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DISSERTATION

RETROVIRAL ENVELOPE GLYCOPROTEINS: MEDIATORS OF NEUROPATHOLOGY IN NERVOUS SYSTEM DEVELOPMENT AND NEURODEGENERATION IN HIV-1 INFECTION

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ANNA DEE FAILS ENTITLED *RETROVIRAL ENVELOPE GLYCOPROTEINS: MEDIATORS OF NEUROPATHOLOGY IN NERVOUS SYSTEM DEVELOPMENT AND NEURODEGENERATION IN HIV-1 INFECTION* BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Advisor

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Abstract of Dissertation entitled

RETROVIRAL ENVELOPE GLYCOPROTEINS: MEDIATORS OF NEUROPATHOLOGY IN NERVOUS SYSTEM DEVELOPMENT AND NEURODEGENERATION IN HIV-1 INFECTION

Better than 90% of AIDS patients have neuropathology identified at autopsy. HIV-1, the causative organism of AIDS, produces neuronal death as a primary viral effect in many AIDS cases, and it does so even though the virus does not infect neurons. The search for the pathomechanisms by which HIV-1 causes neurologic disease has frequently focused on the possible role of the envelope glycoprotein, gp120, as a mediator of excitotoxicity.

Many animal retroviruses of veterinary importance produce neurologic and immunologic syndromes similar to those manifest by HIV-1. The feline retroviruses, feline immunodeficiency virus and feline leukemia virus, are clinically similar to HIV-1 and HTLV-1 (human T-lymphotrophic virus), respectively. Like gp120 of HIV-1, the envelope glycoprotein of FeLV produces lymphocytic and neuronal toxicity.

In the first study of this dissertation, we hypothesized that retroviral envelope glycoproteins can act on advancing growth cones in the developing nervous system, leading to derangement of neuronal connectivity. We tested this hypothesis by applying an oligopeptide derived from the envelope glycoprotein of FeLV to the growth cones of cultured neurons and observing its effect on morphology and calcium regulation. The

oligopeptide produced changes manifested as altered morphology, loss of motility, and increased $[Ca^{2+}]_i$. This suggests a mechanism by which FeLV in particular and retroviruses in general might contribute to abnormal nervous system development in perinatal infections.

Seizures are a common sequela of HIV-1 infection of the brain. In the second study in this dissertation we hypothesized that the presence of gp120 in the hippocampus may produce a pattern of neuronal loss similar to that seen in temporal lobe epilepsy. In this study, we exposed cultured cells of the dentate gyrus (granule cells) and hippocampal gyrus (pyramidal cells) to gp120 to assess their relative sensitivity to its neurotoxicity. Granule cells were resistant to concentrations of gp120 which produce significant cell death amongst pyramidal cells. Such a pattern of differential neuronal sensitivity and cell loss may constitute the neuroanatomic basis for expression of seizures in AIDS patients.

These studies add to the body of evidence that indicates the envelope glycoproteins are important mediators of neuropathology in retroviral infections of the nervous system.

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CHAPTER ONE:
Introduction to Retroviruses, HIV-1, and the Neuropathology of Lentiviral Infection

A Crash Course in Retrovirology

Shortly after the turn of the century and only a few years after the discovery of the first virus (tobacco mosaic virus), French scientists Vallée and Carre isolated the causative organism of Equine Infectious Anemia (EIA) (Vallée & Carre 1904). This discovery made the responsible virus, EIAV, one of the first animal viruses characterized. As later investigation showed, EIAV is also the charter member of the viral family *Retroviridæ*, a group distinguished by a viral genome of positive sense, single-stranded RNA that is reverse transcribed in the host cell into a double-stranded DNA via a viral reverse transcriptase.

Classically, retroviruses have been divided into three subfamilies based primarily on their biological and pathological behavior. These are 1) *Spumavirinæ*, a group of viruses which cause vacuolization of host cells in culture but produce no known disease in vivo; 2) *Oncovirinæ*, a disparate group loosely related on the basis of their tendency to be associated with neoplasia in the host; and 3) *Lentivirinæ*, retroviruses with complex genomes and whose pathological behavior is expressed over an extended course of infection. The International Committee on the Taxonomy of Viruses, however, has recently replaced this traditional triumvirate of viral groups with a familial system based

on genomic and structural relationships. The new parsing of retroviruses divides them into seven redefined genera of which *Spumavirinæ* and *Lentivirinæ* are two (Coffin J, 1996).

The genus *Lentivirinæ* is composed of complex viruses that contain, in addition to the structural genes found in simpler retroviruses, a number of regulatory genes important in viral replication and genetic expression (Clements & Zink 1996, Coffin 1996). EIAV, the animal virus isolated in 1904, is a lentivirus, but until the 1980's no human lentiviruses were known. In 1983 a virus with properties consistent with retroviruses was isolated from a human patient; this retrovirus was linked to the growing pandemic of acquired immunodeficiency in humans (Barré-Sinoussi et al 1983). The virus, subsequently named Human Immunodeficiency Virus Type 1 (HIV-1) (Coffin et al 1986), was eventually identified as a lentivirus based on its behavioral and structural properties. Because of the profound impact HIV-1 has had on human populations in the past two decades, *Lentivirinæ* has been propelled from an inconspicuous group of viruses of primarily veterinary interest to one of preeminent human importance.

Retroviruses are enveloped viruses, 80-130 nm in diameter (Stroop WG 1994). Two copies of the positive-sense, single-stranded RNA genome are contained within a protein capsid whose shape and symmetry are characteristic of the viral species (Stroop WG 1994, Murphy FA 1996). The 7-11 kb viral monomer comprises four genes coding structural proteins in this order (5' to 3'): 1) *gag*, encoding the internal, non-glycosylated structural proteins, 2) *pro*, encoding the protease responsible for cleavage of proprotein products, 3) *pol*, encoding the reverse transcriptase and integrase which generate viral

DNA and insert it into the host genome, respectively, and 4) *env*, encoding the envelope glycoproteins present on the surface of the viral envelope (Coffin 1996). Additional regulatory gene products, not found in the simple retroviruses, are characteristic of *Lentivirinæ* (Barker et al 1995, Clements & Zink 1996). HIV-1 has at least six additional, non-structural gene products. These regulatory proteins are the subjects of intense interest among AIDS researchers, since pharmacological manipulation of their activity may have therapeutic applications.

Retroviruses begin their life cycle (for reviews, see Coffin 1996, Turner & Summers 1999) within the host cell by reverse transcription of the viral RNA into a negative-sense DNA transcript that is subsequently converted into double-stranded DNA. This viral DNA then is integrated as a provirus randomly into the host cell's genome. Once integrated, further transcriptional and translational processes are carried out by cellular machinery. Expression of viral message in permissive cells produces both genomic RNA and mRNA. After production and processing in the cellular ER and Golgi, proteins are targeted to the cell membrane where assembly begins and the virion buds from the host cell. The large precursor proteins produced by translation of viral mRNA are cleaved into definitive structural proteins by the viral protease relatively late in the viral assembly process. Full maturation of the virus (including cleavage of proteins and final packaging of the genome) to an infective virion does not take place until the virion has been released from the host cell. This protracted maturation has been the target of antiviral therapy; the use of protease inhibitors to reduce the release of infective virus has become an important strategy in the medical management of HIV-1 infection.

Retroviruses and Neurologic Disease

In the period from 1940-1965, an epizoötic of respiratory and neurologic disease in the native sheep of Iceland led to the isolation and characterization of the prototypical lentivirus, maedi-visna virus (from the Icelandic words for the shortness of breath and wasting seen in affected sheep) (Sigurdsson et al 1960, Pétursson 1994). Although progressive pneumonia is the most common presentation in affected sheep, some infected animals develop a persistent inflammatory encephalitis and focal demyelination in the central nervous system (Sigurdsson et al 1957, Nathanson et al 1983). This association between lentiviral infection and pathology in the nervous system is a motif exhibited by most lentiviruses and many other retroviruses. Other retroviruses known to produce neuropathology include Caprine Arthritis-Encephalitis Virus (CAEV) (Cork et al 1974), Equine Infectious Anemia Virus (EIAV) (McClure et al 1982), Murine Leukemia Viruses (MuLV) (Andrews & Gardner 1974), Feline Leukemia Virus (FeLV) (Hardy 1981, Hoover and Mullins 1991), Feline Immunodeficiency Virus (FIV) (Phillips et al 1994), Bovine Immunodeficiency Virus (BIV) (Walder et al 1995, Belloc et al 1996) and Simian Immunodeficiency Viruses (SIV) (Hurtrel et al 1991, Lackner et al 1991). The degree to which each of these viruses cause neurologic disease is variable, as are the specific neuropathological lesions associated with each. Yet what remains remarkable is that they are neuroinvasive and neuropathic in spite of having, in nearly every example cited, hematopoietic cells as their primary targets.

The first human retrovirus was identified in 1980. Human T-cell Lymphotropic Virus type 1 (HTLV-1) was isolated from lymphocytes of a patient with cutaneous T-cell lymphoma (Poiesz BJ et al 1980); this virus was later shown to be the causative organism

of Tropical Spastic Paraparesis, a viral disease of the spinal cord (Nakagawa et al 1995). HTLV-1 and the subsequently isolated HTLV-2 were originally categorized as members of the erstwhile *Oncovirinæ*, but are now included in Mammalian C-Type Viruses, a subgroup of the Avian Leukosis-Sarcoma Virus (ALSV) Group. The ALSV Group comprises a large number of simple viruses, many of which have oncogenic properties. Other notable members of the Mammalian C-Type sub-group include the Feline and Murine Leukemia Viruses. Together with HTLV, these three viruses are strongly associated with myelopathies in their hosts, in addition to their lymphoproliferative effects (Andrews & Gardner 1974, Hardy 1981, Haffer et al 1987, Hoover & Mullins 1991, Gessain & Gout 1992, Hollsberg & Hafler 1993, Nakagawa M et al 1995, Wheeler 1997).

The lentivirus HIV-1 is also associated with myelopathy (Navia et al 1986, Dal Pan et al 1994), although this manifestation of HIV-1's neurovirulent behavior affects only 11-22% of AIDS patients (Gabuzda & Hirsch, 1987). More common is a subacute encephalitis that produces a syndrome of progressive psychomotor slowing, cognitive deficits, and dementia.

HIV-1 is an aggressively neurotropic virus. In addition to its cognominal effects on the immune system, infection with HIV-1 is associated with neuropathology in more than 90% of AIDS cases (Gabuzda et al 1987, Nathanson et al 1990, Burns et al 1991, Everall et al 1993). The extant nomenclature for the diverse neuropathological lesions is chaotic, with multiple descriptors for each of the many characteristic lesions (see Navia et al 1986 and Budka et al 1991 for reviews). The classic pathologies are these: 1) HIV

encephalitis (HIVE), characterized by inflammatory cell infiltrates and often the appearance of multinucleated giant cells (Kure K et al 1991, Masliah E et al 1992); 2) HIV leukoencephalopathy, a term which refers to diffuse damage to white matter, "myelin pallor" and a reactive astrogliosis (Smith et al 1990, Burns DK 1991, Masliah et al 1992, Power et al 1993); 3) diffuse poliodystrophy, denoting widespread pathology of cortical and subcortical grey matter (Dal Pan et al 1992, Everall et al 1993); and 4) vacuolar myelopathy, a multifocal inflammatory change in the spinal cord (Harrison & McArthur 1995, Dal Pan et al, 1994).

These lesions are not mutually exclusive, and patients may suffer from any combination of them. The clinical presentation is therefore highly variable, reflecting the heterogeneity of lesions. Some of the most commonly reported signs and symptoms include cognitive impairments (loss of memory, poor concentration, depression, loss of affect) (Navia et al 1986, Portegies 1994, Harrison & McArthur 1995); seizures (Holtzman et al 1989, Wong et al 1990); tremors and loss of fine motor control (Navia et al 1986, Gabuzda & Hirsch, 1987, Glass & Johnson 1996); spasticity, weakness and hyperreflexia in the pelvic limbs (Dal Pan et al 1994, Glass & Johnson 1996); and painful dysesthesias (de la Monte SM et al 1988, Harrison and McArthur 1995). The preeminence of cognitive signs in clinically manifest HIV-1 neuropathology has led to the wide use of the term "AIDS Dementia Complex" (ADC) to describe the constellation of dysfunctions exhibited by infected persons (Navia et al 1986, Harrison & McArthur 1995, Portegies 1994). Other more-or-less synonymous terms include HIV Encephalopathy (HIVE), HIV-Associated Cognitive/Motor Complex, and neuroAIDS.

Regardless of the terminology, it is striking that nearly half of the patients with neurologic signs have no secondary lesions to account for them (Smith 1990, Burns et al 1991, Everall et al 1993). When opportunistic infections and neoplasias (acquired secondary to immunosuppression and decreased immune surveillance) are excluded, a large proportion of neurological signs are directly due to the neuropathic effects of HIV-1 on cells of the nervous system.

How do HIV-1 in particular and lentiviruses in general produce neuropathology? Neurons are clearly lost or injured in ADC (Ketzler et al 1990, Dal Pan et al 1992, Masliah et al, 1992b, Masliah et al 1992c, Everall I et al, 1993; Jernigan TL et al 1993) despite a lack of evidence for significant HIV-1 infection of neurons in vivo (Wiley et al, 1986, Kure et al 1990) and a discordance between the severity of the neuropathology and the small amount of detectable virus in nervous tissue. The answer is likely a complex interaction between the whole virus, individual viral proteins released into the extracellular milieu, and the many cell types of nervous tissue.

Neuroinvasion and Neurotropism

HIV-1 gains access to the nervous system during the often-subclinical meningitis experienced at the first, transient viremia (Goudsmit et al 1986, Hollander & Levy 1987, Gray et al 1996). Neuroinvasion could result directly as free virus penetrates an altered blood-brain barrier, with the choroid plexus being the site most particularly implicated (Falangola et al 1995, Hanly & Petito 1998), or through direct infection of endothelial cells (Moses & Nelson 1994, Poland et al 1995). Most current evidence, however, supports the "Trojan Horse" theory of neuroinvasion as the primary route by which

HIV-1 gains access to the central nervous system. In this proposed mechanism, HIV-1-infected monocytes carry the virus across the blood-brain barrier as a consequence of normal monocytic traffic into the nervous system (Haase 1986, Lawson et al 1992, Georgsson 1994). This ingress may be facilitated by pro-inflammatory signals from infected cells of the blood brain barrier (Tyor et al 1992, Hurwitz et al 1994) or by changes in the expression of cell adhesion molecules at the barrier (Hurwitz et al 1994, Nottet et al 1996).

Precisely which cell types in nervous tissue are routinely infected with HIV-1 in vivo remains disputed, and the receptors for HIV-1 are central to this controversy. The definitive, high-affinity receptor for HIV-1 in the peripheral immune system has been long identified as the CD4 molecule, present on the T-helper lymphocytes and monocytes (Sattentau & Weiss 1988). Different strains of HIV-1 are described as lymphotropic, macrophage-tropic, or dual-tropic depending on their relative tropism for each of these cell types, a specificity dictated solely by the virus' envelope glycoprotein (Hwang et al 1991). The only cells normally found in the nervous system expressing the CD4 marker are the monocyte-derived, phagocytic microglia (and a few, transient monocytes). It is no surprise, therefore, that strains of HIV-1 that are particularly noted for their neuroinvasive properties are those that are macrophage-tropic (Cheng-Mayer et al 1989, Jordan et al 1991, Sharpless et al 1992b, Gonzalez-Scarano et al 1995). The only cells consistently found to support productive infection by HIV-1 in the central nervous system are microglia.

While CD4 is sufficient for binding of the virion to CD4+ target cells, its expression alone is insufficient for fusion between the virus and target cell, an event

requisite for subsequent infection. Certain CD4+ human cells are resistant to infection (Clapham et al 1991) while expression of CD4 on non-human cells does not automatically render them susceptible to infection (Chesebro et al 1990). An additional co-receptor is therefore required for attachment to proceed to fusion. Receptors for chemokines (small cytokines with developmental and pro-inflammatory functions) have recently been identified as important co-receptors for HIV-1; their interaction with the envelope glycoprotein and CD4 is critical for fusion and subsequent infection of the target cell (Deng et al, 1996, Feng et al 1996, Broder & Collman, 1997, Moore et al 1997). It is the interaction between the chemokine receptor and the envelope glycoprotein of HIV-1 which determines the cellular tropism of the viral strain (He et al 1997, Speck et al 1997, Wang et al 1998) and sets in motion the sequential molecular events that lead to fusion of viral and cellular membranes (reviewed in Chan & Kim 1998).

While CD4 remains the definitive receptor for binding of HIV-1 to its target cells (Sattentau 1988), researchers seeking to explain CD4-independent infection have investigated other hypothetical receptors. The membrane glycolipids galactosylceramide (Harouse et al 1991; Cook et al 1994) and sulfatide (van den Berg et al 1992, Cook et al 1994, McAlarney et al 1994) have been proposed as alternative receptors by virtue of the ability of the envelope glycoprotein to bind them. HIV-1 interacts with these membrane glycolipids at an epitope of the surface unit glycoprotein that is distinct from the CD4 binding site (Cook et al 1994, Trujillo et al 1996). These putative receptors could be a route by which HIV-1 binds to and infects CD4-negative neural, endothelial, and colonic cells.

In the central nervous system, HIV-1 proteins and mRNA are localized consistently only to microglia and other monocyte-derived cells (e.g., multinucleated giant cells) (Koenig et al 1986, Kure et al 1990, Watkins et al 1990, Sharpless et al 1992). Nonetheless, it is widely acknowledged that the presence of only a very few copies of provirus and/or severely restricted expression of viral DNA in non-permissive cells may render these at or below the usual threshold for laboratory detection. Accordingly, some studies have detected viral products in astrocytes (Wiley et al, 1986, Nuovo et al 1994, Tornatore et al 1994, Bagasra et al, 1996), neurons (Pumarola-Sune et al 1987, Nuovo et al 1994, Bagasra et al 1996), oligodendrocytes (Nuovo et al 1994, Albright et al 1996) and endothelial cells (Wiley et al, 1986, Moses & Nelson 1994, Bagasra et al 1996). In vitro, a number of glial and neuronal cell lines can be infected, although usually with only little or no production of infectious virion (Brack-Werner et al 1992, Gonzalez-Scarano et al 1995, Albright et al 1996). Ultimately, most reported non-monocytic infections appear to be restrictive, with the infected cells producing only small amounts of viral protein (usually regulatory and not structural) and no whole virions. Accumulating evidence suggests, therefore, that HIV-1 is capable of infecting cells in the nervous system other than microglia, but that such infections are comparatively uncommon in the natural disease and generally result in only restricted expression of viral proteins. While non-permissive infections may not necessarily result directly in death of the host cell, nor contribute to the overall viral burden through production of infectious virus, expression of viral proteins on the surface of and/or dysfunction of infected cells may be important contributors to the neuropathology of HIV.

Mechanisms of Neuropathology

AIDS patients suffer from neurologic disease, sometimes of devastating magnitude. Ample data show loss of neurons, abnormalities in their dendrites (Masliah et al 1992c), and derangement of their ability to conduct information (Iragui et al 1994). And yet, HIV-1 only rarely infects neurons in vivo, and the number of infected cells in nervous tissue is often poorly related to the degree of neuronal dysfunction (for reviews, see Atwood et al 1993, Epstein & Gendelman 1993, Glass & Johnson 1996). Between the neuroinvasive virus and the injured neuron must lie an indirect pathway capable of expressing and amplifying the neuropathic effects of a few virions.

Explanations are legion. Many have focused on infection of the immune cells of nervous tissue and their consequent expression of neurotoxic cytokines and peptides. The HIV-1-infected brain exists in a state of "immune activation," and infected, activated microglia and macrophages have been implicated repeatedly at the center of a maelstrom of inflammatory and neuropathic processes (Tyor et al 1992, Benveniste 1994, Yoshioka et al 1995, Nuovo & Alfieri 1996) (see below).

Another line of inquiry has examined the role of macroglia, both non-infected and restrictively infected. Astrocytes in the HIV-1-infected brain may be implicated in neuronal injury by either loss of their normal housekeeping functions or by active production of excitotoxic substances and cytokines subsequent to exposure to viral proteins (Pulliam et al 1993, Benos et al 1994, Dreyer and Lipton 1995, Vesce et al 1997). Oligodendrocytes are adversely affected by the immune-activated state of the HIV-infected central nervous system (Benveniste 1994, Bernardo et al 1997) and by the

presence of serum, which is permitted ingress into nervous tissue through an altered blood-brain barrier (Power 1993).

Adding to the complexity of these issues is the consideration that immune activation and cellular dysfunction may arise not only from infection by whole virus, but also from contact with individual viral proteins borne on the surface of infected cells and released into the extracellular environment. The regulatory proteins of HIV-1, *Tat*, *Rev*, and *Nef*, are expressed in both productively and restrictively infected cells. Restrictively infected astrocytes may express these regulatory proteins (Saito et al 1994, Ranki et al 1995, Gorry et al 1999), which have been associated with excitotoxicity (Sabatier et al 1991, Magnuson et al 1995, New et al 1997, Cheng et al 1998, Kruman et al 1998), immune activation (Rappaport et al 1999), and upregulation of HIV infection (Cullen 1994, Turner & Summers 1999). Their presence in the extracellular milieu and on the surface of infected cells may be another route by which uninfected neurons are indirectly injured or killed.

The transmembrane glycoprotein, gp41, on the surface of the viral envelope, is another viral product whose individual contribution to neuropathology has been examined. gp41 has been associated with the formation of neurocytokines and induction of nitric oxide synthase (Adamson et al 1996, Adamson et al 1999), conditions which can mediate neurotoxicity in cell culture systems. Additionally, the carboxy-terminal portions of this glycoprotein applied to planar phospholipid bilayers have been shown to produce pores with conductance to a variety of cations and chloride (Chernomordik et al 1994), a mechanism by which affected cells might undergo osmotic swelling and cell death.

Much of the study of individual viral proteins' role in the neuropathology of HIV-1 infection has focused on the effects of the surface unit glycoprotein, a 120-kilodalton molecule known as gp120. gp120 may produce neuronal death through diverse pathways, including astrocytic dysfunction (Pulliam et al 1993, Benos et al 1994, Dreyer & Lipton 1995), competitive inhibition of the function of important neurotrophic molecules (Lee et al 1987, Brenneman et al 1988), and stimulation of cytokine (Yeung et al 1995, Chao et al 1996), eicosanoid (Dreyer & Lipton 1995, Ushijima et al 1995), and free radical (Dawson et al 1993) production in the non-neuronal cellular population. The potential neurotoxicity of gp120 is the subject for the third chapter in this manuscript.

Neuroimmunology:
Cytokines, Eicosanoids and Other Mediators of Neurotoxicity

The resident macrophages of the brain, the microglia, are the only cells of nervous tissue believed to be productively infected by HIV-1. Infection and/or contact with viral antigens in the extracellular environment has the effect of "activating" the quiescent microgliocyte, an immunological process by which the cell is induced to proliferate, begin migration, and increase its expression of pro-inflammatory substances (reviewed in Banati et al 1993). Activated microglia are cytotoxic, by virtue of both their phagocytic behavior and their release of a large variety of free radicals, arachidonic acid metabolites, and cytokines.

Any discussion of immune processes in the central nervous system, therefore, necessarily becomes a description of cytokine and lipid inflammatory mediators. Cytokines in this context are a heterogenous group of small peptide signaling molecules

that act by autocrine or paracrine mechanisms to modulate inflammatory and immune responses. The lipid inflammatory mediators are a group of phospholipid-derived molecules collectively described as eicosanoids and which mostly derive from arachidonic acid metabolism. Like other complex modulatory physiological processes, inflammatory mechanisms involving cytokines and eicosanoids are cascading and possess the ability to amplify the effects of small homeostatic disturbances. It is this property that is evoked to describe how small viral loads can produce global effects in HIVE. HIV-1-infected brains exist in a state of "immune activation," with cytokines and other pro-inflammatory mediators increased within brain parenchyma (Tyor et al 1992, Nuovo & Alfieri 1996) and in CSF (Gallo et al 1989, Grimaldi et al 1991, Perrella et al 1992). In an infectious process like HIV-1 infection, where elimination of the virus is not possible, continuous immune activation may injure bystander cells. In the case of HIVE, those cells are likely to be oligodendrocytes and neurons (for reviews, see Tyor et al 1992 and Benviste 1994).

