55%)
	Multiple	nm (0%)	nm (0%)
	Non-responders	nm (27%)	nm (9%)
1.0	Single spike	227 ± 52 (40%)	358 ± 65 (58%)
	Sustained	86 ± 18 (36%)	95 ± 12 (26%)
	Multiple	nm (0%)	914 ± 344 (6%)
	Non-responders	nm (24%)	nm (10%)
2.0	Single spike	367 ± 40 (41%)	643 ± 66 (57%)
	Sustained	128 ± 11 (36%)	164 ± 32 (21%)
	Multiple	441 ± 43 (10%)	1471 ± 424 (17%)
	Non-responders	nm (13%)	nm (5%)
4.0	Single spike	733 ± 204 (49%)	1008 ± 293 (57%)
	Sustained	88 ± 10 (18%)	237 ± 48 (19%)
	Multiple	613 ± 236 (15%)	1056 ± 374 (21%)
	Non-responders	nm (18%)	nm (2%)

Table 2: Increases in [Ca^{2+}]$_i$ were listed as increases above baseline levels. Numbers in parentheses were percentages of cells that exhibited that particular response profile. nm = non-measurable

single peaks (Fig. 2A), sustained increases (Fig. 2B), and oscillatory (multiple spike) increases (Fig. 2C). Time to maximum [Ca^{2+}]$_i$ for cells with single spike profiles were 3 ± 2 s (neurons) and 3 ± 5 s (astrocytes), and for sustained

increases were 320 ± 83 s (neurons) and 290 ± 64 s (astrocytes). There was little change in time to maximum $[Ca^{2+}]_i$ with increasing doses (data not shown). Peak increases of $[Ca^{2+}]_i$ associated with each response type and their relative occurrence rates (percentages) for neurons and astrocytes are listed in Table 2. Parenthetically, we often found that single spike $[Ca^{2+}]_i$ responses predominated in cells closest to the application site. In astrocytes, the relative frequency of single-spike responses increased from 0.5 to 1.0 pmoles and remained constant from 1.0 to 5.0 pmoles. Oscillations (multiple peaks) were only seen at doses of > 1.0 pmoles in neurons and > 0.5 pmoles in astrocytes. At doses of Tat > 2.0 fmoles in neurons and > 0.5 pmoles in astrocytes we observed a decreased occurrence of sustained increases of $[Ca^{2+}]_i$ in both neurons and astrocytes. At each of the four doses of Tat tested, we observed a greater percentage of non-responding neurons than astrocytes, and for both cell types the percentage of non-responders decreased with increased doses (Table 2). All subsequent studies were conducted on cells exhibiting only single spike initial transient increases in $[Ca^{2+}]_i$.

Neurons and astrocytes desensitized, partially, to repeated applications of 2 pmoles Tat (Fig. 2D). In neurons (n=8), Tat-induced increases in $[Ca^{2+}]_i$ were reduced by up to two-thirds with successive Tat applications. Similarly, about

Figure 2: Three profiles of Tat (2 pmoles) induced initial transient increases of $[Ca^{2+}]_i$ in neurons; single spike (A), sustained (B) and oscillatory (C). All three patterns were observed in neurons and astrocytes; only neuronal responses were illustrated. (D) Representative trace showing partial desensitization of Tat-induced initial transient increases of $[Ca^{2+}]_i$ following repeated application of 2 pmoles Tat onto a single neuron. Arrows indicate Tat applications.

one-third desensitization was observed in astrocytes (data not shown). Superfusion of cells for 30 min with calcium containing buffer did not restore $[Ca^{2+}]_i$ responses to levels achieved with initial applications of Tat (data not shown). These findings are more consistent with partial desensitization of a receptor/signal transduction system than with calcium store depletion.

In addition to the three response patterns of initial transient Tat-induced increases of $[Ca^{2+}]_i$, secondary prolonged increases were observed in 30 of 42 neurons (Fig. 3A). When Tat was applied to cells bathed in nominally calcium-free buffer (nominal calcium) initial transient Tat-induced increases of $[Ca^{2+}]_i$ were not significantly affected and secondary prolonged increases of $[Ca^{2+}]_i$ in 31 of 31 neurons were completely absent thus implicating calcium influx in this delayed event (Fig. 3B). Second applications of Tat in calcium-free buffer produced significantly blunted increases of $[Ca^{2+}]_i$ likely owing to a requirement of extracellular calcium influx to re-fill intracellular pools (Fig. 3B and 3C). When extracellular calcium was re-introduced to cells exposed to Tat, secondary prolonged rises of $[Ca^{2+}]_i$ were again observed in 15 of 17 neurons (Fig. 3C).

*Involvement of extra- and intra-cellular calcium pools in Tat-induced increases of* $[Ca^{2+}]_i$: Under conditions of zero extracellular calcium (0 Ca^{2+} plus 2.0 mM EGTA), Tat-induced initial transient increases of $[Ca^{2+}]_i$ were not significantly different from increases when Tat was applied to cells bathed in calcium containing buffer (Fig. 4A). TMB-8 (100 µM), an inhibitor of intracellular calcium
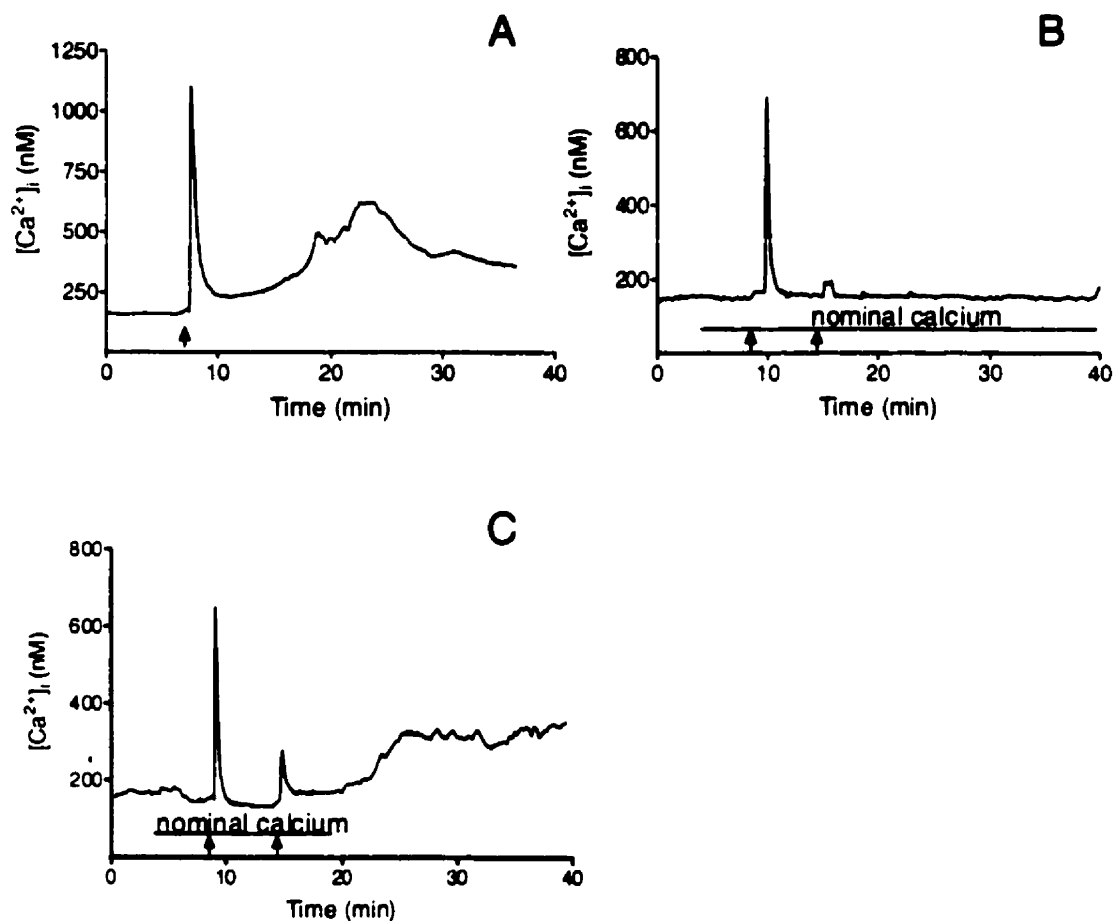
Figure 3: Effects of Tat (2 pmoles) on $[Ca^{2+}]_i$ in human fetal neurons. (A) Initial transient Tat-induced increases of $[Ca^{2+}]_i$ were frequently accompanied by secondary prolonged increases of $[Ca^{2+}]_i$. (B) In the absence of extracellular calcium (nominal calcium), initial transient Tat-induced increases of $[Ca^{2+}]_i$ were slightly diminished, but responses to second applications of Tat were dramatically reduced and secondary prolonged increases in $[Ca^{2+}]_i$ were completely abolished. (C) Re-perfusion with calcium-containing buffer resulted in a re-emergence of secondary prolonged increases of $[Ca^{2+}]_i$. Traces are representative of 15 to 42 neurons per condition. Arrows indicated points of Tat applications.
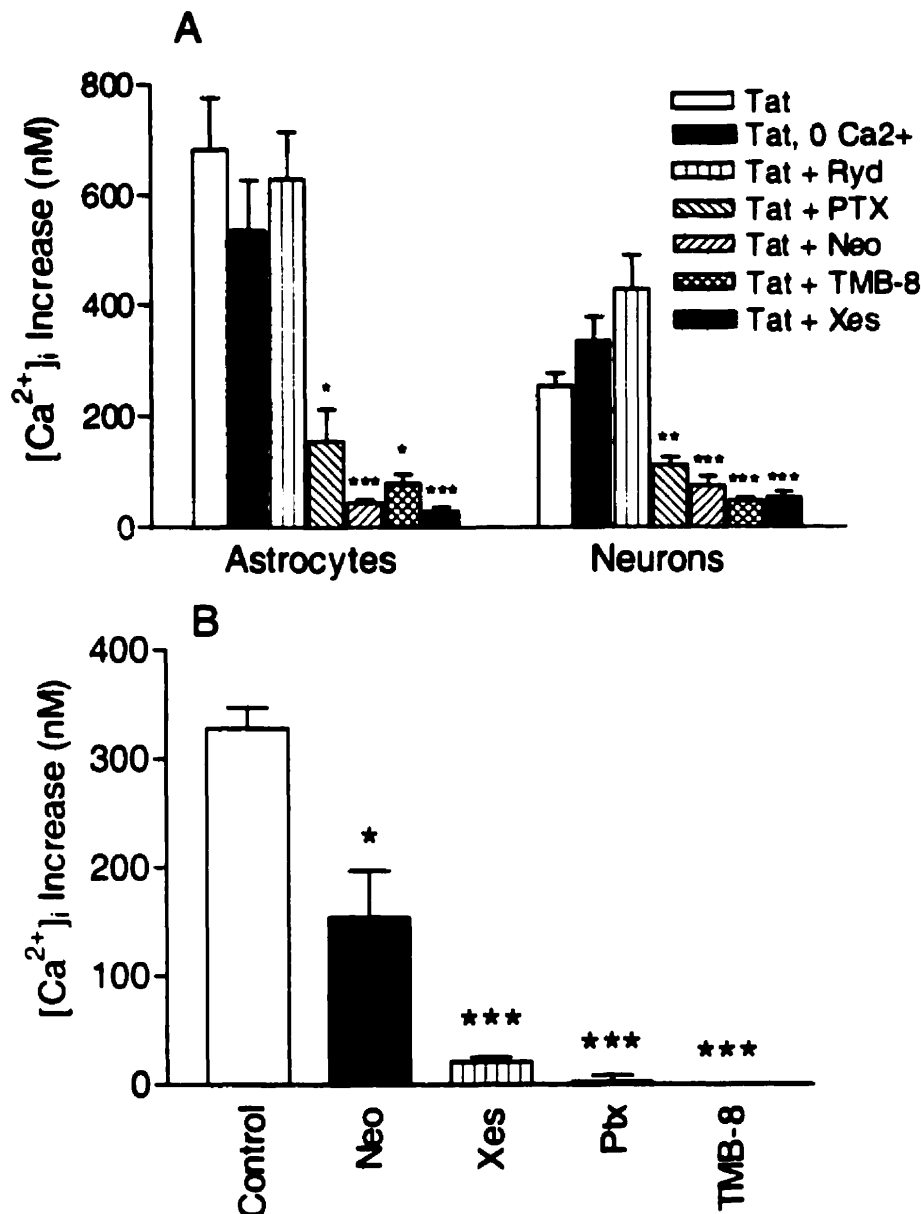
Figure 4: A. Tat (2 pmoles) induced initial transient increases of $[Ca^{2+}]_i$ in human fetal neurons and astrocytes. ADP-ribosylation of G_i with pertussis toxin (PTX), inhibition of phospholipase C activity with neomycin (Neo), and blockade of intracellular calcium release with TMB-8 or xestospongin reduced significantly peak increases of $[Ca2+]_i$ by Tat. Incubation in buffer without calcium ($0\ Ca^{2+}$) or with the intracellular calcium release channel blocker ryanodine (RyD) did not significantly affect peak increases of $[Ca^{2+}]_i$ by Tat. Values are mean and S.E.M. from 21 to 75 cells per condition and were derived from at least 4 different experiments and two separate fetuses per condition. * p < 0.05; ** p < 0.01; ***p < 0.001. B. In neurons, delayed/prolonged secondary increases of $[Ca2+]_i$ induced by 2 pmoles Tat were inhibited when initial transient increases of $[Ca^{2+}]_i$ were prevented. Control values were obtained from the neurons (67 % of total analyzed) that exhibited delayed/prolonged increases in $[Ca^{2+}]_i$ in response to 2 pmoles Tat. Values represent mean ± S.E.M. of 21 - 47 cells derived from at least 4 different experiments and two separate fetuses. * p < 0.05; ***p < 0.001.
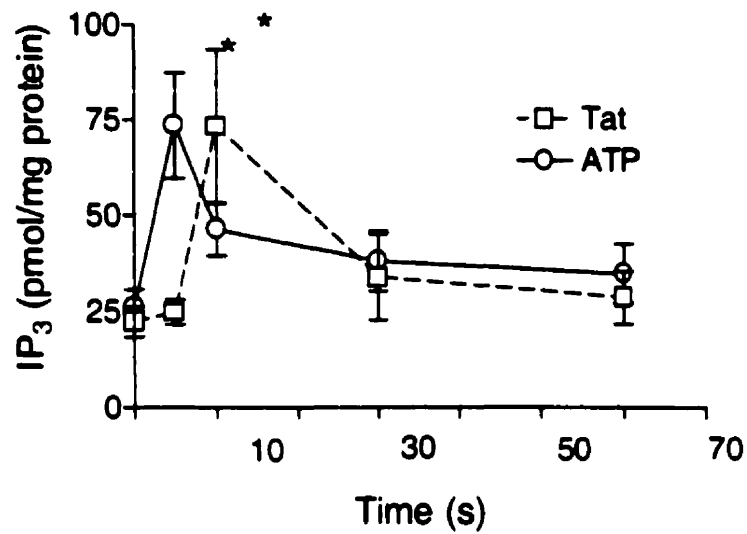
**Figure 5: Time course for Tat- and ATP-induced accumulations of IP₃ in human fetal neurons.** Neurons were incubated in either 700 nM Tat (open squares, solid connecting lines) or 100 µM ATP (open circles, dashed connecting line) for 5, 10, 30 or 60 s. Data were expressed as pmoles IP₃ formed per mg protein. Tat and ATP significantly (* $p < 0.05$) increased IP₃ levels 3.3- and 2.8-fold over controls (0 time), respectively.

release, significantly reduced Tat-induced transient increases of $[Ca^{2+}]_i$ to 48 ±

5.0 nM (p < 0.001) in neurons (n=47) and to 81 ± 16 nM (p < 0.01) in astrocytes

(n=17). The specific inhibitor of IP_3-dependant calcium release, xestospongin

[Gafni et al., 1997], significantly (p < 0.01) reduced Tat-induced initial calcium

transients to 31 ± 7 nM in astrocytes (n=30) and to 54 ± 11 nM in neurons

(n=75). Further implicating IP_3 in the calcium responses to Tat were findings that

neomycin (100 μM), an inhibitor of phospholipase C activity, reduced significantly

(p < 0.001) Tat–induced increases of $[Ca^{2+}]_i$ in neurons (n=25) to 75 ± 17 and in

astrocytes (n=46) to 45 ± 6 nM (Fig. 4A). An additional inhibitor of phospholipase

C, U73122, was used but we found it to interfere with measures of intracellular

calcium in our system. When cells were exposed to pertussis toxin (100 ng/ml)

for 40 min, Tat-induced increases of $[Ca^{2+}]_i$ in neurons (n=21) and astrocytes

(n=9) were reduced significantly to 113 ± 14 (p < 0.01) and 156 ± 57 (p < 0.05),

respectively (Fig. 4A). The inhibitory effects of TMB-8, xestospongin and

pertussis toxin were not due to a general unresponsiveness of the cells because

cells responded to the positive control 50 mM KCl with increases of $[Ca^{2+}]_i$ of 460

± 34 nM in neurons and 525 ± 25 nM in astrocytes that were not significantly

different from those observed in untreated cells (data not shown). Pre-treatment

with ryanodine (10 μM) did not significantly affect Tat-induced increases of $[Ca^{2+}]_i$

in neurons or astrocytes (Fig. 4A). The above results suggested an involvement

of IP_3-regulated pools of intracellular calcium in the observed responses to Tat.

