Cellular Mechanisms underlying HIV-1-Associated Dementia

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ABSTRACT

Many HIV-1-positive individuals suffer from a variety of neurological problems known collectively as the HIV-1 associated dementia. However, as HIV-1 is not thought to replicate in neurones, the molecular mechanism involved in the demise of neurones is unclear. A number of laboratories have suggested that the HIV-1 coat protein gp120 damages neurones by inducing a large cytoplasmic calcium [Ca^{2+}]_c increase.

Thus, began my project to find out what is the exact mechanism by which gp120 causes a rise in neuronal [Ca^{2+}]_c and if there is a way to block the rise and rescue the neurones from dying. I cultured neonatal rat primary hippocampal and cortical cells with or without 5% horse serum (HS) for 18 days or more and imaged [Ca^{2+}]_c with fura-2. In many of these hippocampal cultures grown with HS, gp120 (250 pM) evoked a large rise in [Ca^{2+}]_c in the majority of neurones; in other cultures this [Ca^{2+}]_c signal was absent. Where present the neuronal [Ca^{2+}]_c signal was blocked by the peptide VSLYRCPRFF (50 nM), a competitive inhibitor of the CXCR4 chemokine receptor. Whereas, hippocampal cultures grown without HS, showed a slower rise in [Ca^{2+}]_c in neurones to a moderate level, on addition of gp120. Similarly to above a proportion of these cultures did not respond.

I tested those cultures that did not show any rise in [Ca^{2+}]_c for neurotoxicity to check if gp120 still causes neuronal death. Under these conditions gp120 (250 pM) caused 30% neurotoxicity that was reduced by the blocking peptide VSLYRCPRFF (50 nM) or pertussis toxin (500 ng/ml) preincubation, indicating that CXCR4 activation of G/Go family members plays an important role in neurotoxicity. These results show that gp120-induced neuronal [Ca^{2+}]_c signals are not an essential component of the killing process.

The biggest puzzle in my PhD project was why some cultures show a neuronal [Ca^{2+}]_c response to gp120 while others do not. I investigated the hypothesis that it is the number of microglia which is different in each culture. I stained for microglia in both cultures grown with or without HS. Only those cultures with high microglial density (irrespective of HS) gave a neuronal [Ca^{2+}]_c response to gp120. Thus, microglia play an oblige role in the [Ca^{2+}]_c response to gp120 although the increase in [Ca^{2+}]_c is not necessary for neurotoxicity.
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<td>AAT</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>ABP</td>
<td>AMPA receptor-binding protein</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate</td>
</tr>
<tr>
<td>AMPAR</td>
<td>AMPA receptor</td>
</tr>
<tr>
<td>AP5</td>
<td>2-amino-5-phosphono pentanoic acid</td>
</tr>
<tr>
<td>APAF 1</td>
<td>apoptosis activating factor 1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>β2M</td>
<td>beta 2-microglobulin</td>
</tr>
<tr>
<td>BMVECs</td>
<td>brain microvascular endothelial cells</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>calcium</td>
</tr>
<tr>
<td>[Ca^{2+}]_c</td>
<td>cytoplasmic free calcium concentration</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CD4</td>
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</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHX</td>
<td>cycloheximide</td>
</tr>
<tr>
<td>CIF</td>
<td>calcium influx factor</td>
</tr>
<tr>
<td>CICR</td>
<td>calcium induced calcium release</td>
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Cl\(^-\)  chloride
CNS  central nervous system
CNQX  6-cyano-7-nitroquinoxaline-2, 3 dione
COX 2  cyclo oxygenase 2
CREB  cAMP-response-element-binding-protein
CSF  cerebrospinal fluid
DAB  di amino benzidine
diacyl glycerol
DAG  diacyl glycerol
DHPs  1, 4-dihydropyridines
DiBAC  bis-barbituric acid oxonols
DiBAC\(_{4}(3)\)  DiBAC dye with excitation maxima at approximately 490 nm
DMEM  Dulbecco's minimum essential medium
deoxyribonucleic acid
DNA  deoxyribonucleic acid
EAAT  excitatory amino acid transporter
ER  endoplasmic reticulum
ERK  extracellular signal-regulated kinase
fetal calf serum
FCS  fetal calf serum
GABA  gamma-Aminobutyric Acid
GCs  glucocorticoids
GDH  glutamate dehydrogenase
guanylate kinase
GK  guanylate kinase
GLAST  glutamate/aspartate transporter
GLT  glial glutamate transporter
HIV-1 envelope glycoprotein gp120
gp120  HIV-1 envelope glycoprotein gp120
G protein coupled receptor
GPCR  G protein coupled receptor
GRIP  glutamate receptor interacting protein
GTP  guanosine triphosphate
HAD  HIV-1 associated dementia
HBS  HEPES buffered saline
HD   huntington's disease
HIV-1 human immunodeficiency virus type 1
HIVE HIV-1 encephalitis
H$_2$O$_2$ hydrogen peroxide
HS   horse serum
HVA  high voltage-activated
ICAM-1 intercellular adhesion molecule 1
IFN$\gamma$ Interferon gamma
ILs  Interleukins
IL-1 Interleukin-1
IL-6 Interleukin-6
IL-1$\beta$ Interleukin-1 beta
iNOS inducible nitric oxide synthase
IP$_3$ inositol 1, 4, 5 trisphosphate
JNK c-Jun NH$_2$-terminal protein kinase
K$^+$ potassium
L-NAME N-nitro-L-arginine methyl ester
LTR long terminal repeat
LVA low voltage-activated
MAP-2 microtubule associated protein-2
MAPK mitogen activated protein kinase
MCA middle cerebral artery
MCP-1 monocyte chemotactic peptide-1
MCPG [RS]-alpha-methyl-4-carboxyphenyl glycine
<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MDM</td>
<td>monocyte derived macrophage</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptors</td>
</tr>
<tr>
<td>MGCs</td>
<td>multi nucleated giant cells</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>magnesium</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>MIA</td>
<td>5-(N-methyl-N-isobutyl) amiloride</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>macrophage inflammatory protein-1 beta</td>
</tr>
<tr>
<td>Δψₘ</td>
<td>mitochondrial membrane potential</td>
</tr>
<tr>
<td>NAAG</td>
<td>N-acetyl aspartyl glutamate</td>
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<td>Na⁺</td>
<td>sodium</td>
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<tr>
<td>nAChR</td>
<td>nicotinic acetyl choline receptor</td>
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<td>nicotinamide adenine dinucleotide</td>
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<td>nuclear factor-kappa B</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>NOS</td>
<td>nitric oxide synthase</td>
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<td>neuronal nitric oxide synthase</td>
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<tr>
<td>O₂⁻</td>
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<td>poly ADP ribose polymerase</td>
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<td>inorganic phosphate</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidyl inositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>PSD</td>
<td>post synaptic density</td>
</tr>
<tr>
<td>PTP</td>
<td>permeability transition pore</td>
</tr>
<tr>
<td>Rh123</td>
<td>Rhodamine-123</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress activated protein kinase</td>
</tr>
<tr>
<td>SAP</td>
<td>synapse-associated protein</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>stromal derived factor-1 alpha</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SIV</td>
<td>simian immunodeficiency virus</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>soluble N-ethyl maleimide sensitive factor attachment protein (25 kD)</td>
</tr>
<tr>
<td>SOC</td>
<td>store-operated channel</td>
</tr>
<tr>
<td>SOD</td>
<td>super oxide dismutase</td>
</tr>
<tr>
<td>TCA</td>
<td>tri carboxylic acid cycle</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>T-tropic</td>
<td>T lymphocyte selective</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VOCC</td>
<td>Voltage-operated calcium channel</td>
</tr>
</tbody>
</table>
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Om Bhoor Bhuvassuvah
Om Tat Saviturvarenyam
Bhargo Devasya Dheemahi
Dhiyo Yo Nah Prachodayaat

May there be peace on mortal, immortal and divine planes. I meditate upon the most brilliant splendour of the Sun God. May he stimulate our intellect (so that we are inspired to take the right action at the right time)

(Rgveda –3, 62)
Chapter 1. Introduction

1.1. AIDS as a Disease

Retroviruses were first isolated in the 1910s as the causative agents of leukemia and sarcoma (Gallo, 1995). The first human retrovirus to be isolated was the human T-cell leukemia virus by Gallo et al. in 1981. In that same year, a few scattered case reports of anomalous and overwhelming opportunistic infections in gay men and drug users were reported in the U. S. This new disease entity became known as acquired immunodeficiency syndrome (AIDS). It was shown to be a transmissible, T-cell tropic, immunosuppressive disease of delayed onset. The AIDS causative agent was first isolated by Luc Montaigner et al. in 1983, and was found to be a member of the lentivirae family (*latin* lenti-slow, *virus*-poison) of retroviruses. It is now called the human immunodeficiency virus type 1 (HIV-1).

From initial infection HIV-1 causes immune system dysregulation and gradual erosion of CD4\(^+\)-T cells, which orchestrate cell-mediated immunity in the body (Ho et al., 1995; Wei et al., 1995). Subsequent compromisation of the immune system occurs. The period between infection and disease onset is known as the asymptomatic phase of disease. This virally mediated onslaught usually culminates in a status of immunodeficiency within 8-10 years after initial infection (Pantaleo and Fauci, 1995). This is characterised by the loss of cell-mediated immune response to HIV-1 and other pathogens, leaving the individual vulnerable to opportunistic infections. These patients also have an increased risk of developing cancer or dementia. This symptomatic phase is known as AIDS, and is characterised by less than 200 CD4\(^+\) T cells per \(\mu\)l in plasma and the appearance of opportunistic infections.
1.2. AIDS as a Disease Affecting the Brain

1.2.1. Introduction

Neurological complications in AIDS were first recognised in 1983 (Snider et al., 1983). They include central nervous system (CNS) opportunistic infections, subacute encephalitis, lymphoma, vascular complications and aseptic meningitis with accompanying brain lesions (Snider et al., 1983). Since then, numerous clinical and neuropathological studies have demonstrated that a third of the adults and half of the children with AIDS will inevitably develop neurological impairments directly attributable to HIV-1 infection of the CNS. A range of clinical and pathological disease manifestations of HIV-1 infection of the brain and spinal cord (termed collectively, the HIV-1-associated cognitive/motor complex or HIV-1-associated dementia (HAD)) is a prominent feature of AIDS (Janssen et al., 1989; Price et al., 1988). The disease is often subtle, and includes mental and physical slowing and diminished cognition. Complaints of overt memory loss and difficulties with reading or carrying out simple tasks at work and/or at home can follow. Motor symptoms may occur concomitantly and are manifested by clumsiness progressing to gait disturbances with profound weakness. Inevitably, florid dementia with incontinence, hallucinations, seizures, then coma ensues (Navia et al., 1986).

Surprisingly, mononuclear phagocytes (brain macrophages, microglia, and multinucleated giant cells) in the CNS represent the cell type that is predominantly infected (Koenig et al., 1986). Although infection can occur in astrocytes, it is highly restricted (Saito et al., 1994; Tornatore et al., 1994). Hence, the number of HIV-1-infected cells in the brain is relatively small, consisting predominantly of macrophages and microglia, and therefore the question of how altered function in relatively few cells can produce so much dysfunction of uninfected neurones remains to be
1.2.2. Possible Mechanisms of HIV-1 Entry into Brain

It is thought that HIV-1 enters the brain in association with infected macrophages soon after infection (Edinger et al., 1997). Theoretically, HIV-1 can enter the brain through several different mechanisms that are not mutually exclusive. First, HIV-1 might enter brain tissue by direct infection of brain microvascular endothelial cells (BMVECs) that form the blood brain barrier (BBB). Second, HIV-1 might gain entry into brain tissue via a ‘Trojan horse’ mechanism, inside infected monocytes (Haase, 1986). Third, HIV-1-infected monocytes may induce the expression of adhesion molecules on the BMVECs that allow binding and infiltration of HIV-1-infected monocytes into brain; these same cells might also damage and disrupt the BBB. Studies on the adhesive interactions between monocytes and BMVECs have revealed that HIV-1-infected monocytes selectively induce expression of E-selectin on BMVECs (Nottet et al., 1996). Furthermore, immune activation of the HIV-1-infected monocytes induced even higher levels of E-selectin and vascular cell adhesion molecule 1 (VCAM-1) on BMVECs. As E-selectins and/or VCAM-1 mediate transendothelial migration of monocytes (Carlos et al., 1991), these data support the contention that HIV-1-infected monocytes have a selective advantage for entry into brain tissue. Furthermore, the viral protein Tat induces E-selectins expression on BMVECs (Hofman et al., 1993) and may facilitate macrophage invasion through the BBB (Rappaport et al., 1999).
1.2.3. Neuronal Damage in HIV-1-Associated Dementia

Numerous studies have shown that at the cellular level in HAD there is neuronal death throughout regions of the cerebral cortex (Everall et al., 1994; Everall et al., 1991; Jansen et al., 1991; Wiley et al., 1991) but has not been correlated with severity of disease (Everall et al., 1994). Morphological alterations in the neuritic processes of neurones in both the frontal cortex and hippocampus (Masliah et al., 1992) and damage to dendritic projections in cortical pyramidal neurones have been reported (Everall et al., 1993). Wiley and coworkers have reported atrophy and neuronal loss within the neocortical regions of the HIV-1-infected brain (Wiley and Achim, 1994). Volume loss in the basal ganglia also correlates with the presence of dementia (Aylward et al., 1995; Dal Pan et al., 1992).

Apoptosis is a regular feature of normal animal development and is probably the fate of a substantial fraction of the cells produced in most animals. It is an active, gene-driven process that causes cell contents to be fragmented into vesicular packages that are released by blebbing through the intact cell membrane. This process continues until the cell is entirely packaged in vesicles. These vesicles are quickly digested by local phagocytes with minimal disruption to neighboring cells (Ameisen et al., 1995). In contrast with necrosis, which is a passive form of cell death initiated by noxious external stimuli, such as anoxia, a potent immune response is elicited. In HAD apoptosis seems to be the most prevalent form of cell death triggered by HIV-1 both systemically and within the brain (Adle Biassette et al., 1995). Apoptotic neuronal loss has been well documented in HIV-1 encephalitis (HIVE), which is characterized by the presence of multinucleated giant cells (MGCs or syncytia), composed of infected macrophages and microglia and was detected mainly in the neocortex, limbic system and basal ganglia (Gelbard et al., 1995). Rare apoptotic neurones have been seen in some late HIV-1 asymptomatic cases (An et al.,
1996b), but generally neuronal loss is not thought to occur until the symptomatic phase of HIV-1 infection (Everall et al., 1993; Everall et al., 1991; Gray et al., 1996). These findings are in accordance with an increased viral load seen in the brain at this stage of disease. Neuronal apoptosis, demonstrated in both HIV-1-infected adults and children, is associated with microglial activation and axonal damage, but not necessarily with the occurrence of HAD (Adle Biassette et al., 1999; Adle Biassette et al., 1995; Gelbard et al., 1995). However, a correlation between HIV-1-mediated immune system alterations and progressive peripheral and central sensory tract lesions has been observed (Husstedt et al., 1998). Apoptosis associated with HIV-1 CNS infection has also been demonstrated in vascular endothelial cells, suggesting that programmed cell death may play a role in compromising the BBB (Shi et al., 1996). Other studies have implicated neuronal apoptosis as a factor in both HAD and encephalopathy found in pediatric cases of HIV-1 CNS infection (Adle Biassette et al., 1995; Gelbard et al., 1995). In HIVE, different neuronal populations seem to be selectively vulnerable to damage by apoptotic processes (Everall et al., 1993). These include large pyramidal neurones and interneurones in the cortex (Everall et al., 1993; Everall et al., 1991; Masliah et al., 1992; Wiley et al., 1991), spiny neurones in the putamen (Masliah et al., 1995), and CA3 interneurones of the hippocampus (Wiley et al., 1991). Additional variable areas of atrophy and astrogliosis have also been described (Masliah, 1996; Masliah et al., 1996). In the neocortex and basal ganglia, simplification and reduction of synapto-dendritic connections has been described (Masliah et al., 1995; Masliah et al., 1996). These include a decreased staining for synaptophysin, which labels presynaptic terminals, and a 30-50% decrease in neurone-specific microtubule associated protein-2 (MAP-2) staining, which is predominantly distributed in the cell body and dendrites. Additionally, a loss of synaptophysin and MAP-2 immunoreactivity in neurones was found in gp120 transgenic mice (Toggas et al., 1996). Intracerebral injection of gp120, a major
component of the HIV-1 coat, has been associated with an impairment in memory and learning paradigms in rodents (Hill et al., 1993).

1.2.4. Correlates of HIV-1-Associated Dementia

Since the recognition of neurologic disease associated with HIV-1 CNS infection, numerous groups have attempted to identify correlates of HAD development such as viral load in the brain or immune system. To date these links have not been definitely established (Krebs et al., 2000). In the brain, detection of proviral DNA has been associated with neurologic deficits as well as HIV-1-associated histopathology (Boni et al., 1993). However, results from a similar study that used quantitative polymerase chain reaction (PCR) to detect proviral DNA indicated no correlation between CNS viral load and HAD (Johnson et al., 1996). HIV-1 p24 expression in the brain was also found to be linked to HAD severity (Brew et al., 1995). Studies of brain viral load and HAD may be complicated by a lag between development of HIVE within the CNS and manifestation of clinical dementia (Wiley and Achim, 1994). The severity of dementia has also been associated with the extent of CNS monocyte/macrophage activation (Glass et al., 1995) as well as expression of gp41 and inducible nitric oxide synthase (iNOS) (Adamson et al., 1999; Rostasy et al., 1999). iNOS expression is particularly interesting as a marker for HAD since iNOS synthesizes nitric oxide (NO), a compound implicated as a potential neurotoxin involved in the development of HAD.

Unintegrated DNA, a subset of the total viral DNA load within the brain, may also be associated with HAD. Following conversion of the viral RNA genome to double-stranded DNA through the enzymatic activity of reverse transcriptase, HIV-1 DNA is integrated as a linear molecule into the host genome by the HIV-1-encoded integrase. Circular DNA structures containing either one
or two long terminal repeats (LTRs) are thought not to serve as preintegration intermediates and are considered as a nonproductive offshoot of the replication cycle. Because these circular molecules are not integrated, they accumulate within the infected cell. In vitro, unintegrated HIV-1 DNA accumulates in HIV-1-infected monocyte/macrophage cells to levels lower than those found in productively infected T cells (Sonza et al., 1994). In macrophages and microglial cells, lower levels of unintegrated DNA may be indicative of resistance to superinfection or a more chronic level of replication. One study of HIV-1-infected patients demonstrated that levels of unintegrated linear and circular viral DNA are elevated in postmortem brains of patients with HIVE (Pang et al., 1990). A similar study found that the presence of unintegrated single LTR proviral structures and increased HIV-1 replication were associated with the appearance of MGCs and dementia (Teo et al., 1997). These results parallel a link between the presence of unintegrated viral DNA in the blood and the severity of immunologic disease (Nicholson et al., 1996). Within infected macrophages and microglia in the brain, unintegrated DNA may serve as transcription templates for production of viral RNA and, subsequently, proteins such as gp120 and Tat. These proteins may function as neurotoxins when released into the CNS (Stevenson et al., 1990). In addition, a low level of viral replication from unintegrated templates in macrophages may contribute to the viral load within the brain, as has been demonstrated in vitro in HeLa cells (Cara et al., 1996).

Other groups have identified associations between HAD and markers in the cerebrospinal fluid (CSF) or peripheral circulation. Correlations have been demonstrated between HIV-1 nucleic acids or protein products (RNA or p24) in the CSF and the appearance of clinical disease (Brew et al., 1997; McArthur et al., 1997; Royal et al., 1994). In pediatric CNS disease, viral RNA in the CSF correlates with the cognitive dysfunction (Sei et al., 1996). In HIV-1-infected patients,
levels of HIV-1 RNA in the CSF correlate with viral burden in the brain, indicating that CSF viral RNA may be a surrogate marker for studies of viral load in HAD (Wiley et al., 1998). Also, CSF HIV-1 RNA concentrations correlate well with HAD severity but may also be increased by other CNS infections, such as cryptococcal meningitis (Brew et al., 1997). Peripheral blood p24 levels have also been linked to the appearance of CNS dysfunction (Royal et al., 1994).

1.2.5. HIV-1 Proteins and the Brain

HIV-1 has a number of structural and regulatory proteins that are differentially expressed during its life cycle (Kim and Panganiban, 1996; Ranki et al., 1994). Some of these are putative toxic agents, although in the brain to date the focus has been on three of these proteins. These are the structural HIV-1 envelope glycoprotein (gpl20) and the regulatory proteins Tat and Nef.

1.2.5.1. Gp120

This structural envelope protein mediates binding of the virus to the CD4 and the chemokine receptor complex of the target cell (Deng et al., 1996; Schmidtmayerova et al., 1996). In the brain, this protein is thought to be released extracellularly from infected macrophages, microglia and MGCs depending on an individual's disease stage and progression rate (Lipton and Gendelman, 1995). The mechanism by which gp120 causes neuronal death is discussed in detail later on, so I shall not discuss it here.

1.2.5.2. Tat

Tat is an HIV-1-regulatory protein essential for viral transcription and productive infection of
target cells. Once inside other cells, Tat can be transported to the nucleus where it is capable of transactivating both HIV-1 LTR and cellular promoters (Frankel and Pabo, 1988; Kolson et al., 1994; Rappaport et al., 1999). This is associated with an accumulation of viral mRNAs within the cell. Tat has been shown to transactivate promoters for cytokines such as TGF-β (Cupp et al., 1993; Zauli et al., 1992), TNF-α and IL-2 (Westendorp et al., 1994). Tat seems to have two main roles in the brain, one in promoting HIV-1-infected monocyte entry into the brain and another in neuronal damage within the brain.

Tat is thought to increase BBB permeability directly (Albini et al., 1996; Magnuson et al., 1995) and is speculated to promote the recruitment of HIV-1-infected monocytes into the brain and their migration within the CNS (Cupp et al., 1993; Lafrenie et al., 1996). The evidence for this includes: (1) its ability to upregulate TGF-β1, which is an immunomodulating and potent chemotactic cytokine (Rasty et al., 1996), (2) increasing the expression of β2 integrins in monocytes allowing them to adhere to endothelial cell monolayers much more readily, and (3) the upregulation of protease matrix metalloproteinase-9 which has been associated with the disruption of endothelial barriers (Lafrenie et al., 1996). (4) It is also indicated that Tat induces IL-6 mRNA expression in human brain endothelial cells, which may play a role in altering the BBB (Zidovetzki et al., 1998). (5) Recent studies by Arese et al. (2001) have shown that the HIV-1 Tat protein released by infected cells is a chemotactic molecule for leukocytes and induces a proinflammatory program in endothelial cells by activating vascular endothelial growth factor (VEGF) receptors expressed on both cell types. Lymphomononuclear infiltration elicited by Tat was inhibited by a neutralizing Ab anti-VEGF receptor type 2 and reduced by an anti-monocyte chemotactic peptide-1 (MCP-1) Ab and also by WEB2170, a platelet activating factor (PAF) receptor antagonist. These results highlighted the role of PAF and MCP-1 as
secondary mediators in the onset of lymphomononuclear cell recruitment in tissues triggered by Tat. The consequent BBB disruption is most likely to be detrimental and increase its permeability to HIV-1-infected monocytes.

In monocytes, subnanomolar concentrations of Tat enhance the expression of chemokine receptor CCR5 (Weiss et al., 1999). Tat also recruits monocytes into tissues when injected in the lateral ventricle of the brain (Philippon et al., 1994b). Furthermore, macrophage stimulation by Tat results in the immunosuppressive induction of Fas ligand (Cohen et al., 1999) and impairment of the natural host response by inhibition of NO synthesis (Barton et al., 1996).

In cultured neuronal cells Tat is neurotoxic, causing a rapid Ca\(^{2+}\) influx and subsequent depolarisation through non-NMDA glutamate ionotropic receptors (Magnuson et al., 1995; Nath et al., 1996). Injection of Tat into rats has resulted in cytokine dysregulation, inflammation, astrocytosis and neuronal loss in the gray matter (Philippon et al., 1994a). These pathological findings are suggestive of those seen in HIVE cases. Also, the combination of Tat and TNF-α was shown to induce neuronal apoptosis to a greater extent than either treatment alone (Shi et al., 1998). The given effects of Tat would certainly contribute to the recruitment of infected monocytes into the brain, increased cytokine production and viral load seen in the late stage of asymptomatic HIV-1 brain infection (An et al., 1996a; Gray et al., 1996) and to be neurotoxic \textit{in vitro} (Magnuson et al., 1995; Nath et al., 1996). A recent finding has shown that the expression of endogenous cAMP response element binding (CREB) protein was significantly reduced in PC12 cells by prolonged (24-48 h) treatment with Tat. This decline in the expression of CREB, which plays an essential role in the survival and function of neuronal cells, anticipated a progressive increase of apoptosis in Tat-treated cells. Although obtained in a neuronal cell line,
1.2.5.3. Nef

The HIV-1 protein Nef is an essential modulator of AIDS pathogenesis, endowed with still elusive biochemical and biological properties (Cullen, 1998). Indeed, some long-term nonprogressing AIDS patients are infected with a nef-deleted HIV-1 virus (Deacon et al., 1995), and an intact nef gene is necessary for high titer viral replication in animal models (Kestler et al., 1991). Transgenic nef expression in mouse CD4$^+$ T cells causes the development of an immune syndrome closely resembling human AIDS (Hanna et al., 1998). Furthermore, in T lymphocytes Nef determines the internalization of the CD4 receptor and MHC class I molecules (Garcia and Miller, 1991; Piguet et al., 1999; Schwartz et al., 1996). Nef interacts with tyrosine kinases of the Src family (Lee et al., 1996; Saksela et al., 1995), with serine/threonine kinases (Nunn and Marsh, 1996; Sawai et al., 1994), and with the nucleotide exchange factor Vav (Fackler et al., 1999), thus altering their function (Collette et al., 1996; Greenway et al., 1996; Moarefi et al., 1997). By interfering with the signal transduction machinery, Nef can activate T cells in a variety of experimental models (Du et al., 1995; Hanna et al., 1998; Schrager and Marsh, 1999; Skowronski et al., 1993; Swingler et al., 1999; Wang et al., 2000). Activated T cells become highly susceptible to apoptosis in a process called activation-induced cell death, physiologically relevant for a correct balance of the immune response (Lenardo et al., 1999). Accordingly, prolonged expression of an activating CD8-Nef chimera in Jurkat T cells leads to their apoptotic death (Baur et al., 1994). Interestingly, macaques infected with a nef-deleted SIV do not develop AIDS-like symptoms, due to a dramatic reduction in the apoptotic death of CTL and CD4$^+$ cells (Xu et al., 1997).
1.2.6. Cytokine Dysregulation in the Brain by HIV-1

Cytokines are small immunomodulatory proteins usually released under conditions of tissue stress, like growth, tissue dysregulation, infection and trauma. They comprise a large family of proteins, including interleukins, chemokines TGF-β, TNF-α and neurotrophic factors. They act either at a distance or near the site of release and have multiple and overlapping functions within the body (Hopkins and Rothwell, 1995; Rothwell and Hopkins, 1995). In the brain, they have been associated with the control of behaviour, the release of hormones to the rest of the body and the regulation of the immune response (Hopkins and Rothwell, 1995). The available data on cytokine action indicate the complexity of their interaction, with many opposing roles depending on their concentration, site and duration of action and the presence of other cytokines (Merrill and Benveniste, 1996; Rothwell and Hopkins, 1995).