Cytokines are produced by a wide variety of activated cells and possess the ability to affect not only adjacent cells, but also the cell of origin. In the central nervous system, astrocytes, microglia, and monocytes are particularly noted for their production of cytokines. Their specific effects have been reviewed (Benveniste 1994); these may be summarily described as 1) induction of other cytokines, 2) stimulation of astrogliosis, 3) chemotaxis of inflammatory cells, and 4) increasing the cytopathogenicity of microglia through immune activation. In addition, some cytokines have been shown to affect HIV-1 replication, usually by up-regulation (reviewed in Merrill & Chen 1991, Vicenzi & Poli 1994), and some, most especially the Tumor Necrosis Factors (TNFs) may be directly

cytotoxic to oligodendrocytes (Selmaj & Raine 1988, Selmaj et al 1991) and neurons (Westmoreland et al 1996). Many different cytokines have been implicated as mediators of nervous system dysfunction in HIVE, including Interleukins 1 (IL-1) and 6 (IL-6), Transforming Growth Factor-β (TGF-β), Tumor Necrosis Factor-α (TNF-α), and several colony stimulating factors. In the immune-activated brain, these peptides are produced most notably by microglia, infiltrating macrophages, and astrocytes (Benveniste 1994, Yoshioka et al 1995).

Breakdown of membrane phospholipids produces a variety of important inflammatory mediators, including the eicosanoids (metabolites of arachidonic acid) and platelet activating factor (PAF). Processing of arachidonic acid via the cyclooxygenase and lipoxygenase pathways produces prostaglandins and leukotrienes, respectively, and these, along with PAF, may be expressed by cells of the monocytic line when these are infected by HIV-1 or exposed to gp120 (Wahl et al 1989, Dreyer & Lipton 1995, Ushijimi et al 1995). Eicosanoids and related substances are powerful chemoattractants, increase vasopermeability, and stimulate the release of other pro-inflammatory substances and oxygen radicals. They can lead to increases in extracellular excitatory neurotransmitters through their effects on astrocytes and microglia, and may, in some cases, be directly neurotoxic (reviewed in Lipton et al 1994).

Like some cytokines, a number of lipid inflammatory mediators can also produce abnormalities of myelin and oligodendrocytes (reviewed in Hartung et al 1992); activation of cytokine/arachidonic acid pathways may therefore be an important mechanism by which leukoencephalopathies develop in AIDS Dementia Complex.

Other candidate neurotoxins, the production of which may be stimulated by gp120, gp41, and/or infection by HIV-1, include free radical species such as nitric oxide (NO•) (Lipton et al 1994). Nitric oxide has been identified as an immuno- and neuromodulatory molecule, produced in a variety of cell types through a constitutive, calcium-dependent nitric oxide synthase (NOS) and also by activation of an inducible form of NOS. The expression of this inducible enzyme is known to be upregulated by cytokines in inflammatory conditions of nervous tissue and has been linked to neurotoxicity in ischemic and neurodegenerative disorders (Hewett & Choi 1993, Whittle 1995). Upregulation of NOS has also been demonstrated in neuronal cultures exposed to gp120 (Dawson et al 1993) or gp41 (Adamson et al 1999) and in glial cultures infected with HIV-1 (Bukrinsky et al 1995). Accordingly, increased expression of iNOS has been detected in tissue from AIDS patients suffering from severe dementia (Adamson et al 1996), implicating NO• as another participant in injury to neurons and myelin in the HIV-1-infected brain.

Excitotoxicity

Ionic calcium concentration within resting neurons ($[Ca^{2+}]_i$) is maintained around 5 to 20 nM, a concentration several orders of magnitude less than the approximately 1.3 mM typically seen in the extracellular environment (reviewed in Orrenius et al 1996). Influx of $[Ca^{2+}]$ may occur by a number of means, including 1) the opening of voltage-gated calcium channels in response to membrane depolarization; 2) generation of calcium current through certain varieties of AMPA-type glutamate receptors; and 3) activation of NMDA-type glutamate receptors, concomitant with membrane depolarization sufficient

to remove their magnesium block. Influx of $[Ca^{2+}]$ is a normal consequence of neuronal activity and constitutes an important, pluripotent cellular signal, but when it occurs at supraphysiologic levels, the $[Ca^{2+}]_i$ can exceed the cell's capacity for regulation and set in motion a series of events which lead to death of the neuron (reviewed in Choi 1992 and Orrenius et al 1996). When pathologic increases in $[Ca^{2+}]_i$ are due to excessive membrane depolarization, the process is called "excitotoxicity," and it is widely viewed as one important mechanism by which neurons are ultimately killed in HIV encephalopathy.

There are a number of pathways by which HIV-1 and its gene products might produce excitotoxicity. Early studies of HIV-1-associated neurotoxicity showed that the virus' envelope glycoprotein, gp120, could evoke a rapid, supraphysiologic increase in intraneuronal calcium in cultured rat hippocampal cells (Dreyer et al 1990, Lo et al 1992) and retinal ganglion cells (Dreyer et al 1990, Lipton et al 1991). This increase, which subsequently led to death of neurons, was mediated through NMDA receptors as evinced by the fact that pharmacological blockade of these receptors ameliorated the rise of $[Ca^{2+}]_i$ and the subsequent cell death (Dreyer et al 1990, Lipton et al 1991, Lo et al 1992, Müller et al 1992, reviewed in Lipton 1992a). In these early investigations, it was suspected that gp120 acted as an agonist of glutamate receptors. Later work suggested, however, that rather than acting directly, gp120 behaves as a potentiator of NMDA receptor function, augmenting the excitotoxic properties of endogenous glutamate and other NMDA receptor agonists (Barks et al 1993, Pittaluga & Raiteri 1994, Lannuzel et al 1995, Meucci & Miller 1996, Barks et al 1997).

Recently the regulatory gene product, *tat*, has attracted attention as another possible excitotoxin. *tat* can cause depolarization of cultured rat and human neurons

(Magnuson et al 1995, Cheng et al 1998), can augment hippocampal cell death elicited by NMDA when injected intracerebrally (Wang et al 1999), and can induce increases in $[Ca^{2+}]_i$ that result in neuronal death in murine hippocampal cultures (Kruman et al 1998).

Astrocytic dysfunction may further add to the excitotoxic environment of the HIV-1-infected brain. Astrocytes infected by HIV-1 or exposed to viral proteins in the extracellular milieu may lose important housekeeping functions, particularly uptake of excitatory amino acids (EAAs). Astrocytes exposed in culture to gp120 exhibit decreased uptake (Dreyer & Lipton 1995, Vesce et al 1997) or increased efflux (Benos et al 1994, Vesce et al 1997) of EAAs. Glioma cells exposed to the transmembrane protein, gp41, likewise have reduced uptake and increased discharge of EAAs (Kort 1998). If these effects are realized in the living brain, the endogenous EAAs released as a consequence of normal neuronal activity may accumulate in the extracellular milieu at higher than normal concentrations.

That any of these mechanisms are operative in the living, HIV-1-infected brain is not clear, but evidence exists for the increased presence of endogenous excitatory substances in the CNS of AIDS patients. Ferrarese and colleagues documented a two-fold increase in glutamate in the cerebrospinal fluid of demented HIV patients relative to controls and non-demented HIV patients (Ferrarese et al 1997). Likewise, increases in quinolinic acid, an NMDA agonist produced by activated mononuclear cells, has been detected in the serum (Heyes et al 1991), cerebrospinal fluid (Heyes et al 1991, Achim et al 1993), and brain tissue (Achim et al 1993, Sei et al 1995) of AIDS patients.

Can all these excitatory influences—increased endogenous excitatory substances, excitatory effects of gp120 and *tat*, and loss of astrocytic uptake of EAAs—result in

neuronal death? It appears the answer is "yes," inasmuch as neuronal death due to apoptosis has been documented in association with HIV-1 in vitro and in vivo.

Apoptosis is a form of cell death characterized by fragmentation of DNA, shrinkage and "blebbing" of the cell body and nucleus into membrane-bound fragments, and rapid removal of the cellular remnants by phagocytes with no attendant tissue inflammation (see Hockenbery 1995 and Lombardi et al 1996 for reviews). Apoptotic cell death is distinctive in that its initiation and fulfillment require the active participation (by gene transcription and protein translation) of the cell undergoing it. Apoptosis is a feature of normal organogenesis. It is also seen in a variety of neurodegenerative diseases (Lombardi et al 1996) and is known to be instrumental in the immunopathological manifestations of AIDS (reviewed by Romero-Alvira & Roche 1998). Inasmuch as an increase in [Ca2+]i is an important trigger for initiation of apoptosis, it is logical to suspect that the excitotoxic properties of HIV-1 and its proteins might be triggers that lead to neuronal dysfunction and death.

Neurons are lost in HIV-1 infection of nervous tissue (Dal Pan et al 1992, Masliah et al 1992b, Masliah et al 1992c, Everall et al 1993, Jernigan et al 1993), and at least some of these cells have undergone apoptosis. In primary human brain cultures, infection with HIV-1 (Bin et al 1996, Ohagen et al 1999), exposure to gp120 (Hesselgesser et al 1998, Ohagen et al 1999) or exposure to *tat* (New et al 1997) produce neuronal apoptosis. Neuronal apoptosis is also observed in rat cortical cell cultures (Bagetta et al 1996) and hippocampal slice cultures (Aggoun-Zouaoui 1996) exposed to gp120. Müller and associates were able to prevent apoptosis in murine cortical cultures exposed to gp120 with NMDA blockers, linking the apoptotic effect of gp120 to its

effects at the NMDA receptor (Müller et al 1992). The clinical relevance of these in vitro findings is supported by studies that have documented the presence of apoptosis in the brains of HIV-1-infected patients (Adle-Biassette et al 1995, Gelbard et al 1995, Petito & Roberts 1995, An et al 1996, Bin et al 1996). Where cell type was determined, apoptosis appeared to affect neurons and astrocytes. Although this apoptotic cell death has been most readily detected in patients dying with dementia or other clinically evident encephalopathies, some investigators have found evidence of apoptosis in the brains of HIV-1-positive patients without clinical signs of nervous system disease (An et al 1996, Bin et al 1996).

Human immunodeficiency virus type 1 (HIV-1) is a neuroinvasive and neurovirulent lentivirus. It invades the nervous system early in the course of infection, and in a majority of cases it produces widespread neuropathologies that frequently are manifest clinically as a cognitive-motor syndrome. Inasmuch as HIV-1 infects neurons only restrictively (if at all), a host of neuropathological processes have been proposed to explain the dysfunction and death of neurons. Many of these invoke the participation of glia and other non-neuronal cells in complicated immune and excitotoxic pathways. Some or all of these proposed mechanisms may be operative in some patients at some time in the course of their disease. In the chaos of the extant literature on HIV-1-associated neuropathology, one theme stands out: that the AIDS Dementia Complex is a devastating, complicated syndrome, and it probably results from more than one process in the infected brain. When effective treatment for AIDS Dementia Complex is developed, it will doubtless involve an orchestrated effort on several different cellular fronts.

Therapeutic success will be contingent on a clear understanding of the contributions of each of the many cellular and molecular players that stand between the HIV virion and the neuron.

CHAPTER TWO:
An Oligopeptide of the Feline Leukemia Virus Envelope Glycoprotein Is Associated With Morphological Changes and Calcium Dysregulation In Neuronal Growth Cones

PREFACE AND ACKNOWLEDGEMENTS

The work presented in this chapter has been previously published in the Journal of NeuroVirology [NeuroVir (1997) 3:179-91]. The order and list of authors are as follows: AD Fails, TW Mitchell, JL Rojko, and LR Whalen. Research presented here was executed, recorded, and analyzed by Fails and Whalen. Mitchell and Rojko helped establish the experimental paradigm and developed molecular reagents (CVR5 and antibodies) used herein. This research was supported by a grant from the National Institutes of Health. Dr. John Walrond provided valuable editorial assistance in the preparation of the published manuscript. The technical support of Andrea Mihajlov is also gratefully acknowledged.

ABSTRACT

Neuropathogenic processes that affect the pathfinding properties of neuronal growth cones could account for many of the dysfunctions unique to retroviral infection of developing nervous systems. Pediatric HIV-1 infection, for example, is associated with a

distinctive neuropathogenesis that includes marked cortical atrophy, cognitive disorders, and pyramidal dysfunction. The ability of HIV's envelope glycoprotein, gp120, to produce increased intracellular free calcium ($[Ca^{2+}]_i$)leading to neuronal death has been documented. We hypothesize that gp120 and the envelope glycoproteins of other retroviruses may have similar calcium-increasing effects in advancing growth cones, a property which could disrupt the orderly development of the nervous system. To explore this possibility, we exposed chick ciliary ganglion neurons in culture to a known cytopathic region (CVR5) of the feline leukemia virus' envelope glycoprotein. CVR5 produced ($[Ca^{2+}]_i$)increases and dose-dependent morphological changes in growth cones isolated from their cell bodies by axotomy. These responses of growth cones to CVR5 suggest that the neurotoxic effects of retroviruses could be mediated at the level of the individual growth cone through exposure to envelope glycoproteins and could constitute one mechanism by which these viruses perturb the normal development of the nervous system.

INTRODUCTION

Interest in the pathogenesis of retroviral-induced neurological disease has increased in the past decade as documentation of the neurotoxicity of retroviruses, particularly human immunodeficiency virus (HIV), has grown. The exact mechanisms by which this and other retroviruses cause injury to the nervous system are widely debated, but the neurotoxicity of HIV's envelope glycoprotein, gp120, remains the focus of much scrutiny. It has been demonstrated that application of gp120 to nervous tissue culture causes neuronal death that is associated with marked increases in free intracellular

calcium ion concentrations ($[Ca^{2+}]_i$) (Dreyer 1990, Lipton et al 1991). Such increases have been implicated as the final common pathway in a variety of neurodegenerative disorders (Choi 1992).

Like HIV and other human retroviruses, feline retroviruses (feline leukemia virus [FeLV] and feline immunodeficiency virus [FIV]) have been associated with neurological disease, including myelopathies, behavioral abnormalities, and polyneuropathies (Hardy 1981, Haffer et al 1987, Wheeler et al 1997, Hoover and Mullins 1991, Phillips et al 1994). With its predisposition to produce myelopathies and signs of long-tract disease, the neuropathology of FeLV has been compared to similar clinical syndromes associated with the human T-cell lymphotropic virus type I (HTLV-I), the retroviral cause of tropical spastic paraparesis (Hardy 1981, Hoover and Mullins 1991, Gessain and Gout 1992, Hollsberg and Hafler 1993, Nakagawa et al 1995). Relative to FeLV, FIV is more likely to result in encephalopathy and other suprasegmental disturbances of function which closely resemble the neurological manifestations of HIV encephalopathy (Nathanson et al 1990, Pedersen and Barlough 1991, Hurtrel et al 1992, Phillips et al 1994, Portegies 1994). These feline retroviruses may therefore provide useful models for human retroviral-induced neurologic disease. Like gp120, the envelope glycoprotein of FeLV (gp70) confers target cell specificity upon the virus and dictates its ability to virulently infect cells. Minor variations in gp70 impart major differences in cellular tropism and pathogenicity (Hunter and Swanstrom 1990, Hoover and Mullins 1991, Rigby et al 1992). One of the gp70's variable regions (VR5), which is thought to dictate cytotoxic properties of the virus has been isolated and sequenced. This 15-amino acid sequence as it occurs in highly cytopathic FeLV-C variants differs by only 4 amino acids

from the sequence of the same region in an FeLV variant (FeLV-$A_{Glasgow}$) that exhibits low pathogenicity (Riedel et al 1986, Rigby et al 1992, Phipps et al 1995). This variable region in a highly virulent isolate, FeLV-C_{Sarma}, has been designated CVR5; that of the same region of FeLV-$A_{Glasgow}$ is called AVR5 (Figure 2.1). In its monomeric form, CVR5 has low cytopathogenicity, but when linked as an octomer to a lysine core, CVR5 becomes cytotoxic to lymphocytes in concentrations as low as 3μM (Phipps et al 1995). CVR5 in this configuration (designated CVR5-MAP for multiple antigen peptide and hereafter referred to simply as CVR5) may be presented to the cell membrane in a spatial array more like that which is seen on the surface of the intact virion, a presentation that could be essential to the physiological effect of the peptide.

CVR5	L	C	K	K	T	Q	K	G	H	K	G	T	H	Y	L
AVR5	*	*	N	*	*	*	Q	*	*	T	*	A	*	*	*

Figure 2.1: Amino acid sequences of CVR5 and AVR5.

Work in our laboratory demonstrated significant decreases in neuronal survival and neurite outgrowth in cultured ciliary ganglion neurons exposed to CVR5 at concentrations of 3 μM or greater (Mitchell et al 1994). Additionally, neuronal $[Ca^{2+}]_i$ increased in response to CVR5 exposure, as revealed by a fluorescent calcium indicator dye, fluo-3. These findings implicate calcium dysregulation as a component of the cell injury which results from exposure to this portion of FeLV's envelope glycoprotein; this recalls similar calcium perturbing properties of the envelope glycoprotein of HIV. The particular aspect of retroviral disease of interest to us is the distinctive natural history of

perinatal infection of the nervous system. Infants born with HIV infection are more likely to develop nervous system disease in absence of secondary infections than HIV-infected adults, i.e., as a direct viral effect. Pediatric AIDS is frequently complicated by dysfunction of motor tracts and marked cortical atrophy (Belman et al 1988, Brenneman et al 1990). Neurological effects of *in utero* FeLV infection of kittens are less well-documented, but congenital fetal infection and perinatal death are recognized sequelae of FeLV infection in the viremic, pregnant queen (Hoover et al 1983, Hoover and Mullins 1991).

It is our hypothesis that retroviral envelope glycoproteins can act on the developing nervous system at the level of advancing growth cones, leading to either failure to make or disruption of established neuronal connections. We investigated this hypothesis by observing the morphology and calcium regulation of the growth cones exposed to CVR5 in dissociated chick ciliary ganglion cultures. These cells were chosen because of 1) proven responsiveness of the cell body to CVR5, 2) the relative homogeneity of the neuronal population, and 3) the observation that anisocoria (and hence disturbance of autonomic innervation of the pupil) can be associated with FeLV infection (Brightman et al 1977, Scagliotti 1980). In order to isolate growth cone responses from those originating from the cell body, neurites of active growth cones were transected (axotomized). Cultures were bath-exposed to CVR5 or control solutions, and the axotomized growth cones' morphology was recorded at 15, 30, and 45 minutes post-treatment. In a separate set of experiments, neurons were loaded with a calcium indicator dye, fluo-3, followed by axotomy of dye-loaded growth cones. The relative fluorescence

of the isolated growth cones was recorded for fifteen minutes subsequent to exposure to CVR5.

In this study, we show that CVR5 can act directly on the growth cones of cultured neurons. These actions can be manifested by changes in morphology, loss of motility, and increased $[Ca^{2+}]_i$. These effects are independent of the cell body, as they occur in growth cones that have been isolated from the soma by axotomy. This suggests a mechanism by which FeLV in particular and retroviruses in general might contribute to abnormal nervous system development in perinatal infections.

MATERIALS AND METHODS

Cell Culture

Ciliary ganglia were harvested from 9-day-old embryonic chicks, dissociated by incubation in 1.0 cc of 0.1% trypsin at 37°C for 25 minutes, then the trypsin was inactivated by addition of culture medium containing 10% fetal bovine serum. Following gentle trituration through a flame-polished glass pipette, growth medium (DMEM with 44 mM sodium bicarbonate, 10% fetal bovine serum and 2% chick eye extract [Nishi and Berg 1981]) was added to bring the suspension to approximately one ganglion per milliliter of growth medium. Dissociated cells were preplated in a 35 mm plastic culture dish for three hours in 8.5% CO_2.

Culture dishes were prepared with poly-d-lysine-coated (26 μg/cm) no. 1 glass coverslips applied with silicon-base lubricant to the bottom of drilled plastic culture dishes. The 13 mm well of the dish was fitted with a 2-3 mm high polyethylene collar to provide a confined pool of medium in which to culture and treat cells. Prior to cell

plating, laminin (3 μg/cm) was applied to the well and incubated for one hour before rinsing with growth medium.

Following preplating, a 300 μl aliquot of neuron-enriched supernatant was transferred to each culture dish and incubated at 37°C in 8.5% CO_2. Following removal from the incubator, bicarbonate-buffered medium was replaced with HEPES-buffered medium (DMEM with 25 mM HEPES and 10% fetal bovine serum) through five exchanges of 200 μl aliquots. Plates were coverslipped with glass and removed to a heated microscope stage (37°C). For morphology studies, neurons were viewed under phase-contrast microscopy 3 to 4 hours after final plating.

For calcium studies, cells were grown for two hours then loaded with fluo-3-AM (Molecular Probes, Inc., Eugene, Oregon) (10 μM in HEPES-buffered DMEM) for 45 minutes at 37°C. Plates were then rinsed with HEPES-buffered medium and transferred to the heated microscope stage for an additional 45 minutes of de-esterification prior to study.

CVR5 and AVR5

Lyophilized CVR5 in the MAP form was dissolved in HEPES-buffered DMEM, sterile-filtered (0.2 μm), and frozen in 50 μl aliquots representing individual culture dish treatments. Each aliquot was individually thawed at room temperature prior to application to the culture well.

In control experiments, anti-CVR5 antibodies (polyclonal, ovine) and CVR5 were incubated in HEPES-buffered DMEM at 37°C for two hours before applying to the

cultures. Anti-CVR5 antibodies were present at 0.01 mg/ml and CVR5 at 6 μM in the culture dish well's final volume of 350 μl.

In other control experiments, lyophilized AVR5 in the octomeric, lysine-linked form was similarly dissolved in HEPES-buffered DMEM. This solution was frozen into individual aliquots representing single culture dish treatments; each aliquot was individually thawed at room temperature prior to application to the culture well.

Study of Growth Cone Morphology Under Phase Microscopy

Morphology of growth cones was evaluated with an inverted, phase-contrast microscope (Diaphot, Nikon Corp., Tokyo, Japan) fitted with a 40X (0.85 N.A.) objective. Images were captured using a video camera (MIT 65, DATE-MIT, Inc., Michigan City, Indiana) and led into a video capture board in a Macintosh Quadra 700 computer for storage.

Growth cones were selected for study only if they possessed normal morphology and a neurite length of approximately 100 μm. Neurites that were crossed by others and growth cones that were likely to encounter other cells during the observation period were excluded from study. Neurites were transected (axotomized) close to the cell body using a micropipette pulled for intracellular electrophysiology and mounted on a micromanipulator. For each culture, five transected growth cones with normal post-axotomy morphology and five attached growth cones were selected for study. Morphology and motility of growth cones were evaluated in real time at 15, 30, and 45 minutes following bath-application of CVR5; images of the growth cones were collected at each of these time intervals. Concentrations of CVR5 used were 1, 3, 6, and 12 μM,

and five culture dishes at each concentration were studied. Growth cone morphology was assessed as either "normal" (motile filopodia extending from a growth cone with active elaboration of lamellipodia), "altered" (reduced activity; reduction in numbers and/or blunting and thickening of filopodia; and loss of lamellipodia), or "collapsed" (loss of activity; contraction of entire growth cone into a small, phase-dark mass; and/or loss of filopodia and lamellipodia). Motility was an important criterion by which the growth cones were assigned to a nominal category; each growth cone was viewed in real time so as to detect the movements of filopodia and ruffling of lamellipodia.

To verify that the nominal categories were supported by a quantifiable feature, analysis of filopodial numbers was undertaken. Photomicrographs of 100 growth cones judged to be normal and 100 judged to be altered were randomly selected. An observer, blinded to the original evaluation, reviewed each of these 200 images in random order, and counted the filopodia on each growth cone. In this analysis, the group originally judged to be normal bore 9.2 ± 3.2 filopodia; those originally judged to be altered had 6.0 ± 3.0 filopodia. Based on filopodial number, normal and altered growth cones were statistically different from one another ($p < 1.7 \times 10^{-11}$), confirming that analysis based on the criteria described above separated growth cones into quantifiably different categories.

Control cultures underwent one of three treatments: 1) 50 µl of HEPES-buffered DMEM (n = 6), 2) 6 µM CVR5 incubated with anti-CVR5 antibodies (n = 5), or 3) 6 µM AVR5. Morphology was assessed and recorded as indicated above, and images were collected.

All experiments were done in a single-blind fashion so that growth cone morphology was evaluated and images collected without prior knowledge of the treatment.