Therefore, we determined the effects of Tat and ATP (a positive control) on the

generation of IP_3 in neurons (Fig. 5). We found that neurons responded to Tat

with increases of IP_3 3.3-fold over baseline levels that were comparable to the responses observed with the positive control ATP. When initial transient Tat-induced increases in $[Ca^{2+}]_i$ were prevented with pertussis toxin, neomycin, TMB-8 or xestospongin, secondary calcium influx was prevented (Figure 4B), thus demonstrating that transient release of Ca^{2+} from intracellular stores, in particular those regulated by IP_3, was a pre-requisite for secondary calcium influx following Tat applications.

Initial transient Tat-induced increases of $[Ca^{2+}]_i$ in neurons and astrocytes were not significantly affected by pre-treatment of cells with non-competitive inhibitors of NMDA receptors - dizocilpine (10 and 100 μM) or ketamine (100 μM), a competitive inhibitor of NMDA receptors - AP5 (100 μM), or the non-NMDA receptor blocker - DNQX (100 μM) (Fig. 6). Secondary calcium influx was inhibited by these four receptor blockers; increases in $[Ca^{2+}]_i$ were 38 ± 17 for MK801, 13 ± 5 for AP5, 46 ± 26 for DNQX, and 2 ± 5 for ketamine (Fig. 6). The relative roles of NMDA vs. non-NMDA receptors was not determined in these studies.

Role of intracellular calcium in Tat-induced neurotoxicity: Overnight exposure of fetal brain cells to Tat resulted in aggregation of cells and the appearance of
spoke like neuritic processes (data not shown). Tat increased significantly (p < 0.001) neuronal death to 16 ± 1 % as compared with 3.6 ± 0.2 % in control cultures (Figure 7). Pre-treatment with neomycin, thapsigargin, or xestospongin
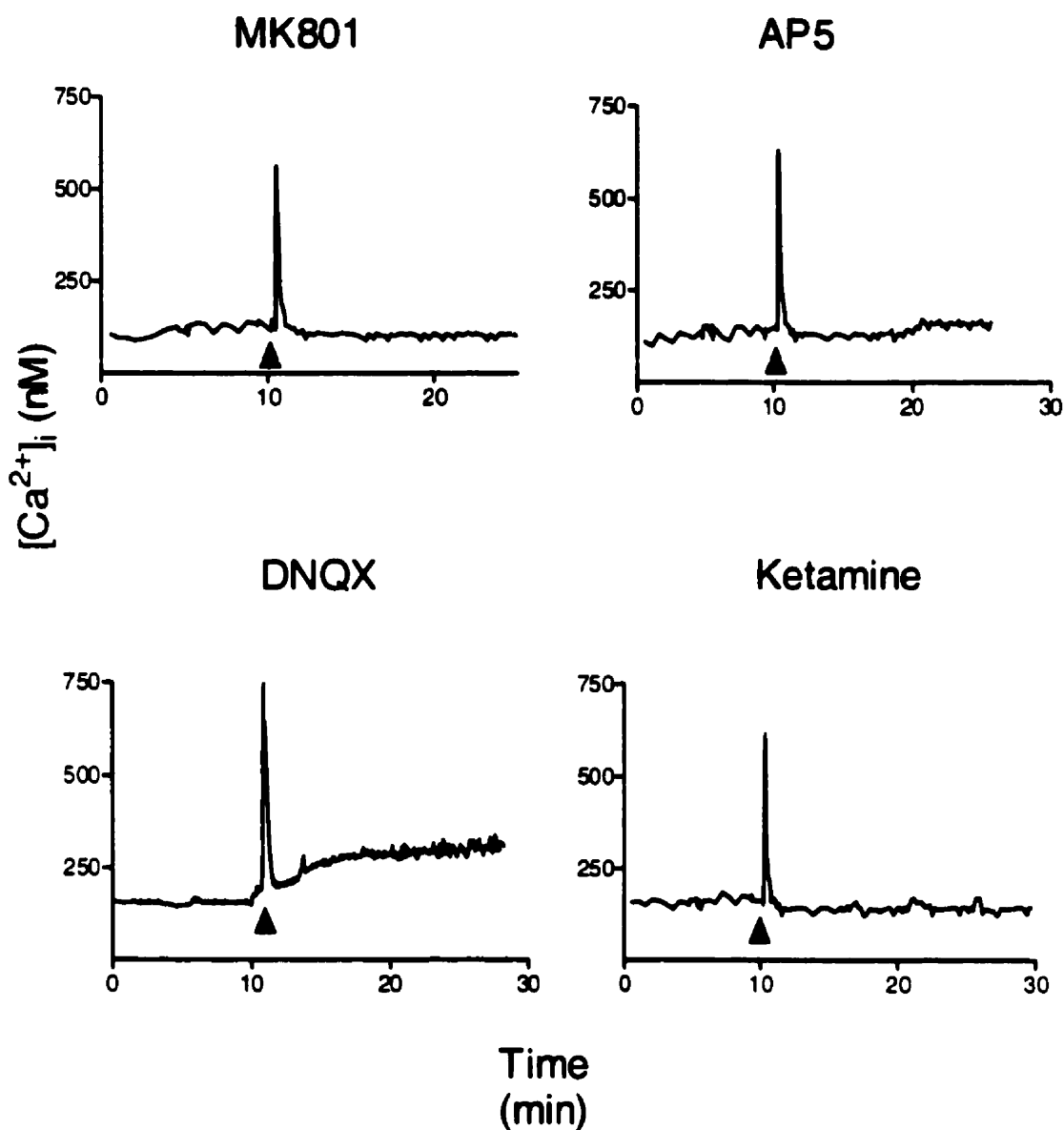
Figure 6. Role of glutamate receptors in Tat-induced initial transient and delayed prolonged increases in [Ca²⁺]ᵢ in neurons. Tat (2 pmoles) stimulation (arrows) resulted in initial transient increases of $[Ca^{2+}]_i$ that were not blocked by glutamate receptor antagonists. Secondary prolonged increases of $[Ca^{2+}]_i$ were inhibited by ketamine (24 of 30 neurons, dizocilipine (25 of 37 neurons), AP5 (15 of 15 neurons) and DNQX (24 of 30 neurons)(each 100 μM). Lower concentrations of the antagonists inhibited secondary increases of $[Ca^{2+}]_i$ but were less efficacious (data not shown). Values shown are mean ± S.E.M. values for delayed prolonged increases in $[Ca^{2+}]_i$ (increases over baseline levels 10 min following Tat applications) from at-least 3 separate experiments and two separate fetuses per condition.
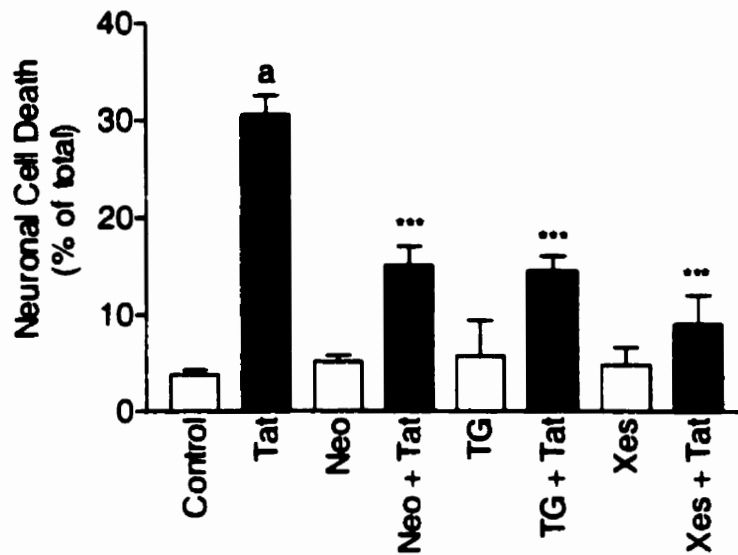
Figure 7: Tat-induced neuronal cell death. Tat (125 nM) was applied to cultures fetal human neurons for 12 hours and cell viability was determined by trypan blue exclusion. Tat significantly decreased cell viability in neurons and the neurotoxicity was inhibited significantly by Pre-treatment with neomycin (Neo), thapsigargin (TG), or xestospongin (Xes). a = p < 0.001 control vs Tat and *** = p < 0.001 compared with Tat.

did not decrease significantly neuronal viability, but reduced significantly (p<0.001) neuronal toxicity induced by Tat. Because overnight treatment with TMB-8 (100 µM) induced significant neuron cell death (p < 0.001), it was not used for determining the potential role of intracellular calcium in Tat-induced toxicity.

DISCUSSION

Loss of select populations of neurons, although not caused by direct HIV-1 infection of neurons *per se*, has been implicated in the pathogenesis of HIV-1 dementia [2]. Two current hypotheses have attempted to explain this unique phenomenon: 1) production/release of neurotoxic HIV-1 proteins and non-viral neurotoxic products from HIV-1 infected cells, and 2) production/release of neurotoxic substances from non-infected bystander cells. The viral proteins implicated to date in HIV-1 neurotoxicity and dementia include gp120, Tat, and to a lesser extent gp160, gp41, Nef, Rev, and Vpr [8, 42, 43]. One mechanism central to HIV-1 protein-induced neurotoxicity, as well as to other conditions such as hypoxia-ischemia, hypoglycemia, sustained seizures and trauma, is increased levels of $[Ca^{2+}]_i$ [8, 44]. Previously, we showed that peptide fragments of Tat, in particular Tat_{31-61}, increased $[Ca^{2+}]_i$ in cultured human fetal neurons and that calcium influx contributed to Tat-induced neuron depolarizations by activation of glutamate receptors [8, 34, 36, 37]. Here, we characterized Tat_{1-72} -induced increases of $[Ca^{2+}]_i$ in cultured human fetal astrocytes and neurons and showed that excitatory amino acid receptor-mediated calcium influx is a secondary/delayed event that is preceded and regulated by Tat-induced intracellular calcium release from IP_3-regulated $[Ca^{2+}]_i$ stores. Inhibition of the initial transient IP_3-mediated increases of $[Ca^{2+}]_i$ either severely attenuated or abolished the delayed increases of $[Ca^{2+}]_i$ and prevented neuron cell death.

Tat is a non-structural viral protein that, in addition to HIV-1 transactivation, has pleiotropic actions including depolarization of neuronal cell membranes [36, 37]. The amounts of Tat required to, increase neuronal death by apoptosis, increase $[Ca^{2+}]_i$, and produce electrophysiological changes have been in the nanomolar range and only transient applications are required to initiate those effects [8, 23, 36, 37]. Circulating levels of Tat have been measured to be about 2.5 nM or 1.6 ng/ml in AIDS patients [11], but concentrations are almost certainly higher in microenvironments around infected cells in brain [8, 59, 60]. Levels of extracellular Tat protein have been difficult to determine in HIV-1 infected human brain tissue likely owing to protein degradation following the typically lengthy time lag associated with obtaining autopsy material. However, we recently reported positive immunohistochemical staining for Tat protein in snap-frozen brains of monkeys infected with a chimeric simian/human immunodeficiency virus and in autopsy brains of HIV-1 infected humans [8]. We showed also that the continuous presence of Tat in vivo was not necessary to produce neuropathological changes. In fact, although Tat could not be detected in vivo by 6 hr following a single injection in rat brain, progressive neuropathological changes including neuronal cell death and gliosis were noted for several days thereafter [35].

Levels of free intracellular calcium are tightly controlled, and complex temporal and spatial fluctuations of $[Ca^{2+}]_i$ can modulate numerous cellular processes including cell survival [38]. Calcium overload triggered by excessive influx through plasma membrane voltage- and receptor-operated channels or by metabotropic

receptor-mediated release of calcium stored in intracellular pools are thought to play important roles in HIV-1 protein-induced neurotoxicity [8, 39, 40]. Mobilization from intracellular calcium pools is an important modulator of apoptosis in a variety of cells including T-cells, ventricular myocytes and cerebellar granule cells [45-49] and has been associated with gp120-induced neuron cell death [50]. Our results clearly show a requirement of IP$_3$-mediated release of intracellular calcium in Tat-induced neuron cell death and we have recently detected mRNA for IP$_3$ receptor subtypes 1 and 3 in cultured human fetal neurons (unpublished observations). Specifically, we showed that secondary increases of $[Ca^{2+}]_i$ are delayed events restricted to neurons and require priming by a transient release of calcium from IP$_3$-sensitive $[Ca^{2+}]_i$ pools. Blockage of glutamate receptors prevented secondary increases of calcium, but the relative contributions of subclasses of glutamate receptors requires further study. It is clear however that a transient release of $[Ca^{2+}]_i$ is a pre-requisite for secondary calcium influx and calcium dysregulation to occur. When IP$_3$-mediated calcium release was blocked at the level of receptor-G-protein coupling with pertussis toxin, prevention of secondary calcium dysregulation was as equally effective as when $[Ca^{2+}]_i$ release was prevented with either TMB-8 or xestospongin. Protection from Tat-induced neuronal cell death by prevention of $[Ca^{2+}]_i$ release with TMB-8, xestospongin or thapsigargin further suggest that $[Ca^{2+}]_i$ release is a pre-requisite to neuron cell death. Yet to be tested is the likely possibility that Tat-induced increases in protein kinase C activity [19, 20] are likewise dependant on this process because diacylglycerol and increased $[Ca^{2+}]_i$ are required for the activation of protein

kinase C, both of which are provided in the phosphoinositol hydrolysis pathway. The cell surface receptor potentially mediating these effects is currently unknown but we have at present ruled out a contribution by metabotropic glutamate receptors (mGluR1 and 5) as well as at least some purinergic receptors (unpublished observations).