Increased levels of cytokine have been demonstrated late in asymptomatic HIV-1 infection prior to putative neuropathological changes (An et al., 1996a; An et al., 1996b; Gray et al., 1996). They have also been detected in the CSF and in particular postmortem brain areas of patients who died of AIDS (Merrill and Martinez-Maza, 1996). In AIDS, it has been suggested that there is a diffusion of cytokines from productively infected areas such as the underlying basal ganglia to the cortex (Merrill and Martinez-Maza, 1996; Tyor et al., 1995; Wesselingh et al., 1993). An excessive production of cytokines is induced by HIV-1 viral proteins, for example gp120 induces TNF-α and IL-6 (Yeung et al., 1995), and Tat induces TGF-β (Cupp et al., 1993).

1.2.6.1. Tumour Necrosis Factor-alpha

Several studies have shown that both tumour necrosis factor-alpha (TNF-α) production and rapid
progression to HAD are linked to the inducible expression of the major histocompatibility complex (MHC) locus (An and Scaravilli, 1997). An individual’s MHC locus determines both the size and rate of their immune response to foreign antigens within the body. In HAD, increased TNF-α mRNA has been shown to be present at significant levels in microglia and macrophages in subcortical white matter (Wesselingh et al., 1993). Moreover, TNF-α has been shown to induce astrogliosis, alter neuronal function by modulating Ca^{2+} currents (Wilt et al., 1995), decrease glutamate uptake in astrocytes (Fine et al., 1996), be neurotoxic through AMPA glutamate receptors (Gelbard et al., 1993), cause demyelination and oligodendrocyte demise (Wesselingh et al., 1993), stimulate the production of other cytokines, activate HIV-1 expression through the nuclear factor (NF)-kappa B transcription factor, and increase the levels of other potential neurotoxins (e.g. leukotrienes, PAF, NO) (Merrill and Martinez-Maza, 1996). TNF-α has been associated with both cell proliferation and apoptosis in different studies. The actual effect of exposure to TNF-α is thought to depend on a multitude of factors, including a cell’s activation state and the presence or absence of numerous stimuli such as other cytokines, growth factors and free radicals (Merrill and Martinez-Maza, 1996). To summarise, TNF-α in the brain during HIV-1 infection seems to increase viral load, be neurotoxic and to cause demyelination.

1.2.6.2. Interleukins

Interleukins (ILs) comprise a large family of proinflammatory cytokines. In HIV-1 infection in the brain the most studied of these include IL-1, IL-6 and IL-1β. IL-1 is pyrogenic and is constitutively expressed at low levels, mainly within the hypothalamus. It stimulates its own production and that of other cytokines (e.g. IL-6, TNF-α, colony-stimulating factors, TGF-β) and HIV-1 replication. It is produced by astrocytes, microglia, infiltrating macrophages and
oligodendrocytes within the brain (Benveniste, 1994). Its presence in the brain during HIV-1 infection is thought to portend a general response to brain infection rather than a specific HIV-1-associated event (Tyor et al., 1992; Vitkovic et al., 1994). Its main role is that of immune activation, possible coincident HIV-1 replication through NF-kappa B activation. It has been found to be expressed in 30-60% of HAD cases (Vitkovic et al., 1994; Wesselingh et al., 1993).

IL-6 is an inducible cytokine with multiple roles, including B cell activation. It is induced by a viral gp120 (Yeung et al., 1995) and other cytokines predominantly IL-1 and TNF-α (Merrill and Martinez-Maza, 1996). IL-6 induces astrogliosis and NGF production in astrocytes which in turn favours neuronal differentiation (Benveniste, 1994). It is also thought to modulate TNF-α levels by acting as a negative regulator of this cytokine. In the brain, IL-6 is thought to be mainly produced by astrocytes and microglia, and has been linked to HIV-1 induction in monocytes, as well as increased T_2 cytokine production from B cells (Benveniste, 1994). These T_2 humoural cytokines favour an increased HIV-1 replication and are associated with high viral load in AIDS (Pantaleo and Fauci, 1995). IL-6 immunoreactivity has been reported in HIV-1-positive and AIDS cases, but there is some discrepancy as to its actual expression levels in HIV-1-infection (Merrill and Martinez-Maza, 1996; Vitkovic et al., 1994).

IL-1β is released by _in vivo_ activation of glia by gp120 (Zielasek and Hartung, 1996). In addition, HIV-1 and gp120 have been shown to activate rat and human microglia and astrocytes _in vitro_ to release IL-1β (Koka et al., 1995; Lipton et al., 1994; Merrill et al., 1992). _In vitro_ gp120 even activates transcription of IL-1β (Benveniste and Benos, 1995). These findings are of note because an adverse role of cytokine activity on learning and memory has recently emerged in the literature. For example, Oitzl et al. (1993) found that performance on the hippocampally-
dependent spatial version of the Morris water escape task was poor after intracerebroventricular injection of IL-1β. Consistent with these findings, Pugh et al. (1999) demonstrated an impairment in hippocampally dependent contextual fear conditioning following central IL-1β administration. In addition, hippocampally dependent learning impairments produced by social isolation stress were blocked by the central administration of interleukin-1β receptor antagonist (Pugh et al., 1999).

The suggestion that increased IL-1β activity may be involved in producing some of the learning impairments associated with HAD is supported by evidence for widespread immune activation in the brains of AIDS patients. Specifically, levels of the proinflammatory cytokines IL-1β and TNF-α are elevated in the CSF of AIDS patients and at postmortem brain examination (Gallo et al., 1989; Tyor et al., 1992).

To summarise, interleukins seem to be involved in the ‘immune activation’ in the brain during the HIV-1 infection and in HAD.

### 1.2.6.3. Transforming Growth Factor-beta

Transforming growth factor-beta (TGF-β) is a potent immunosuppressive cytokine with chemoattractant properties. Its main roles are inhibition of T cell proliferation, MHC expression, proinflammatory cytokines and HIV-1 production (Benveniste, 1994). TGF-β can be upregulated by various cytokines and HIV-1 proteins like gp120 (da Cunha et al., 1995) and Tat (Cupp et al., 1993; Rasty et al., 1996). In HIV-1 infection, as in other brain diseases, TGF-β production and immunoreactivity are tightly linked to levels of IL-1 expression (Vitkovic et al., 1994). TGF-β
and IL-1 levels are particularly high in areas of astrogliosis in AIDS (Vitkovic et al., 1994). TGF-β can modulate the activity of astrocytes and microglia by inhibiting their proliferation and migration to sites of injury (Benveniste, 1994). Thus, TGF-β serves as a powerful modulator of immune activation and suppressor of HIV-1 replication within the brain during HIV-1 infection (Merrill and Martinez-Maza, 1996). Nevertheless, in AIDS when $T_H1 CD4^+$ T cells have been depleted the chemoattractant properties of TGF-β may unwittingly contribute to increased recruitment of HIV-1-infected monocytes into the brain.

1.2.7. Excitatory Amino Acids as a Final Common Pathway for Neurologic Disorders

In many neurologic disorders, injury to neurones may be caused at least in part by overstimulation of receptors for excitatory amino acids, including glutamate and aspartate. The evidence for a contribution of glutamate-receptor stimulation to pathophysiology stems from the fact that in model systems, or occasionally in humans, either a glutamate-like neurotoxin has been identified or glutamate antagonists can ameliorate the disease process to some degree (Lipton and Rosenberg, 1994). These neurologic conditions range from acute insults such as stroke, hypoglycemia, trauma and epilepsy to chronic neurodegenerative states such as Huntington’s disease, the HIV-1-associated dementia, amyotrophic lateral sclerosis and Alzheimer’s disease (Choi, 1988; Lipton, 1994b; Meldrum and Garthwaite, 1990). The evidence for a role of glutamate in neurodegenerative diseases is discussed in section 1.4.5.

Glutamate is the principal excitatory neurotransmitter in the brain, and its interactions with specific membrane receptors are responsible for many neurologic functions, including cognition,
memory, movement and sensation (Gasic and Hollmann, 1992). In addition, excitatory
neurotransmitters are important in influencing the developmental plasticity of synaptic
collections in the nervous system (Lipton and Kater, 1989). However, in a number of pathologic
conditions, including stroke and various neurodegenerative disorders, excessive activation of
 glutamate receptors may mediate neuronal injury or death. Olney coined the termed
'excitotoxicity' for this condition, which may constitute the final common pathway for neuronal
injury due to diseases with diverse pathophysiologic processes (Olney, 1978). This form of
injury appears to be predominantly mediated by excessive influx of Ca^{2+} into neurones through
ionic channels, triggered by activation of glutamate receptors (Choi, 1985). For this reason it is
important to understand the various types of glutamate receptors and the ion channels that they
are capable of activating either directly or indirectly.

Since Ca^{2+} is the final effector in excitotoxicity, I will first review Ca^{2+} homeostasis, then review
excitotoxicity.

1.3. Calcium Homeostasis and Signalling in Neurones

1.3.1. Intracellular Calcium Homeostasis

The 10^3 to 10^4 fold concentration gradient of Ca^{2+} which exists across the plasma membrane of
neurones is maintained by a low passive permeability of the plasma membrane to Ca^{2+} ions, and
active extrusion of Ca^{2+} against a large electrochemical gradient (Fig. 1.1). In addition, all cells
contain various Ca^{2+} stores in intracellular organelles which have very different roles and
dynamics. Ca^{2+} ions participate in a number of tightly regulated signalling pathways within
neurones (Kennedy, 1989). The very high concentration (mM) of free Ca^{2+} in the extracellular
pool as compared to that in the cytosol (sub μM) and the resulting very large Ca\(^{2+}\) electrochemical potential, is particularly suited to its intracellular role as a second messenger, since even minor changes in the Ca\(^{2+}\) permeability of the plasma membrane will produce very significant fluctuations in the free cytoplasmic calcium concentration ([Ca\(^{2+}\)]\(_c\)). Therefore, it is to be expected that a prolonged elevation in [Ca\(^{2+}\)]\(_c\) will activate processes that require high Ca\(^{2+}\).

**Cytosolic Calcium chelation**

At rest, nearly all intracellular Ca\(^{2+}\) is bound to cytosolic Ca\(^{2+}\)-binding proteins and cellular membranes (negatively charged phospholipids), or sequestered within membranous organelles. Consequently, although the total Ca\(^{2+}\) concentration inside neurones can approach or surpass the millimolar level (Carafoli, 1987), the [Ca\(^{2+}\)]\(_c\) is maintained between 20-300 nM (Herman et al., 1990), i.e. at rest no more than 0.1% of the total cell Ca\(^{2+}\) is present in the cytosol as the free ion. Upon Ca\(^{2+}\) entry, diffusion of the Ca\(^{2+}\) in cytosol is very slow, in fact much slower than that of any other second messenger, due to the presence of the large number of Ca\(^{2+}\)-binding sites (Allbritton et al., 1992), which are mostly unoccupied at the sub-micromolar concentrations (Herman et al., 1990). It has been demonstrated that several types of neurones contain high concentrations of one or more of the ‘EF’-hand Ca\(^{2+}\) binding proteins calmodulin, calbindin D\(_{28K}\), parvalbumin and calretinin (Miller, 1995). Of these high affinity Ca\(^{2+}\) binding proteins, Calmodulin, which is highly concentrated in neurones (30-50 μM), can account for a considerable fraction (up to 120-200 μM) of the Ca\(^{2+}\) chelation capacity (Carafoli, 1987; Meldolesi et al., 1988), and serves other messenger functions, while the other Ca\(^{2+}\)-binding proteins appear to primarily act as cytosolic Ca\(^{2+}\) chelators (Miller, 1995). The concentration of these Ca\(^{2+}\) chelators may vary considerably in different neurones. For instance, parvalbumin is present in relatively high concentration in GABAergic neurones, where it may play an important
role in preventing Ca^{2+}-dependent K^+ conductances from reducing excitability (Blaustein, 1988). [Ca^{2+}]_c buffering, although rapid, may be very limited in capacity. Therefore, intracellular organelles are required to sequester Ca^{2+} until the Ca^{2+} load can be extruded across the plasma membrane.

**Plasma membrane Ca^{2+} extrusion mechanisms**

Neurones (Gill et al., 1981), like many other types of cells, have two parallel, independent mechanisms in their plasma membranes for extruding Ca^{2+}: a Ca^{2+}-ATPase, and a Na^+/Ca^{2+} exchange transport system (Fig. 1.1).

The Ca^{2+}-ATPase is a calmodulin modulated, Ca^{2+}-dependent ATPase that removes Ca^{2+} from the cell with high affinity (K_m for Ca^{2+} 0.2-0.3 μM), but with low total capacity (typically 0.5 nmoles of Ca^{2+} transported/mg membrane proteins/sec, (Carafoli, 1987). In neurones, an important property of the Ca^{2+}-ATPase is its stimulation by calmodulin, which increases the V_max of the enzyme and decreases the K_m for Ca^{2+}. This calmodulin dependent stimulation does not involve a phosphorylation event, and is due to a direct interaction of calmodulin with the Ca^{2+} pump.

The Na^+/Ca^{2+} exchanger is particularly active in excitable plasma membranes. Some laboratories have argued that, when nerve terminals are given a small Ca^{2+} load, Ca^{2+} exit is almost entirely dependent upon extracellular Na^+ (Blaustein, 1988). In many cell types, including neurones, the Na^+/Ca^{2+} exchanger removes Ca^{2+} with low affinity (apparent K_m for Ca^{2+} between 0.5-1 μM), but with a high capacity (Blaustein, 1988), leading to the idea that the exchanger is mainly active during recovery after Ca^{2+} transients. The Na^+/Ca^{2+} exchanger removes Ca^{2+} from the cytosol by
exploiting the $\text{Na}^+$ electrochemical gradient generated by the ATP driven $\text{Na}^+, \text{K}^+$-ATPase. Therefore, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is an important point of intersection between $\text{Na}^+$ and $\text{Ca}^{2+}$ homeostasis, explaining how any alteration of the $\text{Na}^+, \text{K}^+$-ATPase has immediate consequences on $[\text{Ca}^{2+}]_c$ homeostasis. The stoichiometry of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is $3\text{Na}^+/1\text{Ca}^{2+}$ exchanged (Carafoli, 1987). Due to its low affinity for $\text{Ca}^{2+}$, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is proposed only to modulate large movements of $\text{Ca}^{2+}$, and not control the fine tuning of $[\text{Ca}^{2+}]_c$.

**Stores of $\text{Ca}^{2+}$ in the ER**

The endoplasmic reticulum (ER) is widely distributed within neurones, being present in dendrites and dendritic spines, axons and presynaptic nerve terminals, and in growth cones (Cheng and Reese, 1985; Dailey and Bridgman, 1989; Deitch and Banker, 1993; Kanaseki et al., 1998; Levesque et al., 1999). It is highly motile, rapidly extending into and retracting from distal regions of growth cones (Dailey and Bridgman, 1989), and congregating in stack-like structures within dendrites in response to stimulation of metabotropic glutamate receptors (Banno and Kohno, 1998). Microtubules and actin filaments appear to have key roles in controlling ER motility, as well as in its structure and function (Tabb et al., 1998; Waterman Storer and Salmon, 1998). The ER is contiguous with the outer nuclear membrane and is often associated intimately with plasma membrane and mitochondria, which suggest functional coupling between these structures (Rizzuto et al., 1998).

The ER is reported to sequester $\text{Ca}^{2+}$ in neurones (capacity = 2.0-3.0 nmol/mg protein (Blaustein, 1988), and is capable of responding to stimuli by rapidly exchanging $\text{Ca}^{2+}$ with the cytosol (Meldolesi et al., 1988). Thus, the ER probably plays an important role in sequestering $\text{Ca}^{2+}$ following neuronal activity (Berridge, 1993; Berridge, 1997). $\text{Ca}^{2+}$ accumulation in the ER is coupled to hydrolysis of ATP by a $\text{Ca}^{2+}$-ATPase that is active at high $\text{Mg}^{2+}$ concentrations. $\text{Ca}^{2+}$
ions do not remain free in the lumen but are bound to specific proteins, this prevents an insoluble Ca\(^{2+}\) phosphate precipitation, and allows Ca\(^{2+}\) to be quickly released upon activation. The two principal channels responsible for releasing Ca\(^{2+}\) from the internal stores are the IP\(_3\) and ryanodine receptors (Berridge, 1993). The IP\(_3\) receptor channel requires both IP\(_3\) and Ca\(^{2+}\) for its opening and in addition, the function of the IP\(_3\) receptor channel appears to be regulated by various mechanisms.

During exposure to glutamate, production of IP\(_3\) following metabotropic receptor activation would be expected to activate Ca\(^{2+}\) release from the ER (Irving et al., 1992). Eventually the fall in the cellular ATP concentration would inhibit the ATPase activity of the ER, and Ca\(^{2+}\) release from the ER could contribute to the excitotoxic increase of [Ca\(^{2+}\)]\(_c\) (Mody and MacDonald, 1995). Accordingly, coactivation of IP\(_3\)-linked muscarinic ACh receptors in hippocampal neurones exacerbates glutamate toxicity (Mattson, 1989), whereas treatment of neurones with agents that suppress ER-mediated Ca\(^{2+}\) release protects against excitotoxicity (Frandsen and Schousboe, 1991). Excitotoxic insults can also result in neuronal apoptosis. For example, induction of epileptiform discharges in entorhinal-hippocampal slices by repeated tetanic stimulation results in apoptosis of CA1 and CA3 neurones (Pelletier et al., 1999); agents that block Ca\(^{2+}\) release from ryanodine-sensitive stores can prevent such excitotoxic apoptosis. In addition, studies of rodent models of stroke have provided evidence for the involvement of ER Ca\(^{2+}\) release in excitotoxic neuronal apoptosis that occurs following cerebral ischemia (Wei et al., 1998).
1.3.2. Sources of Calcium in Cells

The Ca\(^{2+}\) that increases the cytosolic concentration can come from a number of sources. Ca\(^{2+}\) either originates extracellularly and enters via influx pathways or it originates intracellularly and is released into the cytosol. Both Ca\(^{2+}\) influx and release can have several possible mediators, the importance of which differs between cell types. Ca\(^{2+}\) influx can occur via voltage-operated calcium channels, receptor-operated calcium channels, the reverse mode of the Na\(^{+}\)/Ca\(^{2+}\) exchanger and the capacitative influx pathway.

1.3.2.2. Voltage-Operated Calcium Channels

Voltage-operated calcium channels (VOCCs) reside in the plasma membrane and contain a voltage sensor that opens up a pore inside the protein complex that is more or less selectively permeable to Ca\(^{2+}\). Several subtypes of these channels are known and they can be classified pharmacologically and by their activation voltage; they consist of L-type, N-type, R-type, T-type and P/Q-type VOCCs (Birnbaumer et al., 1994; Smith et al., 1999).

Different subtypes of VOCCs have different subcellular distributions and perform different tasks in cells. For example, the N- and P-type channels are found on nerve terminals and are specifically linked to exocytosis of synaptic vesicles (Takahashi and Momiyama, 1993). P-type Ca\(^{2+}\) channels are so called because of their original identification in Purkinje cells of the cerebellum (Llinas et al., 1992). L-type channels are found on the axons of dorsal root ganglion neurones, whereas N-type VOCCs are found on the cell body and provide a strong Ca\(^{2+}\) signal that can regulate gene expression and release large dense core vesicles (Bading et al., 1993; George et al., 1999). L-type channels have also been reported to be concentrated at the base of
apical dendrites of hippocampal pyramidal neurones and at the growth cones of mouse neuroblastoma cells (Sculptoreanu et al., 1995).

Mammalian VOCCs are composed of a pore-forming $\alpha_1$ subunit which has four domains each containing six putative transmembrane segments. From purification studies, these are associated with an intracellular $\beta$ subunit. They also co-purify with an $\alpha_2$ subunit, which is entirely extracellular, linked into the membrane by S-S bonding to a transmembrane $\delta$ subunit (Liu et al., 1996; Witcher et al., 1993). Although the accessory subunits play important roles in channel modulation (Lacerda et al., 1991; Mikami et al., 1989; Varadi et al., 1991), the $\alpha_1$ subunit is primarily responsible for determining the pharmacology and physiology of the resulting current, and heterologous expression of cloned vertebrate $\alpha_1$ subunits has allowed correlation between molecular subtypes and native currents. L-type currents, sensitive to 1,4-dihydropyridines (DHPs), are gated by the $\alpha_{1S}$ subtype found in skeletal muscle (Tanabe et al., 1987) and the $\alpha_{1C}$ (Mikami et al., 1989) and $\alpha_{1D}$ (Williams et al., 1992c) subtypes expressed in heart, brain, and other tissues. DHP-insensitive, non-L-type $\alpha_1$ subunits include the $\alpha_{1B}$ subtype, which is responsible for the $\omega$-conotoxin GVIA-sensitive N-type current (Williams et al., 1992b), the $\alpha_{1A}$ subtype, which gates the $\omega$-agatoxin IVA-sensitive P/Q-type current (Mori et al., 1991), and $\alpha_{1E}$, which gates R-type currents (Soong et al., 1993). In addition to these high voltage-activated (HVA) $\alpha_1$ subunits, the $\alpha_{1G}$ subunit, responsible for the low voltage-activated (LVA) T-type current, has recently been cloned and characterized (Perez Reyes et al., 1998).
<table>
<thead>
<tr>
<th>Calcium channel type</th>
<th>Inhibitors</th>
<th>Activation voltage (mV)</th>
<th>Inactivation voltage (mV)</th>
<th>Conductance (pS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-type</td>
<td>Nifedipine, nimodipine, verapamil, Cd²⁺</td>
<td>Above −10</td>
<td>−50 to −10</td>
<td>25</td>
</tr>
<tr>
<td>T-type</td>
<td>Flunarizine</td>
<td>&gt;-70</td>
<td>−100 to −60</td>
<td>8</td>
</tr>
<tr>
<td>N-type</td>
<td>Omega-conotoxin GIVA, Cd²⁺</td>
<td>&gt;-20</td>
<td>−120 to −30</td>
<td>13</td>
</tr>
<tr>
<td>P/Q-type</td>
<td>Omega-agatoxin</td>
<td></td>
<td></td>
<td>9, 14, 19</td>
</tr>
<tr>
<td>R-type</td>
<td>Not sensitive to any of the above</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1. Properties of voltage-operated calcium channels.

1.3.2.2. Receptor-Operated Calcium Channels

Receptor-operated calcium channels (ROCCs) are Ca²⁺ channels that are activated by the binding of a ligand, usually a neurotransmitter for neurones. Binding of the ligand induces opening of a channel that usually is permeable to Na⁺, K⁺ or Cl⁻, but for a number of channels also has considerable Ca²⁺ permeability. Channels of this type that are Ca²⁺ permeable have very different functions and are listed below.

*N-methyl-D-Aspartate (NMDA)*

NMDA receptors are activated physiologically by binding of glutamate to the receptor and the
simultaneous depolarization of the plasma membrane. This depolarization is needed to relieve the block of the channel pore by Mg$^{2+}$ (Sharma and Stevens, 1996). Opening of the NMDA receptors can be greatly facilitated by the binding of the co-agonist glycine (Mayer et al., 1989). The voltage-dependent blockade of NMDA receptors is of great theoretical interest in that it endows the receptor with a state-dependent activation mechanism. Such a mechanism has been postulated to underlie cellular mechanisms of learning (Hebb, 1949). NMDA receptors have been implicated in many plasticity phenomena within the CNS in vivo, such as long-term potentiation in the hippocampus, long-term depression in the cerebellum, and stimulus-dependent plasticity in the visual cortex (Collingridge and Singer, 1990; Ghosh and Greenberg, 1995).

**Nicotinic acetylcholine receptors**

Nicotinic acetylcholine receptors (nAChRs) are found on synapses at neuromuscular junctions, in the peripheral and central nervous system. Responding to the endogenous neurotransmitter acetylcholine (ACh), nAChRs contribute to a wide range of brain activities that include cognitive functions (Levin, 1992), and neuronal development and degeneration (Zoli et al., 1999). Moreover, the presence of ACh-releasing circuits in embryonic neural tissue suggests a role for ACh and nAChRs during development (Zoli et al., 1995). In addition, nicotine improves vigilance and rapid information processing in humans (Levin, 1992).

**ATP receptors**

ATP receptors are found in the central as well as the peripheral nervous system. They need extracellular ATP for activation, and have been implicated in pain transduction (McCleskey and Gold, 1999).
Neuronal 5-hydroxytryptamine

These receptors open in response to 5-hydroxytryptamine binding and are responsible for modulating depressive states (Grant, 1995).

1.3.2.3. Capacitative Calcium Influx

The generation of $[Ca^{2+}]_c$ signals by metabotropic receptors involves two interdependent and closely coupled components: rapid, transient release of $Ca^{2+}$ stored in the ER, followed by slowly developing extracellular $Ca^{2+}$ entry (Bennett et al., 1998; Parekh and Penner, 1997; Putney and Bird, 1993; Putney and McKay, 1999). Phospholipase C-coupled receptors generate the chemical messenger, inositol 1,4,5-trisphosphate (IP$_3$), that diffuses rapidly from the plasma membrane (PM) to interact with IP$_3$ receptors on the ER that serve as $Ca^{2+}$ channels to release luminal $Ca^{2+}$ and generate the initial $Ca^{2+}$ signal phase (Bennett et al., 1998). The resulting depletion of ER $Ca^{2+}$ serves as the trigger for a message that is returned to the PM, resulting in slow activation of "store-operated channels" (SOCs) or capacitative calcium influx mediating $Ca^{2+}$ entry (Parekh and Penner, 1997; Putney and Bird, 1993; Putney and McKay, 1999). This second phase of $Ca^{2+}$ signals mediates longer-term $[Ca^{2+}]_c$ elevations and replenishes intracellular stores (Putney and Bird, 1993; Parekh and Penner, 1997).

Whereas generation of IP$_3$ and activation of $Ca^{2+}$ release channels mediating the initial $Ca^{2+}$ signaling phase are well understood, the mechanism for coupling ER $Ca^{2+}$ store depletion with $Ca^{2+}$ entry remains a crucial question (Putney and McKay, 1999). Three main hypotheses have been proposed: (i) a diffusible messenger (ii) conformational coupling and (iii) vesicle secretion. The diffusible messenger hypothesis proposes that a messenger molecule is
generated that activates Ca^{2+} entry in response to store depletion. Patch clamp studies in *Xenopus* oocytes provided a very strong support for this hypothesis (Parekh et al., 1993). Numerous molecules have been reported to be acting as the diffusible messenger. These include small G proteins, ATP, GTP, pertussis toxin-sensitive heterotrimeric G proteins, a product of cytochrome P450 activity, arachidonic acid (for references for each see the review by Putney and McKay 1999), cGMP (Kwan et al., 2000) but to date none has been demonstrated very convincingly. Perhaps the most interesting and controversial candidate for second messenger is calcium influx factor (CIF). CIF was initially isolated from Jurkat T cells stimulated to deplete their calcium stores (Randriamampita and Tsien, 1993). It has been partially characterized as a < 500 Da phosphorylated pH-stable anion that is presumably released or generated from the ER or adjacent regions after IP_3_ induces Ca^{2+} release from stores (Clapham, 1993). After these initial studies, further results in favour of CIF came only as late as in 1999 from Richard Marchase’s lab where they provided evidence for the existence of diffusible CIF in yeast cells and in mammalian smooth muscle cells (Csutora et al., 1999; Trepakova et al., 2000). However the exact nature of CIF yet remains unidentified. Clearly, the identification of CIF is the most important prerequisite for this model to gain acceptance.