Study of Growth Cones Under Epifluorescence

To detect alterations in growth cone $[Ca^{2+}]_i$, fluo-3-loaded, axotomized growth cones were viewed using an inverted microscope fitted with a 40X (1.3 N.A.) oil objective. A mercury light source, attenuated by neutral density filters, was used for excitation, and the illumination was limited by computer-controlled opening and closing of a shutter. Fluo-3 fluorescence was obtained using an FITC filter cube. Images were captured using an intensified charge-coupled device camera (Paultek Corp., Grass Valley, CA) and led into the video capture board in the computer. The intensity of growth cone fluo-3 fluorescence was determined using NIH Image software (version 1.59).

Fluo-3-loaded growth cones were selected and transected by the methods described above for morphology studies. A single growth cone with normal morphology (viewed through the microscope's phase optics) and low but detectable fluorescence (viewed on the camera monitor under epifluorescence) was chosen for study approximately 20 minutes post-axotomy to allow time for recovery from axotomy-induced calcium increases. Epifluorescent images were taken every 30 seconds for five minutes prior to treatment to establish a baseline level of fluorescence. Treatment consisted of either control solution (HEPES-buffered DMEM) or 1 μM CVR5. Serial images at 15 second intervals were collected for 10 minutes post-treatment, followed by an additional 5 minutes of observation at 30 second intervals. Following this

observational window, control (medium treated) growth cones were subsequently treated with 2 or 3 μM CVR5, and images collected at 15 or 30 second intervals for an additional seven minutes to demonstrate the responsiveness of these control growth cones. Fluorescence at a given time (t) during the 15 minutes after treatment with either CVR5 or control solution was assessed as the ratio of the fluorescence at that time (F/t) divided by the average fluorescence recorded during the 5 minute pre-treatment period (F/0).

Fluorescence of control-treated growth cones responding to CVR5 applied at the end of the initial observational period was reported as the ratio of the fluorescence at a given time (t) divided by the average fluorescence in the five minutes immediately preceding application of CVR5.

Statistical Analysis

In each culture dish, five attached and five axotomized growth cones were studied. Although no more than one growth cone was studied from any one neuron, it could be argued that the unique microenvironmental conditions within a given dish precluded absolutely random responses from the population of growth cones within that dish. To address this concern, each plate was treated as a single experimental value. Responses of all studied growth cones within that dish were averaged to produce a single data point (% responders) at each observational interval, and these averaged values were used for statistical purposes. Axotomized and intact growth cones were grouped separately for statistical evaluation. Differences in percentage of responders at each dose were evaluated and tested for statistical significance using ANOVA. Filopodial numbers of altered versus normal growth cones were compared using a two-tailed Student's t-test.

For fluorescence studies, growth cones were considered "responders" when the maximum increase in fluorescence was greater than the mean maximum fluorescence of the post-treatment controls plus two standard deviations. Average maximum fluorescence in responding growth cones recorded following application of CVR5 and average maximum fluorescence recorded in the same period for growth cones treated with control solutions were compared using ANOVA.

RESULTS

Most growth cones (80-90%) continued to advance across the culture substrate following axotomy with a micropipette (Figure 2.2). Evaluation of growth cone morphology was made during the experiment by an observer blinded to the applied treatment. Each growth cone was observed in real time for a period sufficient to detect movement of filopodia and/or lamellipodia. The presence or absence of motility and the morphology was recorded, and a still image was captured for each growth cone at each observational period.

Growth cone morphology was evaluated on the basis of the following criteria: motility of filopodia and lamellipodia, number of filopodia, relative area of the P-domain (the actin-dominated cortical region comprising lamellipodia), presence of phase-dark cytoplasmic structures, and the length and density of filopodia (Figure 2.3). Retrospective analysis of 200 randomly selected images (100 each of normal and altered growth cones) verified the statistical difference in filopodial numbers between these two nominal categories.

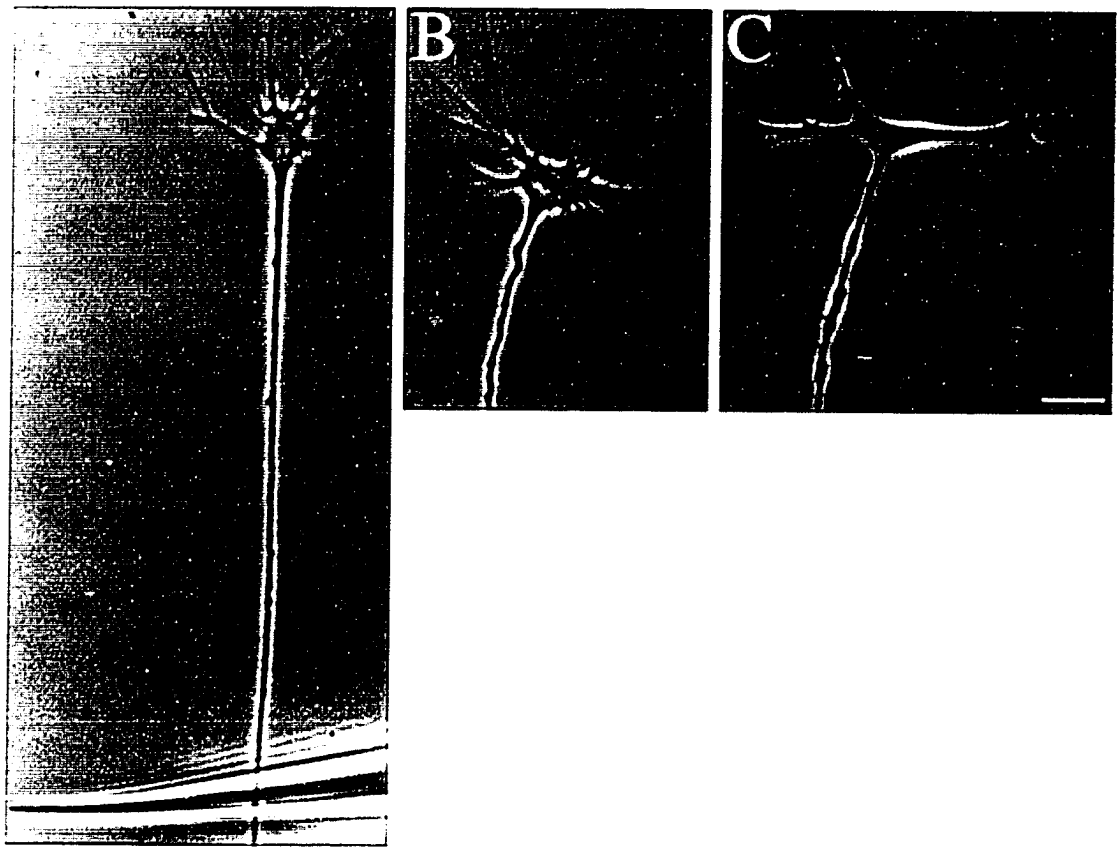


Figure 2.2: Effect of axotomy on a growth cone.
A: Growth cone morphology is normal prior to axotomy, with many, active filopodia and lamellipodia; tip of micropipette is seen at bottom of image.
B: 20 minutes following transection, the axotomized growth cone continues to exhibit normal morphology and activity.
C: 15 minutes later, the growth cone has branched, but its morphology is still judged to be normal based on active extension of multiple filopodia and movement of lamellipodia. Bar = 10 μm

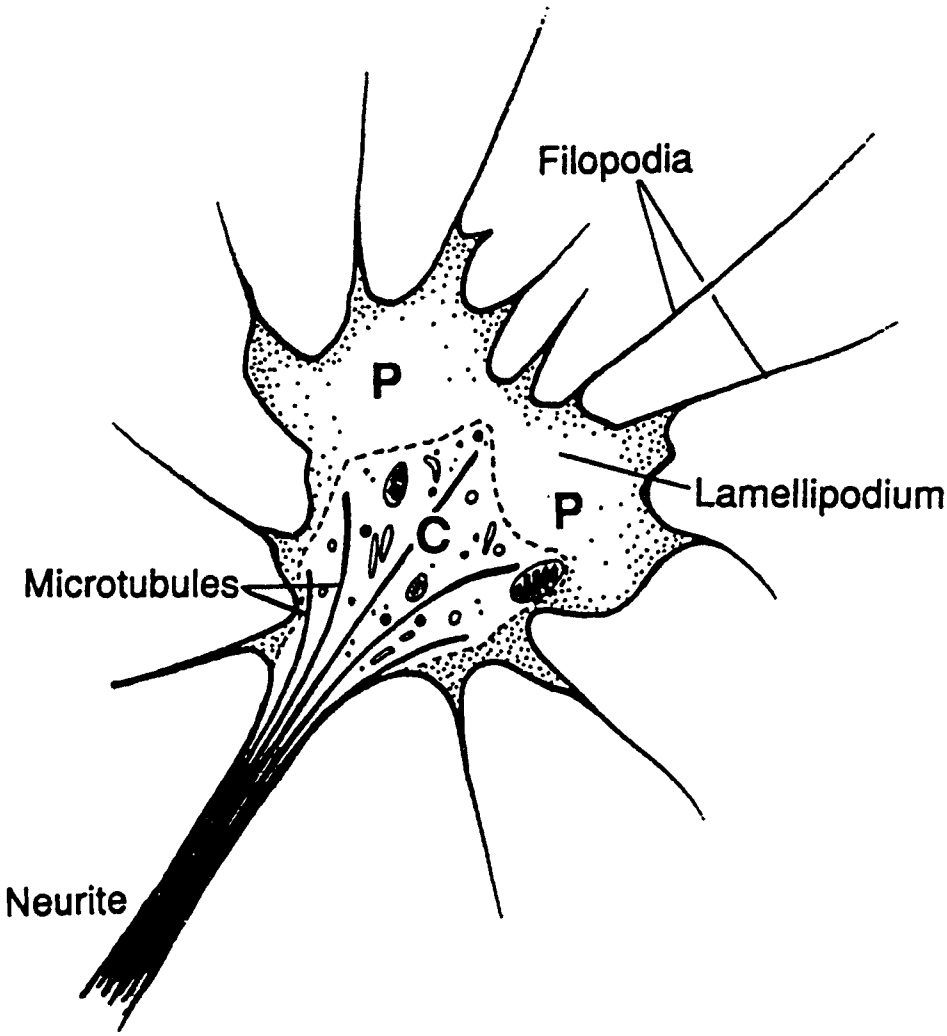


Figure 2.3: Diagrammatic anatomy of a normal growth cone. The peripheral zone (P) is dominated by actin cytoskeletal components and characterized by the active elaboration and retraction ("ruffling") of lamellipodia; multiple actin-based filopodia extend from the peripheral zone. The central zone (C) is the organelle-rich region of the growth cone and is dominated by microtubular components of the cytoskeleton.

"Normal" growth cones exhibited rapidly changing areas with extension, folding and retraction of long, thin filopodia. Elaboration and remodeling of lamellipodia ("ruffling") was observed. This activity was readily detected over a few seconds of real time observations (Figures 2.2 and 2.4).

Growth cones interpreted as "altered" characteristically had reduced numbers of filopodia (generally fewer than seven) which often assumed a short, thickened appearance. Lamellipodia were reduced, and the cytoplasm frequently contained multiple phase-dark inclusions (Figure 2.5). All altered growth cones retained some observable motility of filopodia or lamellipodia, but this movement was less than that seen in normal growth cones (Figure 2.6).

Growth cones which had detached from the substrate or retracted all processes were considered "collapsed," as were growth cones which had lost all motility when observed in real time. Collapsed growth cones usually had few or no filopodia, lacked identifiable lamellipodia, and had phase-dark intracellular accumulations (Figure 2.7).

Axotomized and attached growth cones underwent alteration and/or collapse in a dose -dependent fashion following bath application of CVR5. Percentages of altered and/or collapsed growth cones ("responders") among those axotomized reached statistically significant levels relative to plates treated with DMEM -HEPES at 3 ($p < 0.03$), 6 ($p < 0.004$), and 12 μM ($p < 0.0001$) CVR5 within the first 15 minutes of observation and maintained significant differences throughout the observational period (Figure 2.8). The percentage of axotomized growth cones responding to CVR5 at 1 μM was never significantly different from control values, but was different from 12 μM ($p < 0.03$) at all time intervals. Numbers of responders among attached growth cones were

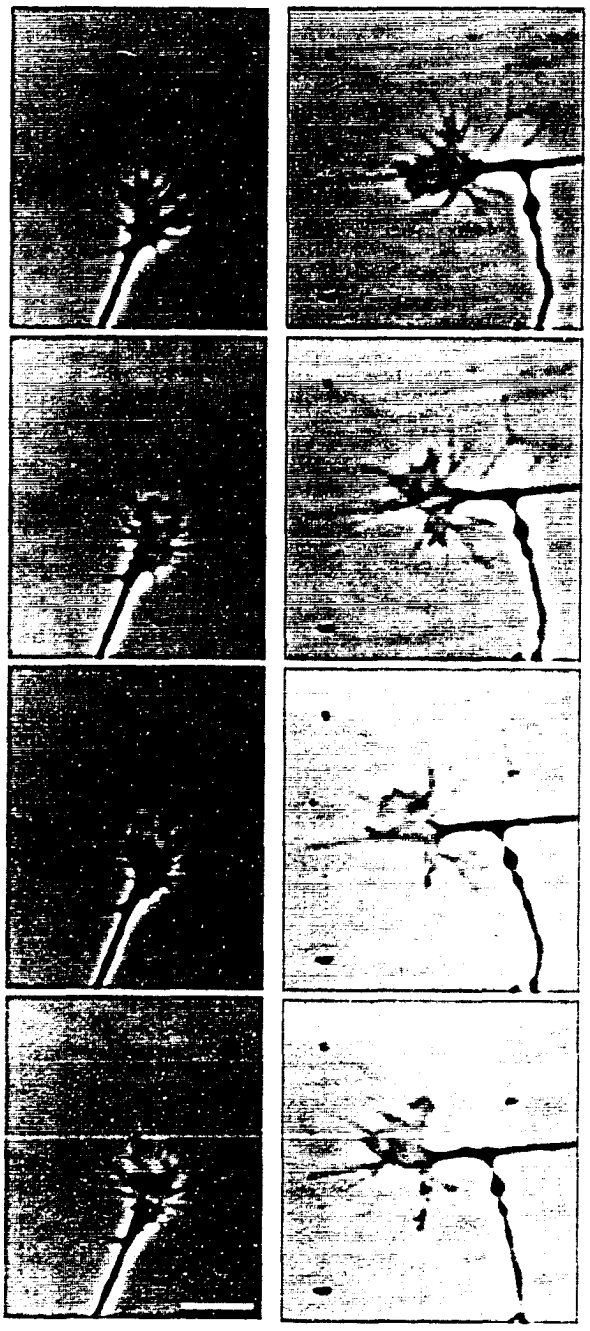


Figure 2.4: Phase micrographs of normal growth cones taken at 30-second intervals. In the left column is an attached growth cone; growth cone on the right is axotomized. Features of normal growth cones are many long, fine filopodia and active lamellipodia. The expanded peripheral area rapidly changes shape with ruffling of lamellipodia.
Bar = 10 μm.

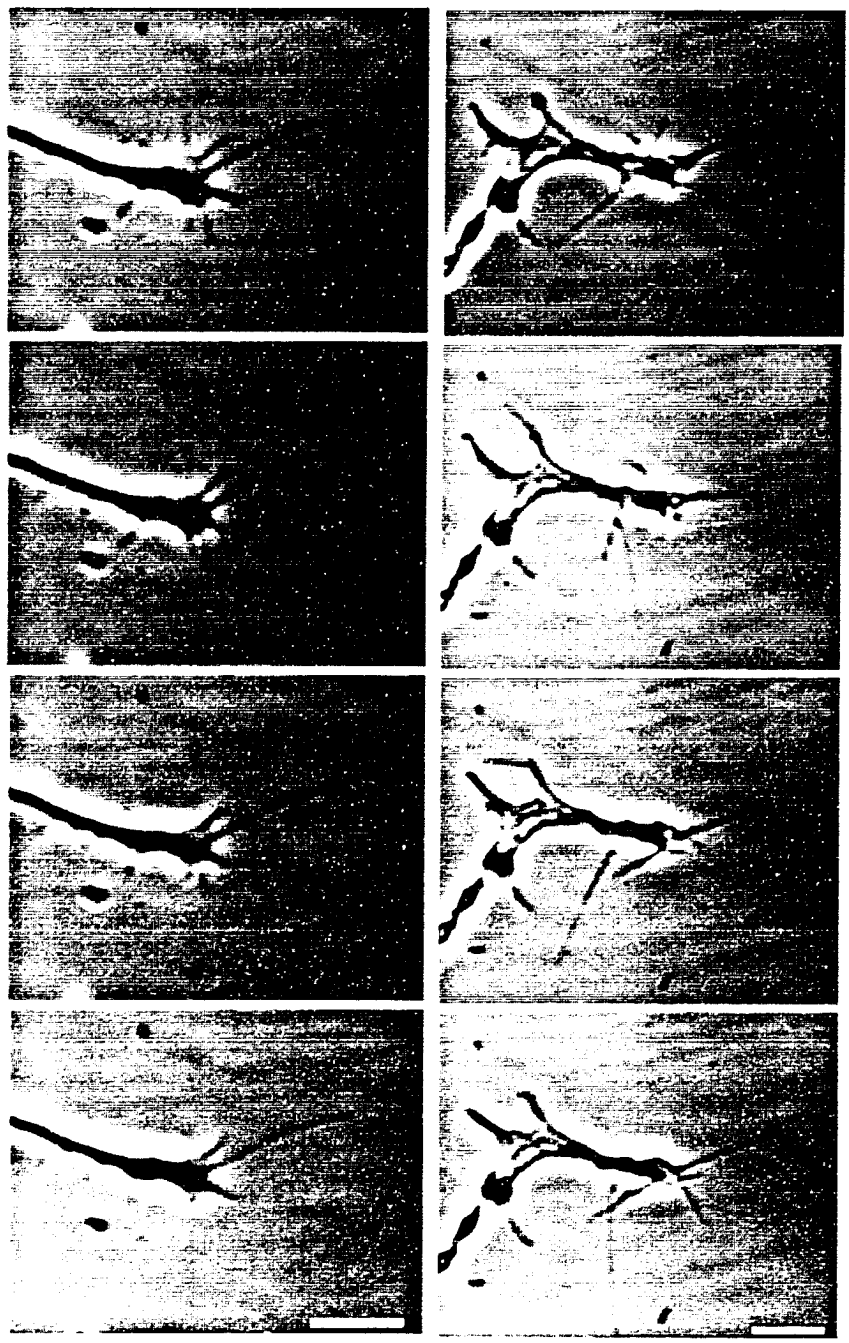


Figure 2.5: Phase micrographs of altered growth cones taken at 30-second intervals. In the left column is an attached growth cone; growth cone on the right is axotomized. Altered growth cones have reduced numbers of filopodia, reduced filopodial and lamellipodial activity, and reduced lamellipodial area. Bar = 10 μm.

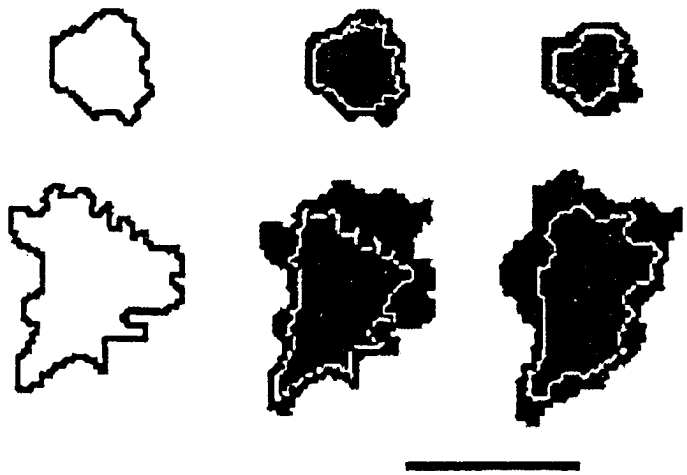


Figure 2.6: Difference tracings of the growth cones in the left columns of Figure2.4 (bottom, normal) and Figure 2.5 (top, altered). Images were generated by superimposition of consecutive frames; areas in black represent changing regions of lamellipodial expansion and retraction. The first portion of the figure corresponds to the outline of the growth cone in the first frame; the second shows the areas of difference between first and second frames; the last portion of the figure shows the difference between second and third frames. Note the reduced motility in the altered growth cone, whose movements are confined to small amounts of retraction over the course of 90 seconds. Bar = 10 μm.

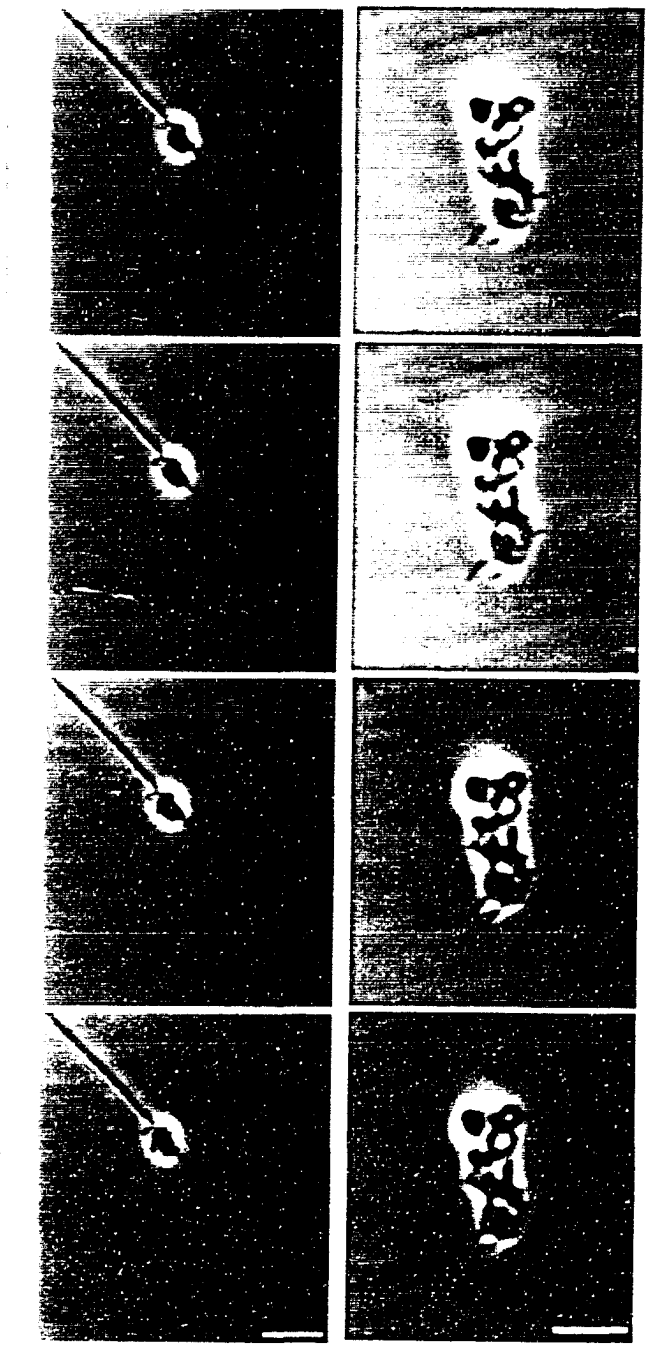


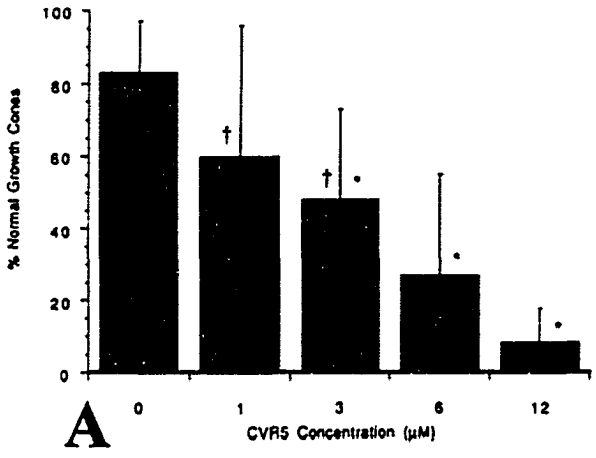
Figure 2.7: Phase micrographs of collapsed growth cones taken at 30-second intervals. In the left column is an attached growth cone; growth cone on the right is axotomized. Collapsed growth cones lack movement; there are no or few filopodia, no lamellipodia, and no identifiable peripheral zone. Bar = 10 μm.