Our findings here that glutamate receptor antagonists blocked delayed prolonged calcium influx suggests that initial transient increases of $[Ca^{2+}]_i$ may initiate, but delayed prolonged increases of $[Ca^{2+}]_i$ instigate, cell death. It is possible that the initial $[Ca^{2+}]_i$ release may play a role in sensitization of glutamate receptors and unmask delayed responses and toxicity[51]. Such sensitization may be mediated by Tat-induced increases in protein kinase C activity[19] because increases in protein kinase C activity can lead to toxicity[46, 52-54] and increases of IP_3 have been found to increase protein kinase C-dependant phosphorylation and sensitization of glutamate receptors[55-57]. These observations are consistent with our previous findings that neurotoxic, but not electrophysiological responses of neurons to Tat could be blocked by the glutamate receptor antagonist AP5[37]. Membrane depolarizations that occurred within seconds of Tat applications correspond temporally to the initial calcium spikes observed in the present study that also could not be inhibited by glutamate receptor antagonists. Neurotoxicity of both the HIV-1 proteins gp120 and Tat can be blocked by glutamate receptor antagonists suggesting that excessive calcium influx through these receptors

represents a final common pathway in the neurotoxicity mediated by both viral proteins.

These results do not however rule out a contribution of factors released from astrocytes in Tat-mediated neuronal cell death. Stimulation of phosphoinositol hydrolysis in astrocytes has been shown to induce glutamate release[58] and we recently found that Tat can induce glutamate release from astrocytes (unpublished observations). Tat has been shown to increase the release of tumor necrosis factor-α[21] and monocyte chemoattractant molecule-1[24], and to activate NF-κB[19], a process controlled by $[Ca^{2+}]_i$ which can regulate cytokine production. Our findings that Tat was both more potent and more efficacious in increasing levels of intracellular calcium in astrocytes when compared to neurons suggests an important role of astrocytes in Tat-induced neurotoxicity and the neuropathological consequences of HIV-1 infection. Hence, it needs to be determined the extent to which initial calcium events in astrocytes not blocked by glutamate receptor antagonists leads to cellular dysfunction.

Inhibiting transient IP_3-mediated elevations of $[Ca^{2+}]_i$ prevented secondary calcium influx and neuron cell death, thus implicating IP_3-mediated $[Ca^{2+}]_i$ release in Tat-induced neuronal cell death. Involvement of IP_3-regulated stores of intracellular calcium in HIV-1 protein-mediated neuronal cell death presents potential new therapeutic targets for the treatment of AIDS-related dementia complex.

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Chapter 4

HIV-1 Tat Potentates Glutamate-Excitotoxicity and Facilitates Glutamate, NMDA and KCl-Induced Increases in Cytosolic Calcium

N. J. Haughey, A. Nath, J.D. Geiger

INTRODUCTION

Disorders of cognition, behavior and motor functions coincident with HIV-1 infection are collectively termed AIDS-related cognitive-motor complex. The basis of this disorder is thought to be a dysfunction in, and the loss of, selected neuronal populations. Despite the pathological appearance of neurons in autopsy brain from HIV-1 infected patients, convincing evidence of direct infection of neurons by HIV-1 has not been demonstrated. Neuronal dysfunction and death are thought to mediated by toxic viral proteins that can be released from HIV-1 infected cells. The viral coat protein gp120 and the *trans*-acting regulatory protein Tat are the two best characterized neurotoxic HIV-1 proteins (reviewed in [1,2])

Tat is released from unlysed HIV-1 infected cells [3] and is present in the serum [4] and brains [1] of HIV-1 infected patients. Exogenous application of Tat results in neuronal cell death [5-8] and at sub-toxic concentrations can dramatically alter the morphology of neurons [9]. Tat-induced neuronal degeneration occurs by calcium dependant mechanisms [10,11] and can be inhibited by antagonists of NMDA, and to a lesser extent, non-NMDA glutamate receptors [10]. We recently reported that a transient application of Tat resulted in a rapid increase of cytosolic calcium from IP_3-sensitive intracellular stores that was followed minutes later by calcium influx through excitatory amino acid receptors. Inhibition of IP_3-mediated calcium release prevented excitatory amino acid receptor-mediated calcium influx and protected neurons from the toxic effects of Tat [11]. Together these findings suggest that Tat-mediated release of calcium from IP_3-sensitive intracellular

calcium pools is a necessary prerequisite for excitatory amino acid receptor mediated calcium influx and that calcium influx via excitatory amino acid receptors is ultimately responsible for neuronal cell death.

Recently it was suggested that Tat could enhance the neurotoxic actions of NMDA *in-vivo* [12]. Here we tested the hypothesis that Tat-facilitates glutamate and NMDA-mediated increases of cytosolic calcium and that Tat and glutamate act synergistically to produce neuronal cell death.

METHODS

Cultured Rat Hippocampal Neurons: The preparation of primary rat hippocampal neurons from 19 day old embryonic Sprague Dawley rats was performed essentially as described by [13]. Hippocampal tissue was disassociated by gentle titration in calcium-free Hank's balanced salt solution and centrifuged at 500 rpm. Cells were resuspended in DMEM/F12 nutrient mixture containing 10 % heat-inactivated fetal bovine serum and 1 % antibiotic solution (10^4 U of penicillin G/ml, 10 mg streptomycin/ml and 25 µg amphotericin B/ml in 0.9 % NaCl)(Sigma). Cells were plated at a density of 100,000 cells/ml on 25 mm poly-D-lysine coated glass coverslips for calcium imaging and at a density of 200,000 cells/ml on 12 mm coverslips for Hoescht staining. After 6 hours, media was replaced with serum free Neurobasal media containing 1 % B-27 supplement (Gibco). Neuronal cultures were used between 10 and 14 days in culture.

<u>Intracellular Calcium Determinations:</u> Concentrations of intracellular calcium

$[Ca^{2+}]_i$ were determined using the Ca^{2+}-specific fluorescent probe Fura-2/AM as

described previously [11]. Cells were incubated for 30 min at 25°C in Krebs-BSA

buffer consisting of (in mM): 111 NaCl, 26.2 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, 4.7 KCl, 1.2

MgCl$_2$, 15 HEPES, 1.8 CaCl$_2$, 5 glucose, 1.5 µM bovine serum albumin (BSA)

and 2 µM Fura-2/AM. Cells were washed with Krebs (no BSA) to remove

extracellular Fura-2 and were incubated at 37°C for 10 min to allow for complete

de-esterfication of the probe. Coverslips containing Fura-2 loaded cells were

placed in a PDMI-2 open perfusion micro-incubator (Medical Microsystems

Corp., Greenvale, NY) where the cells were superfused at the rate of 2 ml/min

with Krebs buffer pre-warmed to 37°C. Cells were excited at 340 and 380 nm,

and emission was recorded at 510 nm with a video-based Universal imaging

system (EMPIX, Missassauga, ON). R_{max}/R_{min} ratios were converted to nM $[Ca^{2+}]_i$

as described previously [14].

To bath apply glutamate (10 and 100 µM), NMDA (100 µM) plus glycine (2 mM)

and KCl (50 mM), buffer flow was interrupted, compounds were applied, and flow

was re-started when peak intracellular calcium levels were achieved. Between

applications, cells were washed for 5 min. Tat was present during Fura2/AM

loading or was pressure applied as described previously [15]. Tat (10, 100 and

1000 nM) incubated during Fura2/AM loading was done in the absence of BSA to

avoid Tat binding to BSA. Controls in the presence of BSA (without Tat) showed

no difference in Fura2/AM loading. Coverslips were washed three times with

buffer before imaging to remove Tat. Memantine (10 μM), xestospongin C (10 μM) and TMB-8 (10 μM) were added 10 min prior to Tat and were present during Fura2/AM loading. Pertussis toxin (100 ng/ml) and cholera toxin (1 μg/ml) were pre-incubated with cells for 40 min at 37°C before the addition of Tat and Fura2/AM loading. None of these procedures interfered with Fura2/AM loading of cells. For pressure applications, Tat (1 μM), ATP (1 μM) and KCl (50 μM) were loaded into glass micropipettes that were positioned approximately 3 cell bodies away from target cells. A pulse pressure of 6 psi was applied three-times for 100 msec each using a Picospritzer (General Valve Corp, Fairfield, NJ); with these parameters it was calculated that 12 pmoles of Tat were applied. Responding cells were defined as those that displayed greater than 50 nM increase of $[Ca^{2+}]_i$ and the levels were determined as the average within the cell soma. Increases of $[Ca^{2+}]_i$ were determined as the peak amplitudes minus basal levels of $[Ca^{2+}]_i$. Significant differences between groups were determined by one-way ANOVA and Tukey's post-hoc comparisons.

Neurotoxicity Assay: The DNA binding dye Hoescht 33342 was used as a measure of apoptosis. Neurons were incubated in Krebs buffer with Tat and glutamate for 12 hours. Cells were fixed with 4 % paraformaldehyde and membranes permeabilized with 0.2 % Trition X-100 for 4 hours at 4°C. Hoescht 33342 was loaded at 37°C for 30 min at a concentration of 1 μM. Nuclei were visualized under epifluorescence illumination (340 nm excitation, 510 nm barrier filter) with a 40X oil immersion objective. Approximately 150 cells were examined

in each of 3 pre-determined fields per experiment and each experiment was conduced in duplicate. Apoptotic cells were considered as fragmented or condensed nuclei and cells in which the DNA was diffusely and uniformly distributed throughout nuclei were considered viable. At the time of counting, the investigator was unaware of the identity of the treatment groups.
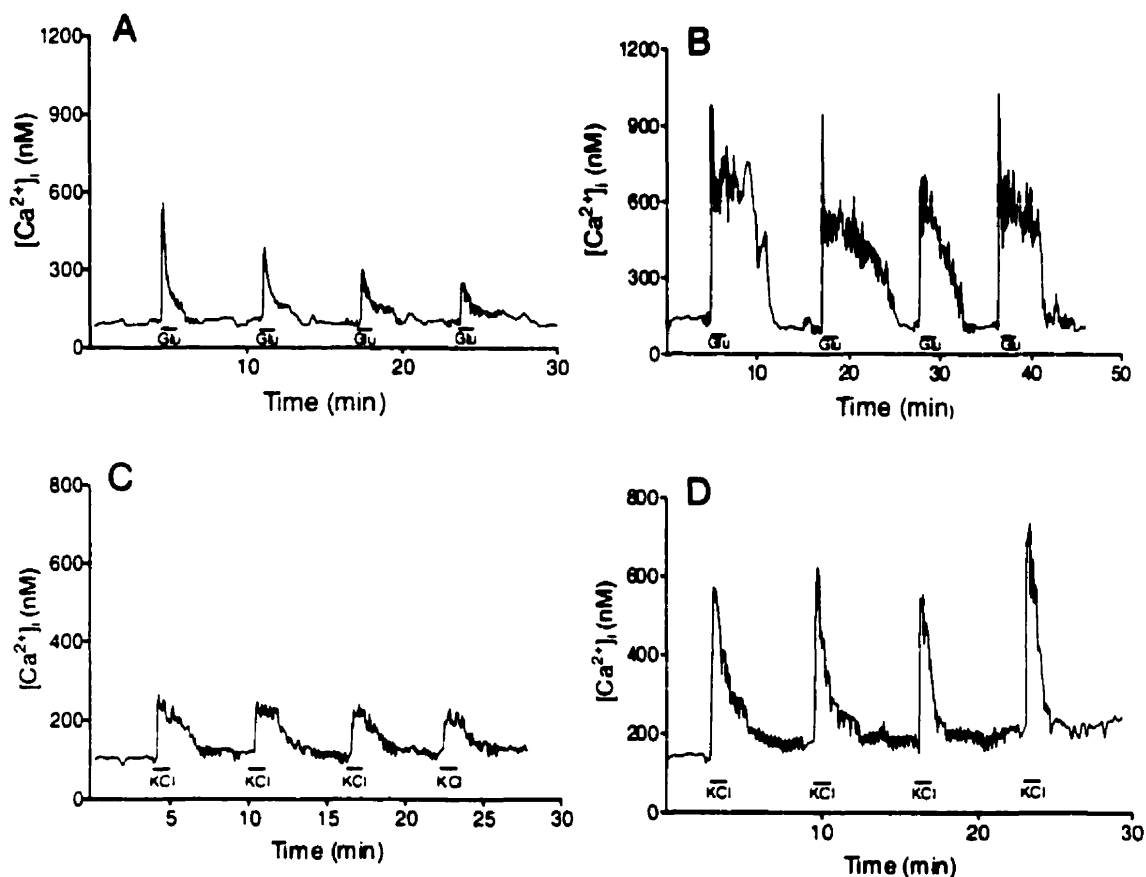
RESULTS

Tat Modulation of Calcium Flux

40 min pre-exposure of neurons to Tat significantly increased glutamate, NMDA and KCl-mediated increases of cytosolic calcium (Fig 1). Four successive applications of glutamate resulted in a progressive decrease in peak calcium responses. Pre-exposure of neurons to Tat prevented this decrease in glutamate-mediated increases of calcium and delayed the return of intracellular calcium levels to baseline following glutamate applications (Fig 2 A, B). Transient (pulse pressure applied) applications of Tat were sufficient to facilitate glutamate-induced increases in cytosolic calcium and resulted in calcium dysregulation in 33 % of the neurons tested (data not shown). Tat has been previously reported to induce the release of calcium from IP$_3$-sensitive intracellular pools [11] and when we inhibited this process with the IP$_3$-regulated calcium pool blocker xestospongin and the G-protein inhibitor pertussis toxin facilitation of glutamate-mediated increases in cytosolic calcium by Tat was reversed (Fig 3).
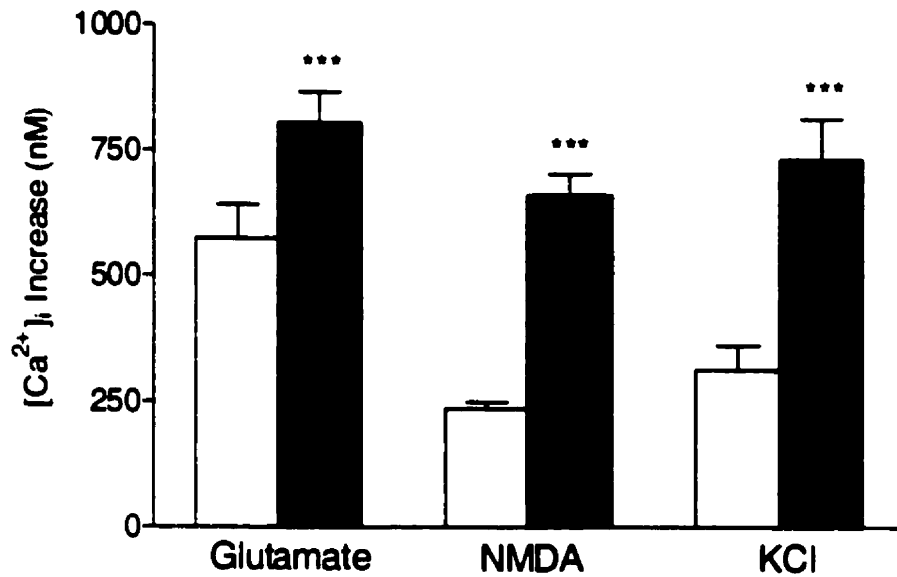
Figure 1: Tat-facilitated glutamate, NMDA and KCL-mediated increases in cytosolic calcium. Glutamate (100 µM), NMDA (100 µM) and KCl (50 µM)-mediated increases in cytosolic calcium (open bars) were significantly increased by pre-exposure to Tat (100 nM) (closed bars). *** = $p < 0.001$.