Alternatively, a direct coupling mechanism involving physical interaction between ER and PM was proposed by Irvine, (1990) and Berridge, (1995), and a possible role of the IP_3_ receptor in such coupling has been provided by (Kiselyov et al., 1998). Consistent with a direct coupling mechanism are studies indicating IP_3_-induced Ca^{2+} store emptying is localized in regions close to Ca^{2+} entry sites (Parekh and Penner, 1997, Petersen and Berridge, 1996). However, one implication of direct coupling would be the expectation of rapid SOC activation. Rather, SOC activation is slow, requiring tens to hundreds of seconds to develop (Hoth and Penner, 1993;
Kerschbaum and Cahalan, 1999; Parekh and Penner, 1997; Zweifach and Lewis, 1993).

The third model for explaining SOC is the vesicle-mediated channel insertion hypothesis in which it is proposed that the decrease in store Ca$$^{2+}$$ causes additional preformed SOC proteins to be inserted in the plasma membrane. Apart from a few pharmacological studies, there were not enough results to support the secretion-like mechanism. Vesicle trafficking inhibitors like primaquine were found to affect thapsigargin-activated calcium entry, with no effect on the IP$_3$ induced Ca$$^{2+}$$ mobilization (Somasundaram et al., 1995). Two recent papers provide fresh evidence in support of this mechanism (Patterson et al., 1999; Yao et al., 1999) as discussed below.

The elusive coupling between ER Ca$$^{2+}$$ stores and PM "store-operated" Ca$$^{2+}$$ entry channels was probed through a novel combination of cytoskeletal modifications by Patterson et al. (1999). Whereas coupling was unaffected by disassembly of the actin cytoskeleton, in situ redistribution of F-actin into a tight cortical layer subjacent to the PM displaced cortical ER and prevented coupling between ER and PM Ca$$^{2+}$$ entry channels, while not affecting IP$_3$-mediated store release. Importantly, disassembly of the induced cortical actin layer allowed ER to regain access to the PM and reestablish coupling of Ca$$^{2+}$$ entry channels to Ca$$^{2+}$$ store depletion. Coupling is concluded to be mediated by a physical "secretion-like" mechanism involving close but reversible interactions between the ER and the PM.

These possibilities are not mutually exclusive. A recent paper by Yao et al. (1999) provides additional evidence for a necessary close physical association between the PM and underlying structures and also provides evidence for the involvement of proteins known to mediate secretion
in other systems. They assayed capacitative Ca\(^{2+}\) entry by examining store depletion-activated whole-cell Ca\(^{2+}\) currents in Xenopus oocytes as well as currents in the membrane underlying giant patch pipettes sealed to the surface of the oocytes. Depletion of Ca\(^{2+}\) stores in Xenopus oocytes activated entry of Ca\(^{2+}\) across the plasma membrane, which was measured as a current I(soc) in subsequently formed cell-attached patches. I(soc) survived excision into inside-out configuration. If cell-attached patches were formed before store depletion, I(soc) was activated outside but not inside the patches. I(soc) was potentiated by microinjection of clostridium C3 transferase, which inhibits Rho GTPase, whereas I(soc) was inhibited by expression of wild-type or constitutively active Rho. Activation of I(soc) was also inhibited by botulinum neurotoxin A and dominant-negative mutants of SNAP-25 but was unaffected by brefeldin A. These results suggest that oocyte I(soc) is dependent not on aqueous diffusible messengers but on SNAP-25, probably via exocytosis of membrane channels or regulatory molecules.

### 1.3.3. The Mitochondria as a Calcium Sink

The idea that mitochondria accumulate large amounts of Ca\(^{2+}\) goes back to the 1950s. Indeed, experiments demonstrated that isolated mitochondria are capable of accumulating over 3 \(\mu\)mol Ca\(^{2+}\)/mg protein (equivalent to 3 mM at 1 mg/ml protein) in the presence of ATP and P\(_i\) (Scarpa and Azzone, 1970). Moreover, Ca\(^{2+}\) accumulation by mitochondria can take precedence over ATP synthesis (Brand and Lehninger, 1975), and was for a time considered an uncoupler of oxidative phosphorylation. Ca\(^{2+}\) entry into the mitochondrial matrix occurs via a Ca\(^{2+}\)-permeable 'uniporter' (presumably a channel) in the inner mitochondrial membrane (Miller, 1991; Nicholls and Akerman, 1982). Normally respiring mitochondria pump protons out of the mitochondrial matrix, resulting in a potential of about -150 to -200 mV across their inner membrane. Thus,
when $[\text{Ca}^{2+}]_e$ increases, it can enter the matrix down a steep electrochemical gradient. The mitochondrial matrix contains several $\text{Ca}^{2+}$-sensitive dehydrogenases that are members of the tricarboxylic acid (TCA) cycle (Denton and McCormack, 1990). Spike-like changes in the $[\text{Ca}^{2+}]_e$ are decoded by the mitochondria to produce long-lasting activation of these enzymes and provide an important way of linking ATP production to energetic demands during physiological activity (Denton and McCormack, 1990; Duchen, 1992; Hajnoczky et al., 1995). However, later it was shown that the mitochondria are not always on the receiving end of $[\text{Ca}^{2+}]_e$ signals and that they might be in a position to participate actively in them as well. One reason for this is that following a period of intense cellular activity, such as a spike train or tetanus, $\text{Ca}^{2+}$ is accumulated by mitochondria, and this $\text{Ca}^{2+}$ has to leave again following cessation of the stimulus (Miller, 1991; Nicholls and Akerman, 1982; Thayer and Miller, 1990). Until recently, the major route of $\text{Ca}^{2+}$ egress from the mitochondrial matrix had been considered to be an $\text{Na}^+/\text{Ca}^{2+}$ exchange system in the inner mitochondrial membrane that probably exchanges two $\text{Na}^+$ for each $\text{Ca}^{2+}$ (Miller, 1991; Nicholls and Akerman, 1982; Thayer and Miller, 1990).

This route of $\text{Ca}^{2+}$ efflux is rather slow and easily saturated. Thus, if $\text{Ca}^{2+}$ influx is relatively fast and extensive, $\text{Ca}^{2+}$ will accumulate in the matrix. Accumulated $\text{Ca}^{2+}$ leaving the matrix will have the effect of buffering the $[\text{Ca}^{2+}]_e$ at relatively high levels until all the $\text{Ca}^{2+}$ has exited, thus increasing the length of the $[\text{Ca}^{2+}]_e$ signal far beyond the cessation of the original stimulus. In effect, the mitochondria can act as a sort of $\text{Ca}^{2+}$ signalling memory-storage device. The importance of this for synaptic function has been demonstrated by Tang and Zucker who studied post tetanic potentiation at the crayfish neuromuscular junction (Tang and Zucker, 1997). Post tetanic potentiation is an enhancement of synaptic transmission that lasts several minutes and occurs following a period of tetanic presynaptic activity. It has been argued that this is due to the
enhancing effects on transmitter release of residual Ca\(^{2+}\) in the nerve terminal that remains following the stimulus. It is now clear that one contribution to this residual Ca\(^{2+}\) is the discharge of mitochondrial Ca\(^{2+}\) accumulated during the preceding period of intense neural activity (Miller, 1998).

Although the role of Na\(^+\)/Ca\(^{2+}\) exchange in mitochondrial function has been explored for many years, another route of Ca\(^{2+}\) release is presently causing a great deal of excitement. Indeed, with the aid of this novel pathway mitochondria can behave as excitable organelles (Ichas et al., 1997; Ichas et al., 1994; Jouaville et al., 1995). When Ca\(^{2+}\) is added to medium containing isolated mitochondria, it is rapidly accumulated. When a certain degree of loading is reached however, addition of further Ca\(^{2+}\) leads to rapid release of accumulated Ca\(^{2+}\), which is followed by re-accumulation. Thus, the addition of Ca\(^{2+}\) at this point produces an 'all or nothing' Ca\(^{2+}\) spike that is reminiscent of the Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) produced by ryanodine receptors. Mitochondrial CICR has now been shown to have some very interesting properties, potentially allowing waves of Ca\(^{2+}\) and concomitant depolarization to propagate from one mitochondrion to another in cellular or transcellular networks. Mitochondrial CICR might co-operate with IP\(_3\)-induced Ca\(^{2+}\) signals and help to amplify them and facilitate the propagation of IP\(_3\)-dependent Ca\(^{2+}\) waves. The juxtaposition of some mitochondria to IP\(_3\)-sensitive Ca\(^{2+}\) stores in the ER might be the anatomical mechanism for such an interaction (Miller, 1991). What are the molecular mechanisms that allow mitochondria to behave in this way? The answer appears to lie with a channel that is found in the mitochondrial membrane called the mitochondrial permeability transition pore (PTP) (Bernardi and Petronilli, 1996; Zoratti and Szabo, 1995). The PTP is a voltage-gated, cation-permeable channel, whose opening is favored by several factors including depolarization, intramatrix Ca\(^{2+}\) and oxidizing agents, and whose closing is favored by protons.
(that is, low matrix pH) and adenine nucleotides. Cyclosporin A is a good blocker of the channel that appears to require interaction with mitochondrial cyclophilin. The channel appears to function in both high- and low-conductance modes. In the high-, but not the low- conductance state, it is also permeable to large molecules of MW up to 1.5 kDa. The PTP has been widely discussed in connection with its role in cell death, a phenomenon generally thought to depend on its high-conductance state (described later). However, little is known about the significance of the low-conductance mode of operation. Ichas et al. have now demonstrated that mitochondrial CICR and associated phenomena are dependent on operation of the PTP in its low-conductance mode (Ichas et al., 1997). The model they suggest, and for which they provide experimental data, goes something like this. Ca^{2+} enters the mitochondrion and protons are pumped out, increasing the matrix pH. High matrix pH facilitates PTP opening. This leads to a collapse of the mitochondrial proton gradient and membrane potential, the outward movement of Ca^{2+} through the channel and acidification of the matrix. Acidification of the matrix leads to closing of the channel. The respiratory chain now restores the proton gradient, and so on. In this way, transient opening of the PTP produces the spike-like CICR observed.
Figure 1.1. Overview of the principle routes of Ca\textsuperscript{2+} entry, efflux, and accumulation by intracellular organelles in neurones.
1.3.4. $[\text{Ca}^{2+}]_c$ and Neurotoxicity

Although excessive $[\text{Ca}^{2+}]_c$ may contribute to a series of membrane, cytoplasmic, and nuclear events that result in neurotoxicity, $\text{Ca}^{2+}$ alone may not be sufficient to trigger this series of events. For example, elevation of $[\text{Ca}^{2+}]_c$ to the same level attained during glutamate application, with use of a metabolic inhibitor such as cyanide or by depolarization of neurones with high concentrations of potassium to allow $\text{Ca}^{2+}$ influx through voltage-gated calcium channels, causes less permanent neuronal damage than does glutamate itself (Dubinsky and Rothman, 1991). The location or mode of entry of $\text{Ca}^{2+}$ may be critical (Bading et al., 1993; Tymianski et al., 1993). In particular, $\text{Ca}^{2+}$-activated NOS is anchored close to NMDA receptors by the linker protein PSD-95 and is therefore preferentially activated by influx through these channels (Sattler et al., 1999). Alternatively, homeostatic mechanisms for handling $[\text{Ca}^{2+}]_c$ overload (such as $\text{Na}^+/\text{Ca}^{2+}$ exchange) may also be compromised by glutamate receptor activation, leading to more prolonged increases in $[\text{Ca}^{2+}]_c$ content within the cell than caused by other influx pathways (Mattson et al., 1989; Randall and Thayer, 1992). Finally, another messenger pathway, as yet unidentified, may have to be triggered coincidently with the rise in $[\text{Ca}^{2+}]_c$ to cause neurotoxicity.

$[\text{Ca}^{2+}]_c$ is important for a number of physiological processes, but excessive amounts may contribute to the overstimulation of normal processes, thus damaging neurones. NMDA receptor activation, increase in $[\text{Ca}^{2+}]_c$, or both can activate a series of enzymes (Fig. 1.2), including protein kinase C, phospholipases, proteases, protein phosphatases, and NOS (Choi, 1988; Dawson et al., 1992; Trout et al., 1993). After phospholipase $A_2$ is activated arachidonic acid, its metabolites, and platelet-activating factor are generated. Platelet activating factor increases $[\text{Ca}^{2+}]_c$, apparently by stimulating the release of glutamate (Bito et al., 1992; Clark et al., 1992).
Arachidonic acid potentiates NMDA-evoked currents (Miller et al., 1992) and inhibits reuptake of glutamate into astrocytes and neurones (Volterra et al., 1992), further exacerbating the situation; oxygen free radicals can be formed during arachidonic acid metabolism (Lafon Cazal et al., 1993), leading to further phospholipase A₂ activation, which represents positive feedback (Au et al., 1985). These processes can cause the neuron to digest itself by protein breakdown, free radical formation, and lipid peroxidation. In addition, one might envisage that in cerebral ischemia, tissue reperfusion increases this damage by providing additional free radicals in the form of superoxide anions (O₂⁻).

Even at lower concentrations, O₂⁻ can participate in reactions to form products that may be toxic to neurones (Lipton et al., 1993). One such reaction involves NO, formed in neurones by NOS. This enzyme is activated by Ca²⁺ influx triggered by NMDA-receptor stimulation (Fig. 1.2). When NMDA receptors are excessively stimulated, NO and O₂⁻ may be produced in increased quantities. Under these conditions, NO and O₂⁻ may react to form a toxic substance called peroxynitrite (ONOO⁻), resulting in neuronal death (Beckman and Koppenol, 1996; Dawson et al., 1991; Iadecola, 1997; Lipton et al., 1993).

In contrast, increased production of NO by endothelial cells can protect the tissue by improving the microcirculation (Huang et al., 1994). Also, NO can be changed to a chemical state that has just the opposite effect, protecting neurones from injury due to NMDA-receptor overstimulation. The chemical state is dependent on the removal or addition of an electron to NO, a condition that can be influenced by the presence or absence of electron donors, such as ascorbate or the amino
acid cysteine. For example, with one less electron, nitric oxide becomes nitrosonium ion (NO\(^+\)), which binds to a regulatory site on the NMDA receptor, a redox modulatory site (Lipton et al., 1993). Such binding results in decreased activity of NMDA receptors, thus affording protection from excessive stimulation. Therefore, depending on the chemical state, the NO group can lead to the destruction or protection of neurones. These findings have led to therapeutic approaches to decrease NMDA-receptor overactivity with the use of drugs resembling NO\(^+\), such as nitroglycerin (Lipton et al., 1993).

In addition to stimulating enzymes of the cell cytosol, \([\text{Ca}\^{2+}]_c\) can activate nuclear enzymes. For example, excessive \([\text{Ca}\^{2+}]_c\) may activate endonucleases that result in condensation of nuclear chromatin and ultimately DNA fragmentation and nuclear breakdown, a characteristic component of apoptosis (Alles et al., 1991). Free radicals may also contribute to DNA fragmentation (Kane et al., 1993).
Excitatory amino-acids, glutamate

Increased intracellular calcium

Protein kinase C
Calcium/Calmodulin-dependent protein kinase 2
Phospholipases
Proteases
Phosphatases
Nitric oxide synthase
Endonucleases
Ornithine decarboxylase
Xanthine oxidase

**Figure 1.2.** Pathophysiologic consequences of the activation of cellular enzyme systems by excitatory amino acid-evoked increase in [Ca$^{2+}$]$_o$
1.4. Excitotoxicity

1.4.1. Types of Excitatory Amino Acid Receptors

Two main subtypes of glutamate receptors have been recognized on the basis of their molecular cloning, electrophysiologic properties, and pharmacologic profile. The two main divisions are ionotropic (receptors that are coupled directly to membrane ion channels) and metabotropic (receptors that are coupled to G proteins and modulate intracellular second messengers such as inositol trisphosphate, calcium and cyclic nucleotides) (Collingridge and Lester, 1989; Monaghan et al., 1989). Ionotropic receptors are divided into three major types based on their selective agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainate.

The three families of ionotropic receptors were first defined by their pharmacology and subsequently by their molecular biology. The receptors appear to be tetrameric (Laube et al., 1998) or pentameric and the subunits that comprise these are specific for each of the three families (Dingledine and Conn, 2000). The subunit composition determines the biophysical properties of the receptor and to a variable extent its pharmacology (Table 1.2).

1.4.1.1. N-Methyl-D-aspartate receptors

NMDA receptors include NR1 and a family of NR2 subunits, probably with a tetrameric stoichiometry (Dingledine et al., 1999; Hollmann and Heinemann, 1994). It seems that in the absence of the NR2 subunit, the NR1 subunit is not integrated into the membrane successfully (McIlhinney et al., 1996). The NR1 subunit appears to be a constituent of all NMDA receptors,
and is vital for function. Transgenic mice in which the murine NR1 gene has been disrupted do not form functional NMDA receptors, and such homozygous mice do not survive (Forrest et al., 1994). It is the NR1 subunit that is thought to bear the binding sites for dissociative anaesthetics and for glycine (Chazot et al., 1992; Grimwood et al., 1995; Lynch et al., 1994). In nonmammalian (Xenopus) cells, expression of NR1 alone produce cation channels that can be opened by NMDA, suggesting that this subunit also bears the glutamate (NMDA) binding site (Moriyoshi et al., 1991). The four NR2 subunits (NR2A–D) have long cytoplasmic tails [644 amino acid residues], the C termini of which end in the conserved sequence –ESDV (NR2A and NR2B) or –ESEV (NR2C and NR2D). A key finding was that this short C-terminal peptide motif mediates binding to the first two PDZ domains of PSD-95/synapse-associated protein (SAP) 90, an abundant protein of the post synaptic density (PSD) (Kornau et al., 1995; Niethammer et al., 1996). PDZ domains are motifs of ~90 amino acids that have been recognized to mediate protein–protein interactions.

*Function.*

NMDA receptors are ligand-gated ion channels with high unitary conductances (40-50 pS) that permit the entry of Na\(^+\) and Ca\(^{2+}\) ions and the efflux of K\(^+\) ions (MacDermott et al., 1986). Furthermore, they display slow deactivation kinetics (Dingledine et al., 1999). At resting membrane potentials, NMDA receptors are blocked by Mg\(^{2+}\) ions, and this prevents them from being activated by glutamic acid. For this reason, NMDA receptors are not thought to be involved in primary synaptic transmission at glutamatergic synapses. However, this blockade by Mg\(^{2+}\) ions is voltage dependent, and thus, NMDA receptors can be activated by glutamate once the neurone has been depolarized by another excitatory mechanism (Mayer et al., 1984; Nowak et al., 1984). NMDA receptors thus may be involved secondarily in glutamatergic transmission once the neurone has been depolarized by the activation of AMPA/kainate receptors. Such a
phenomenon is well characterized in the neocortex, where NMDA receptor activation is responsible for the slow component of excitatory post synaptic potentials at synapses between glutamatergic interneurones and pyramidal neurones (Thomson et al., 1985). Similarly, NMDA receptors appear to contribute to the synaptic excitation following high-frequency firing of Schaffer collaterals in the hippocampus (Herron et al., 1986).

1.4.1.2. \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

AMPA receptors are typically composed of heteromeric combinations of GluR1–4 subunits (Dingledine et al., 1999; Hollmann and Heinemann, 1994). It seems that certain assembly patterns are preferred, for example, in hippocampal pyramidal cells, which express GluR1/GluR2 heteromers and GluR2/GluR3 heteromers, but not GluR1/GluR3 heteromers (Wenthold et al., 1996). The membrane topology of GluR subunits is analogous to that of NMDA receptor subunits, with a C-terminal tail that is cytoplasmically disposed. Interestingly, the GluR2 subunit confers \(\text{Ca}^{2+}\) impermeability on AMPA receptors and is found in the majority of AMPA receptors in principal neurones of the forebrain (Wenthold et al., 1996). Despite coexisting at the same excitatory synapses, AMPA receptors bind to a set of cytoplasmic proteins distinct from those for NMDA receptors (Sheng and Pak, 2000). Nevertheless, continuing the theme established by NMDA receptors, a major set of AMPA receptor interactions is mediated by C-terminal binding to specific PDZ domain containing scaffold proteins. GluR2 and GluR3 subunits share a common C-terminal sequence (–SVKI) that interacts with the 5th PDZ domain of GRIP (now termed GRIPl), a protein containing seven PDZ domains and no other recognizable domains (Dong et al., 1997; Wyszynski et al., 1998). A protein with 6 PDZ
domains [AMPA receptor-binding protein (ABP)] was also isolated by its binding to GluR2/3 (Srivastava et al., 1998). ABP appears to be a splice variant of a GRIP related protein (also called GRIP2) that contains seven PDZ domains (ABP lacking the N terminus and PDZ7 domain of GRIP2) (Bruckner et al., 1999; Dong et al., 1999; Wyszynski et al., 1999).

**Function**

AMPA receptors can be activated by glutamate and AMPA. They are ligand gated cation channels allowing the entry of Na\(^+\) ions and the exit of K\(^+\) ions. AMPA channels have a 5- to 10-fold lower elementary conductance than NMDA receptors (< 10 ps) (Doble, 1999). At many glutamatergic synapses in the brain, Ca\(^{2+}\)-permeable AMPA receptors mediate fast excitatory transmission (Gu et al., 1996; Isa et al., 1996; Mahanty and Sah, 1998; McBain and Dingledine, 1993; Otis et al., 1995).

Studies of native AMPARs by patch-clamp recordings combined with single-cell reverse transcription-PCR (Lambolez et al., 1992) have confirmed the role played by the GluR2 subunit in their permeation (Bochet et al., 1994; Geiger et al., 1995; Jonas et al., 1994), but the identity of the molecular determinants controlling their kinetics is still controversial. Indeed, slow and fast desensitizations were correlated with either GluR2 and GluR4 (Geiger et al., 1995), respectively, or to 'flip' and 'flop' splice variants (Lambolez et al., 1996). The correlation found by Geiger et al. (1995) suggests that permeation and kinetic properties of native AMPARs may not be regulated independently and would range from fast desensitizing-Ca\(^{2+}\) permeable (as found in interneurones) to slow desensitizing-Ca\(^{2+}\) impermeable (as found in pyramidal cells). This is indeed the case for most neural cell types studied so far (Angulo et al., 1997).
1.4.1.3. Kainate receptor

Kainate receptors represent a third class of glutamate-gated ion channel and are made up of subunits (GluR5–7, KA1, and KA2) that are homologous to AMPA receptor subunits. The cytoplasmic domains of GluR6 and KA2 have been shown to bind to the PDZ1 domain and to the SH3 and GK domains of PSD-95, respectively (Garcia et al., 1998), but the in vivo significance of these interactions for kainate receptors is unclear.

Function

Kainate receptors have been difficult to study because they have lower unitary conductances than AMPA receptors and desensitize rapidly to kainic acid. This means that in cells where both AMPA and kainate receptors co-exist, the dominant response to kainic acid is mediated by AMPA receptors. Rapidly desensitising kainate responses, however, have been observed in hippocampal neurones (Lerma et al., 1993). The discovery that compounds such as GYKI 53655 selectively block AMPA receptors has facilitated the characterization of kainate receptors (Lerma, 1997), as indeed has been achieved in hippocampal neurones (Paternain et al., 1995; Wilding and Huettner, 1997). These studies suggest that kainate receptors are not involved in fast synaptic transmission between glutamatergic interneurones (Lerma, 1997), at least in the hippocampus, but do contribute to the excitatory synaptic potential generated by high-frequency stimulation of the mossy fibre input to CA3 neurones (Castillo et al., 1997; Vignes and Collingridge, 1997). Activation of kainate receptors on glutamatergic nerve terminals in the hippocampus apparently leads to a depression of glutamate release (Chittajallu et al., 1996), and a similar inhibition of synaptic transmission subsequently has been observed at hippocampal GABAergic terminals (Clarke et al., 1997).
<table>
<thead>
<tr>
<th></th>
<th>NMDA</th>
<th>AMPA</th>
<th>Kainate</th>
<th>Metabotropic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Family</strong></td>
<td>Ion channel</td>
<td>Ion channel</td>
<td>Ion channel</td>
<td>G-protein linked</td>
</tr>
<tr>
<td><strong>Structure</strong></td>
<td>Oligomeric</td>
<td>Oligomeric</td>
<td>Oligomeric</td>
<td>Monomeric</td>
</tr>
<tr>
<td><strong>Subunits/subtypes</strong></td>
<td>1 NR1 subunit</td>
<td>4 GluR subunits (1-4)</td>
<td>3 GluR subunits (5-7)</td>
<td>8 subtypes known</td>
</tr>
<tr>
<td></td>
<td>4 NR2 subunits (A-D)</td>
<td>‘flip’ and ‘flop’ splice variant</td>
<td>2 KA subunits (I, 2)</td>
<td></td>
</tr>
<tr>
<td><strong>Unitary conductance</strong></td>
<td>Mainly 40-50 pS</td>
<td>Mainly 10-20 pS</td>
<td>Mainly &lt;10pS</td>
<td></td>
</tr>
<tr>
<td><strong>Ion selectivity</strong></td>
<td>Na⁺, K⁺, Ca²⁺</td>
<td>Na⁺, K⁺</td>
<td>Na⁺, K⁺</td>
<td></td>
</tr>
<tr>
<td><strong>Desensitisation</strong></td>
<td>Slow</td>
<td>Rapid (AMPA)</td>
<td>Rapid (kainate)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slow (kainate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Selective agonist</strong></td>
<td>NMDA</td>
<td>Quisqualic acid</td>
<td>Kainic acid</td>
<td>Trans-ACDP</td>
</tr>
<tr>
<td></td>
<td>Quinolic acid</td>
<td>AMPA</td>
<td></td>
<td>Ibotenic acid</td>
</tr>
<tr>
<td></td>
<td>Ibotenic acid</td>
<td>Kainic acid</td>
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<td>Quisqualic acid</td>
</tr>
<tr>
<td><strong>Selective antagonist</strong></td>
<td>2-APV</td>
<td>CNQX</td>
<td>CNQX</td>
<td>Phenylglycines</td>
</tr>
<tr>
<td></td>
<td>Selfotel</td>
<td>GYKI 52466</td>
<td>NS 102</td>
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</tr>
<tr>
<td></td>
<td>MK-801</td>
<td></td>
<td>SYM 2081</td>
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</tr>
<tr>
<td><strong>Regulatory sites</strong></td>
<td>Glycine</td>
<td>Thiazide</td>
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</tr>
<tr>
<td></td>
<td>Polyamine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Trans-ACDP, (+/-)-1-aminocyclopentane-trans-1,3-dicarboxylic acid.

**Table 1.2. Excitatory Amino Acid Receptor Subtypes** adapted from Pharmacol. Ther. Vol. 81, No. 3, pp 163-221, 1999.
### 1.4.1.4. Metabotropic Receptors

Metabotropic glutamate receptors (mGluR) are G-protein-coupled receptors that are not generally thought to be involved in fast synaptic neurotransmission (Pin and Bockaert, 1995; Pin and Duvoisin, 1995; Toms et al., 1995). Eight different receptor subtypes have been identified, which fall into three families. Activation of receptors belonging to Group I (mGluR1 and mGluR5) leads to stimulation of phospholipase C (Nicoletti et al., 1986; Sladeczek et al., 1985), whilst activation of those belonging to Groups II and III (mGluR4, mGluR6, mGluR7, and mGluR8) leads to inhibition of adenylate cyclase activity (Pin and Duvoisin, 1995). It was suggested that metabotropic receptors may modulate NMDA-receptor mediated neurotoxicity (Nicoletti et al., 1996) and that antagonists at Group I receptors or agonists at Group II/III receptors may have neuroprotective effects. Immunochemistry at the electron microscopy level reveals a highly selective expression of mGluR (Lujan et al., 1996; Lujan et al., 1997; Shigemoto et al., 1996), with some occurring presynaptically in close relationship to the presynaptic density (mGluR7, mGluR8) and some occurring on the presynaptic axon, relatively distant from the synaptic cleft (mGluR2, mGluR3).