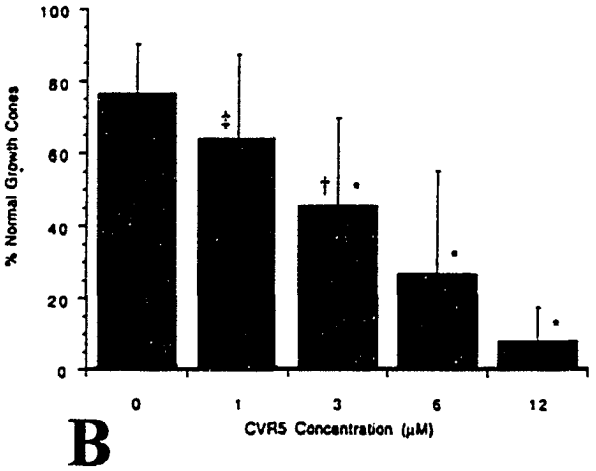
Figure 2.8: Percentages of axotomized growth cones exhibiting normal morphology declines with increasing doses of CVR5.

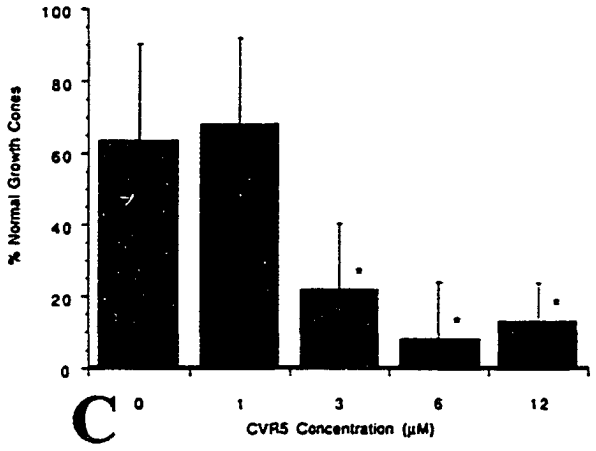
A: At 15 minutes post-treatment, 3, 6, and 12 μM CVR5 are significantly different from control plates (*, $p < 0.02$); 1 and 3 μM CVR5 are significantly different from 12 μM CRV5 (†, $p < 0.03$).

B: At 30 minutes post-treatment, 3, 6, and 12 μM CVR5 are significantly different from control plates (*, $p < 0.04$); 1 μM CVR5 is different from 6 and 12 μM CVR5 (‡, $p < 0.02$); 3 μM CVR5 is different from 12 μM CVR5 (†, $p < 0.02$).

C: At 45 minutes post-treatment, 3, 6, and 12 μM CVR5 are significantly different from both control plates and 1 μM CVR5 (*, $p < 0.02$). For all concentrations and time intervals $n = 5$ culture dishes, each with 5 growth cones. 0 = DMEM (vehicle).







significantly different relative to controls at 6 ($p < 0.006$) and 12 μM ($p < 0.0001$) CVR5 throughout the observational period (Figure 2.9). There was no statistically significant difference in numbers of responders between 6 and 12 μM CVR5 except for intact growth cones at 15 minutes, when fewer growth cones exhibited normal morphology at 12 μM than 6 μM CVR5 ($p < 0.03$). Among growth cones treated with the vehicle, DMEM-HEPES, significantly fewer axotomized than intact growth cones retained normal morphology at the end of the observational period (45 minutes) ($p < 0.05$). Both the rapidity of onset of alterations and the severity of changes (from mild morphological alterations to complete collapse) varied with the dose, with higher doses producing more rapid onset of more severe changes.

Three types of controls were compared to 6 μM CVR5: 1) the vehicle, HEPES-buffered DMEM, in a like volume ($n = 10$), 2) 6 μM CVR5 inactivated with polyclonal antibodies ($n = 5$), and 3) 6 μM AVR5 in the MAP configuration ($n = 5$). The morphological responses of axotomized growth cones treated with these three controls were not statistically different from one another. There was significant difference between plates treated with 6 μM CVR5 and each of the control treatments at all time intervals examined: 1) CVR5 vs. DMEM, $p < 0.006$, 2) CVR5 vs. antibody-treated CVR5, $p < 0.03$, and 3) CVR5 vs. AVR5, $p < 0.02$ (Figure 2.10).

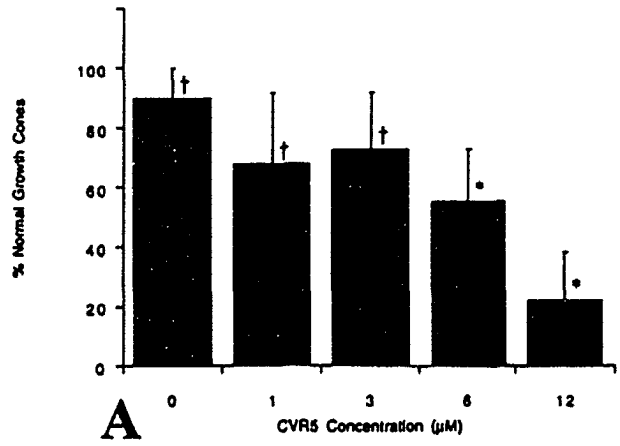
Fluorescence studies with fluo-3 were performed on axotomized growth cones to measure changes in $[Ca^{2+}]i$ in response to CVR5. Growth cones which exhibited an increase of maximum fluorescence at least two standard deviations above the mean maximum fluorescence of medium-treated growth cones were judged to be "responders."

Figure 2.9: Percentages of attached growth cones exhibiting normal morphology declines with increasing doses of CVR5.

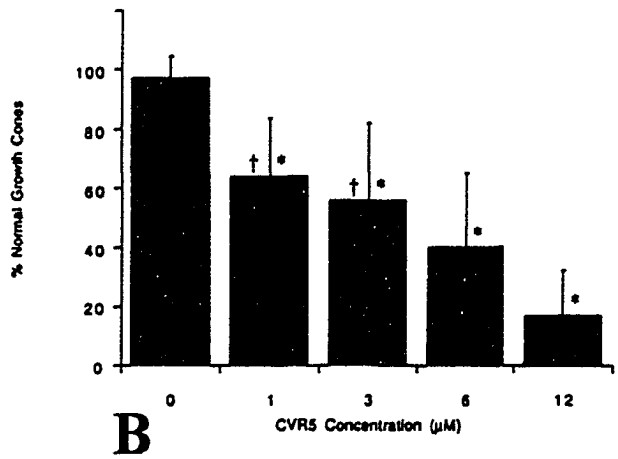
A: At 15 minutes post-treatment, 6 and 12 μM CVR5 are significantly different from plates treated with the vehicle, DMEM (*, $p < 0.01$); 1 and 3 μM CVR5 are different from 12 μM CVR5 (†, $p < 0.03$).

B: At 30 minutes post-treatment, all four concentrations of CVR5 are significantly different from control plates (*, $p < 0.01$); 1 and 3 μM CVR5 are significantly different from 12 μM CVR5 (†, $p < 0.03$).

C: At 45 minutes post-treatment, 6 and 12 μM CVR5 are significantly different from control plates (*, $p < 0.001$); 1 and 3 μM CVR5 are significantly different from both 6 and 12 μM CVR5 (†, $p < 0.006$). For all concentrations and time intervals, $n = 5$ dishes, each with 5 growth cones. 0 = DMEM (vehicle).



A



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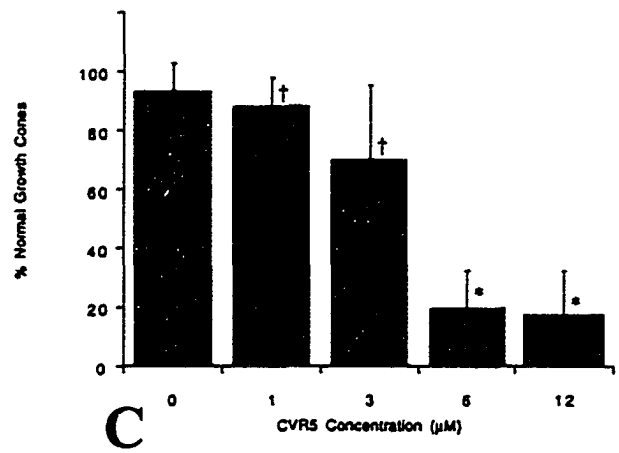

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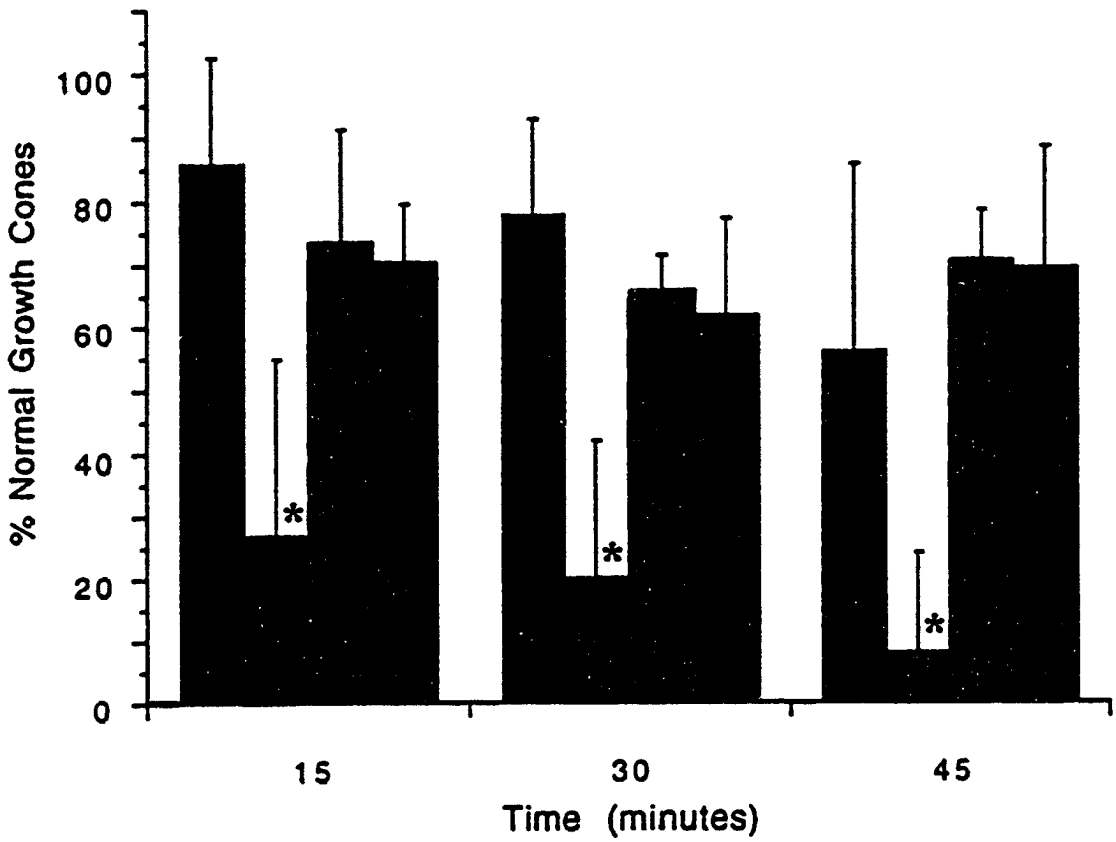


Figure 2.10: Percent of normal axotomized growth cones in cultures treated with HEPES-buffered DMEM (black), 6 μM CVR5 (red), 6 μM CVR5 incubated with anti-CVR5 antibodies (blue), and 6 μM AVR5 (magenta). 6 μM CVR5 is significantly different from cultures treated with DMEM ($p < 0.006$), from those treated with 6 μM CVR5 + antibodies ($p < 0.03$), and from those treated with 6 μM AVR5 ($p < 0.02$) at all time intervals. The morphological responses of growth cones treated with DMEM, those treated with CVR5 inactivated with antibodies, and those teated with AVR5 were not statistically different from one another at any interval.

Among growth cones treated with 1 μM CVR5, 9 out of 16 met this criterion (Figure 2.11). Treatment with DMEM-HEPES never increased $[Ca^{2+}]_i$. To ascertain that these control growth cones could respond with a calcium increase, 2 or 3 μM CVR5 was applied subsequent to the 20 minute observation period. All controls (n = 5) responded with an increase in fluorescence greater than two standard deviations above the mean maximum fluorescence established in the five minutes prior to application of CVR5 (Figure 2.12).

The average maximum increase in fluorescence following application of CVR5 to responding growth cones was 3.11 ± 2.14 (range: 1.63 to 6.30), whereas the average maximum increase in fluorescence following application of DMEM-HEPES to control growth cones was 1.06 ± 0.05 (range: 1.03 to 1.13) ($p < 0.05$). Control growth cones, subsequently treated with 2 or 3 μM CVR5, exhibited an average maximum fluorescence of 1.82 ± 0.34 (range: 1.42 to 2.28). This response is significantly different from the response the same growth cones exhibited after treatment with control solutions ($p < 0.003$).

DISCUSSION

The pathfinding behavior of growth cones is essential to the development of normal connectivity of the nervous system. The process by which the active growth cone chooses one direction over another may be in some cases guided by calcium fluxes generated by receptor interactions with extracellular molecular signals (O'Connor et al 1990, Kater and Mills 1991, Letourneau et al 1994). Studies of growth cones isolated from their cell bodies by axotomy have shown that these growth cones retain the ability

Figure 2.11: Relative fluorescence of a single growth cone treated with 1 μM CVR5 at 5 minutes (open arrow). Fluorescence at any given time is reported as the ratio of fluorescence at that time (F_t) to the average fluorescence (F_0) in the first five (pre-treatment) minutes. Letters on graph correspond to accompanying fluorescent micrographs; intensity of fluorescence in the growth cone is measured against the adjoining greyscale. Bar = 10 μm.

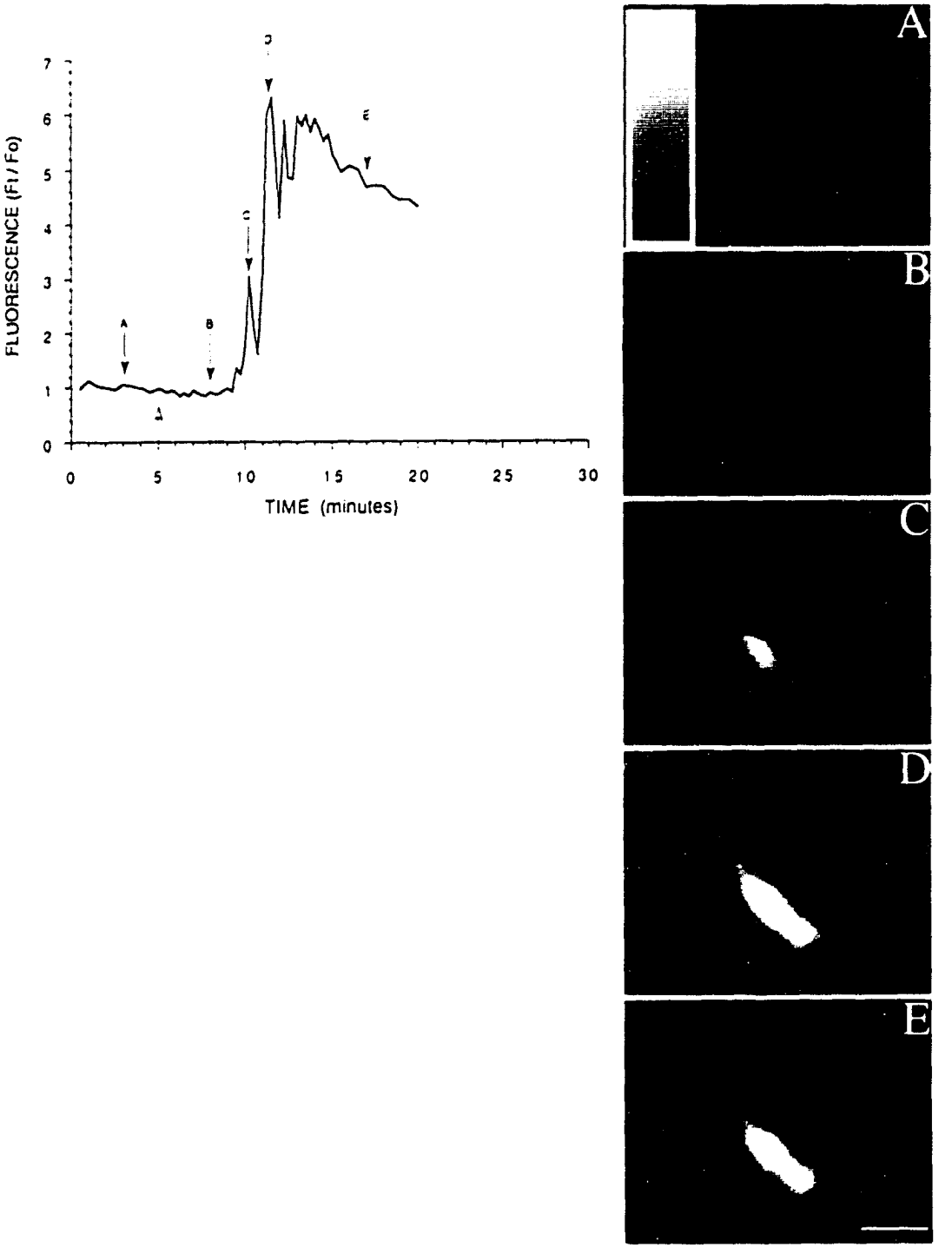
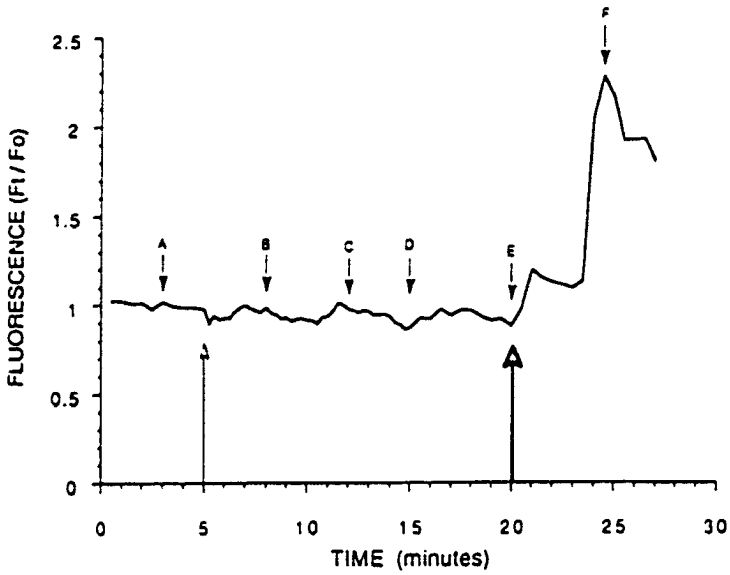
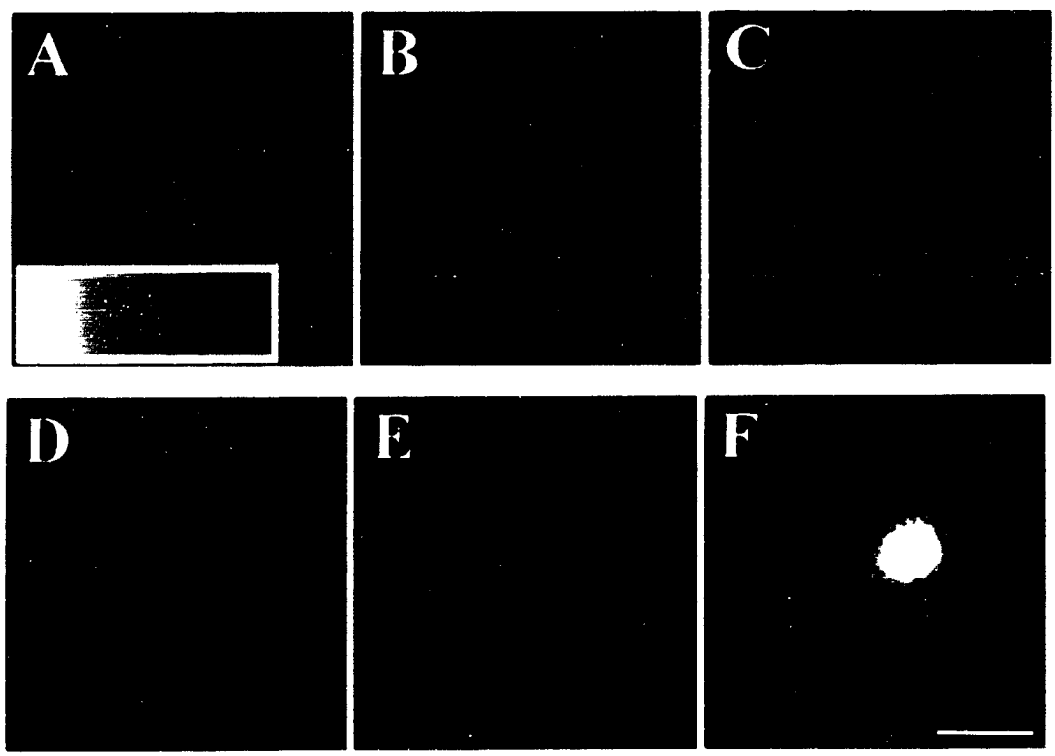


Figure 2.12: Relative fluorescence of a single growth cone treated with HEPES-buffered DMEM at 5 minutes (thin, open arrow). Fluorescence at times between 0 and 20 minutes is reported as the ratio of fluorescence at that time (F_t) to the average fluorescence (F_0) in the first five (pre-treatment) minutes. To demonstrate its ability to respond, the growth cone was subsequently treated with 2 μM CVR5 at 20 minutes (thick, open arrow). Fluorescence after that point is reported as the ratio between fluorescence at that time and the average fluorescence in the five minutes preceding application of CVR5 (i.e., the period from 15-20 minutes). Letters on the graph correspond top accompanying fluorescent micrographs. Bar = 10 μm.





to respond to molecular signals in the environment with increases in $[Ca^{2+}]_I$, indicating that receptors and intracellular messenger cascades exist and can function at a distance from the cell body (Rheder et al 1991). This autonomy of the growth cone is a necessary condition of its long-distance, "target-seeking" function, but may consequently render it sensitive to the presence of viral toxins. The internal free calcium ion concentration in an active growth cone is typically maintained near 200 nM (Connor 1986); supraoptimal $[Ca^{2+}]_i$, however, can lead to collapse of the growth cone, cell dysfunction, or cell death. In this study we examined the ability of a 15-amino acid sequence from a variable region on FeLV-C's envelope glycoprotein (CVR5) to perturb the morphology and calcium regulation of neuronal growth cones in culture.

CVR5 produces increased $[Ca^{2+}]_i$, decreased neurite outgrowth, and decreased survival in cultured chick ciliary ganglion neurons (Mitchell et al 1994). To examine the responsiveness of the growth cone to this oligopeptide, we transected the neurites of active growth cones and bath-exposed the cultures to CVR5. Exposure of isolated growth cones to as little as 3 μM CVR5 produced sustained morphological alterations after 15 minutes. The induction of similar responses in intact neurons required exposure to 6 μM CVR5. In comparisons of intact and axotomized growth cones treated with the vehicle alone, DMEM-HEPES, a larger percentage of isolated growth cones developed abnormal morphologies following this treatment relative to identically treated intact growth cones. It thus appears that the process of axotomy predisposes the isolated growth cone to develop abnormal morphology, even in the absence of exposure to injurious substances. Increased sensitivity to CVR5 is probably a reflection of axotomized growth cones' reduced ability to compensate for even small perturbations of $[Ca^{2+}]_i$ homeostasis.

Fluorescence studies, performed on axotomized growth cones, showed significant increases in $[Ca^2]_i$ in 56% (n = 9/16) of growth cones exposed to 1 μM CVR5. Failure to elicit consistent calcium responses in all growth cones may be explained by three considerations. First, fluo-3-loaded growth cones were in general less likely to sustain normal morphology following axotomy than unloaded growth cones and may therefore have suffered sufficient disruption of their intracellular systems to blunt their calcium responses. Secondly, previous studies with CVR5 have described a subpopulation of neurons in ciliary ganglion cell culture resistant to the $[Ca^{2+}]_i$ increasing properties of this peptide; in these cultures, 20% of the neurons did not respond with $[Ca^{2+}]_i$ to 12 μM CVR5 (Mitchell et al 1997). Finally, the dose of CVR5 used in our calcium imaging studies (1 μM) represents a low concentration associated with less consistent responses of neurons and growth cones. Fluorescent studies of growth cones responding to CVR5 were undertaken early in our study, prior to the majority of the morphology studies, and well before analysis of data that indicated a less consistent and robust response to 1 μM CVR5. Subsequent application of 2 or 3 μM CVR5 to control growth cones was insurance against unresponsiveness; our intent was to show that the failure of these controls to exhibit increased calcium on exposure to control solutions was not due to the inability to produce those responses when exposed to a sufficient concentration of CVR5. Studies of the dose-dependency of the calcium response were not undertaken in this series of experiments.