Figure 2: representative traces depicting facilitation of glutamate and KCl-mediated increases in cytosolic calcium by Tat. Four applications of glutamate (100 μ) resulted in consecutively smaller increases in cytosolic calcium (A). pre-incubation with Tat (100 nM) increased the amplitude and duration of glutamate-mediated increases in calcium (B). Four applications of KCL (50 mM) each resulted in approximaetely equal increases in cytosolic calcium (C) that were increased by pre-incubation with Tat (100 nM).
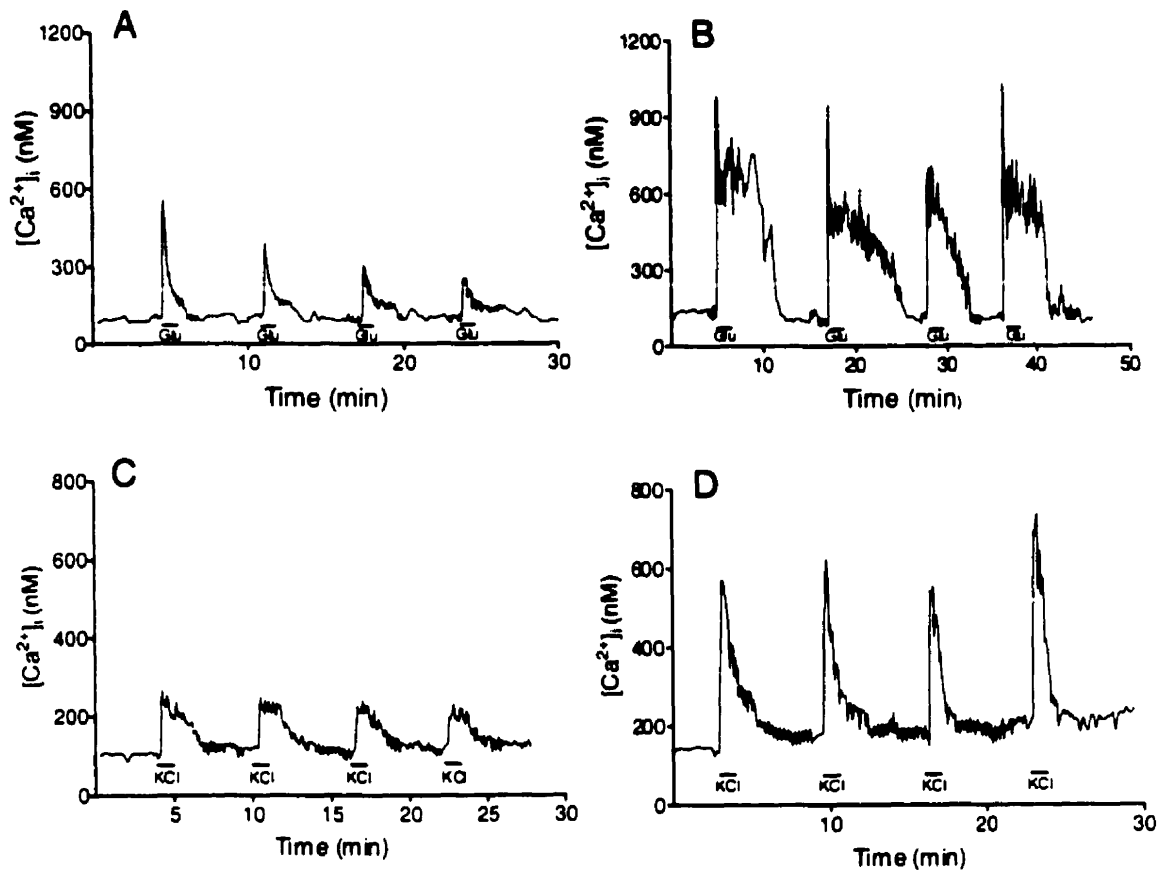
Figure 3: Tat-facilitated increases in glutamate and KCl-mediated calcium flux are dependant on IP₃-receptors. Facilitation of glutamate (100 µM)-mediated increases in cytosolic calcium by Tat (100 nM) were significantly decreased by the IP₃-receptor antagonist, xestospongin and the G-protein inhibitor pertussis toxin (PTX) but not by cholera toxin (CTX). Facilitation of KCL (50 µM)-mediated increases in cytosolic calcium by Tat (100 nM) were significantly reduced by the IP₃-receptor antagonist xestospongin and TMB-8. a = p < 0.001, glutamate vs Tat + glutamate; b = p < 0.001, Tat + glutamate vs Tat + glutamate + xestospongin and Tat + glutamate + PTX; c = p < 0.001 KCL vs Tat + KCl; d = p < 0.001 KCl + glutamate + PTX; d = p < 0.001 KCl + glutamate vs KCl + Tat + xestospongin and KCl + Tat + TMB-8.

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The NMDA receptor antagonist memantine, partially reversed the facilitation of glutamate-induced calcium flux by Tat (Fig 3). Pre-exposure of neurons to Tat significantly increased NMDA-induced increases of cytosolic calcium (Fig 1).

To determine the specificity of Tat-induced facilitation of glutamate and NMDA-receptor mediated calcium flux, we used KCl to activate voltage sensitive calcium channels on neurons. Four consecutive applications of KCl resulted in similar increases of cytosolic calcium. Pre-exposure of neurons to Tat significantly increased all KCl-mediated increases in cytosolic calcium (Fig 2C, D) and a single, transient, application of Tat was sufficient to facilitate all KCl-mediated increases of cytosolic calcium (data not shown). Dysregulation of intracellular calcium homeostasis following KCl applications was not apparent when neurons were pre-exposed to Tat for 40 min or when Tat was pressure pulse applied. Antagonists of IP_3-receptor mediated calcium release, xestospongin or TMB-8, inhibited the facilitation of KCl-mediated increases of cytosolic calcium by Tat (Fig 3).

<u>Correlations between Tat and Agonist-Induced Increases in Cytosolic Calcium</u>

Pressure applications of Tat resulted in a dose related increase of cytosolic calcium that inversely correlated with distance of cells from the application site. We used this phenomenon to determine the effect of Tat, ATP and KCl dose on Insert figure 3 here

increases of cytosolic calcium produced by subsequent applications of 10 μM glutamate. Pressure applications of Tat, ATP and KCl resulted in single, monophasic, increases of cytosolic calcium that returned to baseline levels and remained near baseline for time periods up to 40 min (data not shown).The rank order of correlation strength between peak amplitudes of glutamate mediated increases of cytosolic calcium and agonist-mediated increases was Tat > ATP > KCl (Fig 4 A,B,C). Differential responsiveness of the neurons to Tat, KCl and ATP was not responsible for correlations with Tat-mediated increases in cytosolic calcium because when applied alone, all three compounds produced consistent increases in calcium and the data passed a normality test (Graph Pad Software, Inc., San Diego), that indicated the data corresponded to a normal distribution pattern (data not shown).

Neurotoxicity

Exposure of neurons to glutamate concentrations ranging from 0.01 to 100 μM resulted in dose-related decreases of neuronal survival. Significant decreases in neuronal survival were observed at concentrations of glutamate > 1 μM. A sub-toxic concentration of glutamate or a toxic concentration of glutamate when co-applied with Tat resulted in significant decreases in neuronal survival (Fig 5).
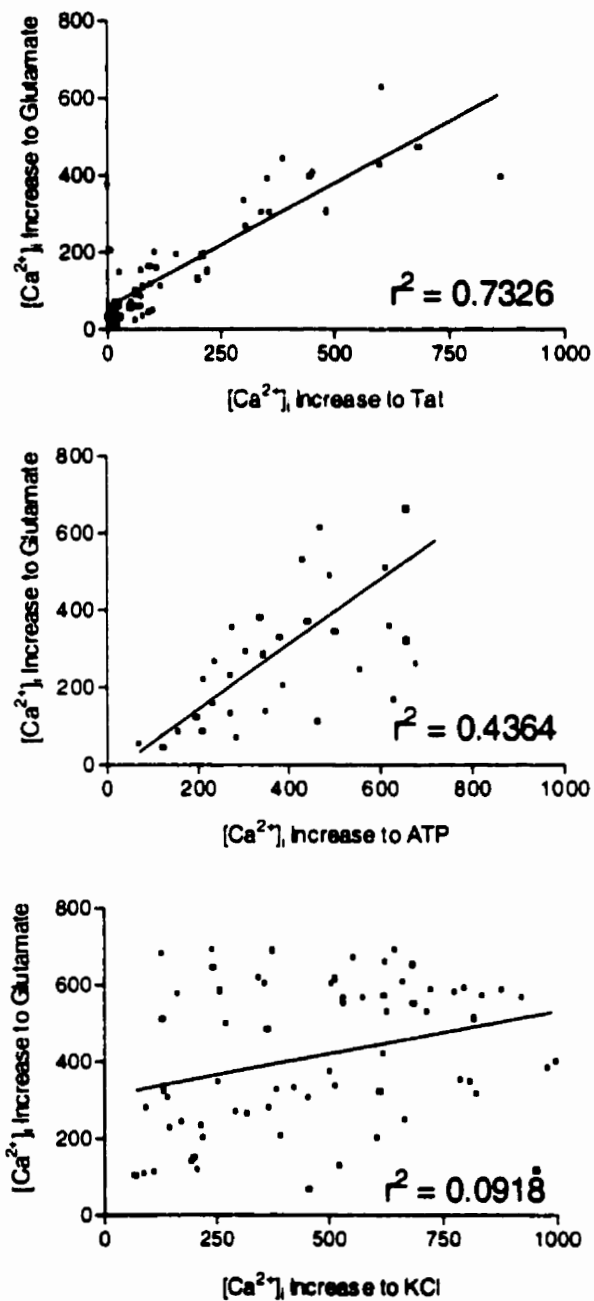
Figure 4: The correlation between increases in cytosolic calcium from transient applications of Tat, ATP and KCl to subsequent applications of glutamate. A. Tat (2 pmoles)-induced calcium release from IP$_3$-sensitive intracellular pools showed stronger correlation with glutamate (10 µM)-mediate increases of cytosolic calcium than did ATP (1 µM) (B) or KCl (50 µM) (C).
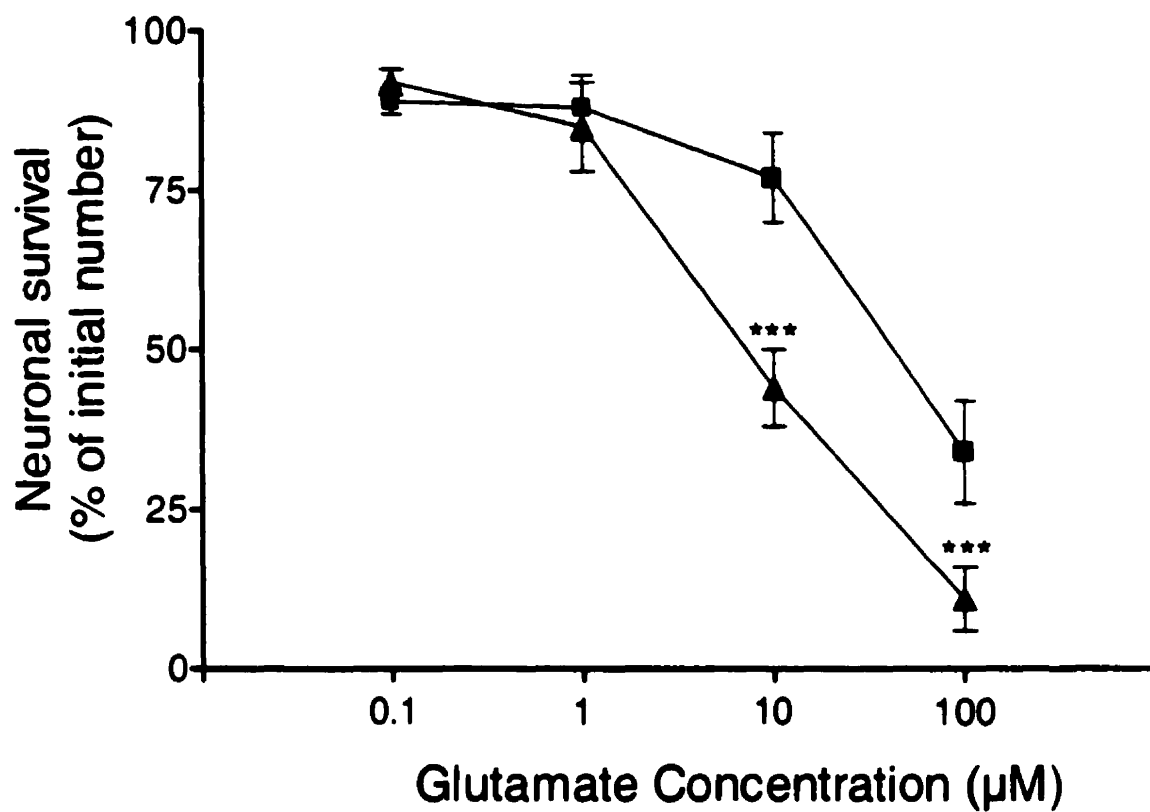
Figure 5: Tat potentiated glutamate-induced decreases in neuronal survival. A sub-toxic (1 µM) or a toxic (10 µM) concentration of glutamate when combined with Tat (100 nM) significantly decreased neuronal survival. *** = p < 0.001.

DISCUSSION

AIDS-related dementia complex occurs in ~ 30 % of the adult and ~ 50 % of the pediatric population infected with HIV-1. Pathological alterations in brain associated with HIV-1 infection include, but are not restricted to, the appearance of multinucleated giant cells, macrophage infiltration, and neuronal cell loss. Neurons are not themselves infected with HIV-1, but can be affected by HIV-1 proteins released from nearby infected glia and macrophages. Tat is known to be released from unlysed, HIV-1 infected cells[3] and has direct effects on neurons [16]. Tat-induced neuronal cell death occurs by mechanisms dependant on IP$_3$-sensitive intracellular calcium release and can be inhibited by blockers of IP$_3$-sensitive stores of intracellular calcium as well as antagonists of glutamate receptors[10, 11]. Here we showed that Tat-facilitated glutamate, NMDA and KCl-mediated increases in intraneuronal calcium by mechanisms that were sensitive to the pharmacological inhibition of IP$_3$-receptors and pertussis toxin sensitive G-proteins.