### 1.4.2. Pathogenesis of Excitotoxicity

#### 1.4.2.1. Mechanisms of Abnormal Glutamate Accumulation

The first sign of glutamate toxicity, which develops within minutes after exposure to glutamate, is neuronal swelling (Choi et al., 1987). This acute toxicity is dependent on the entry of excessive amounts of Na\(^+\) and Cl\(^-\) ions, together with water, into the cell, but it does not necessarily lead to cell death (Choi, 1987). The acute toxicity associated with exposure to
glutamate can be blocked by the removal of Na\(^+\) or Cl\(^-\) from the extracellular medium, but their absence does not protect neurones from delayed toxic effects of glutamate. Neurones die several hours after exposure to glutamate, and this irreversible injury is at least partially dependent on extracellular Ca\(^{2+}\) (Choi, 1985) presumably because excessive Ca\(^{2+}\) influx triggers one or more of the processes described above.

The paradox of excitotoxicity is how or why the mammalian brain evolved with such extraordinary vulnerability to its own excitatory neurotransmitters. What are the events leading to the abnormal glutamate accumulation that triggers excitotoxicity?

The intracellular glutamate concentration in the brain tissue is approximately 10 mmol per liter (Kvamme et al., 1985). Because of the activity of the glutamate transporters, most of the glutamate is intracellular. The extracellular glutamate concentration in brain tissue has been estimated to be approximately 0.6 \(\mu\)mol per liter (Benveniste et al., 1984; Bouvier et al., 1992). Substantial excitotoxic damage to cortical neurones or hippocampal neurones in intact tissue is expected to occur when the glutamate concentration reaches 2 to 5 \(\mu\)mol per liter (Meldrum and Garthwaite, 1990; Rosenberg et al., 1992). Therefore, the ambient concentrations of glutamate are close to those that destroy neurones, and it is important that the extracellular glutamate concentration and compartmentalization be exquisitely controlled to prevent excitotoxicity. On the other hand, since each cell contains 10 mmol of glutamate per liter, the potential for disaster is obviously great.

1.4.2.2. Glutamate Transport

What mechanisms normally maintain glutamate concentrations at low levels in the extracellular
space? Given the sensitivity of neurons to excitotoxicity, a defect in the system for clearing glutamate (or aspartate) would be expected to have disastrous consequences. Glutamate and aspartate are cleared from the extracellular space by Na\(^+\)-dependent, high affinity uptake systems and moved into astrocytes and neurons through transporters (Bouvier et al., 1992; Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992). The high affinity glutamate uptake system uses the energy derived from the Na\(^+\) gradient present across cell membranes to do the concentrative work involved in moving glutamate from the extracellular fluid, in which it is present in micromolar concentrations, into cells, in which it is present in millimolar concentrations. Any defect that impairs the ability of cells to maintain that Na\(^+\) gradient might cause the high-affinity glutamate-uptake system to fail. It is not known what is the margin of safety built into the uptake system, or what degree of failure increases extracellular glutamate concentrations. Support for the hypothesis that glutamate transporters are a potential source of extracellular glutamate comes from studies showing that transporters can run backwards, becoming a source of extracellular glutamate, rather than a sink for it (Szatkowski et al., 1990).

Glutamate as well as other amino acids may also be released into the extracellular fluid as a consequence of swelling of astrocytes (Kimelberg et al., 1990). When cellular energy metabolism is impaired or fails because of ATP depletion, diminished activity of Na\(^+\)/K\(^+\)-ATPase may lead to swelling sufficient to trigger the release of glutamate by this glutamate-transporter. Similarly, hyponatremia may lead to swelling and the release of glutamate.

A variety of subtypes of glutamate transporter proteins have been cloned and sequenced, first in the rat (Bouvier et al., 1992; Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992) and then in human tissue (Fairman et al., 1995). The human transporters are named EAAT-1 to
EAAT-4, whereas the rat transporters have idiosyncratic names. EAAT-1 (GLAST in the rat) is a glutamate transporter protein localised on astrocytes and apparently expressed predominantly in the cerebellum (Rothstein et al., 1994). EAAT-2 (GLT-1 in the rat) is perhaps the most important transporter protein involved in control of synaptic glutamate concentrations. It is located on perisynaptic astrocytes (Danbolt et al., 1992) and expressed widely throughout the CNS. Of all the transporter proteins, EAAT-2 has the highest affinity for glutamic acid.

EAAT-3 (EAAC-1 in the rat) is the principal glutamate transporter protein localised on neurones (Shashidharan et al., 1994). This transporter is thought to be responsible for reuptake of synaptic glutamate back into the nerve terminals of glutamatergic neurones. EAAT-3 is also expressed in peripheral tissues. EAAT-4 (no rat homologue yet identified) is a transporter that is localised exclusively to Purkinje cells in the cerebellum (Fairman et al., 1995), where it is present extrajunctionally and may restrict spill-over of glutamate from glutamatergic synapses (Tanaka et al., 1997).

**Metabolic Shunting of Glutamate in Glial Cells**

Glial cells play an important role in the correct functioning of glutamatergic neurotransmission (Erecinska and Silver, 1990; Hamberger et al., 1981). In particular, astrocytes are responsible for maintaining extracellular concentrations of glutamate at a low level. Released glutamate is removed by uptake into the surrounding glial cells where it is believed to be recycled via the glutamine/glutamate cycle (Fonnum, 1984). The first step in this cycle is amination of glutamate to glutamine via the action of glutamine synthetase, an enzyme exclusively localised in glial cells. It is known that this reaction proceeds at the expense of cellular energy (ATP). Glutamine, thus formed, readily crosses cell membranes and is transported back to the nerve terminals where it can be converted to transmitter glutamate via the action of the neuronal enzyme glutaminase.
In addition, glutamate taken up by glial cells may be transaminated by aspartate aminotransferase (AAT) or oxidatively deaminated by glutamate dehydrogenase to α-ketoglutarate, which can then enter the TCA cycle and be oxidized to carbon dioxide and water, generating ATP (Mason et al., 1992).

### 1.4.2.3. Release of Glutamate from Vesicular Stores

Another possible source of excess glutamate is abnormal release of glutamate from its storage sites in neuronal vesicles. The system of normal excitatory neurotransmission appears to have as one of its features a positive feedback system, with glutamate release causing further glutamate release. This has been demonstrated in the phenomenon of long-term potentiation, a cellular model for learning and memory, in which the amount of glutamate released from excitatory synapses increases after intense stimulation of synapses (Bliss and Collingridge, 1993). In parallel, excitotoxicity has a self-propagating property, since neuronal survival can be enhanced by glutamate (NMDA) antagonists after brief exposure of neurones to glutamate (Rothman et al., 1987). These observations have raised the hope that in patients with stroke, therapeutic intervention after the acute event might limit subsequent damage, by interfering with the self-propagating property of excitotoxicity. The hypothesis that a positive feedback mechanism is involved in the release of vesicular stores of glutamate is the basis for understanding the use of NMDA antagonists after stroke. Furthermore, an in vitro study reported that tetanus toxin blocked excitotoxicity due to hypoxia or hypoglycemia. These results support the hypothesis that a vesicular mechanism of glutamate release has an important role in ischemia, since tetanus toxin inhibits the vesicular release of presynaptic transmitters (Monyer et al., 1992).
1.4.2.4. Injury and Glutamate Release

The simplest cause of excess extracellular glutamate is injury to cells. If each cell contains 10 mmol of glutamate per liter, then the death of even one cell puts all its neighbors at risk, reduced only by their ability to remove the glutamate released on their doorstep. One cause of injury is excitotoxicity itself, and this might be a sufficient explanation for the self-propagating property of excitotoxicity—that is, injured neurones release large quantities of glutamate that in turn injures more neurones. In addition, traumatic injury to neurones may be followed by excitotoxic injury to neighboring neurones; in models of trauma, neuronal death can be blocked by glutamate antagonists, especially those directed against the NMDA receptors (Faden et al., 1989), although non-NMDA-receptor antagonists may also be effective (Wrathall et al., 1992).

1.4.2.5. Role of Energy Failure in Neuronal Injury

Deprivation of oxygen and glucose—for example, during ischemia—decreases the production of high energy phosphate compounds and causes energy failure. However, energy failure itself is not particularly toxic to neurones. What makes it neurotoxic is the activation of glutamate-receptor-dependent mechanisms. If these are blocked by suitable antagonists, then neurones can survive a period of deprivation of oxygen and metabolite substrate (Rothman, 1984). Prolonged energy failure can cause cell death because all essential processes will stop. How does energy failure lead to the excess extracellular accumulation of glutamate?

Focal impairment of cerebral blood flow restricts the delivery of substrates, particularly oxygen and glucose, and impairs the energetics required to maintain ionic gradients (Martin et al., 1994). With energy depletion, ion gradients are lost and neurones and glia depolarize (Katsura et al.,...
1994). Consequently, somatodendritic as well as presynaptic voltage-dependent Ca$$^{2+}$$ channels become activated and excitatory amino acids are released into the extracellular space (Figure 1.3). At the same time, the energy-dependent processes, such as presynaptic reuptake of excitatory amino acids, are impeded, which further increases the accumulation of glutamate in the extracellular space. Activation of NMDA receptors and metabotropic glutamate receptors contribute to Ca$$^{2+}$$ overload (Nehls et al., 1989), the latter via phospholipase C and IP$$^3$$ signalling.

As a result of glutamate-mediated overactivation, Na$$^+$$ and Cl$$^-$$ enter the neurones via channels for monovalent ions (for example, the AMPA receptor channel). Water follows passively, as the influx of Na$$^+$$ and Cl$$^-$$ is much larger than the efflux of K$$^+$$.

The ensuing oedema can affect the perfusion of regions surrounding the core of the perfusion deficit negatively, and also have remote effects that are produced via increased intracranial pressure, vascular compression and herniation.
Figure 1.3. Simplified overview of pathophysiological mechanisms in the focally ischaemic brain. Energy failure leads to the depolarization of neurones. Activation of specific glutamate receptors dramatically increases intracellular Ca^{2+}, Na^+, Cl^- levels while K^+ is released into the extracellular space. Diffusion of glutamate (Glu) and K^+ in the extracellular space can propagate a series of spreading waves of depolarization (peri-infarct depolarizations). Water shifts to the intracellular space via osmotic gradients and cells swell (oedema). The universal intracellular messenger Ca^{2+} overactivates numerous enzyme systems (proteases, lipases, endonucleases, etc.). Free radicals are generated, which damage membranes (lipolysis), mitochondria and DNA, in turn triggering mediators, which activate microglia and lead to the invasion of blood-borne inflammatory cells (leukocyte infiltration) via upregulation of endothelial adhesion molecules.
An increase in the universal second messenger, Ca$_{\text{2+}}$, is thought to initiate a series of cytoplasmatic and nuclear events that impact the development of tissue damage profoundly, mentioned earlier in section 1.3.4. The important role of oxygen free-radicals in cell damage associated with stroke is underscored by the fact that even delayed treatment with free-radical scavengers can be effective in experimental focal cerebral ischemia (Zhao et al., 1994). In addition, the overproduction of radical-scavenging enzymes protects against stroke (Weisbrot Lefkowitz et al., 1998; Yang et al., 1994) and animals that are deficient in radical-scavenging enzymes are more susceptible to cerebral ischaemic damage (Kondo et al., 1997; Murakami et al., 1998).

Mitochondria, which are an important source of reactive oxygen species, are impaired by free-radical-mediated disruption of the inner mitochondrial membrane and the oxidation of proteins that mediate electron transport, H$^+$ extrusion and ATP production (Dugan and Choi, 1994). The mitochondrial membrane becomes leaky, partly owing to the formation of a mitochondrial permeability transition pore, which promotes mitochondrial swelling, the cessation of ATP production and an oxygen free-radical burst (Kristian and Siesjo, 1998). Cytochrome C is released from mitochondria (Fujimura et al., 1998) and provides a trigger for apoptosis.

The haemodynamic, metabolic and ionic changes described above do not affect the ischaemic territory homogeneously. In the centre or core of the perfusion deficit, permanent and anoxic depolarization develops minutes after the onset of ischaemia. Cells are killed rapidly by lipolysis, proteolysis, the disaggregation of microtubules that follows total bioenergetic failure and the ensuing breakdown of ion homeostasis (Siesjo et al., 1998). Between this lethally damaged core and the normal brain lies the penumbra, an area of constrained blood flow with partially
time and without treatment, the penumbra can progress to infarction owing to ongoing
excitotoxicity (see above) or to secondary deleterious phenomena, such as spreading
depolarization, post-ischaemic inflammation and apoptosis (see below). It is, thus, evident that
the prime goal of neuroprotection is to salvage the ischaemic penumbra. Although there is ample
evidence that the penumbra exists in human stroke patients (Furlan et al., 1996; Read et al.,
1998), the extent and temporal dynamics of this area are less well defined: it might be smaller
and exist for a shorter time period in humans (Kaufmann et al., 1999).

1.4.3. Peri-infarct depolarisation

As outlined above, ischaemic neurones and glia depolarize owing to the shortage of energy
supply, and the release of K\(^+\) and glutamate. In the core region of the affected brain tissue, cells
can undergo an anoxic depolarization and never repolarize. In penumbral regions (where some
perfusion is preserved) cells can repolarize, but at the expense of further energy consumption.
The same cells can depolarize again in response to increasing glutamate or K\(^+\) levels, or both,
which accumulate in the extracellular space. Repetitive depolarizations, so-called 'peri-infarct
depolarizations' occur (Hossmann, 1996). Peri-infarct depolarizations have been demonstrated in
mice, rat and cat stroke models, occur with a frequency of several events per hour and can be
recorded for at least six to eight hours. As the number of depolarizations increases, the infarcts
grow larger (Mies et al., 1993). Drugs that reduce the number of depolarizations decrease infarct
size (Iijima et al., 1992). As NMDA- as well as AMPA-receptor antagonists block peri-infarct
depolarizations, some have attributed their neuroprotective potential to this effect. However,
peri-infarct depolarizations have so far escaped detection by electrophysiological or functional
imaging methods in humans, so that their relevance to human stroke pathophysiology remains
1.4.4. Endogenous Glutamate and Acute Neurotoxicity

Glutamate acting on AMPA, NMDA and probably also mGluR1 receptors is thought to play an important role in cell death subsequent to status epilepticus, cerebral ischemia, perinatal asphyxia and traumatic brain injury (Faden et al., 1989; Sherwin, 1999; Wahlestedt et al., 1993; Wong et al., 1999). The role of the ligand-gated channels can be shown by using selective antagonists; thus NMDA receptor antagonists of all types (glutamate receptor competitive antagonists, glycine site competitive antagonists, open channel blockers and selective antagonists acting preferentially on a polyamine site or on the NR2B subunit of the NMDA receptor) protect against ischemic brain damage (Meldrum and Garthwaite, 1990). NMDA receptors have different subunit composition according to their site of expression. Receptors with NR2B subunits are expressed particularly on GABAergic interneurones, so that antagonists acting selectively on these NMDA receptors may have effects differing from those of antagonists acting on NMDA1/NR2A receptors (for review see Dingledine et al., 1999).

A role for glutamate-evoked neuronal injury in ischaemic stroke is supported by data from studies in which rats were injected with antisense oligonucleotides to mRNAs coding for the NMDA receptor (Wahlestedt et al., 1993). In such animals, the expression of this receptor is greatly reduced, and the extent of brain lesions following experimental focal ischaemic stroke is attenuated. This suggests that activation of the NMDA receptor by endogenous glutamic acid released in ischaemia plays a crucial role in determining the extent of neurodegeneration.

The usefulness of NMDA receptor antagonists (and in particular channel blockers) in the clinical
treatment of stroke has been challenged on the grounds that the efficacy of such drugs in models of global ischaemia is, at best, modest (especially when body temperature is controlled) and that the therapeutic time window during which the treatment should be initiated is too short (Buchan et al., 1991a; Buchan, 1990). Although NMDA receptors are highly permeable to Ca^{2+}, there is now general consensus that antagonists of AMPA receptors, such as the quinoxalinedione NBQX, appear to be much more effective than NMDA antagonists in preventing CA1 cell death following severe global ischemia, even when given as late as 24 h after (Buchan et al., 1991b; Nellgard and Wieloch, 1992; Sheardown et al., 1993). These observations indicate that activation of AMPA receptors is necessary, and possibly sufficient, for delayed post-ischemic degeneration and that cells are not irreversibly damaged until well after the ischemic episode.

Transient forebrain ischemia in rodents induces a reduction in expression of mRNA encoding the AMPA receptor subunit GluR2 (Gorter et al., 1997; Heurteaux et al., 1995; Pellegrini Giampietro et al., 1994; Pellegrini Giampietro et al., 1992; Pollard et al., 1993). GluR2 mRNA expression is markedly reduced in the CA1 region (the brain region most vulnerable to ischemia-induced damage), but not in the CA3 region or the dentate gyrus. Analysis of emulsion-dipped sections indicates that the reduction is specific to CA1 pyramidal cells, and reveals that it clearly precedes any histological sign of cell damage. The decline in glur2 mRNA in the CA1 region is first detectable at about 12 h after ischemia, and is about 30% of control at 24 h. GluR3 expression is also reduced, but less so (to about 50% of control), whereas glur1 and nr1 mRNA expression are not significantly changed. The (GluR1 + GluR3)/GluR2 ratio is markedly increased in the CA1 region at 24 h, but not in CA3 neurones or the dentate gyrus. Because the GluR2 subunit limits the Ca^{2+} permeability of AMPA channels, these findings predict the increased formation of Ca^{2+}-permeable AMPA receptors in CA1 neurones after transient global
ischemia at times preceding neurodegeneration. Moreover, timing of the switch is consistent with a causal role for AMPA receptor-mediated Ca$$^{2+}$$ influx in post-ischemic damage (Pellegrini Giampietro et al., 1997).

Reduction in RNA editing at the Q/R site of GluR2 could also increase Ca$$^{2+}$$ permeability of AMPA receptors, but this mechanism is unchanged after global ischemia (Kamphuis et al., 1995; Paschen et al., 1996; Rump et al., 1996).

1.4.5. Chronic Neurodegenerative Disease

It is now clear that excitotoxicity contributes to acute degeneration in conditions such as ischemia, in which rapid glutamate release causes injury that results in part from rapid post synaptic Ca$$^{2+}$$ accumulation. Excitotoxicity probably has a role in neurodegenerative diseases as well (Weiss and Sensi, 2000). This has led to the hope that compounds that interfere with glutamatergic neurotransmission may be of clinical benefit in treating such diseases. However, except in the case of a few very rare conditions, direct evidence for a pathogenic role for excitotoxicity in neurological disease is missing (Doble, 1999). Consistent with an excitotoxic contribution to Alzheimer’s disease and amyotrophic lateral sclerosis, three environmental excitotoxins (beta-N-methylamino-L-alanine, beta-N-oxalylamino-L-alanine and domoic acid) have been described that can reproduce specific features of these conditions in primates (Spencer, 1987; Spencer et al., 1986; Teitelbaum et al., 1990). In addition ß-amyloid peptide, which accumulates in AD, markedly enhances neuronal vulnerability to glutamate-receptor-mediated injury (Koh et al., 1990; Mattson et al., 1992), and deficiencies in glutamate uptake have been observed in spinal-cord tissue from individuals with ALS (Rothstein et al., 1992) and for a process of slow excitotoxicity in Huntington’s disease and Parkinson’s disease. The
The excitotoxic process is probably a secondary pathogenic factor in all these diseases, and the extent to which it contributes to the pathophysiology of the disease is unclear (for review see Doble, 1999 and Meldrum, 2000).

### 1.4.5.1. Amyotrophic lateral sclerosis

The evidence that AMPA receptors on spinal motoneurones are involved in amyotrophic lateral sclerosis (ALS) is of several types (Leigh and Meldrum, 1996; Meyer et al., 1998). There appears to be a reduction in the expression of GLT-1, a glial glutamate transporter, in the spinal cord and brain regions showing loss of motoneurones (Rothstein et al., 1995). In organotypic cultures of spinal cord, glutamate transport inhibitors cause degeneration of motoneurones. This can be prevented by AMPA receptor antagonists such as GYKI 52466 (Hirata et al., 1997; Rothstein and Kuncl, 1995). AMPA receptor antagonists protect against the toxic effects of mutations in Cu/Zn superoxide dismutase in cultured mouse neurones (Roy et al., 1998). Of particular interest are the drugs riluzole and gabapentin which both have proven neuroprotective effects on anterior horn cell degeneration in experimental animals and also have a proven (riluzole) effect on human ALS (Bensimon et al., 1994; Lacomblez et al., 1996; Louvel et al., 1997; Miller et al., 1996). Both compounds interfere with excitatory neurotransmission in the central nervous system, attenuate neuronal excitation and have potent anticonvulsant activity in a number of experimental paradigms (Doble, 1996; Mizoule et al., 1985). The efficacy of riluzole in men has been shown in two large clinical trials (Bensimon et al., 1994; Lacomblez et al., 1996; Louvel et al., 1997) which has demonstrated increased survival for ALS patients treated with riluzole when compared with controls.
1.4.5.2. Huntington’s Disease

Huntington’s disease (HD) may involve a primary metabolic or mitochondrial defect that causes striatal neurones to become vulnerable to excitotoxic effects of NMDA receptor activation (Beal et al., 1993). The pattern of neuronal loss in Huntington’s disease is similar to that seen in animals following experimental excitotoxic lesions to the striatum, suggesting a possible excitotoxic pathogenic factor (Koroshetz et al., 1990). Although the genetic deficit responsible for Huntington’s disease has been identified, the function of the gene product is unknown (MacDonald and Gusella, 1996).

Early reports of reductions in mitochondrial complexes 2 and 4 in the caudate of individuals with HD (Brennan et al., 1985; Stahl and Swanson, 1974) provided the first clues that defects in energy metabolism could be relevant to the pathology of HD. In support of this idea were the observations that damage to basal-ganglia structures occurred in humans who attempted suicide by ingestion of cyanide (Uitti et al., 1985) or by inhalation of carbon monoxide (Klawans et al., 1982) (both of which block complex 4 of the mitochondrial respiratory chain). In the mid 1980s, it was reported that individuals who ingested mildewed sugar cane contaminated with the compound 3-nitropropionic acid (3NP) developed putaminal necrosis that manifested as athetosis and dystonia (He, 1986; Hu, 1987) (3-NP is a suicide inhibitor of the mitochondrial enzyme succinate dehydrogenase). When injected systematically into rodents, 3-NP produces abnormal movements and bilateral striatal lesions that spare the same population of medium aspiny neurones that are resistant to cell death in HD (Hamilton and Gould, 1987; Palfi et al., 1996). Now, decreased ATP synthesis, for example, produced by a mitochondrial toxin, causes a partial depolarization of the neuron that results in the extrusion of Mg$^{2+}$. This leads to the influx of Ca$^{2+}$ and subsequent activation of ‘cell-death’ pathways. This model of neurodegeneration is
called 'weak excitotoxicity' (Albin and Greenamyre, 1992; Beal, 1994). NMDA-receptor-mediated cell death is also implicated in HD because NMDA-receptor agonists, in the same way as mitochondrial toxins, reproduce many of the neuropathological findings seen in HD (Beal et al., 1986). Additionally, NMDA-receptor number (hence 'glutamate-receiving' neurones) is reduced preferentially in the basal ganglia in both symptomatic (Greenamyre et al., 1985; Young et al., 1988) and presymptomatic individuals with HD (Albin et al., 1990).

1.4.5.3. Alzheimer’s Disease

Research on the aetiopathology of Alzheimer’s disease (AD) has focussed on the origins of the characteristic lesions (senile plaques and neurofibrillary tangles) that are observed postmortem in this disease, as well as on the genetic basis for familial forms of AD. These efforts have converged on the hypothesis that the cellular transformation of the membrane protein amyloid precursor protein into insoluble β-amyloid peptides is at the heart of the pathological process (Ashall and Goate, 1994). These β-amyloid peptides not only aggregate to form the principal constituents of senile plaques, but are also toxic to cultured neurones (Yankner et al., 1990). Several studies have investigated the relationship between excitotoxicity and β-amyloid toxicity in vitro (Koh et al., 1990; Mattson et al., 1992), and demonstrated that β-amyloid peptides appear to sensitise cultured neurones to excitotoxic cell death. This may imply that the formation of extracellular β-amyloid may render neighboring healthy neurones vulnerable to excitotoxicity, and provide a mechanism where excitotoxic damage may aggravate neurodegeneration.

1.4.5.4. Parkinson’s Disease

Parkinson’s disease (PD) is characterised by a massive and selective loss of dopaminergic
neurons in the nigrostriatal pathway. The key aetiological event that triggers the onset of this
disease is not known. The selective vulnerability of dopaminergic neurones in Parkinson’s
disease is thought to be related, at least in part, to their high sensitivity to oxidative stress (Hirsch
et al., 1998). Catabolism of dopamine by monoamine oxidase-B can produce large amounts of
reactive oxygen species, which can enter into cycles of free radical generating reactions of the
Fenton type with ferric ions present in large quantities in nigral cells (Gerlach et al., 1994). The
high level of lipid peroxidation seen in the substantia nigra of Parkinson’s disease patients bears
witness to this high level of oxidative stress (Dexter et al., 1989). Such free radical mechanisms
may thus play a key role in the pathological process in the Parkinson’s disease. There is no a
priori reason to suspect that excitotoxic mechanisms may be involved in the primary
aetiopathology of this disorder, although once the nigral cells have been weakened by oxidative
stress, they might become susceptible to slow excitotoxicity, which would proffer the coup de
grace (Doble A., 1999). However, it can be argued that, since striatal neurones are normally
spared in typical Parkinson’s disease, glutaminergic over-activity per se cannot be held
responsible for the neurodegeneration associated with this disease. Instead the premature loss of
nigrostriatal fibers, a feature characteristic of Parkinson’s disease, could be related to factors that
are intrinsic to dopaminergic neurones (see review by Doble, 1999).

On the other hand, Plaitakis and Shashidharan (2000) believe in the indirect evidence suggesting
involvement of glutamatergic mechanisms in the pathogenesis of this disorder. Two major
mechanisms protect neurones from glutamate-induced toxicity: (a) removal of synaptic
 glutamate via a high affinity uptake carried out by cytoplasmic membrane proteins known as
excitatory amino acid transporters (EAAT); and (b) metabolism and recycling of glutamate by
synaptic astrocytes via glutamine synthetase, an ATP-requiring reaction. However, when extra-
cellular glutamate levels are high (0.5-1.0 mM), glutamate metabolism may be shifted toward the ATP-generating oxidative deamination (glutamate dehydrogenase)-TCA cycle pathway. They cloned and characterized two human glutamate dehydrogenases (GDH), one of which is nerve tissue specific. This isoenzyme requires ADP for its activity and it may become functional when cellular energy charge is low. They also cloned three human glutamate transporters. One of these (EAAT3) is neuron specific. In situ hybridization studies using human brain revealed that the pigmented dopaminergic neurones, which degenerate in PD, express EAAT3 at high levels. Primary nerve tissue cultures derived from rat ventral mesencephalon were established and studied for their ability to metabolize glutamate. Results showed that mature cultures expressing high levels of GDH activity were capable of rapidly utilizing glutamate added to the medium at high concentrations (1-1.2 mM). This was associated with little release of aspartate and alanine into the medium. In contrast, immature cultures expressing low GDH activity utilized glutamate at lower rates while releasing substantial amounts of aspartate and alanine into the medium. These data suggest that immature mesencephalic cells metabolize a substantial fraction of the glutamate they take up from the medium via the transamination pathway, compared to mature mesencephalic cultures. Immunocytochemical studies on these cultures revealed that dopaminergic neurones (identified by their tyrosine hydroxylase content) showed intense staining for GDH. Furthermore, inhibition of GDH expression by antisense oligonucleotides was toxic to cultured mesencephalic neurones, with dopaminergic neurones being affected at the early stages of this inhibition. Hence, the dense expression by dopaminergic neurones of proteins involved in the transport and metabolism of glutamate may serve particular biological needs intrinsic to these cells. Further studies are required to test whether these properties render these neurones vulnerable to excitotoxic mechanisms or to abnormalities of glutamate metabolism.
1.4.6. Glutamate metabotropic receptors and Neurodegeneration

The predominant effect of Group I metabotropic glutamate receptor activation is excitatory; agonists acting on mGluR1 or mGluR5 (such as 1S,3R-1-amino-1,3-cyclopentanedicarboxylate and 3,5-dihydroxyphenylglycine) when injected focally into the brain produce epileptic activity and focal neurodegeneration (Sacaan and Schoepp, 1992).