The number of growth cones responding with morphological changes only reached statistical significance at 3 μM CVR5; in contrast, calcium increases of greater than two standard deviations were detected in more than 50% of growth cones treated

with 1 μM CVR5 and in all growth cones treated with 2 or 3 μM CVR5. This suggests that calcium responses are activated at lower concentrations than can produce observable morphological changes. Growth cones encountering low concentrations of the FeLV envelope glycoprotein in vivo could respond with activation of calcium-mediated second messenger systems, a response which could profoundly affect the pathfinding behavior of the growth cone.

Importantly, no growth cones prepared in like manner but treated with vehicle ever showed an increase in fluorescence in response to this treatment. As noted, these control growth cones were subsequently treated with 2 or 3 μM CVR5, and their ability to respond thereby established by the resulting increased $[Ca^{2+}]_i$ in all growth cones so treated. These data demonstrate a convincing link between bath exposure to CVR5 and increases in $[Ca^{2+}]_i$ within the growth cone.

The mechanism by which CVR5 elicits increased calcium in growth cones and neurons is unknown. In other studies in our laboratory (Mitchell et al 1997), neurons were loaded with fluo-3 then treated with CVR5 in the presence of propidium iodide. Propidium iodide undergoes a 40-fold increase in fluorescence when it enters a cell, but is highly impermeant to living membranes. Propidium iodide will therefore produce a fluorescent signal in cells only after loss of membrane integrity. Ciliary ganglion neurons responded to application of CVR5 with increases in $[Ca^{2+}]_i$ revealed by fluo-3 fluorescence, and this signal was either followed by increased propidium iodide fluorescence after a delay of 3-5 minutes or occurred with no subsequent propidium iodide signal. This argues against a loss of membrane integrity as the mechanism by which CVR5 produces increased intracellular free calcium in susceptible neurons. Other

mechanisms by which CVR5 might elicit increases in $[Ca^{2+}]_i$ include receptor interactions and membrane depolarization. Characterization of the roles that these processes might play awaits studies with channel/receptor blockers and electrophysiological techniques.

Morphological responses reached statistical significance in axotomized growth cones at 3 μM CVR5. Calcium increases were recorded in the majority of fluo-3 loaded growth cones exposed to 1 μM CVR5. These concentrations are substantially greater than those of HIV's envelope protein, gp120, used in studies of cultured neurons (Dreyer et al 1990, Lipton et al 1991) and are unlikely to represent concentrations characteristic of in vivo infection with FeLV. This disparity between CVR5 and gp120 may represent a true difference in the calcium-perturbing power of the viral peptides, or may be due in part to the limited resolution inherent to study of individual, isolated growth cones in our system. Detection of morphological changes in growth cones, even by quantitative assessment of criteria such as filopodial numbers or changes in growth cone area, is limited by the resolving power of the imaging system and subject to the vagaries of normal growth cone behavior and appearance. Given the highly ordered process by which the developing nervous system establishes and maintains appropriate connections between distant cells, even very small changes in growth cone steering could have major functional consequences. Inasmuch as a single filopodial contact with an attractive or repulsive molecular signal can dictate the direction of the growth cone's progress (O'Connor et al 1990), contact with a restricted number of viral particles or peptides could generate a subtle intracellular signal that could substantively influences the growth cone's progress. Such signals could be well below the detection threshold of this system.

We are currently undertaking studies to examine the effects of growth cone contact with CVR5 affixed to latex microbeads. This route of molecular exposure is more likely to mimic in vivo conditions of retroviral infection.

A number of retroviruses, including FeLV and HIV, exhibit neuropathogenicity. The envelope glycoproteins of these viruses have been implicated as important causative factors in that neurotoxicity, and have, in the cases of HIV and FeLV, been linked to intraneuronal increases in neuronal $[Ca^{2+}]_i$. The research presented here shows that an oligopeptide of FeLV's envelope glycoprotein, CVR5, is sufficient to produce both changes in morphology and increases in $[Ca^{2+}]_i$ in growth cones isolated from their somata. These findings support the hypothesis that retroviral proteins could affect the developing nervous system by disrupting the functions of neuronal growth cones and by that mechanism contribute to the nervous system dysfunction that is unique to perinatally -acquired infection.

CHAPTER 3:

A Neuroanatomic Substrate for HIV-Associated Synaptic Reorganization: Differential Sensitivity of Neurons in the Hippocampal Formation to gp120

INTRODUCTION

Neurological Disease and HIV

The appearance in the early 1980's of a novel syndrome of human immunodeficiency led to the isolation of a virus with characteristics placing it in the genus Lentivirinæ (Barré-Sinoussi et al 1983). In 1986 the International Committee on the Taxonomy of Viruses suggested the name "Human Immunodeficiency Virus" (HIV) for this lentivirus (Coffin J et al 1986). HIV (later more specifically identified as HIV-1 to distinguish it from the similar but less virulent HIV-2) is the causative organism of a condition characterized by insidious depletion of immune cells, ultimately resulting in the development of acquired immunodeficiency syndrome (AIDS). AIDS is a chronic wasting condition associated with opportunistic infections and neoplasias, and which invariably ends in death.

Early in the epidemiological history of HIV-1, it was recognized that many patients dying of AIDS suffer from clinical neurologic disease. About half of end-stage AIDS patients exhibit clinical signs of nervous system dysfunction, and as many as 90% of patients have neuropathology identifiable at autopsy (see Portegies 1994, Harrison and McArthur 1995, and Glass & Johnson 1996, for reviews). Although opportunistic infections, reactivation of latent infections, and opportunistic neoplasias account for some neuropathology, about half of the cases lack evidence of secondary disease processes. It is now widely acknowledged that HIV-1, like many other animal lentiviruses, is neurotropic and can produce neurologic dysfunction as a direct consequence of the primary viral infection.

Lesions associated with this primary neuropathic effect classically include loss of cortical and subcortical neurons (Dal Pan et al 1992, Masliah et al 1992b, Masliah et al 1992c, Everal et al 1993, Jernigan TL et al 1993); accumulation of inflammatory cells and multinucleated giant cells (Navia et al 1986, Burns DK et al 1991, Kure et al 1991); gliosis (increased numbers of reactive astrocytes) (Smith et al 1990, Martinez et al 1995); and "myelin pallor," a gross change in the appearance of white matter tracts that reflects an abnormality of the associated myelin (Navia et al 1986, Power 1993). The constellation of signs and symptoms associated with these lesions goes by a wide variety of names, including HIV encephalopathy (HIVE), HIV-associated cognitive-motor complex and AIDS dementia complex (ADC). A more encompassing term making an appearance in recent literature is neuroAIDS, a designation that acknowledges the tendency of HIV-associated neurologic dysfunction to be associated with end-stage disease.

This strong association between neurologic disease and HIV-1 infection is remarkable primarily because HIV-1 is not known to infect neurons productively in vivo (Wiley et al 1986, Kure et al 1990). The virus, which is believed to gain access to the nervous system as early as the first, transient viremia, productively infects only the microglia (Koenig et al 1986, Wiley et al 1986, Kure et al 1990, Sharpless et al 1992), resident CNS macrophages that constitute only 1% of the total number of cells in normal nervous tissue. Some evidence suggests that HIV-1 may also infect low numbers of macroglia, especially astrocytes, but this putative astrocytic infection does not appear to result in release of whole, infectious virions nor in death of the infected cell (Pumarola-Sune et al 1987, Nuovo et al 1994, Tornatore et al 1994). Viral proteins are also occasionally detectable in neurons, but, like the infection of astrocytes, these are primarily regulatory, not structural proteins. Neuronal infection by HIV-1, if a feature of the living brain at all, is thought to be restrictive and of ambiguous pathogenicity (Pumarola-Sune et al 1987, Nuovo et al 1994, Bagasra et al 1996).

Somehow, a lentivirus that productively infects only microglia initiates processes that result in global neuronal dysfunction and death. Attempts to decipher this pathway have resulted in scrutiny of the potential contributions of intrinsic immune processes in the brain and of neurotoxicity of viral proteins released into the extracellular milieu and present on the surface of infected cells. Much of the interest in these indirect pathways leading to HIV-1-induced neuronal death has focused on the 120kd glycosylated envelope protein of HIV-1, gp120.

Structure and Biology of gp120

All lentiviruses share the same structural blueprint (for reviews, see Clements & Zink 1996 and Turner & Summers 1999) (Figure 3.1). The genetic material comprises two duplicate strands of RNA, each attended by a magnesium-dependent reverse transcriptase. These are contained within a protein capsid further surrounded by a matrix protein shell adjacent to the inner surface of a lipid bilayer envelope. The viral envelope is acquired from the host cell plasmalemma at budding, and is studded with host-derived molecules and a glycoprotein complex of viral origin.

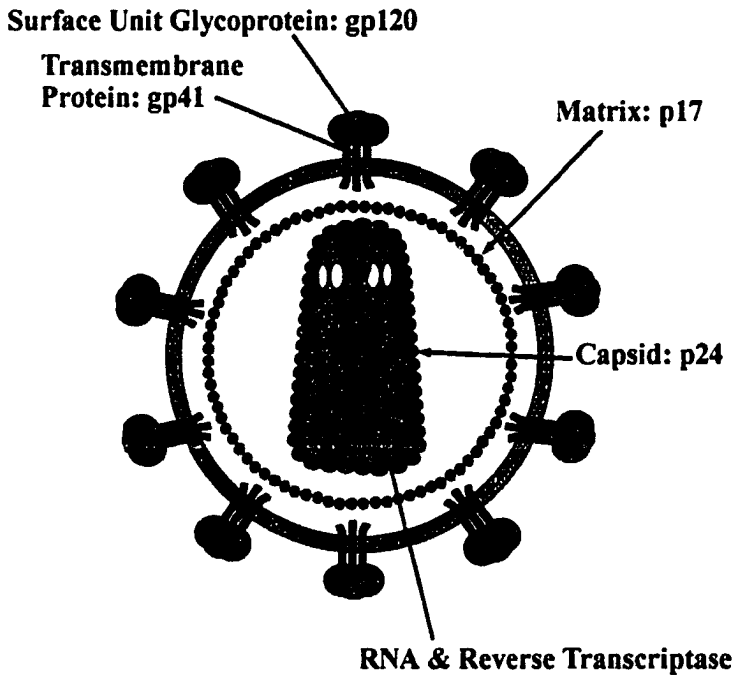


Figure 3.1: Schematic of HIV-1, a lentivirus.

The transmembrane portion (TM) of this viral envelope protein complex is a 41-kd glycosylated protein. TM is attached through non-covalent interactions to a 120 kilodalton, extracellular glycoprotein. In accordance with standard nomenclature, these

glycoproteins are designated "gp" followed by their weight expressed in kilodaltons. Hence, the external glycoprotein is "gp120," and the transmembrane portion is "gp41." These two glycoproteins are generated in the endoplasmic reticulum of the host cell as a single protein product (gp160) that is subsequently cleaved by cellular proteases in the Golgi apparatus (reviewed by Turner & Summers 1999). Both gp41 and gp120 are highly glycosylated, the gp120 amino acid sequence bearing a predicted 24 or more glycosylation sites (Leonard et al 1990). This abundance of sugar moieties provides "immune shielding" for the virus (Wyatt & Sodroski 1998) and has proved a fundamental barrier to crystallization and characterization of the stereochemistry of gp120 (but see Kwong et al 1998 for a recent crystallography report on gp120).

gp120 is about 550 amino acids in length, the exact number being dependent on the strain of HIV-1 (Luciw 1996). Its primary structure is described as five conserved regions interspersed with five hypervariable regions, designated V1 through V5 (Starcich et al 1986); V1 through V4 form loops with disulfide bonded bases. Based on this secondary structure and the characterization of virus-neutralizing antibodies known to recognize discontinuous epitopes of the glycoprotein (Wyatt et al 1998), gp120's functional tertiary conformation probably involves the interaction of widely noncontiguous regions of the globular glycoprotein.

The surface unit glycoprotein gp120 is required for the viral binding to the target cell, and its primary amino acid sequence dictates the type of cell for which the viral strain exhibits tropism. Research has begun to characterize gp120's important domains and their functions: 1) Non-covalent binding with the transmembrane glycoprotein, gp41, is a cooperative function of both the N- and C-termini of gp120 (Helseth et al 1991); 2)

Binding of the cellular receptor CD4 occurs within a protected cleft between inner and outer domains with stability [K_d for the gp120-CD4 interaction is 4 $X10^{-9}$ (Lasky et al 1987)] afforded by a small group of non-contiguous, conserved amino acids (Lasky et al 1987, Kwong et al 1998); and 3) The tropism of the virus—lymphocytic or monocytic or both—is dictated by the third variable loop (V3) through its affinity for the chemokine co-receptor (Speck et al 1997, Wang et al 1999).

The majority of virus-neutralizing antibodies produced against HIV-1 are directed against epitopes of gp120 (Barin et al 1985). The virus parries this immune assault through the mutability of the gp120 primary sequence. Interestingly, the reverse transcriptase of HIV-1 is considered a low-fidelity replicative enzyme, frequently introducing errors into genomic transcriptions (Myers & Pavlakis, 1995). It is estimated that as much as 25% of gp120's 550 amino acid primary sequence is mutable. Infected cells therefore commonly express new and multiple serovars of gp120, generated *de novo*. This viral stratagem allows the virus to evade the immune response and remains an important obstacle to the development of an effective HIV-1 vaccine (for a review, see Nowak 1995).

Evidence suggests that in its virus-associated state, the envelope protein complex is present in an oligomeric form. Findings are inconsistent, though; dimeric, trimeric, and tetrameric arrangements have been proposed. The most current models of viral attachment and fusion with the host membrane postulate a naturally occurring array of at least two, probably three, envelope complexes (Weiss et al 1990, Doms et al 1991, Lu et al 1995, Shu et al 1999). The oligomeric presentation may be important in shielding

virus-neutralizing epitopes of the protein from antibodies and/or in cooperativity during binding to the target cell.

gp120 is bound only non-covalently to gp41 and is therefore readily shed from whole virion and from the surface of infected cells (Schneider et al 1986, Kalyanaraman et al 1990, Earl et al 1991). It has been detected in the serum (Oh et al 1992) and CSF (Oh et al 1992, Keys et al 1993, DiStefano et al 1996) of HIV-1-positive patients. Evidence has accumulated supporting the speculation that in the absence of infective virions, free gp120 can cause dysfunction or death of neurons and neuroglia. Inasmuch as the neuropathology documented to exist in AIDS patients cannot easily be linked to direct infection of neurons, this conjectural role of gp120 has attracted many supporters, much research, and a host of hypotheses to explain its causative role in neuroAIDS.

gp120 as a Neurotoxin

In 1988, Brenneman and colleagues studied the proposition that gp120 shed from HIV-1 and from HIV-1-infected cells might be one mechanism by which HIV-1 infection of the brain results in neuropathology (Brenneman et al 1988). Brenneman's work stemmed from the observation that up to 50% of rat retinal ganglion cells and mouse hippocampal cells in culture were killed by applications of gp120 at as little as 2 pM. Lipton followed this work by showing a rapid, supraphysiologic increase in intraneuronal calcium in hippocampal cells exposed to 200 pM gp120, a rise which augured death of these cells (Lipton et al 1991).

Much effort in the HIV-1 research community was subsequently directed at showing the neurotoxic effects of gp120 in vivo and at elucidating the mechanism(s) by

which it produced calcium influx and neuronal death. Rats injected with gp120 either systemically (Hill et al 1993) or intracerebrally (Bagetta et al 1995, Bagetta et al 1996, Barks et al 1997) exhibited developmental motor retardation and morphological lesions (cell loss and evidence of apoptosis) in their brains. In early investigations, it was suspected that gp120 acted directly at glutamate receptors, especially the NMDA receptor, as an excitotoxin, since glutamate antagonists are usually able to eliminate or attenuate gp120's neurotoxic effects (Dreyer et al 1990, Lipton et al 1991, Lo et al 1992, Müller et al 1992, reviewed in Lipton 1992a). It also appears that gp120 behaves in many systems as a positive modulator of NMDA receptor function, augmenting the excitotoxic properties of endogenous glutamate and other NMDA agonists (Barks et al 1993, Pittaluga & Raiteri 1994, Lannuzel et al 1995, Barks et al 1997).

In 1992, though, Lipton published the important observation that gp120 was *not* neurotoxic in neuronal cultures depleted of macrophages (Lipton 1992b). This argued against a direct effect on neurons. Subsequent work of others echoed this finding, supporting the concept that participation of non-neuronal cells is essential to the neurotoxicity of gp120. Microglia, the resident macrophages of the nervous system, are activated by HIV-1 infection or by exposure to gp120, as evidenced by their release of inflammatory mediators (Merrill et al 1991, Wahl et al 1991, Tyor et al 1992, Benveniste 1994, Giulian et al 1996). The list of postulated and identified mediators is long, but those most often mentioned in the context of HIV-1 neurotoxicity include arachidonic acid (Wahl et al 1989, Dreyer & Lipton 1995), tumor necrosis factor α (TNFα) (Grimaldi et al 1991, Tyor et al 1992, Yoshioka et al 1995), nitric oxide synthetase (Dawson et al 1993, Bukrinsky et al 1995, Adamson et al 1996, Nuovo & Alfieri 1996), and

interleukins (Tyor et al 1992, Benveniste 1994, Yeung et al 1995). The cascading, amplifying effects of these inflammatory mediators have been logically invoked as the means by which a small viral burden can produce global effects.

Macroglia may also suffer derangement of function when exposed to gp120 or other cells activated by gp120. Of particular relevance to neuronal excitotoxicity, astrocytes in culture exhibit efflux (Benos et al 1994, Vesce et al 1997) or decreased uptake (Dreyer & Lipton 1995, Vesce et al 1997) of excitatory amino acids. In some culture systems, astrocytes exhibit a rise in intracellular calcium upon exposure to gp120 (Ciardo & Meldolesi 1993, Cadazzi et al 1995), a perturbation which might reasonably be expected to produce changes in astrocytic function.

In contrast to postulated mechanisms of neuropathogenesis that invoke release of excitotoxins (or potentiation of them), another line of investigation has sought to show that gp120 acts as a competitive inhibitor of intrinsic neurotrophic substances, substances normally expressed and necessary to the maintenance of neuronal function. Best characterized among these is vasoactive intestinal peptide (VIP), which shares some sequence homology with gp120 (Brenneman et al 1988). VIP can prevent the neurotoxic effect of gp120 in dissociated hippocampal cultures (Brenneman et al 1988) and in whole embryo culture (Dibbern et al 1997). Another sequence in gp120 (AA 238-282) has homology with neuroleukin, another neuronal growth factor that promotes neuronal survival in culture and motor neuron regeneration in vivo (Gurney 1987, Lee et al 1987). This process by which a pathogen interferes with normal cellular processes by "imitation" of endogenous substances is called "molecular mimicry." The possible role of molecular mimicry in HIV-1-associated immunopathology is reviewed in Bjork, 1991.

It is now widely accepted that gp120 is not likely to be a direct neurotoxin (but see Lannuzel et al 1997 for a recent dissenting viewpoint). Instead, it probably leads indirectly to neuronal death, most likely through the activation of intrinsic immune processes in the brain and possibly by interfering with the normal "housekeeping" functions of macroglia. It should be appreciated that the many postulated pathways by which neurons are ultimately injured may each play a role under individual circumstances, and that most of them are not mutually exclusive. Whatever their individual contributions, at the center of these disparate, converging pathways lie deranged or dying neurons.

Neuroanatomy of Seizures

Epilepsy is one of the most common chronic neurologic conditions for which neurologists are consulted, second only to headache; its prevalence in developed countries is reported at 4 to 8 cases per 1000 individuals. Of the many types of recurrent seizure disorders, those described as "complex partial seizures" comprise the single largest group (36% of all epilepsies). In terms of the anatomical substrate, complex partial seizures are usually referable to a seizure focus in the temporal lobe, specifically the hippocampus. Indeed, until a revised nomenclature was introduced in 1981, "temporal lobe seizure" was clinically synonymous with "complex partial seizure." Although the two terms are not now considered strictly equivalent, temporal lobe epilepsy (TLE), manifesting as complex partial seizures, remains the single most common type of seizure disorder encountered in adults (reviewed in Williamson and Engel 1997).

Research into the neuroanatomic substrate of temporal lobe epilepsy has shown that the hippocampal formation in human cases and animal models of this seizure disorder frequently exhibits a characteristic pattern of cell loss known as "hippocampal sclerosis." This lesion is distinguished by a reduction, often marked, in the number of cells in the hilar region of the dentate gyrus and commonly extending into the adjacent CA3 pyramidal cell layer of the hippocampal gyrus (Sloviter RS 1994, Mathern et al 1995a, reviewed in Mathern et al 1997). Immunohistochemistry has shown that defined subsets of neurons are preferentially lost in hippocampal sclerosis (de Lanerolle et al 1989, Robbins et al 1991). These are 1) somatostatin-containing interneurons, 2) the large, glutamatergic "mossy cells," and 3) CA3 pyramidal neurons (Figure 3.2).

Loss of hilar interneurons produces deafferentation of dentate granule cells, and loss of CA3 pyramidal cells eliminates specific targets of the granule cells' axons (the "mossy fibers"). In the manner of all neurons deprived of input and targets, the granule cells respond to these changes by producing new axon collaterals.

Granule cells normally project via excitatory mossy fibers to the CA3 pyramidal neurons of the adjacent hippocampal gyrus. In hippocampi removed at surgery from human patients with epilepsy (Sutula et al 1989, Babb et al 1991, Mathern et al 1995b) and in some animal models of epilepsy (Cronin and Dudek 1988), mossy fibers often aberrantly penetrate the granule cell layer and the inner molecular layer of the dentate gyrus. This region of the dentate gyrus normally comprises the dendritic zones of the granule cells, axonal projections from the entorhinal cortex, and a limited number of interneurons. The precise alteration in connectivity that this sprouting constitutes has not been definitively ascertained, but strong evidence exists for the assertion that mossy fiber

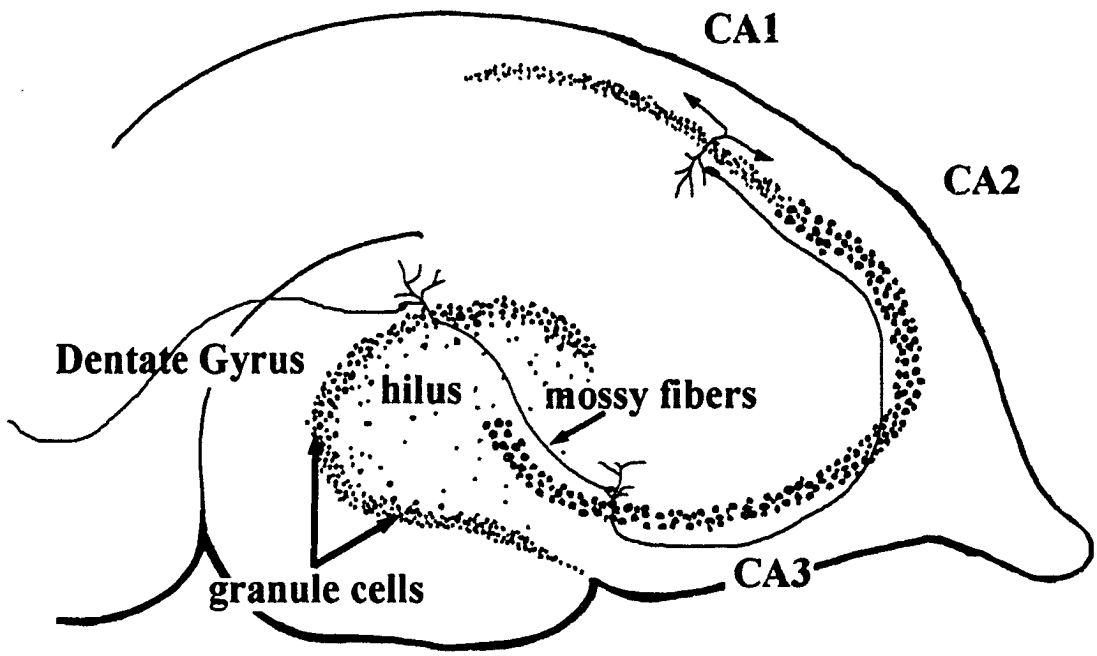


Figure 3.2: Cross-sectional histologic anatomy of the hippocampal formation. Regions CA1, CA2, and CA3 comprise pyramidal cells of the hippocampal gyrus. The arcing layer of granule cells in the dentate gyrus demarcates the region of the hilus. Somatostatin-containing interneurons and mossy cells (a glutamatergic interneuron) are normally found within the hilus. The primary input into the dentate gyrus is via entorhinal projection neurons (purple). Granule cells project via their mossy fibers (red) to the CA3 pyramidal cells of the hippocampal gyrus. These glutamatergic cells send their axons (Schaffer's collaterals, green) to pyramidal CA1, which in turn are the primary output neurons of the hippocampal gyrus.

sprouting can establish abnormal, recurrent excitatory synapses upon granule cells, and that these synapses can predispose the hippocampal formation to seizures (Cronin and Dudek 1988, Dudek et al 1994, Wuarin and Dudek 1996).