We previously showed that a transient application of Tat resulted in the rapid release of calcium from IP$_3$-sensitive intracellular pools and several minutes subsequent to that release an influx of calcium through excitatory amino acid receptors[11]. Tat increased cytosolic levels of inositol 1,4,5-trisphosphate and both IP$_3$-dependant and excitatory amino acid receptor-mediated increases of intracellular calcium were prevented with inhibitors of phospholipase C, antagonists of IP$_3$-regulated pools of intracellular calcium and the G-protein

uncoupler, pertussis toxin [11]. We now demonstrate that transient exposures of neurons to Tat facilitates glutamate, NMDA and KCl mediated increases in cytosolic calcium. Consistent with the metabotropic actions of Tat, pertussis, but not cholera toxins inhibited the ability of Tat to facilitate glutamate-induced increases of intraneuronal calcium. Antagonists of IP_3-regulated pools of intracellular calcium, xestopongin and TMB-8 prevented Tat-facilitation of glutamate-mediated increases in cytosolic calcium. In addition, the degree of increases in intracellular calcium produced by sub-maximal amounts of glutamate was dependant on the dose of Tat. The amplitude of peak increases in cytosolic calcium produced by Tat demonstrated a strong correlation with the degree of intracellular calcium increases produced by subsequent applications of glutamate. These results suggest that intracellular calcium release is an important component of the effect of Tat on glutamate receptor-mediated calcium flux. Tat-facilitated increases of cytosolic calcium in response to applications of KCl showed that the effects of Tat on calcium flux were not specific to the NMDA receptor. It is thus possible that the cation conductance of multiple receptors are increased by Tat.

Previously we reported that, in neurons, glutamate receptor-mediated calcium influx was secondary to, and dependant on, the prior release of calcium from IP_3-sensitive intracellular pools by Tat. Our previous experiments were conducted in mixed neuron and astrocyte cell cultures (excluding microglia). In these mixed cultures, astrocytes also displayed Tat-mediated increases of cytosolic calcium

from IP$_3$- sensitive intracellular pools [11]. In contrast, our work here with cultured rat hippocampal neurons failed to show any evidence for influxes of calcium at the concentrations of Tat tested. We did however observe dysregulation of intracellular calcium when neurons were pre-exposed to Tat and repeatedly stimulated with glutamate. These findings suggest that the release of glutamate in situ may predispose cells to calcium dysregulation and Tat-induced neuronal cell death.

Electrophysiological studies have consistently demonstrated Tat-induced depolarization of neuronal membranes [16-18]. Membrane depolarizations were not inhibited by non-NMDA receptor antagonists [17] or by antagonists of NMDA receptors that act by competitive inhibition of glutamate (AP-5) and glycine binding (5,7-dichlorokynerenate). High concentrations of zinc (1 µM) and to a lesser extent magnesium (1µM) inhibited Tat-mediated membrane depolarizations [19]. Zinc is known to be a potent inhibitor of NMDA-receptor function [20-23] suggesting that Tat may reduce the tonic inhibition of NMDA receptors by zinc.

Calcium flux data suggest that Tat activates metabotropic receptors prior to glutamate-receptor mediated calcium influx. Several metabotropic receptors are known to upregulate NMDA-receptor mediated conductances [24-27] through inositol phospholipid hydrolysis, activation of protein kinase C and tyrosine kinases [24, 28-31]. In particular, tyrosine kinase activity can potentiate NMDA

receptor-mediated currents by reducing tonic zinc inhibition [32]. Tat has been shown to increase cytosolic levels of IP_3, calcium [11] protein kinase C [33, 34] and tyrosine kinase activity suggesting that Tat may upregulate NMDA receptor-mediated currents by activating metabotropic-mediated mechanisms.

The NMDA receptor antagonist memantine inhibited Tat-facilitation of glutamate-mediated increases in intracellular calcium and NMDA-induced increases in calcium were facilitated by pre-exposure of neurons to Tat. Inhibition of Tat-induced facilitations of glutamate-mediated calcium flux by uncoupling of G-proteins and antagonists of IP_3-receptors suggest involvement of the phosphoinositol pathway. Thus, the most likely explanation for inhibition of membrane depolarizations induced by Tat in the presence of high concentrations of zinc, is that Tat acting though tyrosine kinases may reduce tonic zinc inhibition of NMDA-receptors that is overcome by high concentrations of zinc.

Tat-induced neurotoxicity can be prevented by antagonists of NMDA and to a lesser extent non-NMDA receptors [10]. Prevention of Tat-induced intracellular calcium release from IP_3-sensitive pools or block of phosphoinositol hydrolysis inhibited excitatory receptor mediated calcium influx and was neuroprotective [11]. A recent report demonstrated that Tat doubled the size of brain lesions produced by injections of NMDA into rat hippocampus . We now show that sub-toxic concentrations of Tat and glutamate, when combined, result in significant neuronal cell death. Enhanced NMDA receptor-mediated calcium influx by Tat

may provide an explanation for Tat-facilitation of excitotoxic neuronal cell death

and the protection afforded by antagonists of NMDA receptors.

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Chapter 5

Synergistic increases in neurotoxicity by the HIV-1 proteins Tat and gp120 - neuroprotection by memantine

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ABSTRACT:

HIV-1 proteins Tat and gp120 have been implicated in the pathogenesis of dementia associated with HIV infection. Recently, we showed the presence of Tat protein in brains of patients with HIV-1 encephalitis as well as macaques with encephalitis due to a chimeric strain of HIV and simian immunodeficiency virus, and that even transient exposure of cells to Tat leads to release of cytopathic cytokines. In the present study, we report the first demonstration of the presence of gp120 protein in brain of patients with HIV encephalitis. Furthermore, we tested the hypothesis that Tat and gp120 would act synergistically to potentiate each protein's neurotoxic effects. Moreover, we determined the extent to which pharmacological antagonists against processes implicated in HIV-1 neuropathogenesis could block HIV-1 protein-induced neurotoxicity. Sub-toxic concentrations of Tat and gp120, that were sub-threshold for increasing levels of intracellular calcium, when incubated together, caused neuronal cell death and prolonged increases in levels of intracellular calcium. A transient exposure of Tat and gp120 to neurons for seconds was sufficient to initiate neuronal cell death, but maximal levels of neuronal cell death were observed with exposures lasting 30 min. The neurotoxicity caused by Tat and gp120 applied in combination was blocked completely by memantine, partially by amiloride, and not at all by dipyridamole or vigabatrin. Tat and gp120 act synergistically to cause neuronal cell death, and these neurotoxic actions, that can be blocked completely by memantine and partially by amiloride, can be initiated by transient exposure of neurons to these HIV-1 proteins. Thus, only very low and transient levels of HIV-

1 proteins may be sufficient and necessary to contribute towards the neuronal cell death accompanying and underlying HIV-1 dementia.

INTRODUCTION

Infection with the human immunodeficiency virus (HIV) results in neuronal degeneration as observed at time of autopsy and sometimes results in a dementing illness. The neuronal degeneration occurs even though neurons themselves are only rarely infected and this suggests that indirect mechanisms participate in neuronal demise. The cells that are predominantly infected with HIV-1 are invading macrophages and microglia, and these infected cells release neurotoxic substances including the HIV-1 proteins Tat and gp120 [1]. Tat, a non-structural viral protein essential for viral replication, is actively released from unruptured cells [2], has been detected in serum and brain of HIV-infected patients [3-5] and in macaques with encephalitis due to a chimeric strain of HIV and simian immunodeficiency virus [6]. In vitro and in vivo studies have shown that Tat is neurotoxic (reviewed in [1]). Gp120, the HIV-1 viral coat glycoprotein, has been detected in the serum and cerebrospinal fluid of HIV-1 infected patients [7,8] and is neurotoxic both in vitro and in vivo. However, despite findings that env mRNA is elevated in brain of patients with HIV-1 encephalitis [9,10] the role of gp120 in mediating HIV dementia has been questioned because of the inability to detect gp120 protein in brain [11]. Using highly specific polyclonal antisera we now demonstrate the presence of gp120 in brain of patients with HIV-1 encephalitis

and in so doing further establish the role of gp120 in the neuropathogenesis of HIV-1 infection.

Several mechanisms have been implicated in viral protein-induced neurotoxicity including oxidative pathways, excitotoxicity, sodium-proton exchange, calcium dysregulation, release of cytokines, and blockade of glutamate uptake [1]. Based on these observations several drug studies have been initiated for the treatment of HIV dementia. In this study, we first tested the hypothesis that sub-threshold doses of gp120 and Tat when present together would be sufficient and necessary to cause potentiated increases in levels of intracellular calcium and neurotoxicity. We next tested the hypothesis that the synergistic and neurotoxic actions of gp120 co-administered with Tat could be blocked by clinically available pharmacological antagonists and thereby not only potentially identify agents with therapeutic potential but also processes implicated in HIV-1 neuropathogenesis.

METHODS:

Immunohistochemistry:

Paraffin-embedded formalin-fixed sections from the temporal lobe, basal ganglia and hippocampus of three patients with HIV encephalitis were immunostained with a polyclonal goat anti-gp120 antibody generated against recombinant gp120 from HIV-$_{SF2}$ that was provided as a gift to us by Chiron Corporation (Emoryville, CA). This antiserum recognized a single band at 120 kDa by western blot analysis of HIV-infected cell lysates. Briefly, for gp120 staining, sections were deparaffinized and hydrated in serial dilutions of ethanol. Sections were heated in a household microwave in 0.1 M sodium citrate (pH 6.0) for 10 min. Endogenous peroxidase was quenched using 3% hydrogen peroxide. Slides were incubated with the primary antisera (60 µg/ml) for 15 hours at 4°C. Biotinylated goat-anti IgG (Chemicon, Temecula, CA) (1:500) was used as a secondary antibody followed by streptavidin horse raddish peroxidase. Immunostaining intensity was amplified using the TSA system as per manufacturer's protocol (NEN Life Sciences, Boston, MA). Diaminobenzadine was used as the chromogen. Brain tissues from two patients not infected with HIV-1 and without any known neurological complications were used as controls.

Neuronal Cultures:

Brain specimens were obtained from human fetuses of 12-14 weeks gestational age with consent from women undergoing elective termination of pregnancy and approval by the University of Kentucky Institutional Review Board and the

University of Manitoba's Committee for Protection of Human Subjects. Cultures

of human fetal neurons were prepared as described previously [12,13]. Briefly, the

cells were mechanically dissociated, suspended in Opti-MEM with 1% heat-

inactivated fetal bovine serum, 0.2% N2 supplement (GIBCO) and 1% antibiotic

solution (penicillin G 10^4 units/ml, streptomycin 10 mg/ml and amphotericin B 25

μg/ml) and plated in flat bottom 96 well plates. The cells were maintained in

culture for at least six weeks before conducting the neurotoxicity assays.

Chemicals and Recombinant Tat and gp120 proteins:

Fura-2-acetoxymethyl ester (Fura-2/AM) was obtained from Molecular Probes

Inc. (Eugene, OR). Antibodies against the astrocyte marker protein glial fibrillary

acidic protein (GFAP), the neuron marker protein microtubule-associated protein

2 (MAP-2), and the microglia marker protein EBM-11 were purchased from

Chemicon (Temecula, CA.), Boehringer Mannheim (Laval, P.Q.), and Dako

(Denmark), respectively. All other reagents were of analytical grade or the

highest purity available.

Recombinant Tat was prepared as described previously [14] with minor

modifications. The *tat* gene encoding the first 72 amino acids were amplified from

HIV$_{BRU}$ obtained from Dr. Richard Gaynor through the AIDS repository at the NIH

and inserted into an E. coli vector PinPoint Xa-2 (Promega). This construct

allowed the expression of Tat as a fusion protein naturally biotinylated at the

N-terminus. The biotinylated Tat protein was purified on a column of soft release

avidin resin and cleaved from the fusion protein using factor Xa and eluded from the column followed by desalting with a PD10 column. All purification steps contained dithiothreitol to prevent oxidation of the proteins. Tat proteins were >95% pure as determined by SDS-PAGE followed by silver staining. Western blot analysis showed that these preparations contained both monomeric and dimeric forms of Tat. The functional activity of Tat was confirmed using a transactivation assay in HL3T1 cells containing an HIV-1 LTR-CAT construct[14]. gp120 from HIV_{SF2} was obtained as a gift from Chiron Corporation. Recombinant gp120 was made in a chinese hamster ovary cell line and purification yielded a product that was 95% gp120 with the remainder being break down products of gp120 as determined by western blot analysis. The Tat and gp120 preparations contained <1 pg/ml endotoxin as determined using a Pyrochrome Chromogenic test kit (Associates of Cape Cod Inc., Falmouth, MA.). The Tat protein was stored in a lyophilized form and gp120 as a stock solution in water at -80°C in endotoxin free, siliconized microfuge tubes until taken for experimentation. Tat and gp120 were highly susceptible to degradation and loss of biological activity with each freeze-thaw cycle. Therefore, single aliquots were used for each experiment with the remaining solutions discarded.

Neurotoxicity Assay:

At the time of experimental treatment, the culture media was replaced with Locke's buffer (in mM) (154 NaCl, 5.6 KCl, 2.3 $CaCl_2$, 1 $MgCl_2$, 3.6 $NaHCO_3$, 5 glucose, 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES); pH

7.2) and neurons were incubated with Tat, gp120 or both proteins

simultaneously. To determine if a transient exposure of neurons to the viral

proteins was necessary and sufficient to cause toxicity, the viral proteins were

incubated with the neurons for 30 sec, 5 min, 30 min or 15 hours followed by a

complete media exchange. Cell death was monitored by trypan blue exclusion 15

hours after the change to Locke's buffer and the addition of HIV-1 protein(s) as

described previously [12,13]. To determine mechanisms underlying Tat and gp120

neurotoxicity, cells were pre-incubated with either memantine (2 μM), 5-(N-

methyl-N-isobutyl) amiloride (MIA) (10 μM), dipyridamole (10 μM) or vigabatrin

(20 μM) for 30 min prior to addition of Tat (60 nM) plus gp120 (30 pM). MIA was

obtained from Sigma Chemical (St. Louis, MO.) and all other drugs were

obtained from Tocris Cookson (Ballwin, MO.). Cell death was monitored at 15

hours after the addition of the HIV-1 proteins. Neuronal cell counts were

determined from five fields at predetermined coordinate locations. Each field was

photographed, coded and counted. At least 200 cells were counted in each field.

Each experiment was conducted in triplicate wells and at least two independent

experiments were conducted with each pharmacological agent. The means and

standard errors of the mean were calculated and data were analyzed by ANOVA

with Tukey-Kramer post-hoc comparisons. Statistical significance was

determined to be at the 95% confidence interval.