This is probably related to reductions in several potassium conductances producing membrane depolarization. There is also potentiation of NMDA receptor–mediated conductance changes and excitotoxicity (McDonald and Schoepp, 1992). There is, however, one Ca\(^{2+}\) sensitive K\(^+\) channel that is opened by mGluR1 activation leading to hyperpolarization (Fiorillo and Williams, 1998). That activation of group I mGluR contributes to cell death after cerebral ischemia and traumatic brain injury is suggested by reports that Group I receptor antagonists can be neuroprotective in model systems (Mukhin et al., 1996).

In cell cultures, a variety of effects have been described. In hippocampal cell line HT-22 expressing group I mGluR but not ionotropic receptors, a protective effect of glutamate can be demonstrated against oxidative stress and against glucose deprivation (Sagara and Schubert, 1998). Glutamate preexposure has the effect of up-regulating mGluR1 and mGluR3.

Nicoletti and his colleagues, in a remarkable series of studies employing cocultures of neurones and astrocytes, showed that activation of Group II receptors on astrocytes is neuroprotective via release of a neurotrophic factor, TGF-β (Bruno et al., 1998a; Bruno et al., 1997). N-acetylaspartylglutamate (NAAG), the endogenous mGluR3 agonist, is neuroprotective against striatal quinolinate lesions (Orlando et al., 1997) and against NMDA excitotoxicity in mixed
cortical cultures (Bruno et al., 1998b). TGF-β and Group II mGlu agonists also protect against apoptosis induced by β-amyloid (Ren and Flanders, 1996).

1.4.7. Neuronal Cell Death: A Demise with Different Shapes

Brain cells that are compromised by excessive glutamate-receptor activation, Ca\(^{2+}\) overload, oxygen radical or by mitochondrial and DNA damage can die by apoptosis or necrosis (Dirnagl et al., 1999). The decision in part, depends on the nature and intensity of the stimulus, the type of cell, and the stage it has reached in its life-cycle or development (Leist and Nicotera, 1998). Necrosis is the predominant mechanism that follows acute, permanent vascular occlusion, whereas in milder injury, cell suicide becomes unmasked and death resembles apoptosis, particularly within the ischaemic penumbra.

Until recently, developmental neuronal death or neuronal demise resulting from excitotoxicity or exposure to neurotoxins have been considered as conceptually and biochemically different types of death. It had been thought that injury by neurotoxins or glutamate lacked the regulated series of events involved in a cell death programme but rather led invariably to necrosis (Gwag et al., 1995). However, recent evidence suggests that key regulators of apoptosis, i.e. p53 (Xiang et al., 1998), Bax (Miller et al., 1997), Bcl-2 (Martinou et al., 1994) and caspases are also involved in excitotoxic–ischaemic neuronal injury. For example, prevention of neuronal death in cerebral ischaemia by caspase inhibitors (Hara et al., 1997; Loddick et al., 1996) suggests that at least some mechanisms of this death are similar to those of other forms of programmed cell death (Leist and Nicotera, 1998). This concept is directly corroborated in in vitro experiments showing that excitotoxicity can cause both apoptosis and necrosis (Ankarcrona et al., 1995), and that caspase inhibitors significantly reduce the extent of
excitotoxic damage (Du et al., 1997; Leist et al., 1998; Leist et al., 1997c).

Excitotoxicity is further complicated by the prospect that execution of alternative subroutines might differ in different neuronal subpopulations. For example, the contribution of individual receptors could be relevant in the 'decision' of the mode of cell death (Portera Cailliau et al., 1997). The degree of Ca$$^{2+}$$ overload might also convert the mode of cell death in some cases from apoptosis to necrosis (Gwag et al., 1995). Autocrine neuronal apoptosis elicited by synaptic release of excitotoxins seems to favour apoptosis (Leist et al., 1997c), whereas massive glutamate receptor stimulation by exogenous glutamate can additionally elicit rapid necrosis (Ankarcriona et al., 1995). Part of the problem in determining the relevance of apoptosis in neurodegeneration has originated from the assumption that inhibitors of protein synthesis protect cells from the apoptotic, but not necrotic, demise. However, cell death associated with typical apoptotic morphology can occur in the presence of protein synthesis inhibitors (Weil et al., 1996), whereas neither neuronal apoptosis triggered by autocrine excitotoxicity, NO donors, nor Hg$$^{2+}$$ are modified by agents that inhibit protein synthesis (Leist et al., 1997a).

1.4.8. The Shaping of Neuronal Death: Recruitment of Different Subroutines

Contrasting interpretations of the mode of excitotoxic death have originated from the significance attributed to single apoptosis-linked alterations in dying cells. In cerebellar granule cells, glutamate induces typical signs of apoptosis, such as chromatin condensation with the biochemical correlate of high-molecular-weight DNA fragmentation. However, oligonucleosomal DNA laddering and nuclear fragmentation, which are also indicative of
apoptosis, are less apparent (Ankarcrona et al., 1995). On the other hand, cerebellar granule cells exposed to other apoptotic stimuli (for example, staurosporine or 4-hydroxynonenal) exhibit the typical DNA laddering, chromatin condensation and nuclear fragmentation (Koh et al., 1995; Krumen et al., 1997). Because DNA laddering seems to depend on nucleases activated by caspase 3, it is not surprising that, in some forms of cell death where this caspase is not active, its downstream effects are also lacking. Caspase 3-independent apoptosis is well documented in neurones (Kuida et al., 1996; Leist et al., 1998; Leist et al., 1997c; Miller et al., 1997; Stefanis et al., 1996) and other cells (Kuida et al., 1996). Thus, the existence of alternative execution pathways, which become active in certain neuronal populations but not in others, could in part account for the multiple shapes of excitotoxic demise. Execution patterns and the resulting morphology can also depend on the combination of death stimuli or on selective interference of each stimulus with individual subroutines. For example, caspase 3 processing and the downstream subroutines can be impaired by concomitant intracellular ion influx (Hampton et al., 1998). Apoptosis triggered by primary cytoskeletal damage, for example that elicited by colchicine (Bonfoco et al., 1995), obviously gives rise to a different shape of demise than that observed in developmental or receptor-triggered apoptosis, although they could have some execution subroutines in common. Toxic reactions such as those elicited by oxidative or nitrosative stress could at the same time trigger the core death programme and inhibit some of its shape-determining pathways (Leist et al., 1997c). There is evidence that the intensity of the insult also determines the shape of neuronal demise. On exposure to mild excitotoxic insults, neurones die exhibiting the typical apoptotic features. With stronger insults, the degradative processes responsible for apoptotic morphology are often prevented and neurones die by necrosis (Ankarcrona et al., 1995; Bonfoco et al., 1995). This could be due to untimely energy dissipation (Ankarcrona et al., 1995); in fact, recent evidence has revealed that, when energy levels are
rapidly compromised, cells triggered to undergo apoptosis are instead forced to die by necrosis (Eguchi et al., 1997; Leist et al., 1997b).

Assuming that the activation of different pathways for the execution of cell death is determined by the level of injury, it is apparent that caspase inhibitors, for example, might be most effective in areas where the intensity of the excitotoxic insult is low, and ineffective in regions where the stress is more intense. Thus, therapeutic strategies that combine agents to reduce the overall intensity of the insult and the overall lesion size (i.e. NMDA receptor antagonists or selective NOS inhibitors) with agents that block execution of apoptosis (caspase inhibitors) might prove more successful than individual treatments (Ma et al., 1998).

1.4.9. Calcium: Too much or Too little

Survival of neuronal cells appears critically dependent upon an optimal intracellular 'calcium set-point' (Johnson and Deckwerth, 1993), also seen in neurite outgrowth (Lipton and Kater, 1989). Young sympathetic neurones that are heavily dependent on growth-factor support for survival have lower basal $[\text{Ca}^{2+}]_c$ levels than older sympathetic neurones with less growth-factor dependence, suggesting three major $\text{Ca}^{2+}$ states: (1) an inadequate $[\text{Ca}^{2+}]_c$ level (possibly higher in injured cells?) where neurones are at risk for apoptosis and heavily dependent upon trophic support; (2) an intermediate $[\text{Ca}^{2+}]_c$ level where neuronal survival is favoured and dependence upon trophic support is minimal; and (3) an elevated $[\text{Ca}^{2+}]_c$ level that is cytotoxic (Fig 1.4). The dotted line represents the average $[\text{Ca}^{2+}]_c$ in healthy neurones. The horizontal axis represents the situation in undamaged cells. Here, as we pass from zero $[\text{Ca}^{2+}]_c$ through the normal average $[\text{Ca}^{2+}]_c$ level to high $[\text{Ca}^{2+}]_c$, cells are viable until a high $[\text{Ca}^{2+}]_c$ threshold is passed, after which
they die by necrosis. Considering cells at normal \([\text{Ca}^{2+}]_c\), increased injury, represented by increased deviation upwards along the dotted line, induces first successful repair and survival then apoptosis and finally, if damage is severe, necrosis.

![Figure 1.4. Speculative concept diagram of the relationship between an apoptosis-necrosis continuum, the severity of ischaemic injury and \([\text{Ca}^{2+}]_c\). High \([\text{Ca}^{2+}]_c\) and more severe injury promote cell death through necrosis, whereas low \([\text{Ca}^{2+}]_c\) and milder injury promote cell death through apoptosis. Adapted from Nature, Volume 399, Supp, 24 June 1999.](image)

Consistent with the \(\text{Ca}^{2+}\) set-point hypothesis, prolonged exposure to one of several different antagonists of voltage-gated \(\text{Ca}^{2+}\) channels, or medium with reduced \(\text{Ca}^{2+}\), can induce cultured cortical neurones to undergo apoptosis (Koh and Cotman, 1992). Thus, NMDA itself can
attenuate apoptosis; also in cerebellar granule cell culture, neurite growth, differentiation and cell survival are all stimulated by NMDA receptor activation (Burgoyne et al., 1993). Conversely, NMDA-antagonist drugs can enhance neuronal apoptosis in cultures (Hwang and Koh, personal communication) and in the developing rodent brains (Ikonomidou et al., 1999). These drugs support an idea that NMDA-antagonist drugs may be a double-edged sword when applied as a therapy in brains after ischaemic injury. Although the drugs may be beneficial in attenuating neuronal necrosis that is triggered by overstimulation of NMDA receptors and consequent $[\text{Ca}^{2+}]_c$ overload, their benefit may be counterbalanced by a deleterious enhancement of $[\text{Ca}^{2+}]_c$ starvation in neurones prone to apoptosis (Figure 1.5). As noted in figure, antixcitotoxic interventions such as glutamate antagonists reduce necrosis but may exacerbate apoptosis by reducing $\text{Ca}^{2+}$ influx. On the other hand, antagonists used to attenuate apoptosis do not interfere with necrosis. $[\text{Ca}^{2+}]_c$ overload and $[\text{Ca}^{2+}]_c$ starvation are of course opposite states, but both could be triggered by the same ischaemic event, separated in space or time. Excitotoxic $[\text{Ca}^{2+}]_c$ overload may predominate close to the ischaemic core and acutely, whereas further from this core and at later time intervals, $[\text{Ca}^{2+}]_c$ starvation and apoptosis may predominate, at least in certain cells.
Figure 1.5. Ischaemia triggers necrosis and apoptosis in parallel, adapted from Nature, Vol 399, Supp, 24 June 1999. Some anti-apoptotic interventions, such as cyclohexamide or z-VAD.fmk, attenuate apoptosis without affecting necrosis. On the other hand, antiexcitotoxic interventions such as glutamate antagonists may exacerbate apoptosis by reducing $\text{Ca}^{2+}$ influx and $[\text{Ca}^{2+}]_c$.

1.4.10. Mitochondria: Role in Neurodegenerative Diseases

Mitochondria provide ATP that fuels a multitude of ion pumps which produce and maintain critical voltage and ion gradients across neural membranes. $\text{Na}^+$ is actively pumped out of the cells by $\text{Na}^+$/K$^+$-transporting ATPase, which helps set up the resting membrane potential. The $[\text{Ca}^{2+}]_c$ is maintained at a level some 10,000 times less than that outside the cell by means of ATPases that actively move $\text{Ca}^{2+}$ out of the cell or into intracellular storage organelles such as the ER. Most of the ATP produced in the brain is used to restore the ionic gradients that have
been run down by synaptic transmission (Erecinska and Silver, 1989). Morphological and immunohistochemical studies show that glycolytic enzymes of mitochondria are concentrated in dendrites and axon terminals where ion flux occurs associated with synaptic transmission (Knull, 1978).

Evidence that mitochondria have a crucial role in both necrotic and apoptotic cell death is accumulating rapidly. Both these conditions are distinct forms of cell death (as defined morphologically); however, in neuronal populations they can either coexist or be sequential events, depending on the severity of the initiating insult (Ankarcrona et al., 1995). Cellular energy reserves appear to have an important role in these two forms of cell death, with apoptosis favored under conditions with mild insults and preserved ATP levels. Necrosis is frequently induced by toxic insults, such as glutamate excitotoxicity, in which ATP is depleted. An impairment of neuronal energy metabolism resulting in reduced levels of ATP has several consequences. Interferring with the function of the Na⁺/K⁺-ATPase inhibits the repolarization of synaptic membranes after a depolarizing glutaminergic stimulus. Defective repolarization will lead to prolonged or inappropriate opening of some voltage-dependent Ca²⁺ channels, and will decrease the protective, voltage-dependent block of NMDA channels, which as a result will be activated by endogenous level of glutamate. Thus, ion flux, especially the inward movement of Na⁺ and Ca²⁺ through the high-conductance, Ca²⁺ permeable NMDA channels, will be enhanced. A critical result of this is that the [Ca²⁺]c increases dramatically. An increased intracellular Na⁺ concentration, created by increased Na⁺ influx and impaired Na⁺/K⁺-ATPase function, decreases the effectiveness of the Na⁺-Ca²⁺ antiport system, which would normally remove [Ca²⁺]c from the cell. Impaired energy metabolism prevents ATP-dependent extrusion of [Ca²⁺]c and ATP-dependent storage of Ca²⁺ in the ER.
These glutamate-mediated disturbances in ion concentrations might potentiate the metabolic
defect. The enhanced flux of Ca\(^{2+}\) and Na\(^{+}\) through NMDA channels in ATP deficient cells
creates an even greater demand for ATP by the active ion pumps which soon depletes the
cytosolic ATP. At high [Ca\(^{2+}\)_c], mitochondria take up Ca\(^{2+}\) from the cytosol in preference to
generating ATP. In response to glutamate and NMDA there is usually a prominent and
persistent depolarization of the mitochondrial membrane potential (Schinder et al., 1996; White
and Reynolds, 1996), followed by a depletion of energy reserves that results in necrosis
(Bordelon et al., 1997). A requirement for mitochondrial Ca\(^{2+}\) uptake in glutamate-mediated
excitotoxicity has been recently demonstrated (Budd and Nicholls, 1996; Stout et al., 1998).
Activation of NMDA receptors leads to a more-rapid increase in mitochondrial Ca\(^{2+}\) levels and a
greater increase in free-radical production and NO production than does activation of non-
NMDA receptors (Dugan et al., 1995; Peng and Greenamyre, 1998; Reynolds and Hastings,
1995) (Fig 1.6). During the respiration process, O\(_2\) is progressively reduced by a controlled
supply of four electrons to yield water. However, the incomplete reduction of O\(_2\) is possible, and
leads to the formation of chemical entities that are still potent oxidants. These molecules are
known as ROS. Following a one-, two- or three-electron reduction, O\(_2\) may generate
successively O\(_2^-\) (superoxide radical anion), H\(_2\)O\(_2\) (hydrogen peroxide) or OH\(^-\) (hydroxyl
radical). ROS are able to oxidize cellular macromolecules, including DNA, which leads to
activation of poly-ADP ribose polymerase (PARS) which results in ATP and NAD depletion,
which directly contributes to cell death. The production of the various radicals is linked via
chemical or enzymic reactions. Superoxide dismutase (SOD) converts O\(_2^-\) into H\(_2\)O\(_2\), and the
latter can generate OH\(^-\) in the presence of Fe\(^{2+}\) cations (Fenton reaction). Also, NO can also be
oxidized into reactive nitric oxide species, which may show behaviour similar to that of ROS. In
particular the combination of NO and $O_2^{--}$ can yield a strong biological oxidant, ONOO$^-$ (Murphy et al., 1998).

Mitochondria are essential in controlling specific apoptosis pathways (Green and Reed, 1998). The mechanisms by which they exert this function include release of caspase activators, such as cytochrome c, caspase 9 and apoptosis-inducing factor (AIF), and disruption of oxidative phosphorylation (Fig. 1.6). Activated caspases are protein-cleaving enzymes and thereby modify crucial homeostasis and repair proteins that, in turn, disassemble and kill cells. More than 30 proteins can be cleaved, including the DNA-repairing enzyme PARS and the cytoskeletal protein, gelsolin, in addition to presenilins, huntingtin protein and other caspases. It is known from related models that caspases become activated when cytochrome C, released from mitochondria, activates an apoptosome complex [apoptosis-activating factor (APAF1) plus procaspase 9] in the presence of ATP/dATP (Green and Reed 1998). Cytochrome C can enter the cytosol from its location on the external side of the inner mitochondrial membrane. The redistribution of cytochrome c during apoptosis can be prevented by overproduction of the anti-apoptotic protein Bcl2, which is localized to the outer mitochondrial membrane. Oversynthesis of the pro-apoptotic protein Bax triggers cytochrome c efflux from mitochondria (Desagher et al., 1999).

The mitochondrial PTP could be crucial in both necrotic and apoptotic cell death. Its activation increases the inner mitochondrial membrane permeability to solutes with a molecular mass of up to 1.5 kDa. Proposed components of the PTP include the inner mitochondrial membrane adenine nucleotide transporter that interacts with cyclophilin D, the voltage-dependent anion channel in the outer membrane, the peripheral GABA receptor in the outer membrane and mitochondrial
creatine kinase. Bax interacts with the voltage-dependent anion channel to accelerate opening of the PTP, contributing to cytochrome c release. The opening of the channel is favored by Ca\(^{2+}\) and oxidizing agents, whereas closure is favored by protons (low matrix pH) and adenine nucleotides (ADP). Cyclosporin A is an effective blocker of the channel that appears to prevent an interaction of cyclophilin with the adenine nucleotide transporter. It also reduces excitotoxicity \textit{in vitro} (Schinder et al., 1996; White and Reynolds, 1996) and reduces both hypoglycemic and ischemic cell death \textit{in vivo} (Friberg et al., 1998).

A consequence of mitochondrial dysfunction is increased generation of free radicals and oxidative damage, which are strongly implicated in the pathogenesis of neurodegenerative diseases. Mitochondria, the most important physiological source of O\(_2^\cdot\) in animal cells, are estimated to produce 2–3 nanomoles of O\(_2^\cdot\)/min/mg protein (Boveris and Chance, 1973).
Figure 1.6. Involvement of mitochondria in cell death adapted from TINS July 2000, Vol. 23, No. 7, 299 (a) depicts the effects of a severe excitotoxic insult that results in cell death by necrosis, whereas (b) depicts the results of a mild excitotoxic insult that results in apoptosis. After a severe insult (such as ischemia) there is a large increase in glutamate-mediated activation of NMDA receptors, an increase in intracellular $\text{Ca}^{2+}$ concentration, activation of NOS, increased mitochondrial $\text{Ca}^{2+}$ and superoxide generation, followed by the formation of ONOO$^-$. An increase in superoxide generation can also lead to increased $\text{H}_2\text{O}_2$ production, which can then react with transition metal ions ($\text{Fe}^{3+}$ or $\text{Cu}^+$) to generate the highly reactive OH radical. This results in damage to cellular macromolecules, including DNA, which leads to activation of poly-ADP ribose polymerase (PARS). Activation of PARS results in ATP and NAD depletion, which directly contributes to cell death. Both mitochondrial accumulation of $\text{Ca}^{2+}$ and oxidative damage lead to activation of the permeability transition pore (PTP) that is linked to excitotoxic cell death. A mild excitotoxic insult (b) can occur because of impaired functioning of excitotoxicity-amino-acid receptors, which allows more $\text{Ca}^{2+}$ influx, impaired functioning of other ionic channels or impaired energy production, which can allow the voltage-dependent NMDA receptor to be activated by resting levels of glutamate. This can then lead to increased mitochondrial $\text{Ca}^{2+}$ and free-radical production (by mitochondria and via activation of neuronal NOS, which generates ONOO$^-$), yet relatively preserved ATP generation. The mitochondria can then release cytochrome c, caspase 9, apoptosis inducing factor (AIF) and perhaps other mediators that cause apoptosis. The interaction of dATP and Aparf converts pro-caspase 9 to an active caspase, which initiates a caspase cascade that leads to apoptotic cell death. The precise role of the PTP in this mode of cell death is still being clarified but there does appear to be involvement of the adenine nucleotide transporter, which is a key component of the PTP.
Having described excitotoxicity in general, I will now discuss what is happening in AIDS dementia.

1.5. Direct Effects of Gp120 on Neurones

1.5.1. Involvement of NMDA Receptor in Neuronal killing by Gp120

Gp120 was initially observed to be directly neurotoxic, by causing a notable increase in the $[\text{Ca}^{2+}]_\text{c}$ levels through either VOCC or ionotropic receptors (Brenneman et al., 1988; Dreyer et al., 1990; Lipton and Gendelman, 1995; Pittaluga et al., 1996). By virtue of the protective effect of the NMDA receptor and Ca$^{2+}$ channel antagonists it was hypothesised that gp120 caused an overstimulation of the NMDA ionotropic glutamate receptor (Lipton, 1992b). There is evidence for the direct binding of gp120 to the glycine site of the NMDA receptor (Pittaluga et al., 1996). This binding was postulated to cause conformational changes to the receptor, which may allow the ion channel to open for longer periods and increase the incoming ionic flux to such levels as to cause permanent changes within the cell. This may explain the overstimulation of the NMDA receptor and its concomittant excitotoxicity initiated by gp120. This receptor has been implicated in a number of other neurological diseases, like the ischaemic-anoxic brain lesions in patients with stroke (Lipton and Rosenberg, 1994). Moreover, the NMDA receptor is one of the main receptors involved in the process of long-term potentiation, which is thought to be the molecular correlate of short-term memory in the mammalian brain. Overstimulation of this neuroplastic long-term potentiation has been proposed as a basis for neuronal damage (McEachern and Shaw, 1996). Thus, it is possible to envisage that interference with this process by gp120 through NMDA receptor-overstimulation may interfere with short-term memory formation leading to
cognitive impairments and even dementia. In line with this, ventricular gp120 injection in rats caused a learning impairment in the Morris swim maze paradigm (Hill et al., 1993), and retarded the development of complex motor behaviour (Barks et al., 1995).

1.5.2. Involvement of Chemokine Receptor in Neuronal killing by Gp120

A qualitatively different mechanism by which gp120 might kill neurones is suggested by the recent finding that the chemokine receptors CCR5 and CXCR4, which are thought to be the normal chemokine receptors for gp120 binding to macrophages and lymphocytes, are also present on neurones. The identification of chemokine receptors as coreceptors for HIV-1 entry and their differential usage during viral entry has resulted in the reclassification of HIV-1 strains based on their chemokine receptor usage (Berger et al., 1998). Strains of HIV-1 generally use one of two coreceptor molecules in addition to the cell surface receptor CD4 expressed on cells susceptible to infection. It was shown that for infection of macrophages by M-tropic (i.e., macrophage-selective) strains of HIV-1, CD4 and the chemokine receptor CCR5 were required and that the infection of T lymphocytes by T-tropic (i.e., T lymphocyte-selective) strains of HIV-1 used the CXCR4 receptor (Bacon et al., 1995; Baggioolini, 1998; Broder and Collman, 1997; Broder and Dimitrov, 1996; Luster, 1998). The precise determinants of HIV-1 tropism in vivo are clearly very complex and continue to be defined. Thus, some strains of HIV-1 exhibit dual tropism, and others can also use chemokine receptors in addition to CCR5 and CXCR4. The list of the chemokine receptor is expanding and are no less important in studies of HIV-1-associated CNS disease.
Neurones express an array of chemokine receptors. Numerous chemokine receptor messages are expressed in rat hippocampal neurones, including CCR1, CCR4, CCR5, CCR9/10, CXCR2, CXCR4, and CX3CR1 (Meucci et al., 1998). These studies also demonstrated that stromal derived factor-1 alpha (SDF-1α) and other chemokines were able to block gp120-mediated neurotoxicity. 

In vivo, neuronal CXCR4 expression is localized to the hippocampus (Lavi et al., 1997), while CCR5 expression is evident in the hippocampus and cerebellum (Rottman et al., 1997). Neuronal cells produced by differentiation of the human NTera2 cell line express CXCR2, CXCR4, CCR1, and CCR5, which are fully functional in their capacity to bind their respective chemokines and elicit chemotactic responses (Hesselgesser et al., 1997). This same study demonstrated that HIV-1 was antagonistic to SDF-1α binding to CXCR4.

This suggests that gp120 might bind directly to chemokine receptors on neurones and cause death. Binding of gp120 to neurones might initiate several events, including interference with the normal trophic effects of chemokines. These events may be directly neurotoxic or could enhance the sensitivity of neurones to other factors such as glutamate receptor activation (Lipton, 1994a; Meucci et al., 1998; Meucci and Miller, 1996).

The extent of neuronal HIV-1 infection and the contribution to HIV-1-associated neuropathogenesis is not yet clear. Early studies using less sensitive detection techniques suggested that neurones were not infected during HIV-1 infection of the neurones. However, recent investigations using the more sensitive in situ PCR technique have identified small populations of HIV-1-infected neurones (Bagasra et al., 1996; Nuovo et al., 1994). In one study, infected neurones were identified in 17 of the 22 brains examined and the number of infected neurones correlated loosely with the severity of HAD (Bagasra et al., 1996). Furthermore, few
neurones expressed unspliced HIV-1 mRNA, which is indicative of productive viral replication (Bagasra et al., 1996). The relevance of these small populations of HIV-1-infected neurones to the development of HAD has yet to be determined. However, their contribution to the pathogenesis of HIV-1 CNS infection is almost certainly considerably less than the impact of neurotoxicity and neuronal death caused indirectly by production of viral and cellular neurotoxins by other neuroglial cell populations (see review by Krebs, 2000).