Loss of particular cell types in the hippocampal formation may reflect their selective vulnerability to the cellular processes of excitotoxicity (Nitsch et al 1990, Sloviter et al 1991). The initial insult precipitating the development of hippocampal sclerosis and mossy fiber sprouting may be anoxia, trauma, and/or childhood seizures (reviewed in Mathern et al 1997). These injuries are likely to be associated with glutamate dysregulation and exposure of the hippocampus to increased amounts of excitotoxins. Vulnerable neurons are killed, initiating the sprouting of mossy fibers and the development of aberrant circuits. It is not clear that hippocampal sclerosis constitutes a cause or effect of temporal lobe epilepsy. The lesion's close association with the disease, however, is not disputed. The electrophysiologic processes that produce a hyperexcitable hippocampal formation are either directly or indirectly linked to the loss of cells and synaptic reorganization therein.

HIV and Seizures: A Proposed Neuroanatomic Substrate

Of patients with overt signs of HIV-1-associated neurologic disease, 20-50% will exhibit recurrent seizures (Holtzman et al 1989, Parisi et al 1991), and as many as half with this complaint will have no gross brain lesions that might otherwise account for their seizures (Hotlzman et al 1989, Wong et al 1990, Bartolomei et al 1991). The mechanism by which HIV-1 infection predisposes the brain to seizures in the absence of secondary infections or neoplasia is unknown.

We have postulated that the presence of HIV-1 and/or gp120 in the hippocampal formation may produce a pattern of neuronal loss similar to that seen in cases of temporal lobe epilepsy. That is, the neurons that are preferentially lost in TLE might also be lost in HIV-1 infection due to their preferential sensitivity to excitotoxicity. Loss of these cells would stimulate the sprouting of mossy fibers as the granule cells lose afferents and targets. These events could, therefore, recreate the characteristic lesion of temporal lobe epilepsy.

HIV and the Hippocampus

Evidence already exists for a specific neurotoxic effect of HIV-1 and/or gp120 on the hippocampal formation. That gp120 is toxic to hippocampal pyramidal cells has been amply demonstrated in dissociated culture (Dreyer et al 1990, Lo et al 1992, Meucci and Miller 1996, Medina et al 1999), organotypic culture (Aggoun-Zouaoui et al 1996), and in vivo murine models (Barks et al 1993, Barks et al 1997). The hippocampal neurons of transgenic mice expressing gp120 in astrocytes had reduced dendritic fields, as evidenced by a decrease in the volume of neuropil (Toggas et al 1994). This same line of transgenic mice exhibited a marked increase in short term potentiation and a diminution of long term potentiation in the CA1 region, suggesting that gp120 is capable of producing functional alterations of synaptic connections in the hippocampal formation (Krucker et al 1998).

In human tissues acquired at autopsy from HIV-1-infected patients, PCR amplification of sequences from HIV-1's *gag* gene in eight different brain regions revealed highest concentrations of proviral DNA in the hippocampus (Fujimura and Bockstahler 1995). Reyes and associates found immunoreactivity for gp41 in CA2 and

CA3/4 of the hippocampal formation (Reyes et al 1994), and recent application of quantitative HIV-1 RNA assays revealed HIV-1 message in the hippocampus at greater levels than in most other studied regions of the brain (Wiley et al 1998). A decreased density of parvalbumin-positive interneurons has been seen in CA3 (Masliah et al 1992b) of HIV-1-infected brains, as has a reduction in somatostatin-containing interneurons throughout the hippocampal gyrus (Fox et al 1997).

Importantly, Mitchell and colleagues have demonstrated the presence of mossy fiber sprouting in the hippocampal formation of Specific Pathogen Free cats infected with Feline Immunodeficiency Virus (FIV), a lentivirus of felids which, like the related HIV, produces profound immunological dysfunction and frequently neurological disease (Dow et al 1990). The same researchers have likewise documented mossy fiber sprouting in a clinical case of feline epilepsy. The juxtaposition of these findings suggests that the characteristic neuroanatomic lesion of temporal lobe epilepsy may represent a shared feature of seizures due to multiple etiologies across species boundaries (Mitchell et al 1998).

The experimental design described below sought to determine if some neuroanatomical features of HIV-associated seizures and temporal lobe epilepsy might be shared in common. We hypothesized that dentate granule cells are more resistant to the neurotoxic effects of gp120 than hippocampal pyramidal cells. With this differential sensitivity, gp120 in the hippocampus would cause loss of CA3 pyramidal cells, removing targets of the less vulnerable granule cells, and stimulating the sprouting of axon collaterals from the mossy fibers.

METHODS AND MATERIALS:

Cell Culture

Hippocampal Pyramidal Cells

Hippocampal pyramidal cells are a widely employed neuronal cell type in studies of nervous tissue. The technique involves the collection of embryonic tissue at or just after the time of maximal neuronal division in Ammon's Horn, usually at embryonic age 17 or 18 days (E17 - E18) (Bayer 1980). Importantly, embryonic hippocampal tissue collected in the usual manner contains all the neuronal types found in the hippocampal formation, albeit not in their adult proportions. Dentate gyrus granule cells are present in small numbers, as they are represented primarily by a secondary germinal center in the hilus in the fetal brain and will not begin rapid mitosis and differentiation until the first few post-natal days (Cowan et al 1981). Hilar and other interneurons of the hippocampal formation are well represented, as their neurogenesis extends from E14 through to birth (Bayer 1980, Cowan et al 1981). Even the predominant neuronal type, the pyramidal cell, does not occur as a uniform population of neurons; pyramidal cells from all three regions of the hippocampal gyrus (CA1, CA2, and CA3) are present, distinguishable only vaguely from one another in dissociated culture by their size.

Heterogeneity of the neurons in hippocampal culture is not considered in most extant literature making use of this culture technique. It is tempting to speculate that the sub-total lethality of gp120 in studies of its neurotoxicity may in part reflect a differential sensitivity of various neuronal types present in vitro. To reduce the possible effect such heterogeneity introduces, the observer in the studies described here excluded cells that did not meet certain morphologic criteria (described below). Only large pyramidal cells

(most characteristic of CA3) or granule cells were counted in the cell quantification in these experiments.

Hippocampi from E18 Sprague-Dawley rat feti were harvested in Hanks balanced salt solution (HBSS) + dextrose, trimmed of attached entorhinal cortex, and minced into 1-2 mm pieces. These were exposed to 0.125% trypsin and 200 Kunitz units DNAse for 20 minutes, then rinsed and quenched with serum-containing growth medium (DMEM + 10% F12 + 10% heat-inactivated fetal bovine serum + penicillin/streptomycin/amphotericin B). The tissue was then gently triturated through flame-polished Pasteur pipettes in 2.0 ml of growth medium. Cell density was measured with a hemocytometer, the volume adjusted with additional growth medium, and 250 µl aliquots containing 100,000 cells were applied to glass coverslips (22 mm^2) coated with poly-d-lysine at 26 $\mu g/cm^2$. After 2-3 hours in a 37°C incubator with 8% CO_2, an additional 1.0 ml growth medium was added to each well. Cultures were maintained at 37° and 8% CO_2 for 14 days prior to experiments. One ml of growth medium from each well was exchanged twice a week. At approximately 7 days in vitro (div), cultures were exposed to 5µM cytosine arabinoside for 24 hours to arrest multiplication of glia. Subsequent to this antimitotic treatment, medium was changed to Neurobasal supplemented with B27 and antibiotics.

Dentate Gyrus Granule Cells

Granule cells of the dentate gyrus appear relatively late in development of the CNS; at birth (E23/P0), only 5-10% of the adult number of granule cells are present (Fricke and Cowan 1977, Bayer 1980, Cowan et al 1981). Neurogenesis of granule cells

occurs in a secondary germinal center, established in the presumptive hilus around E14. The peak of granule cell neurogenesis occurs during the first postnatal week, with an estimated 50,000 new cells appearing daily during the period between P5 and P7 in the rat pup (Schlessinger et al 1975). Granule cell culture therefore differs from pyramidal cell culture in several ways. The tissue is harvested postnatally, near the peak time of mitosis (P6 to P8) so as to collect a maximum number of granule cells within 24-48 hours of their final division. Because the hippocampal gyrus is well formed at that time, the dentate gyrus is micro-dissected from the Cornu Ammonis so as to produce cultures enriched for granule cells. The dissociated cells are centrifuged through 4% bovine serum albumen to remove the cellular fragments that occur in greater quantity when postnatal tissues are subjected to dissection and trituration. Finally, antimitotics are added to cultures earlier, at 4-5 div, because postnatal tissues are populated with astrocytes that rapidly proliferate under culture conditions. Cultures prepared in the manner described below were enriched for granule cells (est. 70%), with lesser numbers of pyramidal cells (est. 20%) (from that portion of CA3—also called CA4—that extends into the region between the blades of the granule cell layer) and interneurons (est. 10%), identified on the basis of their morphology and lack of dynorphin immunoreactivity.

Hippocampi were harvested in HBSS + dextrose from P6-8 Sprague-Dawley rat pups. Pups were anesthetized with IP pentobarbital, decapitated, and their brains removed. The intact hippocampus was rapidly dissected, then sectioned transversely into 1000 μm slices on a McIlwain tissue chopper. The dentate gyrus was isolated from these slices under a dissecting microscope, taking care to exclude as much of CA3 as possible. Collected dentate gyri were exposed to 0.11% trypsin and 200 Kunitz units DNAse for 20

minutes, then rinsed with bovine serum albumen (BSA)-containing HBSS. The tissue was then treated with soya bean trypsin inhibitor and another 200 Kunitz units DNAse, and gently triturated through flame-polished Pasteur pipettes in 1.0 ml HBSS. Tissue was allowed to settle then layered with 4% BSA and centrifuged 15 min at 500g to assist in removal of cellular fragments. The cells were decanted and the pellet resuspended in serum-based growth medium (DMEM + 10% F12 + 10% fetal bovine serum + penicillin/streptomycin/amphotericin B). Cell density was calculated with a hemocytometer, the volume adjusted with additional growth medium and 250 µl aliquots containing 10,000 cells were applied to glass coverslips (22 mm^2) coated with poly-d-lysine at 26 $\mu g/cm^2$. After 2-3 hours in a 37° incubator with 8% CO_2, an additional 1.0 ml growth medium was added to each well. Cultures were maintained at 37° and 8% CO_2 for 14 days prior to experiments. One ml of growth medium was exchanged twice a week. When astrocytes approached a monolayer (at approximately 4 - 5 div), cultures were exposed to 5µM cytosine arabinoside for 24 hours to prevent continued growth of glia. Subsequent to this antimitotic treatment, medium was changed to Neurobasal supplemented with B27 and antibiotics.

Cell Treatments

The envelope glycoprotein of HIV-1, gp120, was acquired from two sources. For pyramidal cell experiments (themselves positive control for the subsequent granule cell experiments), recombinant products of the IIIB strain (Intracell) and SF2 strain (a kind gift of Doug Brenneman) were used and found equally efficacious. gp120 was stored at -70°C in working aliquots that were thawed immediately prior to application to cell

cultures. After 14 days of incubation, growth medium was exchanged in all cultures just prior to treatment. $gp120_{IIIB}$ was applied to cultures at 10 pM and 200 pM. $gp120_{SF2}$ was applied at 10 pM. The negative control condition was application of the vehicle, the defined growth medium. Cultures were incubated an additional 5 to 6 days, without another exchange of medium, then subjected either to labeling with carboxyfluoroscein for counting, or fixation and staining for cell identification.

For granule cell experiments, the IIIB strain of gp120 was utilized at 10 and 200 pM. Just prior to treatment, all cultures underwent an exchange of medium. The negative control condition consisted of exposing a working aliquot of $gp120_{IIIB}$ to 100°C for 20 minutes then applying the denatured glycoprotein to control cultures at 200 pM. All cultures were then incubated an additional 5 to 6 days, after which they were either labeled with carboxyfluoroscein for counting, or fixed and immunostained for cell identification.

Glutamate was bath-applied to a series of granule cell cultures to document the differential sensitivity of pyramidal cells versus granule cells to this excitatory neurotransmitter. As previously indicated and discussed below, granule cell cultures, while enriched for this primary neuron of the dentate gyrus, also contain smaller numbers of interneurons and CA3/CA4 pyramidal cells. This series of experiments exploited that fact by quantifying both granule cells and pyramidal cells in the same glutamate-treated culture. L-Glutamic Acid (Sigma Chemical Co.) in growth medium (pH of medium plus glutamic acid = 7.2) was applied to cultures at 10, 50, and 100 μM. The control treatment consisted of growth medium only. Cultures were incubated an additional four to five days following treatment, then loaded with carboxyfluoroscein for quantification.

Cell Counting

To accurately count neurons in culture required that three criteria be met: 1) Only live neurons should be counted, 2) the area sampled on a given coverslip should be large enough that the normal variation in cell density across the coverslip does not randomly affect the total count, and 3) only neurons of the subtype of interest (pyramidal or granule cells) should be counted.

To ensure that only live, healthy neurons were counted, cultures were loaded with 1 μM carboxyfluoroscein-AM dye (Molecular Probes). The AM-ester form of this fluorescein derivative is actively taken up by all live cells. Within the cell, constitutively expressed cytoplasmic esterases remove the AM-ester, rendering the fluorochrome impermeant to the cell membrane. After 30 minutes of cellular loading in the incubator, cultures were rinsed with HEPES-buffered DMEM, brought to the heated microscope stage of the inverted, phase contrast microscope (Nikon Diaphot), and allowed to continue de-esterification for an additional 30 minutes prior to imaging. Using an FITC filter cube, cultures were illuminated with epifluorescence. Only live cells successfully took up and de-esterified the fluorescent probe. The astrocytic bed, comprising a near-monolayer of flattened cells, assumed a faint fluorescent background against which the more spherical neurons fluoresced much more brightly.

For gp120-treated cultures, a protocol was developed that sampled the central region of each coverslip in a square of 100 contiguous microscopic views at 20X. This protocol covered an area of approximately 62 mm^2 in the center of the coverslip; as the aliquot of suspended cells was applied to the center of the coverslip at plating, it is

estimated that the sample represented somewhat greater than half the neurons present in the culture. For glutamate-treated cultures, a protocol sampling fifty contiguous microscopic frames was utilized.

A mercury light source, attenuated by neutral density filters, was used to excite the loaded cells. The fluorescent images were captured using an intensified charge-couple device camera and displayed on a monitor. The real-time camera image was recorded on VHS videotape as the microscopist moved the stage through contiguous fields in a pre-determined 10 X 10 pattern (or 10 X 5 pattern for glutamate studies), pausing at each field for a few seconds in order to record a stable, focused image.

Subsequently, a trained observer, blinded to the treatment of each culture, reviewed the videotape to record neuronal numbers. The observer was taught to differentiate large pyramidal and granule cells on the basis of somatic size and neuritic pattern. The number of fluorescing cells with morphology consistent with the neuronal type of interest in each field was recorded. Somata of pyramidal cells were generally oval or polygonal, around 20 - 30 micrometers in diameter, and had a few prominent neurites. Granule cells had smaller (ca. 10 μm), round somata and exhibited a radial array of many fine neurites discernible for only a short distance from the cell body (Figure 3.3).

The sum of 100 (gp120 studies) or 50 (glutamate studies) fields represented the total number of cells recorded for a given coverslip and constituted one replicate.

Immunostaining

Pyramidal cells are large multipolar neurons that use glutamate as their neurotransmitter. They lack a distinguishing antigenic marker that can be used to

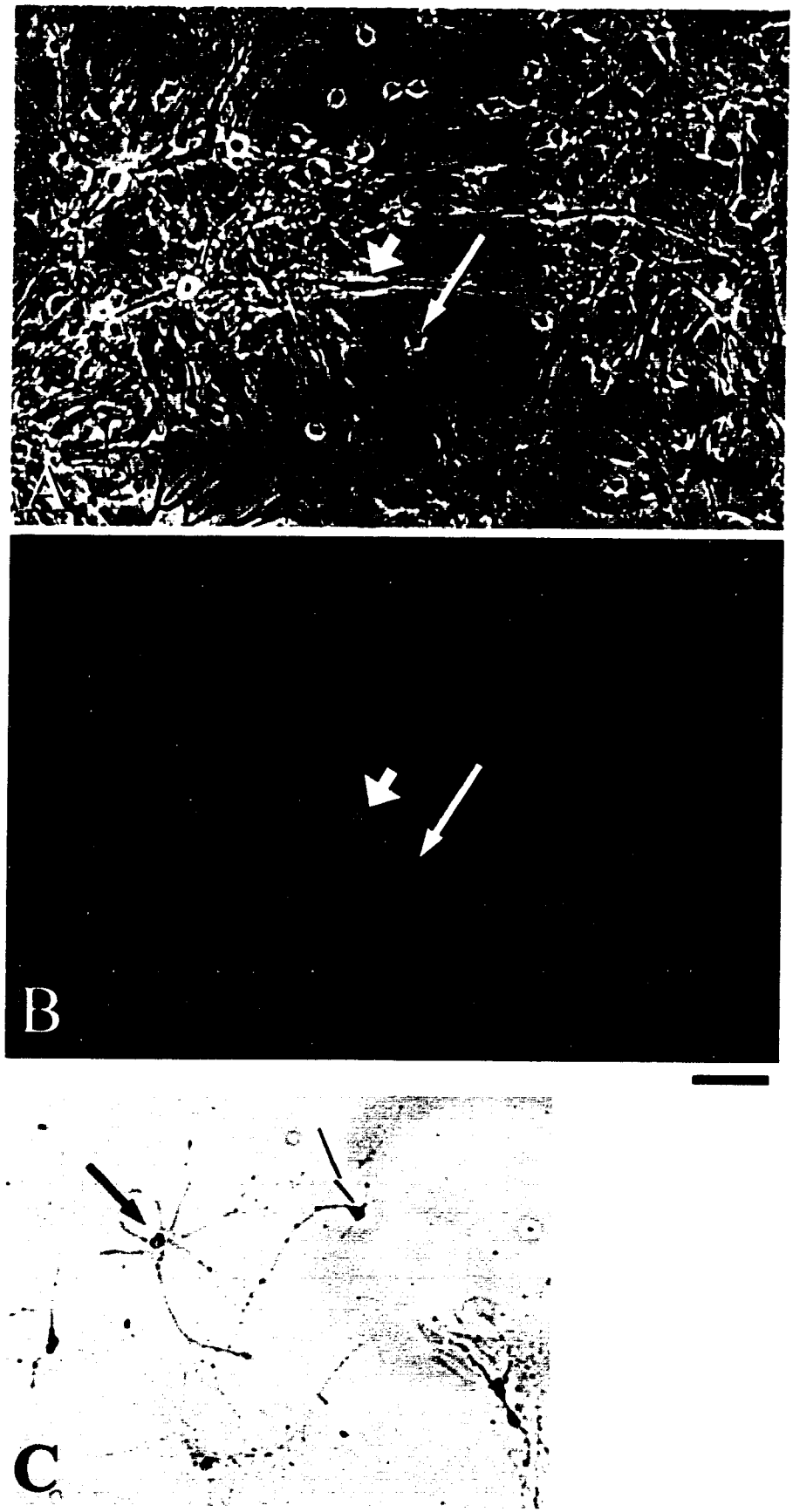
Figure 3.3: Cultures treated with glutamate were populated by a variety of neuronal types.

A: Under phase-contrast microscopy, pyramidal cells were identified as large, phase-bright cells with several prominent neurites (short, thick arrow). Granule cells appeared as small, round, phase-dark neurons with many fine neurons (long, thin arrow).

B: The same field viewed under fluorescence with cells loaded with carboxyfluoroscein enhances the morphological distinction between the two primary neuronal types.

C: Immunolabeling for a granule cell marker, Dynorphin A, shows that morphological criteria used under fluorescence distinguish granule cells (white arrow) from pyramidal cells (black arrow), which do not stain for this marker. Reaction products are revealed with DAB.

Bar = 0.1 mm



differentiate them from other glutamatergic neurons. Granule cells, in contrast, are small multipolar neurons which, in addition to glutamate, also express dynorphin, an opioid peptide that functions as a co-transmitter at mossy fiber synapses (Hoffman and Zamir 1984). Some cultures were immunostained with neurofilament, an intermediate filament found predominantly in the processes of neurons, to demonstrate the presence of neurons in general. Some cultures prepared from the dentate gyrus were immunostained with dynorphin A to document the presence of granule cells and demonstrate their characteristic morphology in vitro.

Astrocytes are present in the cell suspension prepared from brain tissue at the time of explantation. In serum-based growth medium, astrocytes proliferate and rapidly assume a monolayer on the coverslip. In both pyramidal and granule cell culture, astrocytes were permitted to grow to near-confluence (at about 7 and 4 days, respectively) at which time the cultures were treated with cytosine arabinoside to arrest astrocytic mitosis. Astrocytes may participate in the neurotoxic effects of gp120 and HIV infection (Benos et al 1994, Dreyer & Lipton 1995, Vesce et al 1997). Therefore, their presence was confirmed through immunocytochemical staining for their distinctive molecular marker, GFAP (glial fibrillary acidic protein).

Another non-neuronal cell of likely importance in mediating gp120-induced cell death is the resident macrophage, the microgliocyte. Microglia are necessary for gp120 to manifest neurotoxicity in culture (Lipton 1992b). These cells express a variety of cell surface markers considered useful for their immunocytochemical identification; in these experiments, the presence of microglia was confirmed by staining for CD11b (Integrin αM).

Immunostaining techniques were modified from Mitchell 1998. Briefly, cultures were fixed in cold 4% formaldehyde for 10 minutes, then washed in 0.1 M phosphate buffer prior to processing for the immunomarker of interest. Cells intended for labeling with intracellular markers (GFAP, dynorphin, and neurofilament) were permeablized through exposure to 0.3% Triton. Primary antibodies were used as indicated: neurofilament200 (Sigma #N4142), rabbit polyclonal, at 1:1000; GFAP (Sigma #G-9269), rabbit polyclonal, at 1:200; dynorphin A 1-8 (Pennisula #RIN 8697), rabbit polyclonal, at 1:1000; CD11b (Chemicon #MAB1405), mouse monoclonal, at 1:500. Primary antibodies were applied to coverslips and incubated overnight at 4°C.

A biotinylated secondary antibody was applied on the second day, followed by exposure to the ABC reagents of the Vectastain Elite ABC kit (Vector Laboratories) according to the manufacturer's instructions. To visualize the reaction product, cultures were incubated with diaminobenzidine (DAB) and glucose oxidase, producing the characteristic brown-black precipitate on the labeled cells.

Statistical Analysis

Each "n" represented the total number of cells counted in 100 (gp120 studies) or 50 (glutamate studies) fields of a single coverslip. All statistical analyses were performed using SAS 6.12 software (SAS Institute Inc., Cary, North Carolina). Analysis of variance (ANOVA) with multiple comparisons was performed to determine if significant differences in the number of cells/culture existed between treatments and controls. Significance for all tests was accepted when $P<0.05$, and all interactions were performed

a priori. The R-Studentized residual was used for testing potential outliers (see results and discussion below).

RESULTS

Pyramidal cell cultures were characterized by a nearly confluent bed of astrocytes (Figure 3.4), a mixture of neuronal cell types dominated by pyramidal neurons (Figure 3.5), oligodendrocytes, and the occasional microgliocyte (Figure 3.6). The predominant neuronal type, based on positive staining with neurofilament and typical morphology, was the pyramidal neuron.