Intracellular Calcium Determinations:

Levels of intracellular calcium ($[Ca^{2+}]_i$) were determined in human fetal neurons

plated in 33 mm diameter coverslips using the Ca^{2+}-specific fluorescent probe

Fura-2/AM. Cells were incubated for 30 min at 25°C in Krebs-BSA buffer

consisting of (in mM): 111 NaCl, 26.2 $NaHCO_3$, 1.2 NaH_2PO_4, 4.7 KCl, 1.2

$MgCl_2$, 15 HEPES, 1.8 $CaCl_2$, 5 glucose, 1.5 μM bovine serum albumin (BSA)

and 2 μM Fura-2/AM. Cells were washed 3-times with Krebs (no BSA) to remove

extracellular Fura-2 and were incubated at 37°C for 5 min to allow for complete

de-esterification of the probe. The coverslips containing Fura-2 loaded cells were

placed in a LU-CSD Leiden coverslip dish situated in a PDMI-2 open perfusion

micro-incubator (Medical Microsystems Corp., Greenvale, NY) and cells were

superfused at 2 ml/min with KREBS buffer pre-warmed to 37°C. Cells were

excited at 340 and 380 nm, and emission was recorded at 510 nm with a video-

based Universal imaging system (EMPIX, Missassauga, ON). R_{max}/R_{min} ratios

were converted to nM $[Ca^{2+}]_i$ as described previously [42] and used calcium

standards obtained from Molecular Probes (Eugene, OR). All images were

acquired by real time averaging of 16 frames of each wavelength that included a

background reference subtraction from each of the acquired images. gp120, Tat

or a combination of the two HIV-1 proteins were loaded into glass micropipettes

(1.0 mm OD, 0.78 mM ID) pulled to a final outer tip diameter of < 1.0 μm.

Micropipettes were positioned approximately 3 cell bodies away from target cells

and HIV-1 proteins were pressure-applied to cells (3 X 100 msec, 6 psi) using a

Picospritzer (General Valve Corp, Fairfield, NJ). Cells within 5 cell body widths of

the micropipette were monitored for time periods up to 1 h. Peak increases in $[Ca^{2+}]_i$ were determined by subtracting the maximum $[Ca^{2+}]_i$ achieved during a 5 min period following Tat or gp120 applications from baseline $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ were averaged over the cell body and large processes. Calcium dysregulation was defined as an inability of cells to maintain or re-establish calcium homeostasis 15 min after gp120 and/or Tat application. For all calcium experiments, the amount of Tat and gp120 pressure-applied to neurons was calculated in moles.

RESULTS

<u>Detection of gp120 in the brain of patients with HIV encephalitis</u>:

Prior to testing the hypothesis that Tat was capable of synergistically increasing

the neurotoxicity of gp120 we determined, using a new antibody against gp120,

the presence of gp120 in brain of patients with HIV-1 encephalopathy. This was

an important first step because the previous inability to detect gp120 in brain

tissue of HIV-1 infected patients or in mice that transgenically overexpressed

gp120 [11] raised questions as to whether gp120 played an important role in HIV-1

neuropathogenesis. The lack of staining of control tissues with this antisera

indicated clearly a lack of immunoreactivity against normal cellular proteins.

Gp120 positive cells were present in all HIV-1 infected patients tested and in all

brain regions examined. Cells staining for gp120 were most often observed in

basal ganglia and perivascular cells (Figure 1). Multinucleated giant cells were

easily identified and were immuno-positive for gp120 (Figure 1 A,-C). Gp120

positive cells with microglial morphology were found in close proximity to neurons

(Figure 1A) and were scattered in focal areas within white matter and basal

ganglia (Figure 1C and D). In some brain areas, gp120 immunoreactive cells

were observed in the perivascular matrix and in cells in the perivascular region

(Figure 1D). Occasionally gp120 positive cells were noted in the lumen or wall of

blood vessels (Figure 1E and F).

<u>Neurotoxicity of Tat and gp120 is synergistic</u>:

Tat (Figure 2A) and gp120 (Figure 2B) dose-dependently increased neuronal cell

death, however gp120-induced neurotoxicity at concentrations about three orders

of magnitude less than Tat. For Tat, statistically significant and maximal levels of

neurotoxicity were observed at concentrations starting at 125 nM (Figure 2A). For

gp120, statistically significant and close to maximal levels of neurotoxicity were

observed at concentrations starting at 500 pM (Figure 2B). To determine if the

neurotoxic properties of Tat and gp120 could be synergistic, we used the sub-

threshold gp120 concentration of 30 pM and the sub-threshold Tat

concentrations of 15, 30 or 60 nM. As expected from our previous studies, gp120

at 30 pM or Tat at 60 nM did not significantly increase neuronal death (Figure 3).

Significant neuronal cell death was observed when neurons were exposed to 30

pM gp120 in the presence of 60 nM Tat (Figure 3). There was a gradual decline

in neuronal cell death with 30 and 15 nM concentrations of Tat in the presence of

30 pM gp120 (Figure 3). In all instances, <20% cell death was observed and the

dead cells were randomly scattered throughout the culture dishes. This suggests

that a select population of neurons is susceptible to viral protein-induced
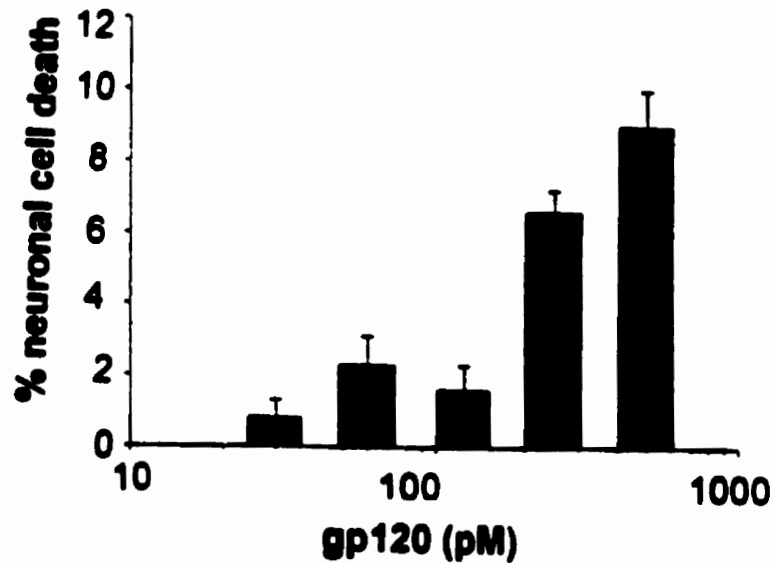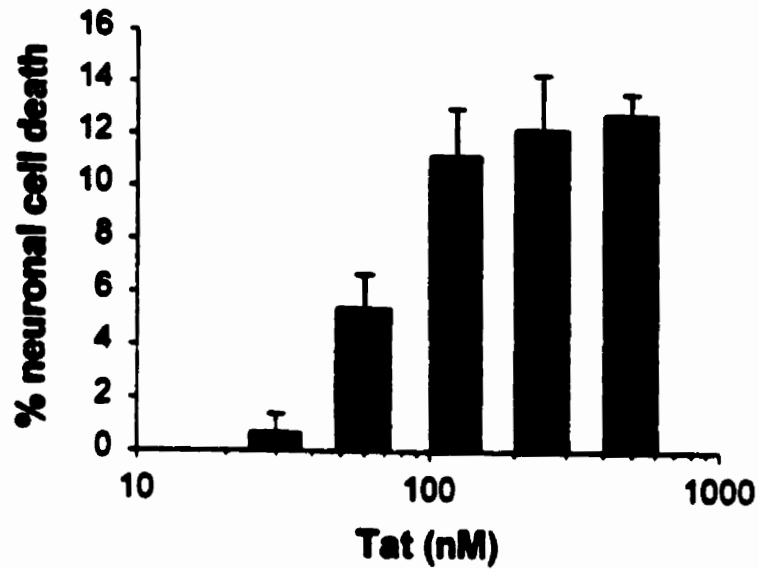
neurotoxicity.

<u>Intracellular calcium changes induced by Tat were synergistic with gp120 and</u>

<u>glutamate</u>:

Tat (n=45) at a dose of 200 fmoles and gp120 (n=53) at a dose of 0.01 fmoles

did not produce any significant changes in $[Ca^{2+}]_i$ in neurons (Figure 4), When
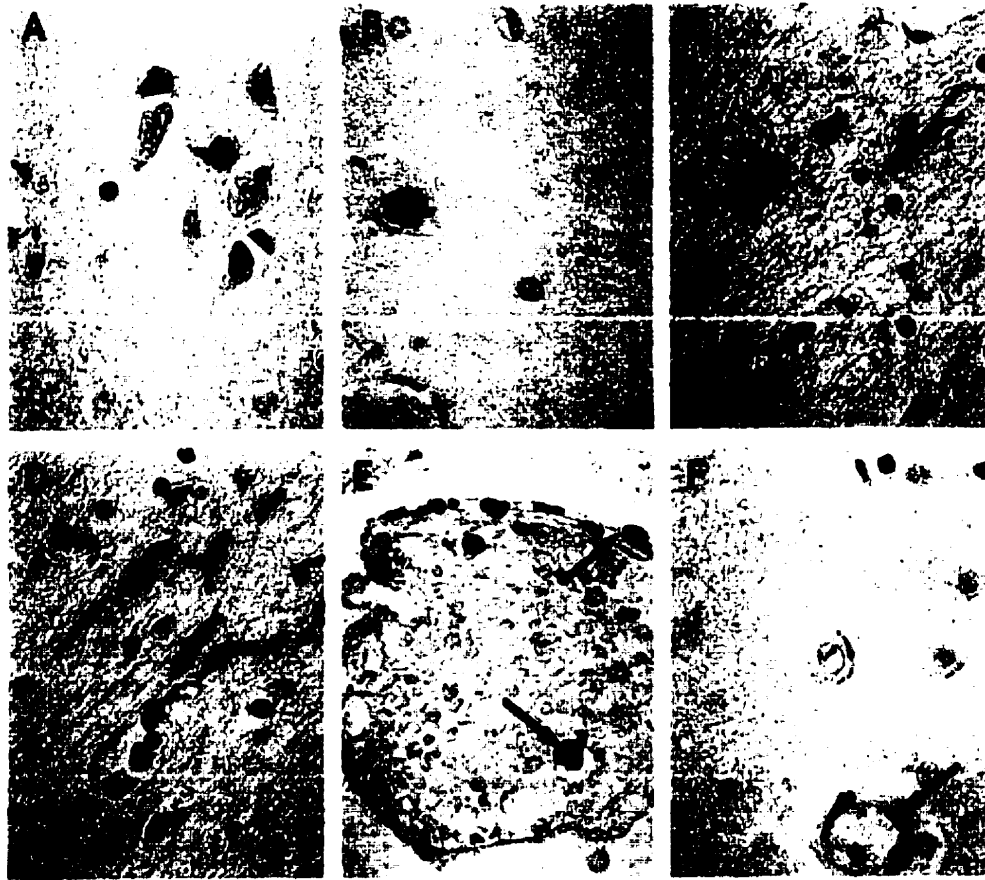
Figure 1: Immunolocalization of gp120 in patients with HIV encephalitis.
Paraffin embedded formalin fixed section from the basal ganglia.
hippocampus and temporal cortex of four patients with HIV encephalitis were
immunostained with a polyclonal goat antisera to raised against highly
purified recombinant gp120. Diaminobenzadine was used as a chromogen.
(A) gp120 positive cells in close proximity to neurons in the basal ganglia. (B)
Multinucleated giant cell immunostaining for gp120. (C) Several glial cells in
the basal ganglia show immunostaining for gp120. (D) Several perivascular
cells and the perivascular matrix shows immunostaining for gp120 (E) A
capillary in the basal ganglia shows gp120 positive cells in the lumen and in
the perivascular region (F) A small blood vessel in the hippocampal region
shows gp120 positive cells.

Figure 2: Dose response of Tat and gp120-induced neurotoxicity:
Cultures of human fetal neurons were treated with either Tat (30-500 nM) or
gp120 (30-500 pM) and cell death was monitored as described in methods.
All data represent % neuronal cell death above control, calculated as mean
± S.D. from three independent experiments done in triplicates. Significant
cell death ($p < 0.05$) was noted with $\geq$125 nM Tat (A) and 500 pM gp120
(B).
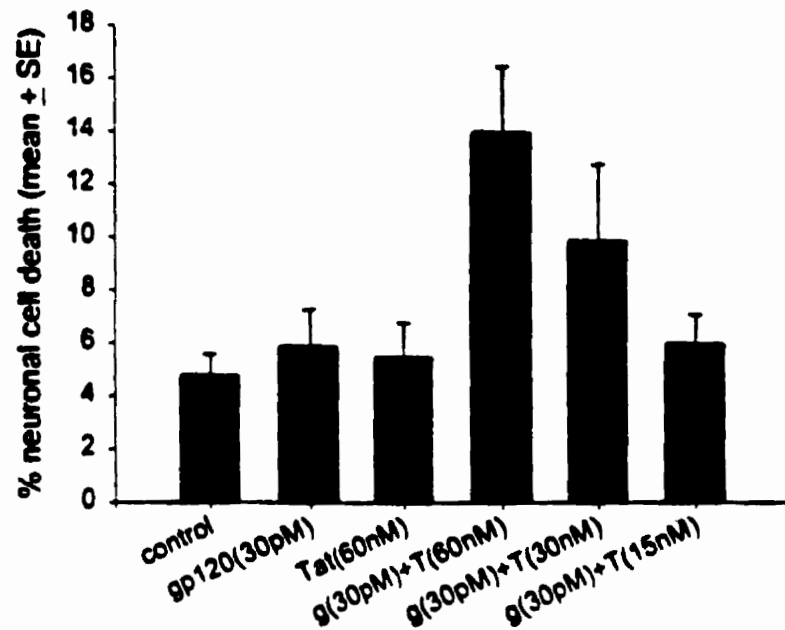
Figure 3: Synergistic response of Tat and gp120-induced neurotoxicity: Significant cell death (p < 0.05) was noted when human fetal neuronal cultures were treated with gp120 (30 pM) and Tat (60 nM) in combination while neither dose produced significant toxicity when added independently. Progressively less neuronal cell death was noted when gp120 (30 pM) was added with either 30 nM or 15 nM of Tat.
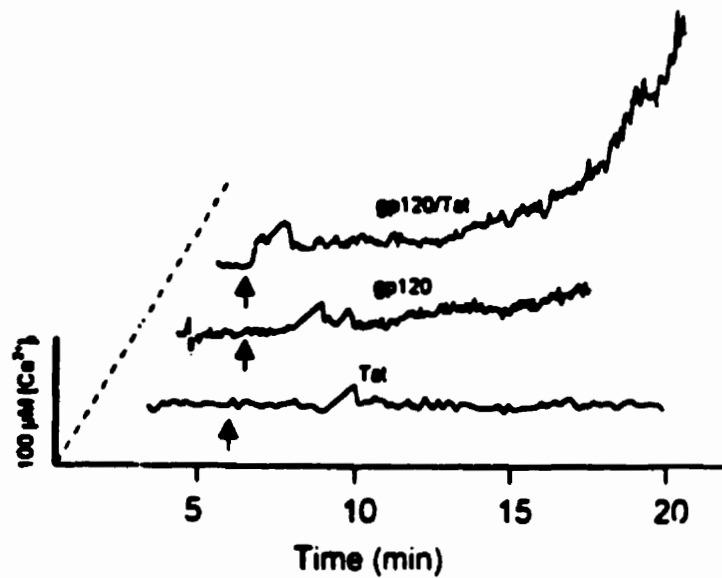
Figure 4: Synergistic effects of Tat and gp120 on $[Ca^{2+}]_i$ in cultured human fetal neurons. Tat (200 pmoles) and gp120 (0.01 fmoles) by themselves or in combination were loaded into micropipettes and pulse pressure applied onto neurons. Tat or gp120 when applied alone produced small increases in $[Ca^{2+}]_i$. In contrast, when gp120 and Tat were applied together, significantly larger, transient and secondary/prolonged increases in $[Ca^{2+}]_i$ were observed. The calcium transients illustrated were representative or 45 neurons tested with Tat, 53 neurons tested with gp120, and 39 neurons tested with Tat and gp120 in combination.