**1.6. Brain Macrophage-Mediated Neuronal Injury in HAD**

A paradox exists between the small numbers of productively HIV-1-infected brain macrophages and microglia and the severe clinical cognitive and motor deficits that some patients with AIDS experience. It seems as though some sort of amplification mechanism is required to explain these observations. Moreover, the secretion of neurotoxins by HIV-1-infected macrophages is likely to be regulated by a complex series of intracellular interactions between several different brain cell types, including mononuclear phagocytes, astrocytes and neurones (Lipton, 1992b). HIV-1-infected brain mononuclear phagocytes, especially after immune activation, secrete substances that contribute to neurotoxicity (Genis et al., 1992; Giulian et al., 1996; Nottet et al., 1995; Pulliam et al., 1991). These include arachidonic acid and its metabolites, PAF, proinflammatory cytokines (TNF-α and IL-1β), amines, free radicals such as NO and O₂⁻ and the glutamate like agonist, cysteine (Bukrinsky et al., 1995; Gelbard et al., 1994; Genis et al., 1992; Giulian et al., 1996; Yeh et al., 1994). In a similar fashion, macrophages activated by gp120 release arachidonic acid and its metabolites, TNF-α, IL-1β, and cysteine, which can lead to NMDA receptor mediated neurotoxicity (Wahl et al., 1989; Yeh et al., 1994).
Numerous studies have demonstrated elevated levels of cytokines TNF-α (Franciotta et al., 1989; Perrella et al., 1992), IL-6 (Gallo et al., 1989; Perrella et al., 1992), IL-1β (Gallo et al., 1989), and IL-1α (Perella et al., 1992) in the CSF of HIV-1-infected patients. Elevated levels of TNF-α, IL-1β, and TGFβ mRNA expression in HIV-1-infected brains have also been reported (Wahl et al., 1991; Wesselingh et al., 1993). Increased expression of TNF-α has also been associated with the diagnosis of HAD (Wesselingh et al., 1993). Also, increases in beta 2-microglobulin (β2M) and neopterin, considered to be markers of immune activation, have been observed in the CSF of HIV-1-infected patients with HAD (Brew et al., 1990; Brew et al., 1992; Brew et al., 1996). β2M, the light chain of the major histocompatibility class 1 (MHC-1) cell surface molecule, is upregulated by a number of cytokines, including IFNγ (Price, 1994). PAF, secreted by HIV-1-infected monocytes (Genis et al., 1992), is also found at elevated levels in HAD patients (Perry et al., 1998).

Elevated levels of cytokine expression during HIV-1 CNS infection have numerous effects on the normal function and architecture of the brain. TNF-α can cause damage to myelin and myelin-producing oligodendrocytes (Selmaj et al., 1990; Wilt et al., 1995) as well as influence HIV-1 gene expression in monocytes (Vitkovic et al., 1990) and glial cells (Tornatore et al., 1991). TNF-α, produced in high levels by HIV-1-infected monocytes cocultured with astrocytes (Genis et al., 1992), is also toxic to primary human neurones (Gelbard et al., 1993). Also, TNF-α can induce iNOS expression with consequent NO production in cultured astrocytes (Simmons and Murphy, 1993). PAF, which is also found in the CSF during HIV-1 infection, is also toxic to primary neurones (Gelbard et al., 1994) as well as the NTera 2 neuronal cell line (Westmoreland et al., 1996). Exposure to PAF can result in neuronal apoptosis (Perry et al., 1998), which may be mediated by signal transduction pathways that differ from those used by
TNF-α (Pulliam et al., 1998). Arachidonic acid and oxygen radicals can lead to increased release or decreased re-uptake of glutamate, which can also contribute to this type of neuronal damage (Volterra et al., 1994). Quinolinate can also act as a glutamate-like agonist to injure neurones (Heyes et al., 1992). Although TGF-β, IL-1β, and IL-6 are all elevated during HAD or HIV-1 CNS, their impacts on neuroglial function/survival and their participation in HAD are not as clearly defined (Kolson et al., 1998).

Primary macrophages and the U-937 monocytic cell line both release increased levels of the CC chemokine MCP-1 in response to HIV-1 infection, suggesting that the same may occur in microglial cells (Mengozzi et al., 1999). Elevated levels of MCP-1 in the CSF, which correlate with the appearance of HIVE (Cinque et al., 1998), may result in recruitment of greater numbers of HIV-1-susceptible mononuclear immune cells to the CNS. In addition, during the progression of HAD and the decline of CD4+ T lymphocytes in the peripheral circulation, infected macrophages and microglia may take on the role of maintaining macrophage-tropic viral strains that predominate in the brain. These cells produce and release soluble gp120, Tat, and Nef (Lipton, 1992c; Sabatier et al., 1991; Werner et al., 1991) which are also potential neurotoxins. Gp120 shed by infected microglia may also indirectly impact neuronal function by binding to oligodendrocytes and causing demyelination (Kimura Kuroda et al., 1994).

Despite the level of neuronal degeneration in HAD suggested by the large body of clinical findings, HIV-1 infection within the CNS is supported not by neurones, but by microglial cells. Numerous studies have demonstrated that infected microglial cells, macrophages, and MGCs were present in HIV-1-infected brains at numbers that greatly exceeded the populations of any other infected cell type (Epstein et al., 1984; Gabuzda et al., 1986; Stoler et al., 1986; Wiley et
al., 1986). Even when PCR was employed to detect low copy numbers of HIV-1 nucleic acids (Bagasra et al., 1996; Nuovo et al., 1994), cells identified as microglia and macrophages were shown to be the predominant HIV-1-infected cell type. HIV-1 infection of microglial cells may contribute indirectly to neurotoxicity through interactions between gp120 and CXCR4 expressed on microglial cells, which lead to macrophages/microglial cell activation and release of soluble mediators that induce neuronal apoptosis (Kaul and Lipton, 1999). This hypothesis is corroborated by detection of neuronal apoptosis coincident with the regional appearance of MGCs and p24 expression in HIV-1-infected brains (Adle Biassette et al., 1999).

The importance of macrophages/microglia in brain (as explained above) has been explored in detail in primary cultures as well. The fact that gp120 no longer kills neurones in primary cultures when the culture is selectively depleted of microglia shows an obligate role of microglia in neuronal killing induced by gp120 (Lipton, 1992c).

On the other hand, Meucci and colleagues grew primary hippocampal cultures free from any microglial contamination (Meucci et al., 1998). In these cultures they observed a rise in $[\text{Ca}^{2+}]_c$ on addition of gp120 and also delayed neurotoxicity induced by gp120. Several chemokines were able also to block gp120-induced apoptosis of hippocampal neurones, both in the presence and absence of the glial feeder layer. They thus emphasize that chemokine receptors may directly mediate gp120 neurotoxicity.

1.7. Astrocytes

The contribution of HIV-1 replication in astrocytes to CNS viral burden and neuropathogenesis, the recent subject of an extensive review (Brack Werner, 1999), is still a topic of active debate.
Early studies of patients with HIV-1-associated encephalopathy indicated that HIV-1 was located predominantly in MGCs, macrophages, and microglia, but could also be identified to a lesser extent in astrocytes (Epstein et al., 1984; Stoler et al., 1986; Wiley et al., 1986). Investigators in subsequent studies have demonstrated that the presence of HIV-1-specific nucleic acids and antigens in cells identified biochemically and morphologically as astrocytes within postmortem tissue samples from AIDS patients with encephalopathy or HAD (Bagasra et al., 1996; Nuovo et al., 1994; Ranki et al., 1995; Saito et al., 1994). Supporting in vitro investigations have shown that human astrocytes in both primary cultures and continuous cell lines are susceptible to HIV-1 infection (Cheng Mayer et al., 1987; Chiodi et al., 1987; Dewhurst et al., 1987; Harouse et al., 1989; Tornatore et al., 1991). Infection of these cells results in low levels of HIV-1 replication characterized by limited expression of viral antigens and nucleic acids, no changes in cell mortality or morphology, and very low to undetectable levels of progeny virus production (Cheng Mayer et al., 1987; Chiodi et al., 1987; Dewhurst et al., 1987). Studies have proposed that the restrictive level of replication in astrocytes may be due not only to the lack of CD4 expression, but also to cell type-specific blocks in the replicative cycle (Neumann et al., 1995; Niikura et al., 1996). Astrocytes supporting restricted HIV-1 replication may act as viral reservoirs in the CNS, producing elevated levels of HIV-1 following reactivation by cytokines associated with CNS infection. Astrocyte infection may also be enhanced by the presence of co-infecting pathogens like cytomegalovirus (McCarthy et al., 1998).

Holden and colleagues, 1999 found that increase in \([\text{Ca}^{2+}]_c\) in neurones appear to be due to gp120 activation of astrocytes and a vicious cycle that includes Na\(^+/\text{H}^+\) exchanger, NMDA-type excitatory amino acid receptor and L-type calcium channel activation. Stimulation of Na\(^+/\text{H}^+\) exchanger on astrocytes causes glutamate and K\(^+\) release, cause neuronal depolarization, activate
neuronal VOCC and excitatory amino acid receptors, and participate in gp120 neurotoxicity.

Gp120-induced increase in $[Ca^{2+}]_c$ in astrocytes and neurones were blocked by the amiloride analog MIA (inhibitor of Na$^+/H^+$ exchanger), as well as by the absence of Na$^+$ ions. These findings are consistent with previous results that TTX, a Na$^+$ channel blocker, protected against gp120 neurotoxicity. This group, also found that AP5, a competitive NMDA receptor antagonist, and memantine, a non-competitive NMDA receptor antagonist, blocked gp120-induced increases in $[Ca^{2+}]_c$ in neurones as well as astrocytes. These findings suggest that, subsequent to gp120 application, NMDA receptor-channel complexes are activated, extracellular Ca$^{2+}$ enters and glutamate release may be enhanced. Neurones and astrocytes express NMDA and non-NMDA type ionotropic glutamate receptors (Huntley et al., 1997; Verkhratsky and Kettenmann, 1996) and stimulation of neuronal NMDA receptors by gp120 is secondary to the production of arachidonic acid, inhibition of glutamate re-uptake and increased accumulation of extracellular glutamate (Dreyer and Lipton 1995). These events may lead to excitation of, and injury to, neighboring neurones. However, this cycle was overridden by the open channel blocker memantine. Third, they found that a broad spectrum of L-type VOCC blockers, but not N-, P-, or Q-type blockers, significantly reduced responses to gp120 in neurones, but not in human fetal astrocytes. This is consistent with the finding that astrocytes do not express functional L-type VOCC.

In particular, virally encoded proteins such as gp120 may trigger an increase in glutamate efflux (Benois et al., 1994). This effect may be exacerbated by a decline in astrocyte uptake of glutamate, through the action of arachidonic acid (Barbour et al., 1989), reactive oxygen intermediates (Piani et al., 1993), and TNF-α (Fine et al., 1996) all these are known to be
produced by activated, HIV-1-infected, monocytes (Genis et al., 1992). Thus, there may be an important role for astrocyte-directed therapeutic interventions in the treatment of HIV-1 dementia, perhaps including the use of specific cytokine blockers as well as trophic factors and specific activators of glutamate transport.

1.8. Oligodendrocytes

Although oligodendrocyte infection has been demonstrated in vitro (Albright et al., 1996), substantial evidence supporting in vivo infection has yet to be reported. Although oligodendrocyte abnormalities have been described in examinations of HIV-1-infected postmortem brains (Esiri et al., 1991), the cellular damage was likely caused by indirect mechanisms of cytotoxicity and not by direct infection. Using in situ PCR, Bagasra and colleagues demonstrated infection of oligodendrocytes in numbers less than either astrocytes or neurones (Bagasra et al., 1996). The relevance of these findings has yet to be fully understood.

1.9. Cells at the CNS Periphery: Endothelial and Choroid Plexus Cells

Endothelial cells, which form the first barrier between the peripheral circulation and the CNS, may provide an entry portal through which HIV-1 may enter the CNS. HIV-1 replication in brain capillary cells may lead to virus budding into the interior of the brain and infection of adjacent neuroglial cells within the brain by direct cell-to-cell contact or the spread of cell-free viral progeny. In vitro and in vivo studies suggest that BMVECs can support HIV-1 replication. In vitro, human BMVECs are susceptible to HIV-1 infection (Moses et al., 1993) facilitated by CD4-independent viral entry. In vivo, HIV-1 has been detected using in situ PCR in
microvascular endothelial cells at levels exceeding 65% in postmortem CNS tissue of AIDS patients (Bagasra et al., 1996). Endothelial cells are also infected in animal models of HIV-1 infection. HIV-1 infection of the cerebral vascular endothelium was demonstrated 60 weeks after intravenous inoculation of pigtailed macaques with T-tropic strain LAI (Frumkin et al., 1995).

HIV-1 may also enter through replication in epithelial cells of the choroid plexus, as has been observed with visna virus (Johnson, 1982). Choroid plexus cells have been shown to be susceptible to HIV-1 infection (Bagasra et al., 1996; Falangola et al., 1995; Harouse et al., 1989). This port of entry may contribute to the regional distribution of HIV-1 observed within the brain, particularly within deep white matter and diencephalic and mesencephalic structures, but not within the cortex (Dickson et al., 1991; Kure et al., 1990; Price, 1994).
Chapter 2. Materials and Methods

2.1. Materials

Neurobasal (NB) medium, Catalogue no: 21103-049, Minimum Essential Medium Eagle (MEM) with Glutamax-1, Catalogue no: 41090-028, L-glutamine, Catalogue no: 15032-014, and B-27, Catalogue no: 17504-044 were purchased from Gibco Life Technology. Heat inactivated horse serum (HS), fetal calf serum (FCS), cytosine arabinoside and poly-D-lysine were from Sigma. Cover glasses, 22 x 40 mm (Thickness number 1) were from BDH. Trypsin 1:250 (T 4799) was used for primary cell culture, and trypsin-EDTA solution (1X) (T 3924) was used for the cell line, both were purchased from Sigma. Dulbecco’s Modified Eagle’s Medium (DMEM), Catalogue no: D 6046 was bought from Sigma.

Fura-2 AM, Rhodamine 123 and Propidium Iodide were purchased from Molecular Probes. Hoechst was bought from Sigma. IFNγ was purchased from Peprotech.

For microglia staining normal horse serum (S2000), ABC Kit (PK4001), anti mouse IgG (rat adsorbed, made in horse), and biotinylated BA2001 were purchased from Vector Labs. Primary antibody ED1 was from Serotec. Mouse anti rat ED1 (MCA341R), diaminobenzidine (DAB), and haematoxylin were from Sigma.

Xylene, ethanol and DPX mountant were from BDH.

HIV-1 envelope glycoprotein gp120 was obtained from MRC (UK) AIDS Research and Reagent programme and the peptide VSLSYRCPRFF was purchased from Alta BioScience, Birmingham, U.K. Gp120 was provided as 1.72 mg/ml in citrate buffer (40mM Na+ citrate, 280
mM NaCl). Aliquots of 2 µl were made. These aliquots were diluted further in HBS to give intermediate stock at 10 µg/ml (added 342 µl of HBS to each 2 µl aliquot of 1.72 mg/ml gp120). Working solution of gp120 at 100 ng/ml or 250 pM were made at the time of experimenting from the intermediate stock (20 µl of intermediate stock to 2 mls of HBS). For control, 2 µl of citrate buffer was added to 342 µl of HBS and were mixed to make "vehicle control". In control experiments, I added 20 µl of this vehicle control to 2 ml of HBS and then performed the experiment.
2.2. **Cell line SK-N-SH human neuroblastoma**

SK-N-SH human neuroblastoma cells were a kind gift of Professor Ian Everall, Institute of Psychiatry, London. These cells were derived from a 4-year-old female Caucasian suffering with neuroblastoma. These cells were cultured in DMEM plus 7% each of FCS and HS in culture flasks and were grown in a humidified atmosphere of 5% CO₂ and 95% air at 37° C and fed every 4-5 days with fresh DMEM +7% FCS and HS, until they had grown to confluence. At this stage the cells were split 1:10 with trypsin-EDTA solution (Sigma, 1X), 5 minutes, 37°C. One day after plating onto laminin-coated petriperm dishes in a differentiation medium of DMEM plus 2% of each serum, the dishes were used for [Ca^{2+}]ₐ imaging experiments.

2.3. **Primary Cell Culture**

2.3.1. **Hippocampal Culture in MEM**

Rat hippocampal neurones were grown in primary culture. One day old rat pups were killed by cervical dislocation. Hippocampi were dissected and placed in HEPES buffered solution (HBS) containing 0.1% trypsin for half an hour at 37° C. HBS was composed of the following (in mM); HEPES, 20; NaCl, 120; CaCl₂, 1.8; MgCl₂, 1; KCl, 5.5; glucose, 2.5; pH 7.2. After half an hour the suspension was centrifuged at 1000 rpm for 3 min and the supernatant removed. The pellet was washed first in HBS then twice in MEM supplemented with 10% HS, as previously described (Peudenier et al., 1991). The cells were dissociated by trituration through a 5 ml serological pipette and then a flame narrowed Pasteur pipette. Dissociated cells were then plated onto autoclaved 22 x 40 mm cover glasses (Number 1), that had been coated with poly-D-lysine (0.1 mg/ml) and washed with sterile water. The neurones were grown in a humidified
atmosphere of 5% CO\textsubscript{2} and 95% air at 37° C and fed every 4-5 days with MEM plus 10 % HS. Cytosine arabinoside (10 \textmu M) was added to the cultures within 48 hours of plating. Cells employed for these experiments were grown in culture for a minimum of 2 weeks.

A significant problem with the above preparation was that glia would suddenly increase in number on day 12 and it became very difficult to detect neurones. Adding cytosine arabinoside for the second time (when the culture is 10 days old) helped, but the success was still only 20%. Hence, I researched the possibility of another protocol for hippocampal culture that promoted neuronal survival without much glial growth.

**2.3.2. Hippocampal culture in NB medium**

Gibco advertised their serum free NB medium, which they claimed would support the growth of neuronal cultures for 3-4 weeks when supplemented with 2% B-27 (a serum substitute) and 1% glutamine. There was no need to add a toxic compound such as cytosine arabinoside to inhibit glial growth and glial cells would represent less than 5% of the total cell number. Unlike other cell culture media, NB medium does not contain glutamate, and thus does not cause excitotoxic damage to cells expressing NMDA receptors.

Thus, I moved to trying NB medium by Gibco. I used two different protocols. I grew neuronal cultures in a) NB medium + 2% B27 + 1% glutamine or b) NB medium + 2% B27 + 1% glutamine + 5% HS. In the second case, cytosine arabinoside was added when the culture was 5 days old then removed after 48 hours (the whole medium was removed and replaced with fresh NB medium + 5% HS). In both preparations, medium was changed twice in a week, each time half the old medium being replaced with new medium.
2.3.3. Cortical Cultures

Primary cortical cultures were also used. Rat pups were killed as above. Cerebral cortices were placed in ice-cold Neurobasal medium and triturated 20-30 times till the suspension became cloudy (Prothero et al., 1998). The suspension was then plated on coverglasses coated with poly-D-lysine as before.

2.4. Fluorescent Imaging of Calcium

2.4.1. Fura-2

\([\text{Ca}^{2+}]_c\) can be measured using intracellularly trapped fura-2 (Gryniewicz et al., 1985). The acetoxyethyl ester form of the dye (fura-2 AM) diffuses across the plasma membrane and non specific intracellular esterases rapidly hydrolyse the ester bonds generating the membrane impermeant free acid, fura-2, which will not leak out of the cell after extracellular fura-2 AM is removed (Tsien, 1981). Fura-2 is currently the most popular \(\text{Ca}^{2+}\) indicator for microscopy of individual cells, it is a bright fluorophore which has excitation wavelength compatible with modern glass microscope optics. While the free dye has an excitation maximum at around 370 nm, \(\text{Ca}^{2+}\)-loaded fura-2 has an excitation maximum of 335 nm. In the present study, fura-2 is excited at 350 nm and 380 nm and the emission is monitored at > 505 nm. At 350 nm there is an increase in fluorescence on binding \(\text{Ca}^{2+}\), while at 380 nm there is a decrease, i.e. on binding \(\text{Ca}^{2+}\) there is a shift in the peak of the fura-2 excitation spectrum reflecting the increased relative amount of \(\text{Ca}^{2+}\)-loaded dye. Using the ratio of fluorescence at 350 nm and 380 nm cancels out variations in dye loading and local optical path length and compensates for absolute changes in illumination intensity (Tsien, 1988). In the present study, cultured primary hippocampus and cortical cells were loaded in 5 \(\mu\)M fura-2 AM for 30-40 min at room temperature in HBS. It is
worth noting that when cells were loaded in growth medium they would not generate a subsequent response to glutamate application, suggesting that trace amounts of glutamate in the nominally glutamate free medium caused inactivation of the receptors.

Fura-2 has the disadvantage of becoming trapped inside organelles (Connor, 1993) and of releasing the hydrolysis by-products acetate, protons and formaldehyde that are of potential toxicity to living cells. However, the low concentrations of fura-2 AM used here should not result in the excessive presence of these by-products or excessive $[\text{Ca}^{2+}]_c$ buffering by the probe. Moreover, fura-2 AM loading seems less problematic in neurones than in other cell types (Tsien, 1989) where compartmentation is a major problem (Sheu and Jou, 1994).

### 2.4.2. Calibration of Fura-2 Signal

The 350/380 ratio of fluorescent intensity is related to $[\text{Ca}^{2+}]_c$ by the Grynkiewicz equation:

$$[\text{Ca}^{2+}]_c \ (\text{nM}) = \frac{K_d \cdot S \cdot (R - R_{\text{min}})}{(R_{\text{max}} - R)}$$

$K_d$ = Dissociation constant for the dye

$R$ = The ratio of the fluorescence ($F_{340}/F_{380}$)

$R_{\text{min}}$ = The ratio of the fluorescence when the dye is completely free of $\text{Ca}^{2+}$

$R_{\text{max}}$ = The ratio of the fluorescence when the dye is completely saturated with $\text{Ca}^{2+}$

$S$ = The ratio of saturated to free fluorescence at 380 nm excitation ($F_{\text{sat}}/F_{\text{free}}$ at 380 nm excitation)

The value of $R_{\text{min}}$ and $R_{\text{max}}$ were measured on the fluorescence imaging system by a calibration
using two standard solutions, zero Ca\(^{2+}\) (NaCl 120 mM, glucose 25 mM, KCl 5.5 mM, MgCl\(_2\) 2.8 mM, EGTA 2 mM, HEPES 20 mM, pH 7.2) and 1.8 mM Ca\(^{2+}\) (NaCl 120 mM, glucose 25 mM, KCl 5.5 mM, MgCl\(_2\) 2.8 mM, CaCl\(_2\) 1.8 mM, HEPES 20 mM, pH 7.2). Solutions were loaded into Camlab (Cambridge, UK) microcuvettes with path length 50 \(\mu\)m and imaged on the stage of the microscope. Typical values were \(R_{\text{min}} = 0.352\), \(R_{\text{max}} = 12.412\) and \(S = 8.54\) and the \(K_a\) was taken to be 236 nM for the Grynkiewicz equation (Groden et al., 1991) allowing the [Ca\(^{2+}\)]\(_c\) to be calculated.

2.4.3. \([\text{Ca}^{2+}]_c\) Measurement

2.4.3.1. Human Neuroblastoma cells

SK-N-SH human neuroblastoma cells were cultured in DMEM plus 7% each of FCS and HS. One day after plating onto laminin-coated petriperm dishes in a differentiation medium of DMEM plus 2% of each serum, cells were loaded with Ca\(^{2+}\) indicator by incubation for one hour in 4 \(\mu\)M Fura-2 AM plus 5 mg/100 ml Pluronic F127 in differentiation medium at room temperature. Pluronic, a non-ionic detergent, stabilizes micelles of hydrophobic fura-2 AM in aqueous medium. Dishes were then rinsed with fresh differentiation medium and placed on the stage of an imaging fluorescence microscope. Temperature was maintained at 36°C and the pH of the medium was maintained with 5% CO\(_2\). Images were acquired from a field of cells during illumination at each of 350 nm and 380 nm, allowing calculation of [Ca\(^{2+}\)]\(_c\) in each of a number of cells.
2.4.3.2. Primary Cultures

Cells were loaded with the Ca\(^{2+}\) indicator fura-2 by incubating for 30-40 min at room temperature in 5 μM of the AM ester in HBS. Cover slips were rinsed three times in fresh HBS then mounted in a rapid perfusion bath (RC26G, Warner, Connecticut, USA) and placed on the stage of a Zeiss IM inverted microscope fitted with an illumination system (Sutter, California, USA) and intensified CCD camera (Extended Isis, Photonic Science, UK). 350 nm/380 nm fluorescence image pairs were acquired at a repetition rate of 4 per minute under control of Kinetic Imaging software (Liverpool, UK). Fluorescence values were corrected for background light, estimated from an area of the field without cells, then converted to \([\text{Ca}^{2+}]_c\) values by applying \([\text{Ca}^{2+}]_c = K_d S (R - R_{\text{min}})/(R_{\text{max}} - R)\) where \(R = \text{I}_{350}/\text{I}_{380}\), \(R_{\text{min}}\), \(R_{\text{max}}\) and \(S\) were measured from in vitro solutions and the \(K_d\) was taken to be 236 nM (Groden et al., 1991).

2.5. Electrophysiological Recording

Patch-clamp recordings of hippocampal neurones were performed in the whole-cell configuration using standard procedures (Hamill et al., 1981). The bath solution contained HBS. HBS was composed of the following (in mM); HEPES, 20; NaCl, 120; CaCl\(_2\), 1.8; MgCl\(_2\), 1; KCl, 5.5; glucose, 2.5; pH 7.2. The patch pipette contained internal solution of the following composition (in mM); KCl, 130; NaCl, 10; EGTA, 10; CaCl\(_2\), 1; MgCl\(_2\), 1; MgATP, 5; HEPES, 7.5; pH 7.1. Electrophysiological measurements were performed at room temperature with patch pipettes of resistance 5-15 MΩ. The patch pipette was pressed gently against the membrane, and some suction was applied. The membrane sealed to the glass, raising the overall resistance to between 2 and 5 GΩ. The pipette potential was then clamped at -50 mV and sharp negative pressure applied to the pipette to rupture the plasma membrane and establish continuity with the
inside of the cell. As soon as the whole cell configuration was achieved I terminated the voltage clamp and allowed the membrane voltage to move to its natural value (I=0 current clamp mode). Membrane voltage was then monitored during changes of bathing solution. Most cells had resting voltage of about -40 mV; I rejected cells with resting voltages more positive than -35 mV.

2.6. Neuronal Death

Neurones were plated on glass coverslips and cultured. Propidium iodide (60 μM) and Hoechst 33342 (10 μg/ml) were used to stain the cells; nuclei of dead cells had bright red propidium stain while live cells showed only blue Hoechst stain. Alive and dead neurones were counted in each microscopic field and the percentage of dead neurones was calculated. Viable cells can be stained by incubation with the bis-benzimidazole, Hoechst 33342. This compound binds to the AT-rich regions in the minor groove of DNA. Upon excitation with the UV light, the dye-DNA complexes fluoresce blue. The emission spectrum is broad and extends through into the red part of the spectrum.

2.6.1. Measurement of Viability

In this context, a viable cell is defined as having an intact plasma membrane. It does not mean that the cell is capable of long term survival. The simplest method of measuring viability is to add propidium iodide (PI) and to measure red fluorescence. This dye which stains DNA is excluded by an intact plasma membrane. The method can be enhanced by incubating briefly with Hoechst. Dead cells will be red +ve; viable cells blue +ve, red –ve. Cells undergoing apoptosis
whose plasma membrane are still intact are recognised by the intense blue Hoechst fluorescence of their condensed nuclei (Delaney et al., 1997; Iwakura et al., 2000). I did not observe apoptotic cells in this study. I therefore only sorted into two categories, alive (Propidium Iodide -ve) and dead (Propidium Iodide +ve). Neuronal viability was evaluated after 48 hr. Treatments with gp120 and/or chemokines were started when the culture was at least 22 days old and were carried on for 48 hours. Cultures were treated for 2 hours with chemokines before the addition of gp120.