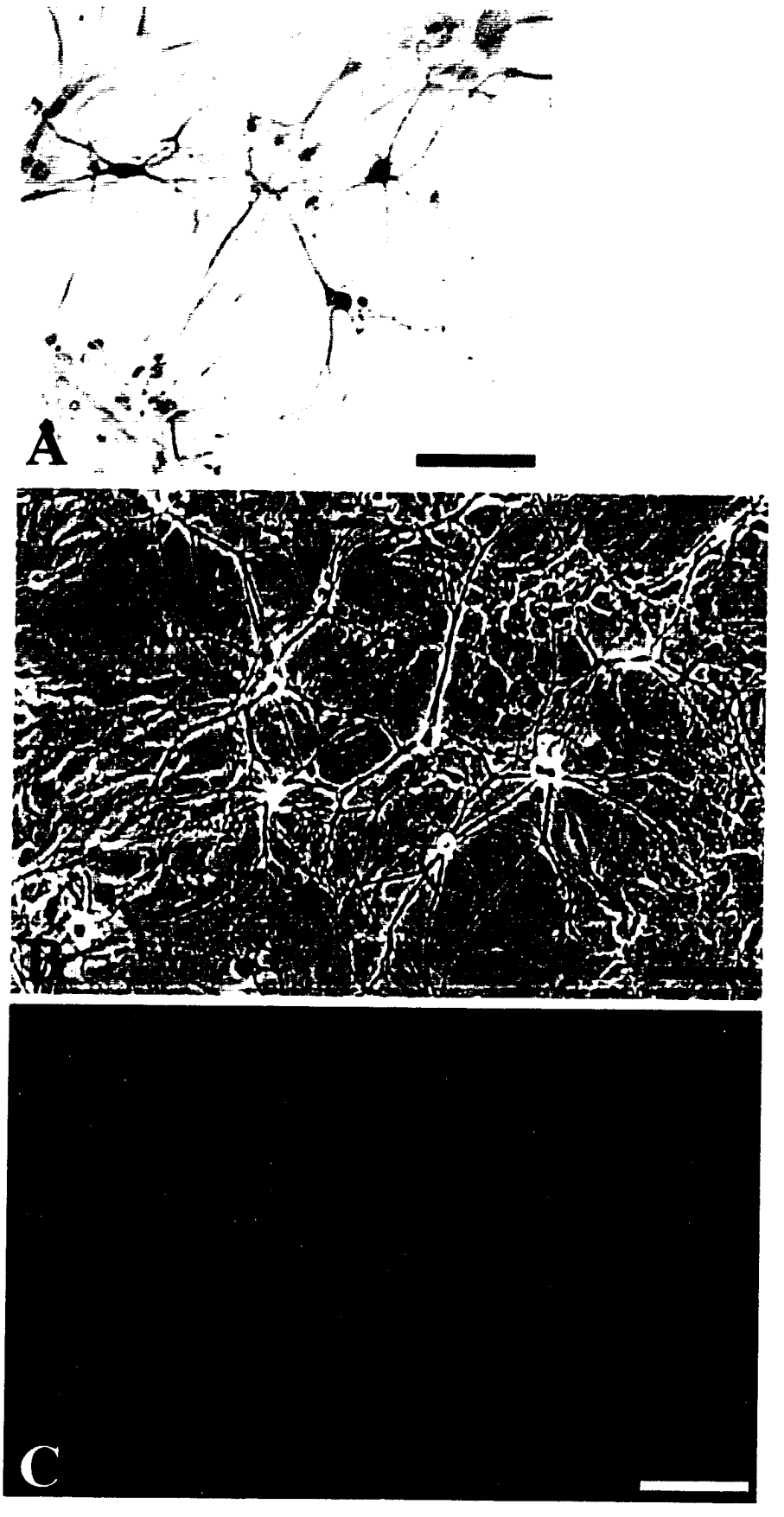
Pyramidal cell cultures treated with $gp120_{IIIB}$ at 10 pM (n = 12) exhibited a 39.78% reduction in numbers of pyramidal cells relative to control treatment (n = 15). This reduction was statistically significant at p = 0.0001. Cultures treated with $gp120_{IIIB}$ at 200 pM (n = 6) exhibited a reduction of pyramidal cell numbers of 44.91% (p = 0.0004). $gp120_{SF2}$ applied to cultures at 10 pM reduced pyramidal cell numbers by 28.65% (p = 0.0036). None of the treatments were statistically different from one another. These results (Figure 3.7) are consistent with published data (Dreyer et al 1990, Lo et al 1992, Meucci and Miller 1996) and confirm that 1) both serovars of gp120 are capable of producing neurotoxicity at these doses, and 2) our culture and imaging system is capable of detecting the sub-total cell lethality produced by gp120.

Granule cell cultures (Figure 3.8) exhibited a nearly-confluent layer of astrocytes, a variety of neuronal cell types, oligodendrocytes, and occasional microglia (Figure 3.9). Approximately 75% of the neurons present were determined to be granule cells based on their size and dendritic pattern, a morphological assessment supported by

Figure 3.4: Pyramidal cell culture at 14 days in vitro (div) immunolabeled for GFAP. Reaction products are revealed with DAB. Bar = 0.1 mm.

Figure 3.5: Pyramidal cells in culture.

A: The presence of neurofilament (NF200) identifies cells as neurons. Reaction products revealed by DAB.
B: Phase-contrast micrograph of pyramidal cells on a confluent bed of astrocytes.
C: The same field as in B with live cells loaded with carboxyfluoroscein reveals characteristic morphology.
Bar = 0.1 mm.



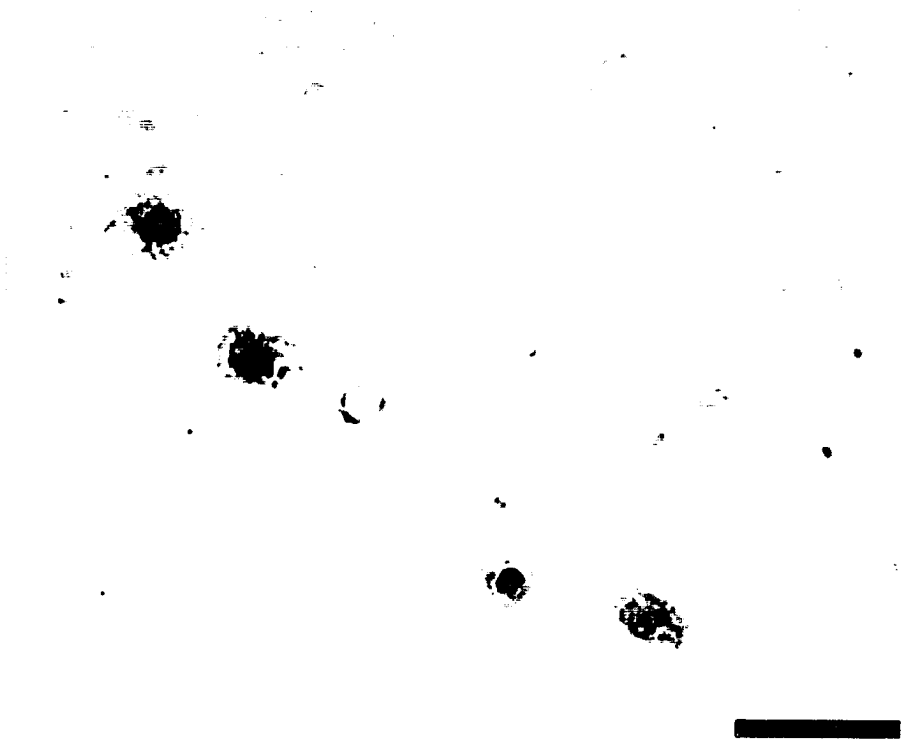


Figure 3.6: Activated microglia are present in hippocampal pyramidal cell cultures. Presence of a specific microglial marker, CD11b, is revealed by DAB. Bar = 0.1 mm.

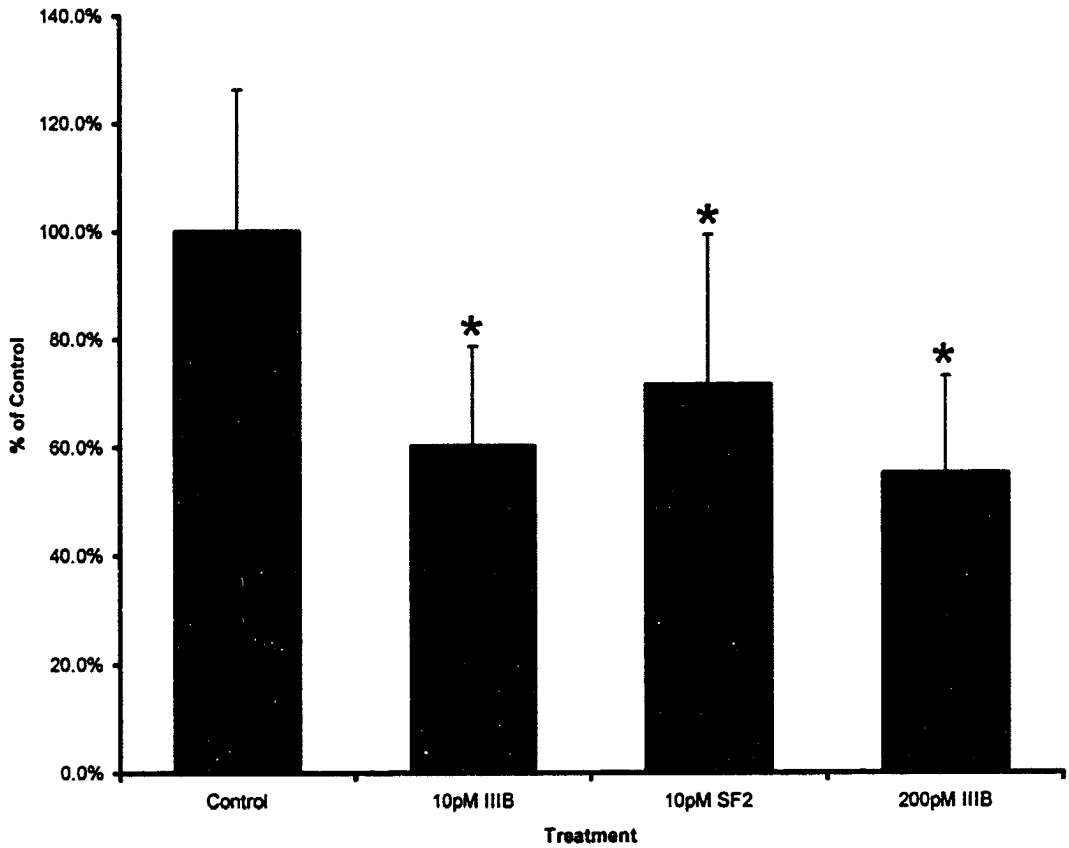
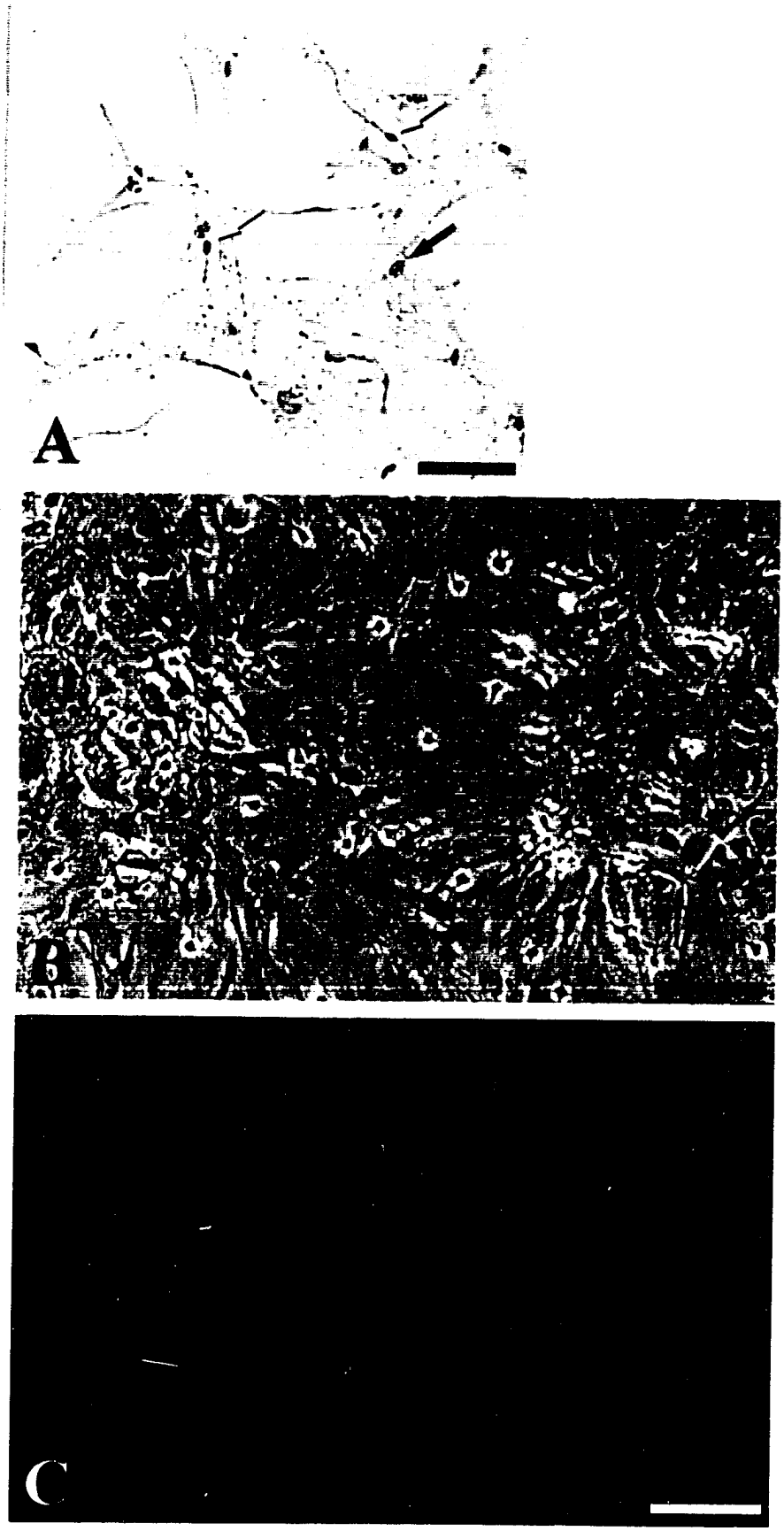


Figure 3.7: Pyramidal cell survival after exposure to gp120, expressed as a percentage of control cultures. The control condition was treatment with the vehicle (growth medium). Treatment with 10 pM $gp120_{IIIB}$ produced a 39.78% reduction in pyramidal cell survival ($p = 0.0001$) relative to control cultures. Treatment with 10 pM $gp120_{SF2}$ resulted in a 28.65% reduction in pyramidal cell survival ($p = 0.0036$). $gp120_{IIIB}$ at 200 pM reduced pyramidal cell numbers by 44.91% ($p = 0.0004$). The three gp120 treatments did not differ statistically from one another.

Figure 3.8: Granule cells in culture.

A: Immunolabel for Dynorphin A reveals granule cells (white arrow). Note adjacent, unlabeled astrocyte (black arrow). Reaction products are revealed by DAB.
B: Phase-contrast micrograph of granule cells on a confluent bed of astrocytes.
C: The same field as in B with live cells loaded with carboxyfluoroscein reveals the characteristic morphology of the granule cells.
Bar = 0.1 mm.



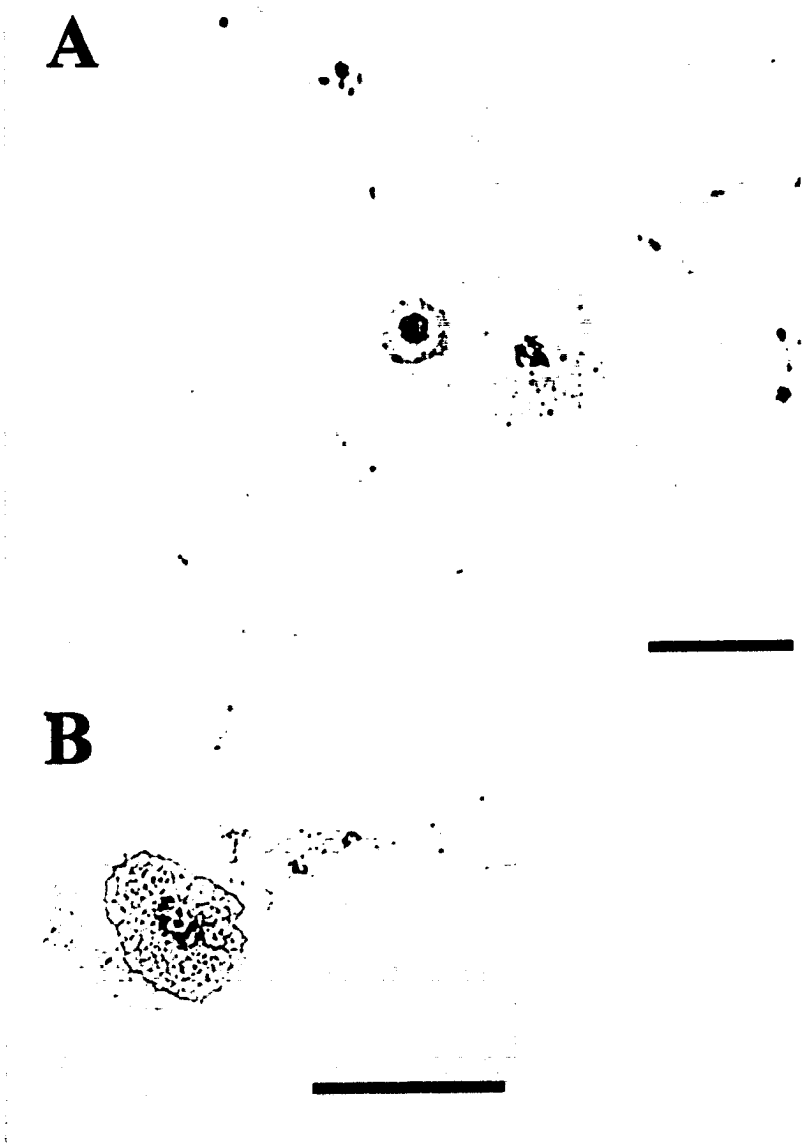


Figure 3.9: Activated microglia are present in granule cell cultures.
A: Presence of a specific microglial marker, CD11b, is revealed by DAB.
B: Higher power view of labeled microgliocyte illustrates the foamy characteristic of the activated cell.
Bar = 0.1 mm.

immunocytochemistry for dynorphin, an opioid co-transmitter unique to granule cells in the hippocampal formation (Hoffman and Zamir 1984).

When all collected data points are considered (Figure 3.10), the number of granule cells in cultures treated with $gp120_{IIIB}$ at 10 pM (n = 16) was not statistically different from the control condition (n = 16) (p = 0.065). Likewise, the number of granule cells in cultures treated with $gp120_{IIIB}$ at 200 pM (n = 17) did not differ from control (p = 0.10). The means of each of these groups, however, exhibited an intriguing and unexpected trend.

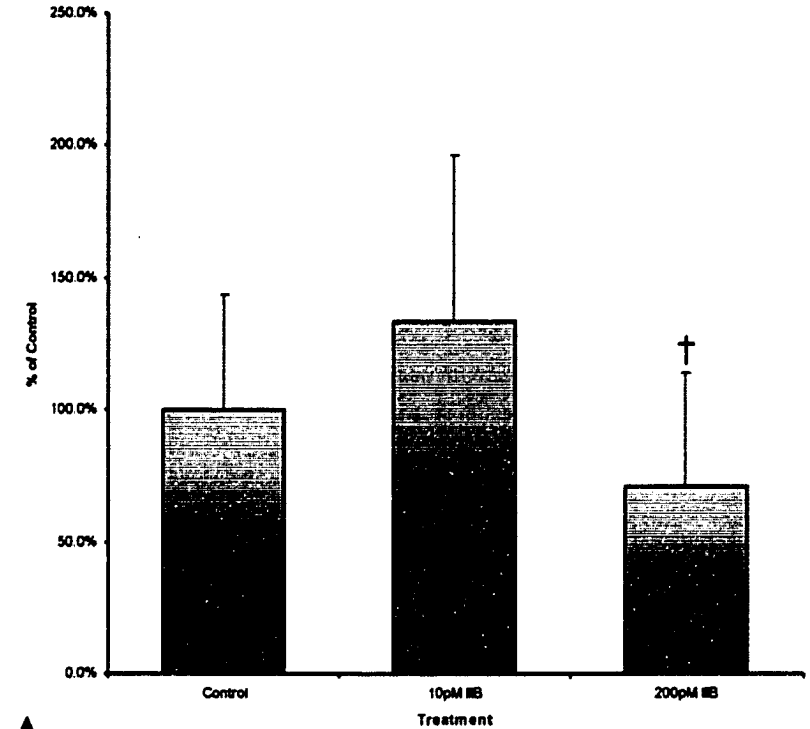
The mean number of granule cells in control cultures was 394.25 (SD = 172.05); that of cultures treated at 200 pM was 287.13 (SD = 172.08), a trend toward reduced numbers of neurons that was not statistically significant. The mean number of granule cells in cultures treated at 10 pM, however, was 527.19 (SD = 246.78). This number was not statistically different from that seen in the control condition, but suggested an *increased* survival (or in vitro neurogenesis) of granule cells exposed to $gp120_{IIIB}$ at 10 pM. This anomalous finding was further supported by the strongly significant difference that treatment with 10 pM exhibited relative to cultures treated with 200 pM (p = 0.0008). The eccentricity of this finding led us to examine the individual data points in each set, and subject them to an R-Studentized residual to test for potential outliers.

Amongst cultures treated with $gp120_{IIIB}$ at 10 pM, one data point (1124) was well beyond two standard deviations of the mean and came very close to satisfying an R-Studentized residual tested with a Bonferroni adjusted t-value. Simply put, that point is statistically so different from the others that we may justifiably believe it to be an error and remove it from the data set.

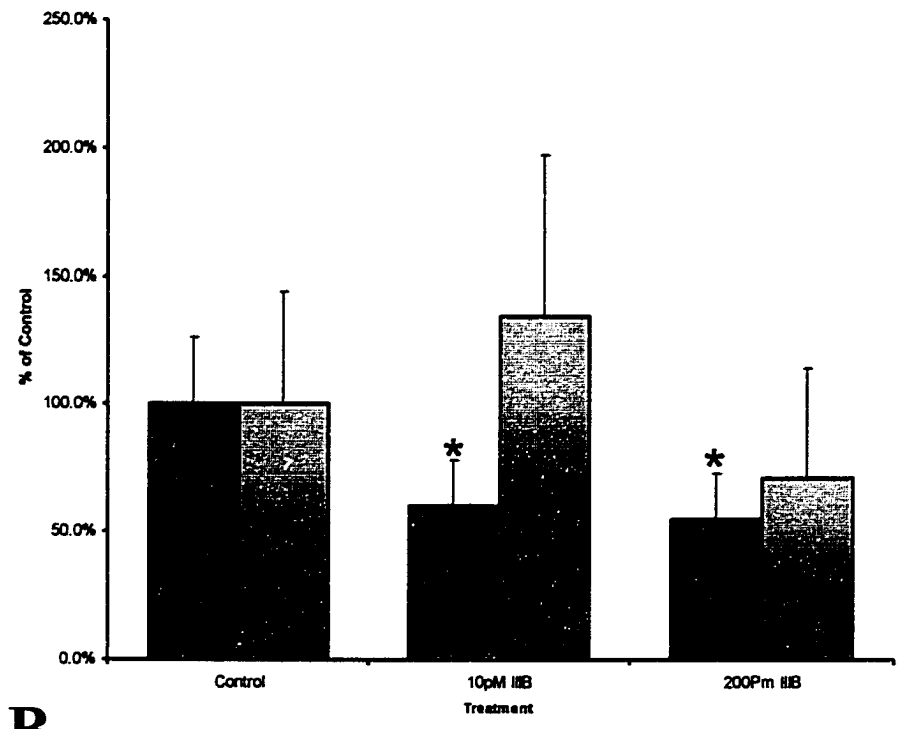
Figure 3.10: Granule cell survival after exposure to gp120.

A: Graph includes all data (no points excluded). Granule cell survival is expressed as a percentage of the control condition; control cultures were treated with 200 pM heat-inactivated $gp120_{IIIB}$. Survival in cultures treated with $gp120_{IIIB}$ at 10 pM was not statistically different than the control condition ($p = 0.065$). Cultures treated with $gp120_{IIIB}$ at 200 pM did not differ from control cultures ($p = 0.1$). $gp120_{IIIB}$ at 10 pM produced cell loss that was statistically different than that in cultures exposed to 200 pM (†, $p = 0.0008$).