these same doses of Tat and gp120 were combined and applied to neurons together, immediate, transient increases in $[Ca^{2+}]_i$ of 173 ± 34 nM (n=39) were observed (Figure 4). In 33% of these neurons, these initial increases in calcium were followed by dysregulation of calcium homeostasis apparent as secondary/prolonged increases in $[Ca^{2+}]_i$ (Figure 4). Dysregulation of $[Ca^{2+}]_i$ was not observed when subthreshold amounts of gp120 or Tat were applied alone. Pre-incubation of neural cells with gp120 followed by pressure applications of Tat resulted in an increase of $[Ca^{2+}]_i$ that was followed by oscillating calcium levels in discrete sub-cellular domains. These intracellular "hot spots" were the focus for intracellular calcium waves. Gp120 applied alone did not result in oscillations of intracellular calcium (Fig 5 A, B)

To determine whether the synergistic responses observed between Tat and gp120 were specific to these two proteins or could be mimicked by a general excitotoxic stimulus like glutamate we determined whether even a brief exposure of neurons to Tat would increase responses to 100 µM glutamate. As a control, we repeatedly exposed neurons to glutamate and found a steady decrement in calcium responses (Figure 6 A). Initial peak $[Ca^{2+}]_i$ responses were 451 ± 52 nM, followed by 291 ± 30 nM, 225 ± 23 nM and 174 ± 15 nM. However, after a transient exposure of neurons to 2 pmoles of Tat, glutamate-induced increases in $[Ca^{2+}]_i$ were significantly larger - 671 ± 64 nM, 548 ± 43 nM, 638 ± 34 nM and 459 ±84 nM (P < 0.01) and thus did not decrease with repeated exposures and
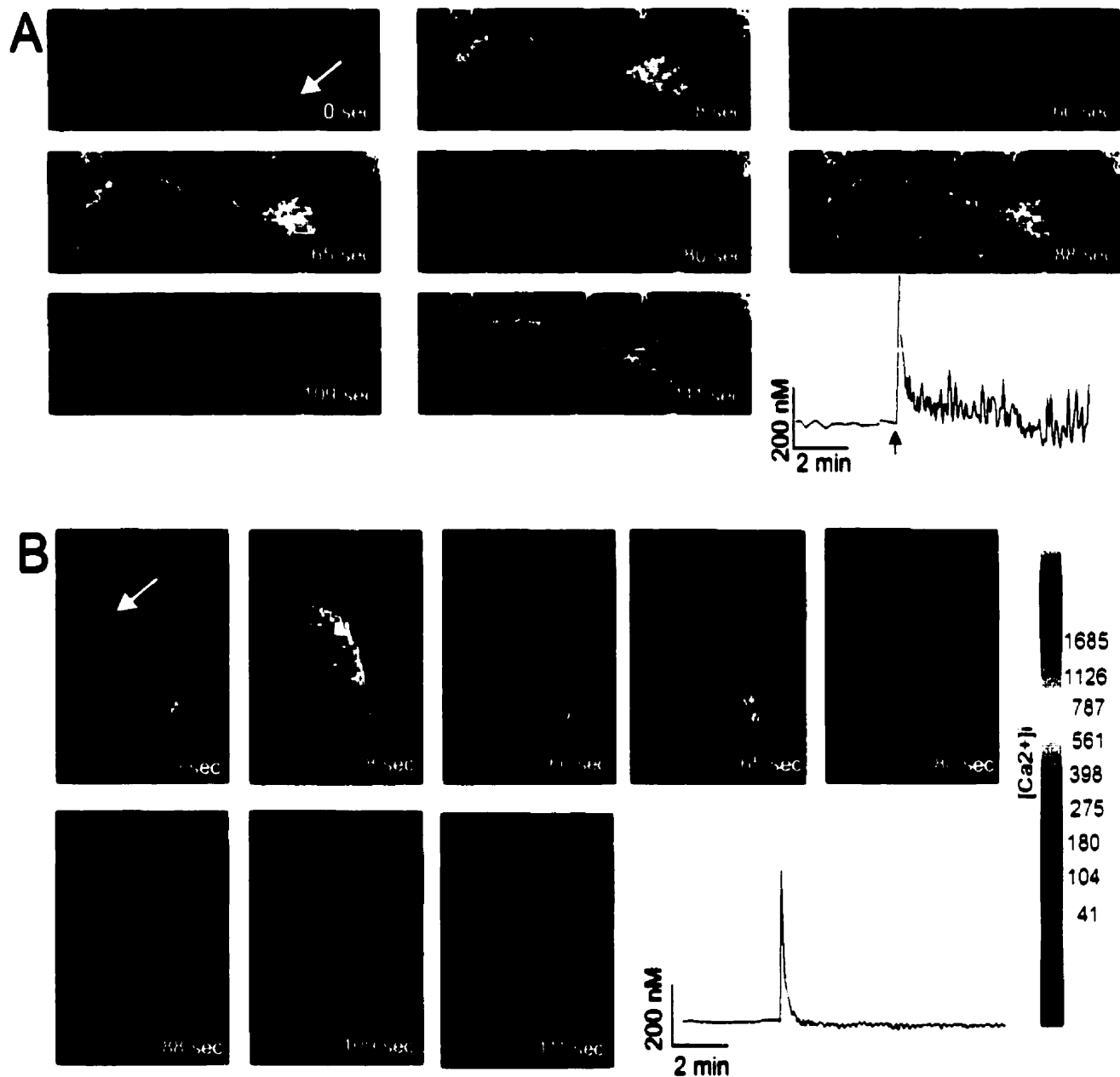
Figure 5. A. Human neural cells pre-exposed to gp120 (250 pM) for5 min followed by pressure application of Tat (2 pmoles) resulted in the appearance of intracellular calcium "hot spots" that were the focal points of intercellular calcium waves. B. Pressure application of gp120 alone (500 pM) resulted in a transient spike of $[Ca^{..}]$ without calcium oscillations. Arrows indicate regions of cells graphically represented in the lower right quadrants of image panels.

instead in 17 of the 52 cells tested resulted in a large and dysregulated increase in $[Ca^{2+}]_i$ (Figure 6B).

Neurotoxicity to Tat and gp120 requires only a transient exposure:

To determine the length of time neurons need to be exposed to viral proteins for neurotoxicity, we incubated human fetal neurons with a combination of Tat and gp120 for 30 sec, 5 min, 30 min or 15 hr and neuronal cell death was monitored 15 hours later after the proteins were applied to the neurons. Increased neuronal cell death was observed with even a 30 sec exposure and longer durations of exposure produced increased amounts of cell death. A 30 min exposure was sufficient to produce nearly maximal neuronal cell death (Figure 7).

Pharmacological characteristics of Tat and gp120 toxicity:

Excitatory amino acid receptors, sodium-proton exchangers, and free radicals, and GABA have all been implicated in pathogenesis and treatment of HIV-1 dementia. Accordingly we tested the ability of pharmacological inhibitors of these implicated systems to block the neurotoxicity induced by Tat in combination with gp120. The glutamate receptor antagonist memantine completely blocked HIV protein-induced neurotoxicity (Figure 7). The sodium-proton exchange blocker, MIA partially blocked the neurotoxicity. On the other hand, neither the free radical scavenger dipyridamole nor the GABA agonist vigabatrin significantly decreased the HIV-1 protein-induced neurotoxicity (Figure 8).

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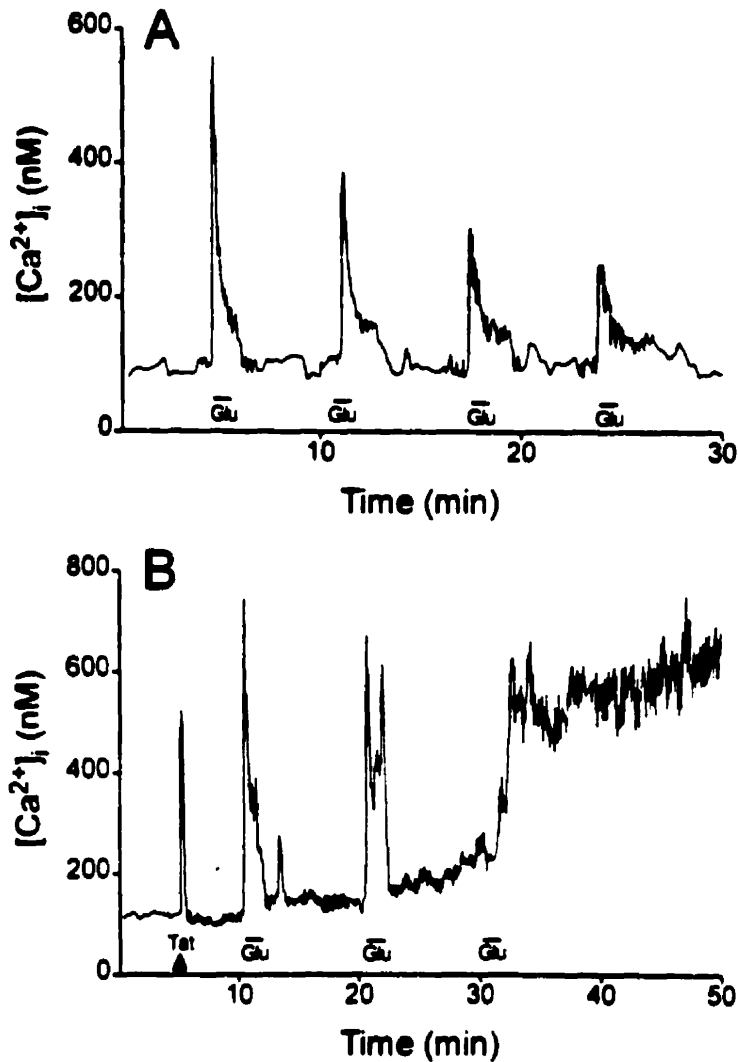
Figure 6. Increases sensitivity to glutamate by transient exposure to Tat. (A) Repeated applications of glutamate (100 µM), interspersed by 10 min wash periods, resulted in progressively smaller increases in levels of intracellular calcium. (B) Pressure application of 50 pmoles Tat increased sensitivity and resulted in massive dysregulation of calcium responses to glutamate. These illustrations are representative of 30 neurons tested with glutamate alone and 5 neurons pre-exposed to Tat followed by glutamate.
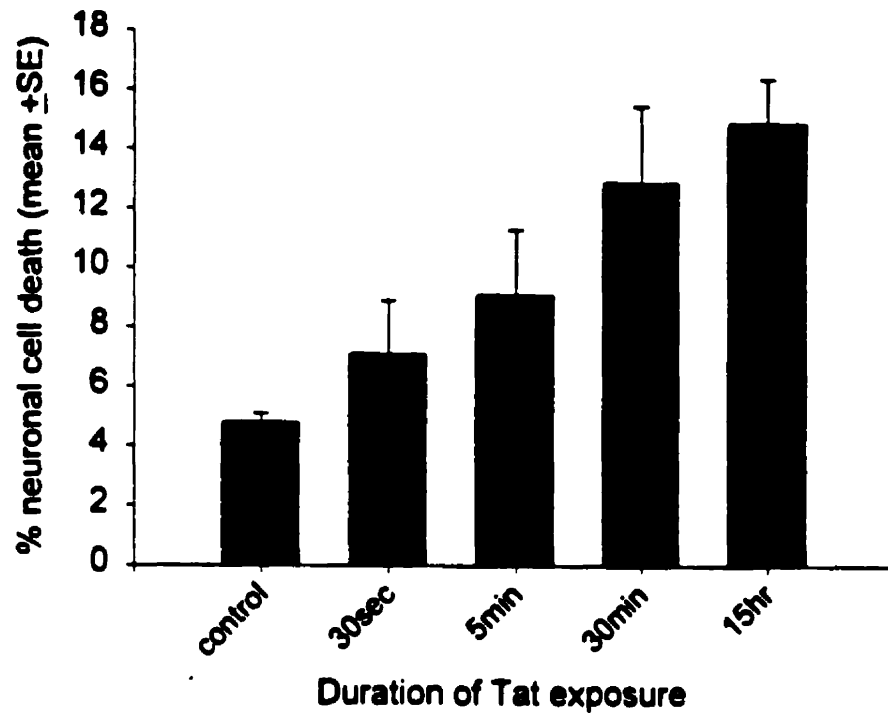
Figure 7: Neurotoxicity Induced by transient exposure to Tat and gp120: Neuronal cell death was assessed at 15 hours following exposure to Tat (60 nM) and gp120 (30 pM) for variable duration's of time. The amount of neuronal cell death correlated with the duration of Tat plus gp120 exposure. A 30 min exposure was sufficient to cause significant neurotoxicity. * $p < 0.05$; ** $p < 0.005$
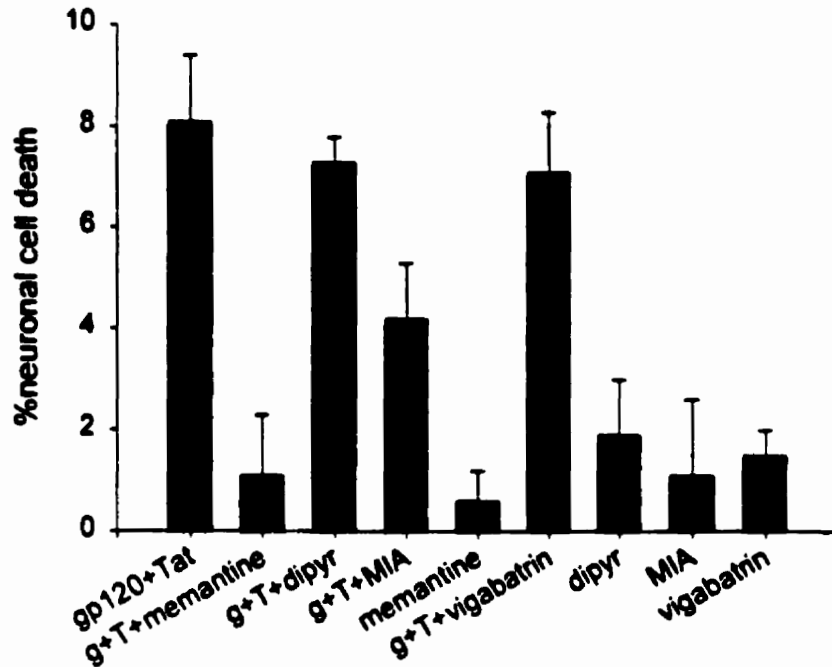
Figure 8: Pharmacological manipulation of Tat and gp120-induced neurotoxicity: Neurotoxicity of Tat (60 nM) plus gp120 (30 pM) was completely inhibited by memantine (2 μM) (p < 0.01), partially inhibited (p < 0.05) by methylisobutylamiloride (10 μM), and was not significantly affected by dipyridamole (10 μM) or vigabatrin (20 μM).