2.7. Immunocytochemical Staining of Microglia

2.7.1. Method

I fixed cover glasses kept in 60 mm tissue culture dishes by adding methanol at -20°C for 15 min. After fixing, slides were washed twice, 5 minutes each time in PBS. I then wiped off the excess PBS and placed the slides in a wet slide box. I then added 100 µl of normal HS (1:40) to each slide to block nonspecific sites and incubated for 30 minutes at room temperature. After incubation I tipped off the blocking serum and wiped slides. 100 µl of primary antibody ED1 (1:500) was then added to each slide. The slides were incubated overnight at 4°C in a wet box.

Next day, the slides were washed three times, 5 minutes each time in PBS, excess PBS was wiped off and the slides were placed back in the wet box. 100 µl of biotinylated (secondary) antibody for ED1 (1:1000) was then added to each slide and slides incubated for 1 hour at room temperature.
In the mean time I made a 1:100 dilution of ABC solution in PBS 30 min before it was required, where Reagent A is Avidin, Reagent B is Biotinylated Horseradish Peroxidase. This technique employs unlabeled primary antibody, followed by biotinylated secondary antibody and then a preformed Avidin and Biotinylated horseradish peroxidase macromolecular Complex. This has been termed the ABC technique.

After 1 hour incubation with the secondary antibody, the slides were washed three times, 5 minutes each time in PBS, excess PBS was wiped off. 100 µl of ABC was added to each slide and the slides incubated for a further 1 hour at room temperature followed by three five minute washes in PBS.

Next, a 3, 3'-Diaminobenzidine tablet set was dissolved in distilled water (1 ml) to yield a buffered solution containing DAB and urea hydrogen peroxide. Each Sigma Fast Dab tablet set contains the following when dissolved in 1 ml water: DAB Sigma product No. D-9167 (0.7 mg/ml); Urea hydrogen peroxide, Sigma product No. U-5005 (1.6 mg/ml); Tris buffer 0.06 M. 100 µl of this solution was added to the slides and incubated for 10 minutes. The slides were then washed in PBS for at least 5 min for DAB to develop. Hematoxylin (2 mM) was then added to each slide for 20 seconds followed by washing in tap water for 2 minutes. Slides were then dehydrated by dipping in the following reagents:

90% ethanol  45sec
100% ethanol  45sec
100% ethanol  45sec
Xylene 100%  45sec
Xylene 100% 3min

Slides were then mounted using DPX and allowed to dry for at least 10 minutes.

Using this protocol active microglia stain an intense brown while inactive microglia stain light brown and the neurones and astrocytes pick up the blue/purple hematoxylin stain.

2.8. Measurement of Membrane Potential using Slow Dye

2.8.1. DiBAC (Bis-oxonol) Dyes

The three bis-barbituric acid oxonols, often referred to as DiBAC dyes, form a family of spectrally distinct potentiometric probes with excitation maxima at approximately 490 nm (DiBAC$_4$(3)), 530 nm (DiSBAC$_2$(3)) and 590 nm (DiBAC$_4$(5)). The dyes enter depolarized cells where they bind to intracellular proteins or membranes and exhibit enhanced fluorescence and red spectral shifts (Epps et al., 1994). Increased depolarization results in more influx of the anionic dye and thus an increase in fluorescence. DiBAC$_4$(3) reportedly has the highest voltage sensitivity (Brauner et al., 1984). The DiBAC dyes are excluded from mitochondria because of their overall negative charge, making them superior to carbocyanines for measuring plasma membrane potentials by flow cytometry.

2.8.2. Methodology

The cells were loaded in 1 μM DiBAC$_4$(3) in HBS and were mounted on the stage of the microscope for imaging. Excitation was at 490 nm and emitted light was measured at 520 nm (Schroeder and Neagle, 1996). Various test agents were then applied by superfusion, 1 μM
DiBAC₄(3) being present throughout.

2.9. Simultaneous Imaging of [Ca²⁺]ᵣ and Mitochondrial Membrane Potential

Cells were loaded for 30 min at room temperature with 5 µM fura-2 AM (Molecular Probes) and 0.005 % pluronic in normal HBS. The mitochondrial membrane potential (Δψₘ) indicator rhodamine 123 (Rh123, 10 µg/ml Molecular Probes) was added to the culture during the last 15 min of the fura-2 loading period. The cells were then washed extensively. Under these conditions, Rh123 appears to re-equilibrate, resulting in a signal that the Duchen lab has routinely found to give a reproducible signal in response to a wide range of manipulations and in many cell types in line with changes in Δψₘ predicted from chemiosmotic theory (Keelan et al., 1999). Rh123 loading under these conditions has only a small effect on the fura-2 signals, which were qualitatively the same in cells loaded with only a Ca²⁺ indicator and cells loaded with both Ca²⁺ indicator and Rh123. There was no evidence of toxicity of Rh123 under these conditions. Most fluorescence measurements were made on an inverted epifluorescence microscope using a × 20 fluorite objective lens to view a field of cells. [Ca²⁺]ᵣ and Δψₘ were monitored in single cells using excitation light provided by a xenon arc lamp, the beam passing sequentially through 340, 380 and 490 nm filters (10 nm bandwidth) housed in a computer-controlled filter wheel (Cairn Research Ltd, UK). Sequential excitation of cells at 340 and 380 nm allowed ratiometric measurement of fura-2 fluorescence and excitation at 490 nm allowed measurement of Rh123 fluorescence; a complete set of three images was acquired every 10 s. The images were acquired using a frame transfer 800 × 1200 pixel array (800 × 600 active area), cooled CCD camera and digitised to 12 bit resolution (Digital Pixel Ltd, UK). All imaging data were collected and
analysed using Kinetic Imaging software (Liverpool, UK). A computer-controlled shutter kept photodynamic damage of the cells to a minimum by allowing exposure to excitation light only when required for imaging.
Chapter 3. Variability

3.1. Introduction

3.1.1. Gp120 Does not Always Evoke a Neuronal $[\text{Ca}^{2+}]_c$ Signal

A lot of work has been done on studying the role of HIV-1 coat protein gp120 in killing neurones in rodent brain cultures as well as human embryonic brain cultures. Lipton has proposed that $[\text{Ca}^{2+}]_c$ elevation represents a final common pathway by which HIV virus, stroke, trauma and epilepsy trigger neuronal death (Lipton, 1992b). In fact, since then many laboratories have spent a lot of time in finding out the various mechanisms that are triggered by gp120 which contribute to cell death. Hence, when I began my PhD work the aim of my project was to find the exact mechanism by which gp120 produces a rise in $[\text{Ca}^{2+}]_c$ in neurones and if there is a way to block this rise and rescue the neurones from dying.

A number of publications have described acute $[\text{Ca}^{2+}]_c$ elevations in cultured hippocampal neurones upon addition of gp120 (Holden et al., 1999; Lipton et al., 1991; Meucci et al., 1998). A common feature of these papers is that the cultures are maintained for two weeks or more before use. In contrast laboratories studying synaptic transmission between neurones in culture, and laboratories studying responses of neurones to glutamate or NMDA receptor activation, typically use the cultures 7 days after dissection. I found that primary hippocampal cultures grown in MEM + 10% HS, showed a neuronal $[\text{Ca}^{2+}]_c$ response to glutamate/NMDA after 7 days in culture. These cultures never survived in good condition beyond 12 days. In some cases, a small fraction of the neurones did survive to 13 days, and in these I usually saw a potentiation by
gp120 of the NMDA induced rise in $[Ca^{2+}]_{e}$. This phenomenon is described in more detail in section 3.2.2. However, when the primary hippocampal cultures were grown in NB medium + 5% HS, the neuronal $[Ca^{2+}]_{e}$ response to NMDA/glutamate appeared after 12 days in culture, while only those cells cultured for over 18 days showed a neuronal $[Ca^{2+}]_{e}$ response to gp120 (see section 3.2.3). Those cultures that were 18 days old also showed a neuronal $[Ca^{2+}]_{e}$ response to NMDA. Also, I found that primary hippocampal cultures grown in NB medium -HS, showed a neuronal $[Ca^{2+}]_{e}$ response to glutamate/NMDA when 15 days old, but gave a neuronal $[Ca^{2+}]_{e}$ response to gp120 only when the cells were over 22 days old (see section 3.2.3).

A requirement for some time in culture following tissue dissociation suggests that enzymatic dissociation removes critical cell surface receptors that need to be resynthesized or some other process that changes with culture. Cultures dissociated without the use of enzymes offer the possibility of testing this hypothesis. Therefore, I also performed non-enzymatic dissociation of neonatal rat cortex (Prothero et al., 1998) hoping that with this preparation I should see the neuronal $[Ca^{2+}]_{e}$ responses to gp120 even in young cultures. Since gp120 kills these neurones too (see chapter 5), it seemed reasonable to suppose that it might elicit neuronal $[Ca^{2+}]_{e}$ responses in these cultures. However, 10 independent cortical cultures tested at 4 or 30 days after dissociation showed no neuronal $[Ca^{2+}]_{e}$ response to gp120.
3.2. Results

3.2.1. Gp 120 Induces a Rise in $[Ca^{2+}]_{c}$ in Human Neuroblastoma Cells

I first began my work with testing gp120 on SK-N-SH human neuroblastoma cells for $[Ca^{2+}]_{c}$ responses. Gp120 was added at the indicated time to a final concentration of 250 pM. Parallel experiments conducted with vehicle alone caused no significant $[Ca^{2+}]_{c}$ change (average $[Ca^{2+}]_{c} = 80.78 \pm 4.56$ nM, n = 70, from four independent platings) (Figure 3.1). Addition of 250 pM gp120 to SK-N-SH cells produced individual, non-correlated oscillations in these cells and caused a rise in the level of $[Ca^{2+}]_{c}$ from a resting level $= 83.47 \pm 2.3$ nM, rose to $= 162.73 \pm 21.75$ nM, n = 75 from four independent platings, $p < 0.0001$ compared to vehicle.

I then moved on to trying primary hippocampal cultures as these neuroblastoma lines were not considered to be a good model of studying excitotoxicity (Yoshioka et al., 1996). For this reason, further work was done not on the cell line but on primary rat central neurones.
Figure 3.1. HIV-1 coat protein gp120 induces oscillatory [Ca$^{2+}$]$_{o}$ responses in human neuroblastoma SK-N-SH cells (A), whereas in (B) addition of vehicle caused no significant rise in the level of [Ca$^{2+}$]$_{o}$.
3.2.2. Potentiation of NMDA Induced Rise in $[Ca^{2+}]_c$ by gp120

Gp120 does not always produce a rise in $[Ca^{2+}]_c$ in neurones, however it potentiated the NMDA-induced $[Ca^{2+}]_c$ rise in embryonic cultures of rat hippocampal neurones (Meucci and Miller, 1996) or human CNS neurones (Lannuzel et al., 1995).

Figure 3.2 shows the typical response I observed on the addition of NMDA (50 μM) + glycine (20 μM) to hippocampal cultures maintained for 13 or more days in MEM + 10% HS- a rapid rise of $[Ca^{2+}]_c$ in neurones (282.61 ± 19.31 nM, n = 57 cells from 3 independent cultures) which returned to basal level after agonist removal.

In neurones from the same cultures preincubated for three minutes with 250 pM gp120, the increase in $[Ca^{2+}]_c$ produced by 50 μM NMDA + 20 μM glycine was significantly larger (794.52 ± 104.61 nM, p < 0.001).
Potentiation of NMDA-induced rise in $[\text{Ca}^{2+}]_c$ by gp120

Figure 3.2. Potentiation of NMDA induced rise in $[\text{Ca}^{2+}]_c$ by gp120 in neurones. Lower trace shows $[\text{Ca}^{2+}]_c$ rise on addition of 50 μM NMDA + 20 μM glycine while the upper trace shows $[\text{Ca}^{2+}]_c$ peak on addition of 50 μM NMDA + 20 μM glycine when the culture is preincubated with 250 pM gp120 for 3 minutes. Mean of 57 and 60 neurones respectively from 3 independent cultures maintained in MEM + 10% HS.
3.2.3. Hippocampal cultures in Neurobasal Medium

Because cultures maintained in MEM + 10% HS rarely survived to an age where gp120 responses could be detected, I next tried NB medium, supplemented with 2% B27 and 1% glutamine, a defined culture medium developed by Brewer et al. (1993) to specifically promote the survival of central nervous system neurones. Medium was changed every 3-4 days. Hippocampal neurones survived in a healthy condition for at least 4-6 weeks in this medium, and showed a \([Ca^{2+}]_c\) response to NMDA on the 15\(^{th}\) day in culture (Figure 3.3A). These cultures usually failed to show any response to gp120, neither a \([Ca^{2+}]_c\) rise, nor a potentiation of NMDA responses. Occasionally gp120 evoked a progressive rise of \([Ca^{2+}]_c\) in neurones of the type illustrated in Figure 3.3 and 3.5B in cultures maintained for 22 days or more in NB + 2% B27 + 1% glutamine. In the 68 neurones that showed responses of this type, \([Ca^{2+}]_c\) had risen to an average of 316.39 ± 59.65 nM by the \((28 ± 3.2)\)th minute after addition of gp120. However, most cultures maintained in NB +2% B27 failed to respond to gp120 (Table 3.1).

I therefore examined the effect of adding 5% HS to the NB medium supplemented with 2% B27 and 1% glutamine. Neurones survived well in this medium, as in the absence of HS. These cultures showed a neuronal \([Ca^{2+}]_c\) response to NMDA when 12 days old (Figure 3.4). As in the cultures without HS, gp120 failed to elicit a neuronal \([Ca^{2+}]_c\) response in the majority of cultures. However, I did observe a neuronal \([Ca^{2+}]_c\) response to gp120 in some of the cultures when 18 days old or more, and of these responses about 90% took the form of massive, abrupt \([Ca^{2+}]_c\) increases. In this restricted subset of cultures \([Ca^{2+}]_c\) rose from a resting level of 68.5 ± 1.5 nM to a peak of 762.93 ± 159.04 nM (93 neurones) after gp120 addition (Figure 3.4 and 3.5D) (Table 3.1).
Figure 3.3. Neuronal \([\text{Ca}^{2+}]_c\) responses to gp120/NMDA by hippocampal cultures in NB medium - HS. (A and B): the neurones gave a \([\text{Ca}^{2+}]_c\) response to 50 \(\mu\text{M}\) NMDA + 20 \(\mu\text{M}\) glycine when 15 days old, but did not give a \([\text{Ca}^{2+}]_c\) response to 250 \(\text{pM}\) gp120. (C and D): the neurones were 22 days old and gave a \([\text{Ca}^{2+}]_c\) response to both NMDA and gp120.
Figure 3.4 Neuronal $[Ca^{2+}]_c$ responses to gp120/NMDA by hippocampal cultures in NB medium + 5% HS. (A and B): the neurones gave a $[Ca^{2+}]_c$ response to 50 μM NMDA + 20 μM glycine when 12 days old, but did not give a $[Ca^{2+}]_c$ response to 250 pM gp120. (C and D): the neurones were 18 days old and gave a $[Ca^{2+}]_c$ response to both NMDA and gp120.
**Figure 3.5** gp120-evoked \([Ca^{2+}]_c\) signals in neurones. A: Typical field of neurones cultured in NB medium +2% B27 with no added HS. Neurones are visualized by the fluorescence of intracellular fura-2 (excited at 380 nm). Fluorescence images are displayed using a colour scale in which progressively brighter regions are displayed using progressively warmer colours from black through orange to white. B: \([Ca^{2+}]_c\) signals from the neurones of A as 250 pM gp120 was added. Each cell indicated with a number in A is plotted in this graph. C: Typical field of neurones cultured in NB medium +2% B27 + 5% HS (same magnification as A). D: Massive, sustained \([Ca^{2+}]_c\) response recorded from the neurones of C after addition of 250 pM gp120. Each neurone indicated with a number is plotted in this graph. As noted in the text, \([Ca^{2+}]_c\) responses of the form shown here appeared in only the minority of cultures.
Figure 3.5

A

B

C

D
3.3.3. Cortical cultures

The requirement for a long period in culture before hippocampal cells would respond to gp120 suggested that a membrane receptor, necessary for gp120 action, was destroyed during the enzymic dissociation process and was then slowly re-expressed or some other process that changes with culture. I therefore examined the effect of gp120 on $[\text{Ca}^{2+}]_c$ in cultures of cortical neurones prepared without the use of proteolytic enzymes. Neurones in these cultures responded to 50 $\mu$M NMDA + 20 $\mu$M glycine when 4 days old: $[\text{Ca}^{2+}]_c$ rose from a resting level of $71.70 \pm 1.61$ nM to $305 \pm 28.07$ nM, $n = 62$ from 3 independent cultures (Figure 3.6) and this responsiveness was maintained for up to 30 days in culture. Nevertheless, when tested at 4 days in culture, these neurones never showed a $[\text{Ca}^{2+}]_c$ response to gp120 (average $[\text{Ca}^{2+}]_c = 63.54 \pm 2.26$ nM, $n = 65$ from 3 independent cultures), nor showed a potentiation of NMDA induced rise in $[\text{Ca}^{2+}]_c$ by gp120 (resting $[\text{Ca}^{2+}]_c = 68.89 \pm 3.21$ nM, rose to $= 298.53 \pm 32.56$ nM, $n = 62$ from 3 independent cultures) (Figure 3.6).

I also tested primary cortical cultures when 30 days old. Again, they showed a neuronal $[\text{Ca}^{2+}]_c$ response to 50 $\mu$M NMDA + 20 $\mu$M glycine (resting $[\text{Ca}^{2+}]_c = 70.67 \pm 1.30$ nM, rose to $353.67 \pm 36.02$ nM, $n = 62$ from 3 independent cultures). These neurones again did not show a $[\text{Ca}^{2+}]_c$ rise upon addition of gp120 (average $[\text{Ca}^{2+}]_c = 65.57 \pm 3.21$ nM, $n = 68$ from 3 independent cultures) nor showed a potentiation of NMDA induced rise in $[\text{Ca}^{2+}]_c$ by gp120 (resting $[\text{Ca}^{2+}]_c = 72.89 \pm 6.8$ nM, rose to $355.42 \pm 60.37$, $n = 65$ from 3 independent cultures) (Figure 3.7).
Figure 3.6. Primary cortical cultures in NB medium - HS, 4 days old. A) the neurones showed a \([Ca^{2+}]_c\) response on addition of 50 μM NMDA + 20 μM glycine. B) in a sister culture to that shown in A there is no potentiation of NMDA induced rise of \([Ca^{2+}]_c\) by 250 pM gp120. C) the neurones also did not show a rise of \([Ca^{2+}]_c\) upon addition of 250 pM gp120.
Figure 3.7. Primary cortical cultures in NB medium - HS, 30 days old. A) the neurones gave a \([\text{Ca}^{2+}]_c\) response upon addition of 50 \(\mu\text{M}\) NMDA + 20 \(\mu\text{M}\) glycine. B) in a sister culture to that shown in A the neurones gave no potentiation of NMDA induced rise in \([\text{Ca}^{2+}]_c\) by 250 pM gp120. C) the neurones also did not give a rise of \([\text{Ca}^{2+}]_c\) on addition of 250 pM gp120.
3.3. Discussion

As I have gone through my work, the readers can see that it was very difficult for me to obtain a neuronal $[Ca^{2+}]_e$ response to gp120. However, when I started my PhD and even in the literature today involving gp120 and $[Ca^{2+}]_e$, no laboratory talks of how difficult it is to obtain these neuronal $[Ca^{2+}]_e$ responses. This raises the question of whether cultures in other laboratories do in fact always show a neuronal $[Ca^{2+}]_e$ response to gp120, and my emphasis to readers would be not to be misled by their work. I will go through the main papers individually and discuss their work.

Dreyer and coworkers 1990, measured $[Ca^{2+}]_e$ in postnatal rat retinal ganglion cells by digital imaging microscopy with fura-2. Application of 200 pM of highly purified gp120 from a recombinant source produced a striking increase in $[Ca^{2+}]_e$. Compared to control levels $[Ca^{2+}]_e = 63 \pm 4$ nM, mean ± SEM, $n = 42$, obtained before the addition of coat protein, levels increased 33-fold within 7 min of gp120 application ($2100 \pm 330$ nM, $n = 10$). Other preparations of gp120 purified from natural isolates (RF2 and 3B) produced similar results. Similar effects were seen when gp120 was applied to hippocampal neurones (Rosenberg and Aizenman, 1989). Overall, 200 pM gp120 produced an increase in $[Ca^{2+}]_e$ in 76% of the neurones tested ($n = 75$). Highly purified recombinant gp120 envelope protein produced these effects in a dose-dependent fashion at picomolar concentrations. Immunoprecipitation with antibody to gp120, but not with control immunoglobulin-containing serum, depleted solutions of the viral envelope protein and also prevented both the rise in $[Ca^{2+}]_e$ and neuronal toxicity. The gp120-induced increase in $[Ca^{2+}]_e$ was abrogated by transiently lowering extracellular $Ca^{2+}$ or by adding the dihydropyridine calcium channel antagonist nimodipine (100 nM). $Ca^{2+}$ channel antagonists also prevented gp120-induced neuronal injury. In addition, intracellular stores appeared to contribute
Lipton and coworkers 1991, observed that exposure of rat retinal cultures to HIV-1 coat protein gp120 for several minutes increased \([Ca^{2+}]_c\) in approximately half of the ganglion cells. They found that in multiple experiments (n = 9, separate days), \([Ca^{2+}]_c\) in retinal ganglion cells of cultures treated with 20-600 pM gp120 was significantly higher than in controls (p < 0.01). The addition to retinal cultures of either NMDA antagonists or enzyme to degrade endogenous glutamate prevented both the rise in \([Ca^{2+}]_c\) and neuronal death. They therefore suggested that a small influx of \(Ca^{2+}\) via NMDA receptor operated channels, in addition to voltage-activated \(Ca^{2+}\) channels, resulted in mobilization of intracellular \(Ca^{2+}\) stores and the subsequent lethal action of gp120. Since, under standard conditions in these cultures, neither glutamate nor a low picomolar concentration of gp120 is deleterious on its own, they suggested that the neurotoxicity was synergistic.

Lannuzel and coworkers 1995, found that gp120 and gp160 were toxic for human neurones, potentiated the NMDA-induced \([Ca^{2+}]_c\), increases without affecting NMDA activated currents or voltage-sensitive \(Na^+\) or \(Ca^{2+}\) currents, and elicited, in a subpopulation of spinal neurones, an increase in \([Ca^{2+}]_c\), (an increase in \([Ca^{2+}]_c\) in the presence of gp120 or gp160 alone was observed in 12 of 30 spinal neuronal clusters tested between days 15 and 24 but in none of the 14 prosencephalic neuronal clusters tested between days 15 and 30). They reported gp120 and gp160 (20-250 pM) induced potentiation of the NMDA-induced \([Ca^{2+}]_c\) responses in 77% of cases (55% in prosencephalic neurones, n = 20, 93% in spinal neurones, n = 28). I therefore, thought that may be potentiation of NMDA-induced rise in \([Ca^{2+}]_c\) responses would be more reliable than looking for \([Ca^{2+}]_c\) responses induced by gp120 alone. But, that was not the case as

substantially to the increase in \([Ca^{2+}]_c\) elicited by gp120.
clearly evident from table 3.1, when my cultures did not show \([Ca^{2+}]_c\) responses to gp120, they also did not show a potentiation of NMDA-induced rise in \([Ca^{2+}]_c\).

Meucci and coworkers, 1998 also observed \([Ca^{2+}]_c\) responses to gp120. They used a bilaminal culture system in which pure populations of hippocampal neurones were cultured on coverslips in close juxtaposition to an astrocyte feeder layer, although the experiments were carried out in the absence of glial feeder layer. Fura-2-based \([Ca^{2+}]_c\) imaging showed that various chemokines produced either \([Ca^{2+}]_c\) oscillations or single \([Ca^{2+}]_c\) spikes in these cultures. Gp120 (200 pM) also increased \([Ca^{2+}]_c\) in neurones but this was by no means a consistent finding (26 of 55 neurones showed \([Ca^{2+}]_c\) increases by gp120).

Klein and coworkers, 1999 found that the chemokine receptors CCR3, CCR5, and CXCR4 are present in a subpopulation of macaque and human neurones, and CCR5 and CXCR4 are present on astrocytes immediately ex vivo and continue to be expressed on neurones and astrocytes for at least 2–3 wk in culture. Chemokine receptors were shown to respond to their appropriate chemokine ligands with increases in \([Ca^{2+}]_c\) that, in the case of neurones, required predepolarization with KCl. Data were representative of two to five separate experiments with different neuronal preparations. Fetal macaque neurones responded to recombinant SIVmac239 gp120 with increases in \([Ca^{2+}]_c\) and again required predepolarization with 20 mM KCl for maximum response (representative of four separate experiments).

Holden and coworkers, 1999, pressure-applied gp120 on to cultured human fetal neurones and astrocytes and, by using single-cell calcium imaging, determined the mechanisms responsible for gp120-induced increases in the levels of \([Ca^{2+}]_c\). Positive responses to gp120 were defined as
increases in $[Ca^{2+}]_c$ at least two standard deviations above basal levels; 83 ± 1% of neurones and 85 ± 1% of astrocytes responded positively to gp120. Basal $[Ca^{2+}]_c$ of 154 ± 1 nM in 1619 astrocytes analysed (n = 1619 from 21 separate fetuses) was not significantly different from that of 155 ± 1 nM in neurones (n = 1749 from 25 separate fetuses). Pressure application of 25 nM gp120 (HIV-1IIIB or HIV-1SF2) near neurones resulted in a 10-15-fold increase in $[Ca^{2+}]_c$ {Data were mean ± S.E.M. values (above background $[Ca^{2+}]_c$) for at least 40 different cells from two separate fetuses}. Similar results were obtained for astrocytes. When astrocytes and neurones were in close proximity in a single field, astrocyte responses preceded neuronal responses by seconds to minutes.

The authors do not explain why when observing basal $[Ca^{2+}]_c$ levels in neurones and astrocytes 21/25 separate fetuses were used, but for studying the effect of gp120 only two separate fetuses were used. In all the experiments they performed with gp120, they have shown data from only two separate fetuses. This raises the possibility that $[Ca^{2+}]_c$ responses were not observed in cells from the other 23 fetuses.

Medina and coworkers, 1999 report that in postnatal (Postnatal day 2; P2) rat hippocampal cultures maintained in vitro for more than 15 days, gp120 induced a dramatic and persistent rise in $[Ca^{2+}]_c$ in neurones. This rise was entirely due to the release of $Ca^{2+}$ from intracellular stores, as it was fully blocked by drugs that either deplete stores or prevent CICR, and was not affected by antagonists of NMDA receptors or calcium channels. During experiments, neurones were continuously perfused with external solution containing 1 μM TTX and 10 μM CNQX. A brief 1-s pulse of NMDA (50 μM) with glycine (20 μM) through a micropipette induced a fast rise in $[Ca^{2+}]_c$-dependent fluo-3 fluorescence that recovered to resting levels within 10-30s. A similar
focal application of gp120 through a micropipette did not have any significant effect on $[\text{Ca}^{2+}]_c$.

In contrast, bath application of gp120 (200 pM) induced a slow increase in fluorescence in 12 out of 19 experiments (the average fluorescence change was $179 \pm 39\%$, $n = 19$).

Overall a careful reading of the literature gives the clear impression that $[\text{Ca}^{2+}]_c$ responses to gp120 are variable and often absent.
I also found the neuronal \([\text{Ca}^{2+}]_c\) response to gp120 to be highly unreliable. If I draw a graph showing the date of dissection and whether I got a \([\text{Ca}^{2+}]_c\) response to gp120 or not, it will be clearer to see how difficult it was to obtain a \([\text{Ca}^{2+}]_c\) response. For the sake of simplicity I am only including the data from Jan 2000 to May 2000. For details of the medium used, see table 3.1.

\[\text{[Ca}^{2+}]_c\text{ responses to 250 pM gp120, yes or no}\]

*Figure 3.8* Hippocampal cultures grown in the period of Jan 2000-May 2000. \([\text{Ca}^{2+}]_c\) imaging was performed to see whether the neurones showed a \([\text{Ca}^{2+}]_c\) response to gp120 or not.
Table 3.1 tabulates all the results over three years and again shows the variable appearance of the $[\text{Ca}^{2+}]_c$ responses to 250 pM gp120. The reason why some cultures showed a $[\text{Ca}^{2+}]_c$ response to gp120 while others did not is addressed in chapter 6.