B: Comparison of granule cell survival and pyramidal cell survival after five days exposure to $gp120_{IIIB}$ at 10 and 200 pM. Neither treatment produced significant changes in granule cell survival relative to the control condition. Pyramidal cell numbers were significantly reduced by both 10 pM ($p = 0.0001$) and 200 pM ($p = 0.0004$) $gp120_{IIIB}$.



A



B

Amongst cultures treated with $gp120_{IIIB}$ at 200 pM, one data point (796) also lay beyond two standard deviations of the mean, although it did not come as close to satisfying rigorous statistical tests for outliers. Nonetheless, one could arguably exclude this one point from the set based on its residual plot.

If the data are reconsidered while excluding only the 1124 data point, the mean of the cultures treated with 10 pM gp120 is reduced to 487.4 (SD = 195.23). It is still somewhat higher than (although not statistically different from) the control condition ($p = 0.15$). Even with this modification, however, $gp120_{IIIB}$ at 10 pM produces values statistically different from treatment at 200 pM ($p = 0.0019$).

If the data is reinterpreted while excluding both suspect data points (1124 and 796), the mean of cultures treated with 200 pM gp120 is reduced to 246.4. Under these considerations, $gp120_{IIIB}$ at 200 pM results in a 37.5% reduction in numbers of granule cells, a diminution relative to control conditions that is statistically significant ($p = 0.0133$). The significant difference between 10 and 200 pM is likewise increased ($p = 0.0002$).

Under any of the above-proposed schemata, including consideration of all collected data without exclusion, the results are consistent with the hypothesis that granule cells are resistant to the neurotoxic effects of gp120 relative to hippocampal pyramidal cells.

There is evidence to suggest that differential sensitivity to the processes of excitotoxicity largely dictates the differential sensitivity of various neuronal types to neurodegeneration in the HIV-1-infected brain. To support this posited role of excitotoxicity in our culture systems, we exposed neuronal cultures to 10 μM ($n = 6$), 50

μM (n = 6), and 100 μM (n = 3) glutamate and quantified the number of surviving granule cells and large pyramidal cells.

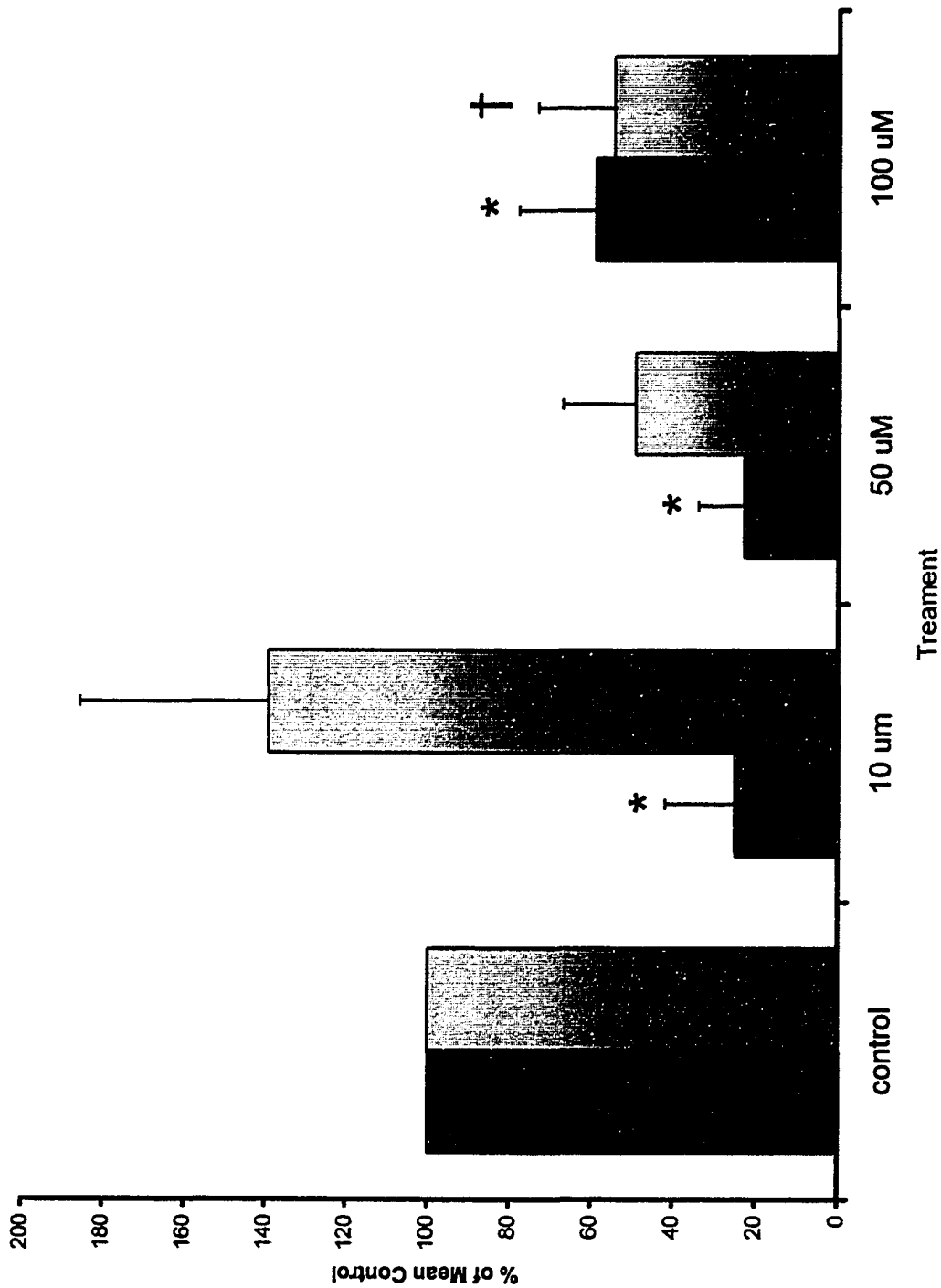
Pyramidal neurons showed a statistically significant decrease in survival relative to the control condition (n = 6) when exposed to as little as 10 μM glutamate (24.96% of control, $p = 0.0003$), a significance that persisted through 50 and 100 μM ($p < 0.03$). In contrast, granule cells did not show a statistically significant reduction in survival until exposed to 100 μM glutamate (54.61% of control, $p = 0.05$). Under our culture conditions, granule cells were more resistant to the excitotoxic effects of glutamate than pyramidal cells (Figure 3.11).

DISCUSSION

The processes that transpire between entry of HIV-1 into the CNS and the dysfunction or death of neurons are likely to be multiple and circuitous. Many of the proposed pathomechanisms invoke viral proteins in the external milieux and activation of intrinsic, cascading inflammatory processes. Most theories ultimately converge on glutamate receptors and excitotoxicity as the final common pathway by which HIV-1 (or its protein products) kills or deranges neurons. Because it is liberally shed from virus particles and infected cells (Schneider et al 1986, Kalyanaraman et al 1990, Earl et al 1991), much research has targeted the role of the surface unit glycoprotein, gp120, in HIV-1-associated neurotoxicity.

Under many experimental conditions, gp120 appears to augment the excitotoxic properties of glutamate, possibly by modulation of neuronal NMDA receptor function (reviewed in Lipton 1992a) or through its effects on a variety of non-neuronal cells.

Figure 3.11: Granule cells are resistant to the excitotoxic effects of glutamate relative to pyramidal cells. Pyramidal cells (blue) had significantly decreased survival at all concentrations of glutamate (*, $p < 0.03$). Numbers of granule cells (tan) were not significantly decreased relative to control conditions until cultures were exposed to 100 μM glutamate (†, $p = 0.05$).



Whatever the precise mechanism(s), certain neurons exposed to gp120 experience a rapid rise in $[Ca^{2+}]_i$ upon exposure to gp120 in culture, and this rise is associated with subsequent cell death.

Not all neurons are equally susceptible to gp120. The heterogeneous vulnerability of different neuronal types in vivo and in vitro may be related to either their unique assemblage of excitatory amino acid receptors or to the individual cell's resistance to the $[Ca^{2+}]_i$–mediated processes of excitotoxicity. Neurons vary in their intrinsic ability to control increased $[Ca^{2+}]_i$; hippocampal pyramidal cells of CA1 and CA3, for instance, are preferentially lost in a variety of neurodegenerative disorders, and this susceptibility has been linked to unbuffered increases in $[Ca^{2+}]_i$ (Mattson et al 1989, Mattson et al 1991). The relative resistance or susceptibility of a given neuron to the neurotoxic effects of gp120 might therefore be directly linked to its ability to buffer an increase in $[Ca^{2+}]_i$. Concordantly, cultured cortical neurons expressing the calcium-binding molecule Calbindin D28K are resistant to gp120 neurotoxicity (Diop et al 1995).

HIV-1 is known to target the hippocampus. Expression of viral DNA and RNA (Reyes et al 1994, Fujimura and Bockstahler 1995, Wiley et al 1998) and neuronal loss (Jernigan et al 1993, Reyes et al 1994) have been reported in the hippocampus in neuroAIDS. Hippocampal pyramidal cells exhibit increases in $[Ca^{2+}]_i$ upon exposure to gp120 (Dreyer et al 1990, Lo et al 1992) but the differential sensitivity of individual primary neuronal types within the hippocampal formation has not been reported. Conceivably, loss of pyramidal cells with relative sparing of the granule cells during HIV-1 infection of the brain could reproduce the histological lesion characteristic of temporal lobe epilepsy. Under this hypothesized pattern of selective cell death, the

enduring granule cells, deprived of their normal targets, would sprout axon collaterals. The resultant synaptic reorganization could constitute the neuroanatomic substrate for the recurring seizures that are often seen in neuroAIDS.

In the work described herein, we exposed the two primary cell types of the hippocampal formation to gp120 in order to compare their relative sensitivity to the neurotoxic effects of this glycoprotein. The specific hypothesis under investigation is that dentate gyrus granule cells are resistant to the neurotoxicity of gp120 at concentrations that kill hippocampal pyramidal cells. In a related study, neuronal cultures were exposed to glutamate to document the differential sensitivity of granule and pyramidal cells to this excitatory amino acid.

Because of the suspected role of glia in gp120-associated neurotoxicity, immunostains were employed to document the presence of astrocytes and microglia in the cultures. Both types of glia were present in cultures of hippocampal pyramidal cells and in granule cell cultures.

Pyramidal cell cultures exposed to two different serovars of gp120 (IIIB and SF2) exhibited a 39.78% and 28.65% loss, respectively, of pyramidal neurons at a concentration of 10 pM. Cultures exposed to 200 pM $gp120_{IIIB}$ experienced a 44.91 loss of pyramidal cells. These data, all statistically significant at $p < 0.004$, are consonant with published reports (Brenneman et al 1988, Lipton et al 1991, Meucci and Miller 1996).

Granule cell cultures were exposed to $gp120_{IIIB}$ under the same culture conditions as the pyramidal cells. At 10 pM, the number of granule cells after 5 or 6 days of exposure was not statistically different than control cultures. At 200 pM, the number of granule cells after 5 days of exposure was not statistically different than control cultures.

These results are consistent with the hypothesis. Granule cells were resistant to concentrations of gp120 that produced significant death of hippocampal pyramidal cells.

Interestingly, however, treatment with 10 pM gp120 resulted in granule cell survival statistically higher ($p = 0.0008$) than that in cultures exposed to 200 pM. Moreover, although it did not achieve statistical significance, the average number of surviving granule cells in cultures treated with 10 pM gp120 was greater than that seen in control cultures (527.19 vs. 394.25). Even after discarding a single data point that fit statistical qualifications for being an "outlier" (see Results), treatment with 10 pM gp120 continued to support a higher average number of surviving cells relative to control cultures (487.4 vs. 394.25, $p = 0.1544$), and continued to show statistically higher cell survival relative to treatment with 200 pM gp120 ($p = 0.0019$).

One possible interpretation of this unexpected finding relates to the observation that certain neuronal types benefit from depolarization in culture. Exposure of cerebellar (Gallo et al 1987), sympathetic (Lampe et al 1995), ciliary ganglion (Collins et al 1991), and dorsal root ganglion (Scott 1977) neurons to increased concentrations of K^+ enhances the survival of these cells in culture. The beneficial effect of the depolarization induced by increased K^+ is linked to increased $[Ca^{2+}]_i$ in these neurons. It is not difficult to conceive that increased $[Ca^{2+}]_i$ wrought by exposure to low (10 pM) concentrations of gp120 could similarly enhance survival of dentate gyrus granule cells. Investigation of this proposal could proceed through use of calcium-indicator dyes such as Fura-2 and blockers of calcium currents to document changes in intracellular calcium and the nature of these changes. Interestingly, in the glutamate-exposure studies also undertaken in the described work, granule cells exposed to 10 μM glutamate enjoyed increased survival

over control cultures (139% of mean control). This increase did not reach statistical significance ($p = 0.085$), but the trend is strikingly reminiscent of the pattern of survival exhibited by granule cells exposed to 10 pM gp120. A small sample size may have prevented detection of statistical significance. Nonetheless, the trends toward increased granule cell survival in low concentrations of both gp120 and glutamate are supportive of the postulate that moderate depolarization of granule cell neurons enhances their survival in vitro.

Modest, "bufferable" elevations of $[Ca^{2+}]_i$ in gp120-exposed granule cells could stimulate axon collateral outgrowth. Outgrowth of neuronal processes in cultures responds to changes in $[Ca^{2+}]_i$, with the optimal outgrowth occurring at elevated levels (Kater and Mills 1991). The calcium-increasing properties of gp120 present in the hippocampal formation may thereby not only encourage survival of granule cells simultaneous with loss of their target pyramidal cells, but also encourage the subsequent sprouting of aberrant mossy fibers.

Several data points amongst those acquired in the gp120-exposed granule cell experiments described above may meet statistical criteria to be considered "outliers." In addition to the statistical outlier (1124) identified amongst the granule cell cultures treated with 10 pM gp120, one culture in the group treated with 200 pM fit some criteria for exclusion from consideration. This data point (796) lay beyond two standard deviations of the mean, lying far afield in the residual plot of data. If it is excluded as an outlier, the mean number of cells in cultures treated with 200 pM gp120 becomes 246.4, and the survival of granule cells in this group is statistically less than that of control cultures by a decrement of 37.5% ($p = 0.0133$). Because the cultures treated with 10 pM

gp120 remain statistically no different than the control condition, these results still support the hypothesis that granule cell cultures are resistant to gp120 at a concentration (10 pM) which is neurotoxic to hippocampal pyramidal cells, and that granule cell death results only when the concentration is increased to greater than 10 pM.

CAVEATS AND CONCERNS

One of the primary technical difficulties in neurotoxicity studies of this nature is the variability of neuronal density on and between coverslips plated with neurons. With subtotal cell loss (reported here as a statistically significant loss of as little as 28%), the measurable decrement in neurons can be overwhelmed by variations in cell numbers. An unlucky small sampling of a given coverslip could readily give an unrepresentative number, if the distribution of neurons within the culture is—as often is the case—uneven.

This concern led to the development of an exhaustive sampling protocol in this study. In these gp120 studies, a series of one hundred contiguous microscopic fields from the center of each coverslip was sampled and evaluated. Since cell suspensions are aliquoted onto each coverslip at or near the center, it is estimated that this sampling encompassed about half the neurons within the culture.

To reduce variability between coverslips, cell density was evaluated with a hemocytometer each time a new cell suspension was prepared, and equal aliquots of 100,000 cells (for hippocampal pyramidal cell culture) or 20,000 cells (for granule cell culture) were applied to each coverslip. Attention was devoted to adequate trypsinization and trituration so that a uniform, single-cell suspension was created for plating purposes.

An important consideration in quantifying surviving neurons in these cultures was the extent to which more than one neuronal type was represented in the culture. This is a facet of gp120 studies in the hippocampal formation that has not been addressed in published literature. Cultures of the hippocampus prepared at E18 (as is typical in most studies) are populated by pyramidal cells of all subfields, interneurons of Ammon's horn and the hilus, and by small numbers of granule cells. Cultures prepared from dentate gyri dissected from the rest of the hippocampal formation at P5-7 have a substantial number of large pyramidal cells (CA4) and hilar interneurons admixed.

Since differences in gp120 sensitivity between these groups was the focus of this study, particular care was taken to quantify only the cells of interest. An observer, blinded to the treatment protocol, was instructed in recognition of individual cell types based on the morphology revealed under carboxyfluoroscein labeling. Cells were evaluated by size of soma, number and diameter of cell processes, and pattern and length of neurites. Immunostaining was used to confirm that the morphological criteria corresponded to actual cell type.

Another criticism that might be made of the experimental design described herein and in other studies making use of neuronal cell culture is that the process of harvesting, dissociating, and incubating primary nervous tissue may have the unintended effect of selecting for a subset of cells from the cultured tissue. Preparing a cell suspension from whole tissue is inherently traumatic. If, as hypothesized, gp120 produces death of those neurons less able to regulate increases in $[Ca^{2+}]_i$, then many of the very cells most likely to be sensitive to gp120-induced neurotoxicity may be eliminated from the experimental system. Such a cell loss will bias the results in favor of producing no effect. Less

traumatic culture techniques (i.e., organotypic culture) and/or in vivo experiments would obviate this concern and allow evaluation of cells expected to be most susceptible to injury.

The majority of studies on the neurotoxicity of gp120 utilize murine systems; it bears noting that HIV-1 cannot infect murine cells. Arguably, such systems are so exotic (vis-à-vis natural infection) that they lose clinical relevance. Equally arguable is the position that this makes rat and mouse systems ideal for investigating gp120 in that neuropathological effects attributable directly to the molecule are therefore isolated from effects due to infection by the virus.

The bulk of extant evidence makes it clear that gp120, when introduced into experimental systems (both in vitro and in vivo), is capable of producing neuropathology. What is not clear is the degree to which this property is relevant in clinical HIV-1 infection. While there is evidence that gp120 is released from whole virions and is expressed on and shed from the surface of infected cells, the extent to which this happens in brain parenchyma during natural infection is mostly speculative.

The mutability of the glycoprotein and therefore its variability between HIV-1 strains could also influence the degree to which a given serovar can be expected to produce in natural infection those effects attributed to it in experimental settings. It is curious to note that the two strains used in this study (SF2 and IIIB) are both lymphotropic, and are therefore not expected to be representative of primary strains infecting the human nervous system. Both are widely used in published studies of gp120-associated neuronal effects (e.g., calcium increases, neuronal death), and—even more curiously—the majority of studies utilizing gp120 in experimental settings to produce

neuronal effects do so with lymphotropic strains. This is anomalous in the face of the well-established fact that most CNS infections are initiated by macrophage-tropic (M-tropic) strains of HIV-1 (Cheng-Mayer et al 1989, Jordan et al 1991, Sharpless et al 1992b, Gonzalez-Scarano et al 1995). Over a clinical course, the cellular tropism of strains recoverable from the CNS of an AIDS patient expands, just as it does in the peripheral blood (Atwood et al 1993). Nonetheless, Power and associates found a strong bias toward M-tropic consensus sequences amongst clones derived from the brains of patients dying of AIDS, even when non-macrophage-tropic strains were readily recoverable from the spleen of the same patient (Power et al 1994). It remains to be proven, therefore, that the neurotoxic effects shown here and in other studies are also a feature of the M-tropic strains that are more likely to be isolated from naturally occurring infections.

FUTURE DIRECTIONS

The data presented in this chapter are supportive of the broader hypothesis posited to explain the prevalence of seizures as a sign of neuroAIDS. Further investigation is required to demonstrate that what is a possibility (i.e., that resistant granule cells lose their pyramidal cell targets and sprout aberrant mossy fiber connections) is a de facto consequence of HIV-1 infection of the brain.

One central feature of gp120 neurotoxicity is its calcium-increasing effect on susceptible cells. Future studies making use of the calcium-imaging dye Fura-2 could demonstrate that the $[Ca^{2+}]_i$ fluctuations of granule cells exposed to gp120 are quantitatively and qualitatively different than those of pyramidal cells. The hypothesis

predicts that granule cells will have a smaller $[Ca^{2+}]_i$ increase than pyramidal cells and/or a more rapid return to baseline levels. Pharmacological dissection of calcium changes in exposed cells (with a variety of channel blockers, for instance) may be employed to characterize the nature of calcium fluxes.

Neuronal death in HIV-1 infection is thought in many instances to be mediated through apoptosis (Adle-Biassette et al 1995, Gelbard et al 1995, Petito and Roberts 1995, An et al 1996, Bin et al 1996). A variety of morphologic and immunologic techniques can be employed to demonstrate apoptosis in tissue and culture, and these may be applied to both pyramidal and granule cell cultures to detect this process amongst gp120-exposed cells.

Ultimately, the processes that are examined in dissociated cells in cultures enriched for individual cell types should be re-examined under more physiologic conditions. Intracerebroventricular injection of gp120 has been studied in mice and rats where neuropathology due to the glycoprotein's neurotoxicity has been detected. The hippocampus could be specifically examined, with attention to the quantitation of different cell types of the hippocampal formation and the presence or absence of mossy fiber sprouting. Acute (i.e., living) hippocampal slices from such animals can also be examined electrophysiologically to correlate anatomical changes with the physiologic ones that are associated with epileptogenesis.

Should all these experimental conditions support the hypothesis that gp120 in the hippocampus leads to the development of the lesion of temporal lobe epilepsy, the logical culmination to this line of investigation is examination of human tissues. Do AIDS patients exhibiting seizures have hippocampal sclerosis? Do their hippocampi have

mossy fiber sprouting? The experiments described in this chapter represent the initial step in a series of investigations that could lead from the description of an effect of gp120 on an individual neuronal type in vitro to a comprehensive understanding of the neuroanatomical substrate underlying seizures in AIDS patients.

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APPENDIX:

Numbers of pyramidal cells per coverslip (total in 100 contiguous frames) in pyramidal cell cultures exposed to gp120.

	Control (vehicle)	$gp120_{IIIB}$ at 10 pM	$gp120_{SF2}$ at 10 pM	$gp120_{IIIB}$ at 200pM
	328			
	211			
	376			
	198	232	368	
	245	232	244	
	403	112	290	
	269	237	300	
	402	204	280	
	334	257	140	
	356	103	220	158
	239	144	180	105
	431	274	376	253
	476	123	160	234
	264	226	175	204
	396	230	80	132
Total	4928	2374	2813	1086
n	15	12	12	6
Mean	328.5	197.8	234.4	181.0
SD	86.3	60.2	91.7	58.7
% control	100	60.22	71.35	55.09

Numbers of granule cells per coverslip (total in 100 contiguous frames) in granule cell cultures exposed to gp120.

	Control (heat-inactivated gp120)	$gp120_{IIIB}$ at 10 pM	$gp120_{IIIB}$ at 200 pM
			181
	384	381	796
	773	633	149
	338	809	374
	258	605	227
	451	594	198
	510	231	279
	293	231	419
	429	382	221
	296	191	142
	605	273	283
	127	667	209
	204	1124	167
	607	716	171
	359	489	251
	203	590	527
	471	519	145
Total	6308	8435	4881
n	16	16	17
Mean	394.25	527.19	287.13
SD	172.05	246.78	172.08
% of control	100	133.7	72.8

	granule cells in control cultures	granule cells at 10 μM glutamate	granule cells at 50 μM glutamate	granule cells at 100 μM glutamate	pyramidal cells in control cultures	pyramidal cells at 10 μM glutamate	pyramidal cells at 50 μM glutamate	pyramidal cells at 100 μM glutamate
	754	282	155	192	65	56	22	78
	429	493	285	282	141	37	37	93
	367	598	166	254	160	31	14	42
	414	836		309	148	8		65
	232	515		205	81	14		57
	256	693		97	46	14		44
Total	2452	3417	606	1339	641	160	73	379
n	6	6	3	6	6	6	3	6
Mean	408.67	569.5	202	223.17	106.83	26.67	24.33	63.17
SD	187.59	188.98	72.09	76.16	48.59	18.2	11.68	19.83
% of Control	100	139.6	49.43	54.61	100	24.96	22.78	59.13

Numbers of pyramidal cells and granule cells per coverslips (total in 50 contiguous frames) in granule cell cultures exposed to glutamate. Neuron types were identified on the basis of morphology, and both granule cells and pyramidal cells were counted within each frame. Total value per coverslip (one replicate) is the sum of the cell type of interest in 50 frames.