DISCUSSION

Tat, gp120 and other HIV-1 proteins can cause neurotoxicity in vitro as well as in vivo. However, demonstration of the presence of these proteins in brain of HIV-1 infected patients has been difficult and it remains unclear as to whether sufficient quantities of these proteins are present to produce neurotoxicity in vivo. Some progress in these regards has been made including reports by several independent laboratories that the HIV-1 Tat protein was present in brain of patients with HIV-1 encephalitis [4,5] (Khallili, MCP Hahnemann University, personal communication). We now demonstrate immunohistochemically the presence of gp120 in microglial cells, multinucleated giant cells and mononuclear perivascular cells in brain of patients with HIV-1 encephalitis. This breakthrough was likely aided by the availability of a polyclonal antisera directed against the glycosylated form of gp120 and the use of a technique to amplify immunohistochemical staining. These observations provide an important missing link in establishing the role of these proteins in mediating HIV-1 associated neuronal dysfunction. We further demonstrate that only very small concentrations of HIV-1 proteins are required to induce neurotoxicity and that continuous exposure of neurons to these proteins is not needed to produce neurotoxicity.

Previous studies from our laboratory have shown that Tat and gp120 use different mechanisms for causing at least initial increases in levels of intracellular calcium in neurons although in both instances, these increases in intracellular calcium lead to cell death. Tat increases the release of calcium from IP_3-

regulated intracellular pools and subsequent to this, calcium fluxed into cells mediated by excitatory amino acid receptors. On the other hand, gp120 acted first on sodium-proton exchange channels and subsequent to that action calcium fluxed into cells mediated by L-type calcium channels and excitatory amino acid receptors [15,16]. Although the initial actions of Tat and gp120 were different, the convergence onto similar mechanisms for increasing levels of intracellular calcium led us to hypothesize that their combined actions might be more than additive (i.e. synergistic). In this study, we observed that, Tat and gp120 when incubated together at sub-toxic and sub-threshold doses produced significant dysregulation of calcium and neuronal cell death. These synergistic responses were of such magnitude that toxic concentrations of Tat and gp120 in combination were about 10-fold lower than the concentrations of Tat or gp120 necessary to decrease neuronal viability.

Previously, it was reported that the neurotoxic effects of gp120 and glutamate were synergistic [17]. Now, we demonstrate that in the presence of Tat, glutamate produced massive calcium dysregulation in neurons. These observations suggest that viral proteins can synergize with one another and with other neurotoxic substances to cause significant derangement of neuronal function at much lower concentrations than previously envisioned.

We demonstrated that exposure to viral proteins for seconds to minutes may be sufficient to cause neurotoxicity several hours later. This delayed response

following a transient exposure to Tat is consistent with our previous observations. For example, exposure of glial cells and monocytes to Tat for a few minutes was sufficient to induce the expression of cytokines implicated in neurotoxicity several hours later [18]. Also, Tat injection intracerebroventricularly in rats produced progressive neuropathological changes even though Tat could not be detected in brain beyond two hours [19]. Thus continuous exposure to viral proteins may not be necessary and a "hit and run" phenomenon may be operative.

Our initial studies showed that Tat-induced neurotoxicity occurred in the 0.5 to 1 μM range [12]. That study used Tat from a commercial source. We have since determined that several technical factors accounted for these high concentrations; Tat activity is highly susceptible to freeze thaw cycles, Tat is easily oxidized, and Tat sticks to serum proteins, glass and plastic with high affinity. Some of these factors are now controlled for and procedural changes have been implimented, the result being lower concentrations of Tat required to produce neurotoxicity. For example, we now prepare Tat in our own laboratory, we do not expose the Tat to freeze-thaw cycles, we keep the Tat from being oxidized, and we do our incubations in buffer without serum. Factors that we are still unable to control are sticking of Tat to plastic dishes, metal needles of syringes and some degradation of the molecule during purification and storage. We thus conclude that the actual *in vivo* concentrations for Tat-induced effects in the brain are probably much less than that needed for the *in vitro* experiments. Despite this, however, we were able demonstrate effects of Tat for example

cytokine and chemokine induction with concentrations of 10-100 ng/ml of Tat[20,18] which are similar to the concentrations found by others to alter physiological effects [4,21-27,28-32]. Furthermore, the concentrations used by others and us are not too dissimilar from the concentrations of 1 ng/ml of Tat that were shown to be present in the serum of patients with HIV-1 infection[3] and 4 ng/ml in conditioned medium of HIV-1 infected cells[33]. Even so, levels of Tat directly adjacent to Tat-producing cells will be much higher than what is measured in biological fluids. Therefore, the levels of Tat necessary to cause neurotoxicity in vitro are close to levels found in vivo but likely are underestimates of levels in close proximity to HIV-1 infected/Tat producing cells.

In this study, using cortical neuronal cultures, we noted that select populations of cells are susceptible to viral protein-induced neurotoxicity. These observations support previous in vivo studies in patients with HIV encephalitis, which show that large cortical neurons, interneurons in the hippocampus and nigrostriatal fibers are preferentially lost[34-36]. Further studies are necessary to determine the biochemical characteristics of these neurons.

We evaluated the neuroprotective role of several pharmacological agents with diverse mechanisms of action to determine underlying mechanisms for Tat and gp120 neurotoxicity. We found that memantine, a wide-spectrum glutamate antagonist, completely blocked neurotoxicity produced by Tat and gp120 applied in combination. A previous study showed that memantine inhibited gp120-

induced neurotoxicity [37]. Memantine is currently in clinical trial for the treatment of

HIV-1 dementia. The effects of gp120 on intracellular calcium have been shown

to be mediated via sodium proton exchange channels which can be blocked by

amiloride [38]. Hence we examined the ability of MIA, a potent analog of amiloride

to block Tat and gp120 induced neurotoxicity. This drug only partially blocked the

neurotoxic effects of the HIV proteins. To determine if inhibition of free radical

production was an effective strategy for blocking Tat plus gp120 induced

neurotoxicity we used dipyridamole which is widely available as an anti-platelet

agent but also has antioxidant properties. We previously reported that

dipyridamole blocked gp120-induced free radical production in monocytes [39].

Tat has also been shown to increase free radical production by causing

mitochondrial dysfunction in neurons and inhibiting manganese superoxide

dismutase [40,41]. However, dipyramidole did not have any effect on Tat plus gp120

induced neurotoxicity. This does not however, exclude the possibility that other

antioxidants may have a role in neuroprotection against the HIV proteins. Finally,

we evaluated the role of a GABA agonist vigabatrin to block Tat plus gp120-

induced neurotoxicity because patients with HIV encephalitis exhibit loss of γ

aminobutyric acid (GABA) containing interneurons in the hippocampus [34] and we

have previously shown a relative increase in GABA levels in animals injected

with Tat presumably as a compensatory mechanism for the neurotoxic effects of

the protein [19]. No neuroprotective effect of vigabatrin was noted.

In conclusion, both Tat and gp120 are present in the brain of patients with HIV encephalitis, their neurotoxic effects are synergistic and require only a transient expression. Further, the neurotoxicity induced by these products can be effectively blocked by memantine. Our studies support the exploration of this and similar drugs in the treatment of HIV dementia.

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General Discussion

Neurological complications often accompany infection by HIV-1 [1,2]. Organic-based psychological disturbances include changes in affect, memory, and cognition. Motor functions can deteriorate and changes in gait, distal tremors and generalized muscle weakness have all been noted.

A viral correlate for the appearance and severity of neurological symptoms in HIV-1 infected patients has been difficult to determine. Viral load assessed in blood or cerebral spinal fluid, does not always predict the onset, or the severity, of neurological symptoms [3,4]. The degree of HIV-1 infected macrophage infiltration into the brain seems to be the best correlate to disturbances of neurological function [5-7]. Macrophages and microglia are the predominant cell types infected in brain; astrocytes are infected by HIV-1, but replication is severely restricted due to a poorly understood translational block of structural components of the virus [8]. This limited infection is insufficient to account for the widespread neuropathology seen in HIV-1 infected brains, that includes, multinucleated giant cell formation, astrocytosis, dendritic pruning, synaptic simplification and the loss of selected populations of neuronal cells [9,10]. Neuronal cell death cannot be due to the processes of lytic viral infection of these cells, because direct infection of neurons by HIV-1 has only rarely been reported [6,11], and likely represents the PCR amplification of unincorporated, circular DNA. Viral

proteins, appropriately termed "virotoxins", have been implicated as the agents responsible for neuronal dysfunction and death [12, 13]. The coat protein gp120 and the viral transactivator Tat are, to date, the best characterized of these toxins.

Gp120 and Tat, both of which are neurotoxic, have been detected in the serum and cerebrospinal fluid of HIV-1 infected patients [14-16]. Tat immunoreactivity has been detected in the brains of HIV-1 infected patients and in macaques with encephalitis due to infection with a chimeric strain of HIV and simian immunodeficiency virus [17]. The *in vitro* detection of Tat protein provided additional evidence in support of the hypothesis that it may be a neuropathogenic agent. Using highly specific gp120 polyclonal antisera, we demonstrated here, for the first time, the presence of gp120 in the temporal lobe, basal ganglia and hippocampus of patients with HIV-1 encephalitis. Our detection of gp120 protein in brain further suggests a role of this protein in the neuropathogenesis of HIV-1 infection.

Gp120 is thought to negatively affect neuronal function and survival as a result of interactions with, macrophages, microglia and astrocytes [13]. Lipton, and others have consistently shown that macrophages and microglia are a necessary component of gp120-induced neuronal cell death in mixed cell cultures [18, 19]. Removal of these cell types with leucine methyl ester or inhibition of their function with the tripeptide TKP protects neurons [20]. This suggests that toxic factors released from these cells, that may include, arachidonic acid, platelet-

activating factor, free radicals (NO and O_2^-), glutamate, quinolinate, cysteine and amines are important contributors to neuronal demise [13].

Previous reports demonstrated that gp120 inhibited glutamate uptake and induced glutamate release from astrocytes by calcium dependent mechanisms [21-23]. Presumably, these actions would affect neurons that are in close proximity to gp120-stimulated glial cells. Using single cell calcium imaging and immunohistochemical techniques, we demonstrated gp120 increased levels of $[Ca^{2+}]_i$ first in astrocytes and seconds to minutes later in neurons [24]. The increases in $[Ca^{2+}]_i$ were due to activation of Na^+/H^+ exchange. In neurons, calcium influx was inhibited by antagonists of L-type calcium channels and NMDA-receptors. However, in astrocytes, calcium influx was unaffected by L-type calcium channel antagonists and NMDA-receptor blockade only blunted gp120-induced increases of $[Ca^{2+}]_i$. These findings demonstrated a temporal relationship between gp120-mediated increases of calcium in astrocytes and neurons. Our results, in conjunction with previous reports (see [12, 13, 19] for reviews), suggest the following scenario: Gp120 shed from infecting virus or released from lysed cells may interact with astrocytes and activate Na^+/H^+ exchange. The increase in intracellular pH may inhibit the transport of the excitatory amino acid glutamate and the increased concentrations of extracellular glutamate would overstimulate excitatory amino acid receptors, increase calcium influx, and cause membrane depolarization and opening of voltage-sensitive calcium channels. The combined effect would be a large increase in cytosolic calcium, possibly enough to trigger apoptotic processes. Our findings thus

support the hypothesis that gp120-induced neuronal cell death occurs by indirect means.

Tat is a well-known and well-characterized transcription factor that is necessary for the replication of HIV-1. Tat is the only HIV-1 transcript that is actively exported from unlysed, HIV-1 infected cells [25, 26]. Once exported, Tat is available to interact with neurons, astrocytes and microglia through paracrine and autocrine pathways. Tat is unique among HIV-1 proteins in that it interacts directly with neurons [27, 28] and is neurotoxic [29]. The effects of this secreted and toxic protein may provide a more complete explanation for the wide spread pathology seen in the brains of AIDS patients who demonstrate limited central nervous system infection.

The mechanism of Tat-induced neurotoxicity involves intracellular calcium [27-31]. We showed that in neurons, Tat increased intracellular levels of inositol 1,4,5-trisphosphate (IP_3) and activated biphasic calcium events involving first the release of calcium from IP_3-sensitive intracellular pools and minutes later calcium influx through excitatory amino acid receptors. The delayed event of calcium influx was absent when calcium release from IP_3-sensitive stores was inhibited, suggesting a link between the two events. Indeed, Tat-induced neurotoxicity was negligible when IP_3-mediated calcium release was prevented. Calcium release from IP_3-sensitive internal stores is thus an important contributor to, and may be the primary trigger for, Tat-induced neurotoxicity.

Overstimulation of glutamate and voltage-sensitive receptors are well known

mediators of neurotoxicity. At-least one HIV-1 protein, gp120, facilitated

glutamate-induced neuronal cell death [32], presumably by increasing extracellular

glutamate concentrations and via sensitization of NMDA-receptors by cysteine

and nitric oxide [13]. Glutamate receptor mediated ion flux can be enhanced by

phosphorylation of carboxy terminal sites on the cytoplasmic portion of these

receptors by mechanisms that can involve phosphoinositol hydrolysis and MAP-

kinase [33-35]. These same pathways are stimulated by Tat, suggesting that Tat

may act on neurons to facilitate glutamate-mediated calcium flux. Indeed we

demonstrated that Tat-facilitated glutamate, NMDA and KCl-induced calcium

influx in a dose-dependant manner. These findings suggest that a sensitization of

selected neuronal receptors by Tat may mediate neurotoxic effects. In support of

this contention are results that demonstrated increased infarct size when Tat and

NMDA were co-injected into rat hippocampus [36]. These findings provide an

explanation for the neuroprotection afforded by antagonists of NMDA and non-

NMDA receptors following exposure to Tat, and suggest an important role for

calcium released from IP_3-sensitive intracellular pools in Tat-mediated neuronal

degeneration.

Central to the mechanisms of Tat and gp120-mediated neurotoxicity are

increases of intracellular calcium. Each of these proteins however, increases

calcium in neurons by (apparently) separate mechanisms. It was thus possible

that the effects of the two proteins on calcium flux would combine to produce super increases of intracellular calcium and synergistic effects on neuronal death. Amounts of gp120 and Tat that resulted in negligible calcium transients in neurons and astrocytes produced significant increases of $[Ca^{2+}]_i$ when combined. When neurons were pre-exposed to gp120 followed by transient application of Tat, calcium oscillations were noted in discrete sub-cellular domains that formed the focal points for intercellular calcium waves [37]. A similar synergy of effect was observed in neurotoxicity assays. Sub-toxic amounts of gp120 and Tat when combined, even transiently, resulted in robust toxicity [38]. Based on the effects of Tat and gp120 discussed thus far, we suggest the following: gp120 interacts with astrocytes and macrophage/microglia by a mechanism involving Na^+/H^+ exchange to induce the release of neurotoxic factors including, but not limited to, arachidonic acid, cysteine and glutamate. Arachidonic acid and cysteine can sensitize NMDA receptors. A further sensitization of glutamate and voltage sensitive receptors by Tat would result in overstimulation and massive calcium influx. This experimental approach demonstrated, for the first time, synergy between neurotoxic HIV-1 proteins.

These findings may provide an explanation for the failure of current pharmacological approaches to the treatments of HIV-1 dementia that target only glutamate or voltage sensitive-mediated calcium flux. Our results suggest that effective treatment of HIV-1-related dementia would require attenuation of both receptor types. In addition, we have identified IP$_3$-sensitive intracellular calcium

pools as important mediators of HIV-1 protein mediated neurotoxicity. Our findings provide a rational basis for the development of therapeutic agents that target intracellular calcium pools for the pharmacological treatment of HIV-1 related dementia.

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