My results are set out in the following chapters as follows:

**Chapter 4** addresses the signaling processes underlying the neuronal $[\text{Ca}^{2+}]_c$ response to gp120. The dataset is restricted to the subset of cultures that did indeed respond to gp120 with a neuronal $[\text{Ca}^{2+}]_c$ signal. The problem of why only a small subset of cultures showed this response is not addressed in this chapter.

**Chapter 5** describes experiments on gp120 neurotoxicity. These experiments were performed on those cultures that failed to show an acute neuronal $[\text{Ca}^{2+}]_c$ response to gp120.

**Chapter 6** addresses the question of why only a small subset of hippocampal cultures showed an acute neuronal $[\text{Ca}^{2+}]_c$ response to gp120.
Table 3.1. Tabulation of all primary cultures with responses observed.

<table>
<thead>
<tr>
<th>Date of dissection</th>
<th>Medium</th>
<th>Type of Response to 250 pM gp120</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>4/09/97</td>
<td>MEM + 10% HS</td>
<td></td>
<td>Culture died when 12 days old</td>
</tr>
<tr>
<td>18/09/97</td>
<td>MEM + 10% HS</td>
<td></td>
<td>Culture died when 12 days old</td>
</tr>
<tr>
<td>25/09/97</td>
<td>MEM + 10% HS</td>
<td></td>
<td>Culture died when 12 days old</td>
</tr>
<tr>
<td>2/10/97</td>
<td>MEM + 10% HS</td>
<td>Not determined</td>
<td>Infected</td>
</tr>
<tr>
<td>9/10/97</td>
<td>MEM + 10% HS</td>
<td></td>
<td>All dishes died when 12 days old</td>
</tr>
<tr>
<td>23/10/97</td>
<td>MEM + 10% HS</td>
<td></td>
<td>All dishes died when 12 days old</td>
</tr>
<tr>
<td>30/10/97</td>
<td>MEM + 10% HS</td>
<td></td>
<td>All dishes died when 12 days old</td>
</tr>
<tr>
<td>6/11/97</td>
<td>MEM + 10% HS</td>
<td>Not determined</td>
<td>Infected</td>
</tr>
<tr>
<td>14/11/97</td>
<td>MEM + 10% HS</td>
<td>Potentiation of NMDA induced rise in ([Ca^{2+}]_c) by gp120</td>
<td></td>
</tr>
<tr>
<td>27/11/97</td>
<td>MEM + 10% HS</td>
<td>Potentiation of NMDA induced rise in ([Ca^{2+}]_c) by gp120</td>
<td></td>
</tr>
<tr>
<td>Date of dissection</td>
<td>Medium</td>
<td>Type of response to 250 pM gp120</td>
<td>Comment</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------</td>
<td>---------------------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>14/01/98</td>
<td>MEM + 5% HS</td>
<td>Not determined</td>
<td>All cells died on or before day 12</td>
</tr>
<tr>
<td>21/01/98</td>
<td>MEM + 5% HS</td>
<td>Potentiation of NMDA induced rise in $[Ca^{2+}]_e$ by gp120</td>
<td></td>
</tr>
<tr>
<td>29/01/98</td>
<td>MEM + 5% HS</td>
<td>Potentiation of NMDA induced rise in $[Ca^{2+}]_e$ by gp120</td>
<td></td>
</tr>
<tr>
<td>6/02/98</td>
<td>MEM + 5% HS</td>
<td>Potentiation of NMDA induced rise in $[Ca^{2+}]_e$ by gp120</td>
<td></td>
</tr>
<tr>
<td>All the cultures from Feb to September 98 died</td>
<td>MEM + 5% HS</td>
<td>Not determined</td>
<td>All cells died on or before day 12</td>
</tr>
<tr>
<td>Date of Dissection</td>
<td>Medium</td>
<td>Type of Response to 250 pM gp120</td>
<td>Comment</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------</td>
<td>---------------------------------</td>
<td>-------------------------------------------------------------------------</td>
</tr>
<tr>
<td>21/09/98</td>
<td>NB-HS</td>
<td>Potentiation of NMDA induced rise in $[Ca^{2+}]_c$ by gp120</td>
<td>MEM + 10% HS All cells died on or before day 12</td>
</tr>
<tr>
<td></td>
<td>MEM + 10% HS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28/09/98</td>
<td>NB-HS</td>
<td>Slow, steady increase in $[Ca^{2+}]_c$ Potentiation of NMDA induced rise in $[Ca^{2+}]_c$ by gp120</td>
<td></td>
</tr>
<tr>
<td>5/10/98</td>
<td>MEM + 10% HS</td>
<td>Not determined</td>
<td>All cells died when 12 days old</td>
</tr>
<tr>
<td>26/10/98</td>
<td>NB-HS</td>
<td>Slow, steady increase in $[Ca^{2+}]_c$</td>
<td></td>
</tr>
<tr>
<td>3/11/98</td>
<td>NB-HS</td>
<td></td>
<td>Infection</td>
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<tr>
<td>16/11/98</td>
<td>NB-HS</td>
<td></td>
<td>Infection</td>
</tr>
<tr>
<td>26/11/98</td>
<td>NB-HS</td>
<td>Slow, steady increase in $[Ca^{2+}]_c$</td>
<td></td>
</tr>
<tr>
<td>18/01/99</td>
<td>NB-HS</td>
<td></td>
<td>INFECTION</td>
</tr>
<tr>
<td>Date of Dissection</td>
<td>Medium</td>
<td>Type of Response to 250 pM gp120</td>
<td>Comment</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------</td>
<td>----------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>ALL DISSECTIONS IN JAN, FEB 99 INFECTED</td>
<td>NB-HS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAR, APRIL, MAY 99 DISSECTIONS ALSO SUFFERED FROM INFECTION</td>
<td>NB-HS</td>
<td></td>
<td>Infected</td>
</tr>
<tr>
<td>1/06/99</td>
<td>NB-HS</td>
<td>No $[Ca^{2+}]_c$ response to gp120, No potentiation of NMDA induced $[Ca^{2+}]_c$ rise by gp120</td>
<td></td>
</tr>
<tr>
<td>7/06/99</td>
<td>NB-HS</td>
<td>No $[Ca^{2+}]_c$ response to gp120, No potentiation of NMDA induced $[Ca^{2+}]_c$ rise by gp120</td>
<td></td>
</tr>
<tr>
<td>14/06/99</td>
<td>NB-HS</td>
<td>No $[Ca^{2+}]_c$ response to gp120, No potentiation of NMDA induced $[Ca^{2+}]_c$ rise by gp120</td>
<td></td>
</tr>
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All the dissections, hippocampal and cortical in NB-HS performed in June, July, August, Sep 1999 did not show a $[Ca^{2+}]_c$ response to gp120
<table>
<thead>
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<th>Type of Response to 250 pM gp120</th>
<th>Comment</th>
</tr>
</thead>
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<td>11/10/99</td>
<td>NB-HS</td>
<td>NO RESPONSE</td>
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</tr>
<tr>
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<td>NB+5% HS</td>
<td>Massive abrupt [Ca^{2+}]_c increase</td>
<td></td>
</tr>
<tr>
<td>25/10/99</td>
<td>NB+5% HS</td>
<td>Massive abrupt [Ca^{2+}]_c increase</td>
<td></td>
</tr>
<tr>
<td>1/11/99</td>
<td>NB-HS</td>
<td>NO RESPONSE</td>
<td></td>
</tr>
<tr>
<td>8/11/99</td>
<td>NB+5% HS</td>
<td>Massive abrupt [Ca^{2+}]_c increase</td>
<td></td>
</tr>
<tr>
<td>15/11/99</td>
<td>NB+5% HS</td>
<td>Massive abrupt [Ca^{2+}]_c increase</td>
<td></td>
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<tr>
<td>25/11/99</td>
<td>NB+5% HS</td>
<td>Massive abrupt [Ca^{2+}]_c increase</td>
<td></td>
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<td>Date of dissection</td>
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<td>------------</td>
<td>----------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>31/01/00</td>
<td>NB-HS</td>
<td>NO RESPONSE</td>
<td></td>
</tr>
<tr>
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<td>NB-HS</td>
<td>NO RESPONSE</td>
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<tr>
<td></td>
<td>NB + 5% HS</td>
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<td>NB-HS</td>
<td>NO RESPONSE</td>
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<td>NB + 5% HS</td>
<td>NO RESPONSE</td>
<td></td>
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<td>NB +5% HS</td>
<td>NO RESPONSE</td>
<td></td>
</tr>
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<td>6/03/00</td>
<td>NB +5% HS</td>
<td>NO RESPONSE</td>
<td></td>
</tr>
<tr>
<td>13/03/00</td>
<td>NB-HS</td>
<td>NO RESPONSE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NB + 5% HS</td>
<td>NO RESPONSE</td>
<td></td>
</tr>
<tr>
<td>20/03/00</td>
<td>NB-HS</td>
<td>Delayed and moderate rise in $[Ca^{2+}]_c$</td>
<td>First time did microglia staining with ED1 and saw lots of microglia</td>
</tr>
<tr>
<td></td>
<td>NB + 5% HS</td>
<td>Delayed and moderate rise in $[Ca^{2+}]_c$</td>
<td></td>
</tr>
<tr>
<td>27/03/00</td>
<td>NB +5% HS</td>
<td></td>
<td>Infected</td>
</tr>
<tr>
<td>Date of dissection</td>
<td>Medium</td>
<td>Type of Response to 250 pM gp120</td>
<td>Comment</td>
</tr>
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<td>-------------------</td>
<td>-------------------</td>
<td>----------------------------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>5/04/00</td>
<td>NB-HS</td>
<td>NO RESPONSE</td>
<td></td>
</tr>
<tr>
<td>17/04/00</td>
<td>NB-HS</td>
<td>Delayed and moderate rise in [Ca^{2+}]_c</td>
<td></td>
</tr>
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<td>NB-HS</td>
<td>NO RESPONSE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NB + 5% HS</td>
<td>NO RESPONSE</td>
<td></td>
</tr>
<tr>
<td>2/05/00</td>
<td>NB-HS</td>
<td>NO RESPONSE</td>
<td></td>
</tr>
<tr>
<td>8/05/00</td>
<td>NB + 5% HS</td>
<td>NO RESPONSE</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 4. Calcium and Other Immediate Responses

4.1. Introduction

Modulation of $[\text{Ca}^{2+}]_c$ is an important means of regulating diverse neuronal functions. $[\text{Ca}^{2+}]_c$ can be elevated by influx via voltage-sensitive (Tsien, 1983) and receptor operated (Mayer and Westbrook, 1987) $\text{Ca}^{2+}$ channels, mobilization from intracellular stores (Pozzan et al., 1994), or by reversal of the electrogenic $\text{Na}^+$/Ca$^{2+}$ exchanger (Khodorov et al., 1993). Removal of $[\text{Ca}^{2+}]_c$ after stimulation of $\text{Ca}^{2+}$ influx is performed by multiple processes; 1) mitochondrial $\text{Ca}^{2+}$ uptake 2) $\text{Ca}^{2+}$ binding by cytosolic proteins 3) ATP dependent $\text{Ca}^{2+}$ efflux or sequestration and 4) $\text{Ca}^{2+}$ efflux via $\text{Na}^+$/Ca$^{2+}$ exchange (Carafoli, 1987; Pozzan et al., 1994).

It is now generally accepted that $\text{Ca}^{2+}$ is an important ion in mediating the transition to neuronal death in the case of gp120 toxicity, because use of NMDA receptor antagonists not only blocks the rise in $\text{Ca}^{2+}$ but also rescues the neurones from dying (Lipton et al., 1995). However, AMPA, kainate and NMDA receptor channels also mediate the influx of $\text{Na}^+$, which can further contribute to the elevation of $[\text{Ca}^{2+}]_c$ and directly cause damage to neurones. In agreement with this, blockade of voltage sensitive $\text{Na}^+$ channels with TTX reduces gp120 neurotoxicity (Diop et al., 1994).

In the work described here, I will be considering two cell surface receptors that could potentially cause a rise of neuronal $[\text{Ca}^{2+}]_c$: a glutamate receptor and the chemokine receptor CXCR4, a seven transmembrane receptor signalling to trimeric G proteins. All results are from that subset of cultures that generated neuronal $[\text{Ca}^{2+}]_c$ responses to gp120.
Exposure of hippocampal neurones to gp120 potentiates the $[\text{Ca}^{2+}]_c$ increase generated by a subsequent exposure to NMDA receptor agonists (Figure 3.2, and Lannuzel et al., 1995). However, both Lipton and coworkers and Lannuzel et al. report that in voltage clamped cells gp120 has no effect on the currents generated by subsequent exposure to NMDA receptor agonists (Lannuzel et al., 1995; Lipton et al., 1991). One possible explanation of this paradox is that gp120 depolarizes unclamped neurones. This would then increase the $\text{Ca}^{2+}$ influx through activated NMDA receptors, because depolarization relieves the blockage of the channel by extracellular Mg$^{2+}$ ions (Schneggenburger et al., 1993). This phenomenon would not be observed in voltage clamped cells. I therefore performed experiments to examine the effect of gp120 on the membrane voltage of hippocampal neurones.

4.2. Results

4.2.1. Block of Gp120 Induced Rise in Neuronal $[\text{Ca}^{2+}]_c$ by Chemokine Receptor Antagonist peptide VSLSYRCPCRFF

Two protein targets for gp120 have been identified in brain tissue. Gp120 can bind to the glycine site on the NMDA receptor, favouring opening (Pittaluga et al., 1996; Pittaluga and Raiteri, 1994; Xin et al., 1999). Gp120 can also bind to the chemokine receptor CXCR4, which is expressed on neurones, astrocytes, and microglia (Albright et al., 1999; Hesselgesser et al., 1997; Klein et al., 1999). The role of these two targets were tested in cultures grown with HS where untreated neurones showed robust $[\text{Ca}^{2+}]_c$ responses to gp120.

VSLSYRCPCRFF, a peptide derived from the chemokine SDF-1α, has been shown to protect HeLa cells against infection and to block the $[\text{Ca}^{2+}]_c$ response to SDF-1α without triggering a
[Ca\textsuperscript{2+}]\textsubscript{c} response itself (Heveker et al., 1998). I examined the effect of VSLSYRCPRFF (50 μM) on the neuronal [Ca\textsuperscript{2+}]\textsubscript{c} response in primary hippocampal cultures. Cultures were treated with this peptide for 15 minutes, followed by the addition of 250 pM gp120 in the continued presence of VSLSYRCPRFF. In 82 neurones in 6 fields from 3 independent cultures, no [Ca\textsuperscript{2+}]\textsubscript{c} rise to gp120 was observed. While a [Ca\textsuperscript{2+}]\textsubscript{c} rise to gp120 was seen in 75 of 76 sister neurones in 6 fields from the same 3 cultures not treated with VSLSYRCPRFF (Figure 4.1).

4.2.2 Block of Gp120 Induced Rise in Neuronal [Ca\textsuperscript{2+}]\textsubscript{c} by Glutamate Receptor Blockers

Antagonists of glutamate receptors have previously been reported to block the [Ca\textsuperscript{2+}]\textsubscript{c} response to gp120 in rat neurones (Lipton, 1991; Lo et al., 1992).

I incubated the hippocampal cultures for 30 minutes in the presence of glutamate receptor blockers (20 μM MK-801, 20 μM CNQX and 500 μM MCPG) followed by the addition of 250 pM gp120 in the presence of these blockers. In 58 neurones in 6 fields from 3 independent cultures no [Ca\textsuperscript{2+}]\textsubscript{c} response to gp120 was observed while a [Ca\textsuperscript{2+}]\textsubscript{c} response to gp120 was seen in 60 of 60 sister neurones in 6 fields not treated with glutamate antagonists (Figure 4.2).
**Figure 4.1.** The chemokine receptor mediates the neuronal \([Ca^{2+}]_e\) response to gp120. **A:** All neurones imaged in a culture maintained in Neurobasal + 2% B27 +5% HS respond to gp120. Overall 75 of 76 neurones in 6 fields from 3 independent cultures responded to gp120. **B:** In a second field from the same culture incubated with CXCR4 antagonist peptide VSLSYRCPCRFF, no neurone responded to gp120. Overall 82 neurones in 6 fields from the same 3 cultures were tested, none responded to gp120.
Figure 4.1

A

B

+ peptide

[Ca^{2+}]_c (nM)

Time (min)
Figure 4.2. A glutamate receptor mediates the neuronal $[Ca^{2+}]_c$ response to gp120. A: All neurones imaged in a culture maintained in NB medium + 2% B27 +5% HS respond to gp120. Overall 60 of 60 neurones in 6 fields from 3 independent cultures responded to gp120. B: In a second field from the same culture incubated with cocktail of glutamate receptor antagonists (20 μM MK-801, 20 μM CNQX, 500 μM MCPG) no neurone responded to gp120. Overall 58 neurones in 6 fields from the same 3 cultures were tested, none responded to gp120.
Figure 4.2

A

B

+ cocktail of glutamate receptor antagonists
4.2.3. Effect of Gp120 on Membrane Voltage

The resting membrane voltage of hippocampal neurones was recorded by the whole cell patch clamp method, using a pipette solution with an ionic composition similar to that of cytosol. Application of 250 pM gp120 in a rapid perfusion bath caused no significant change of membrane voltage (Figure 4.3).

A possible problem with these experiments was that an intracellular messenger or other critical solute might be lost from the cells through the patch pipette. I therefore repeated the experiment using a fluorescent dye technique, rather than an electrophysiological approach, to measure membrane voltage.

![Graph showing effect of gp120 on membrane voltage.](image)

**Figure 4.3.** Effect of gp120 on membrane voltage. A neurone from a hippocampal culture grown in MEM + 10% HS, 13 days old bathed in HBS was whole cell patch clamped through a pipette containing internal solution (see section 2.5 for details). Immediately after attaining the whole cell configuration the amplifier was switched to \( i = 0 \) mode, that is, the system measured membrane voltage without injecting current. After 2 minutes of recording the bathing solution was rapidly changed to HBS containing 250 pM gp120. No effect on membrane voltage was seen in this or nine other similar cells.
4.2.4. Gp120 Block of Potassium Depolarization

In order to study the effect of gp120 on neuronal membrane potential, I used the slow membrane potential dye DiBAC$_4$(3). In control experiments, the dish was first incubated with HBS + DiBAC$_4$(3) (1 μM), followed by perfusion of high potassium saline + DiBAC$_4$(3) (1 μM), in which K$^+$ replaced Na$^+$ to a total concentration of 68 mM. High potassium depolarized the neurones and caused an increase in fluorescence as shown in Fig 4.4A, (resting fluorescence = 52.58 ± 1.40 arbitrary units, rose to = 84.34 ± 5.35 arbitrary units, n = 58 from 3 independent cultures). Other dishes exposed to gp120 + DiBAC$_4$(3) (1 μM), showed no change in fluorescence and therefore membrane potential, confirming the results obtained electrophysiologically. Surprisingly, when these gp120-treated neurones were then superfused with high potassium saline + DiBAC$_4$(3) (1 μM), no depolarization occurred (n = 62 from 3 independent cultures) (Fig 4.4B). All these cultures were grown in MEM + 10% HS.

I observed a similar result when measuring neuronal [Ca$^{2+}$]$_c$ responses to high potassium saline. I treated cultures with high potassium saline in the presence of cocktail of glutamate receptor blockers (MK-801 20 μM, CNQX 20 μM, MCPG 500 μM), and applied high potassium for 2 second. As expected [Ca$^{2+}$]$_c$ rose dramatically in neurones (resting [Ca$^{2+}$]$_c$ = 52.78 ± 2.46 nM, rose to = 262.91 ± 25.39 nM, n = 40, from 3 independent cultures) due to influx through voltage gated calcium channels (Fig 4.5A). When gp120 was applied to hippocampal cultures in the presence of glutamate receptor blockers there was no neuronal [Ca$^{2+}$]$_c$ rise, as expected in the presence of the receptor blockers. However, when high potassium saline was added 5 minutes later there was no [Ca$^{2+}$]$_c$ rise in neurones (n=58, from 3 independent cultures) (Fig 4.5B).
Figure 4.4. gp120 block of potassium depolarisation. A: All cells in a culture depolarized in response to raised extracellular potassium. Overall 58 neurones in 6 fields from 3 independent cultures showed increase in fluorescence on addition of high potassium (68 mM) HBS. B: In a second field from the same culture treated with gp120 no neurone showed a change in membrane potential. Subsequent superfusion with high potassium HBS did not evoke depolarisation. Overall 62 neurones in 6 fields from the same 3 cultures were tested, none showed depolarisation by high potassium HBS applied after gp120.
Figure 4.4
**Figure 4.5.** Gp120 block of potassium induced rise in neuronal $[\text{Ca}^{2+}]_c$. A: All neurones in a culture showed a $[\text{Ca}^{2+}]_c$ peak on addition of high potassium HBS in the presence of glutamate receptor blockers (20 $\mu$M MK-801, 20 $\mu$M CNQX and 500 $\mu$M MCPG). Overall 40 neurones in 6 fields from 3 independent cultures showed a $[\text{Ca}^{2+}]_c$ peak on addition of high potassium HBS in the presence of glutamate receptor blockers. B: In a second field from the same culture incubated with gp120 + glutamate receptor blockers no neurone showed $[\text{Ca}^{2+}]_c$ peak on addition of high potassium HBS. Overall 58 neurones in 6 fields from the same 3 cultures were tested, none showed $[\text{Ca}^{2+}]_c$ peak.
Figure 4.5

A

B

gp120 + cocktail of glutamate receptor antagonists
4.3. Discussion

4.3.1. involvement of Chemokine Receptor CXCR4 in the Rise of Neuronal \([Ca^{2+}]_c\)

There is evidence that activation of neuronal chemokine receptors for example, in sensory (Bolin et al., 1998), and hippocampal neurones (Meucci et al., 1998) does produce \([Ca^{2+}]_c\) transients. VSLSYRCPCRFF, a peptide derived from SDF-1α, has been shown to protect HeLa cells against infection and to block the rise of \([Ca^{2+}]_c\) to SDF-1α without triggering a \([Ca^{2+}]_c\) response itself (Heveker et al., 1998).

I examined the effect of VSLSYRCPCRFF on the neuronal \([Ca^{2+}]_c\) response to gp120 in my primary hippocampal cultures and found that when cultures were treated with the peptide for 15 minutes, the gp120 induced rise in neuronal \([Ca^{2+}]_c\) was completely abrogated. Thus, the CXCR4 receptor plays an obligate part in the \([Ca^{2+}]_c\) response of cultured hippocampal neurones to gp120.

4.3.2. Involvement of Glutamate Receptor in the Rise of Neuronal \([Ca^{2+}]_c\)

Use of NMDA receptor antagonists can block the gp120 induced rise in \([Ca^{2+}]_c\) and also rescue the neurones from dying (Lipton, 1992a). In agreement with this report, I found that when the rodent primary hippocampal cultures were preincubated with a cocktail of glutamate receptor blockers (20 μM MK-801, 20 μM CNQX and 500 μM MCPG) the rise of \([Ca^{2+}]_c\) in neurones
upon addition of gp120 was completely absent showing the involvement of a glutamate receptor in the rise in neuronal $[Ca^{2+}]_e$.

4.3.3. The Neuronal $[Ca^{2+}]_e$ Rise is Delayed yet Concerted

Although the majority of cultures failed to show a neuronal $[Ca^{2+}]_e$ response to gp120, in those that did respond, $[Ca^{2+}]_e$ in all the neurones within the field of view remained low for a few minutes after gp120 addition and then rose suddenly in all the neurones (e.g. Fig 4.1A and Fig 4.2A). The simplest explanation for these observations is that the onset of the $[Ca^{2+}]_e$ rise represents the onset of concerted electrical activity in the neurones. Hippocampal neurones in culture form mutual excitatory synapses (Cattabeni et al., 1999). Under certain conditions, e.g. when inhibitory GABAergic synapses are blocked, a rise in electrical activity causes an increased glutamate release from synapses leading to further electrical activity in a positive feedback mechanism that progresses to massive depolarization of all the neurones. On this model, gp120 acts in some manner to increase the probability of such massive self-reinforcing depolarization setting the neuronal network into an unstable state where at any time a small, random increase in electrical activity will rapidly progress to massive, uniform depolarization.

The network model, can explain why Lipton and Lannuzel failed to see an effect of gp120 on NMDA currents (Lannuzel et al., 1995; Lipton et al., 1991). An important point to be noted is that both groups applied NMDA/glutamate to the patched cells only and therefore would not have triggered network activity.
4.3.4. Gp120 Blocks Potassium induced Depolarization

The result illustrated in Figure 4.5, that gp120 blocks the neuronal \([\text{Ca}^{2+}]_c\) rise to high \(K^+\) applied in the presence of glutamate receptor blockers, could be explained by proposing that gp120 blocked voltage gated calcium channels. However, the result illustrated in Figure 4.4 shows that this is not the explanation, rather, gp120 is blocking the depolarizing effect of the high \(K^+\). My explanation for this surprising result of section 4.2.4 is based on the fact that hippocampal cultures contain both glutamatergic and GABAergic neurones (Hoch and Dingledine, 1986). I propose that gp120 acts either directly or indirectly to cause the release of all transmitters from all the neurones. When glutamate receptors are blocked, the GABA effect, an opening of chloride channels, is unmasked. The membrane voltage is clamped close to its resting value, so that when high potassium saline is applied, the neurones do not depolarize. The network model explained above fits nicely with ‘gp120 blocks \(K^+\) depolarization’ phenomenon.
Chapter 5. HIV-1 Coat Protein Gp120 and Neuronal Injury

5.1. Introduction

Purified gp120 has been shown to cause significant neuronal cell loss in rodent hippocampal cultures (Brenneman et al., 1988; Dreyer et al., 1990). This is not the result of direct viral infection because neurones themselves are only rarely, if ever, infected by the virus. Lipton and coworkers have suggested that immune-activated, HIV-1-infected or gp120-stimulated brain macrophages and microglia release a number of factors that act upon neurones to produce excessive stimulation of NMDA receptors. Ca\(^{2+}\) entering the neurones through the NMDA receptors and through voltage gated calcium channels then triggers either apoptosis or necrosis (Dawson et al., 1993; Dreyer et al., 1990; Lipton et al., 1991). In confirmation of this hypothesis, the NMDA antagonist APV completely prevented gp120-induced neurotoxicity (Lipton et al., 1991; Lipton, 1994b). The protein inhibitor cycloheximide (CHX) also reduced gp120-induced neurotoxicity. The protective action of CHX could be caused by interference with some active processes in the cell-death program triggered by gp120, as reported for other cases of apoptosis (Galli et al., 1995; Martin et al., 1988) or by prevention of oxidative stress-induced apoptosis (Ratan et al., 1994). Alternatively, CHX might merely decrease the production and release of gp120-induced neurotoxins by non-neuronal cells (Giulian et al., 1993). A qualitatively different mechanism by which gp120 might kill neurones is suggested by the recent finding that the chemokine receptors CCR5 and CXCR4, which are thought to be the normal chemokine receptors for gp120 binding to macrophages and lymphocytes, are also present on neurones. Chemokines (chemotactic cytokines) are a family of related proteins that have been shown to be essential for information transfer and signalling between different types of immune cells (Baggiolini, 1998). Chemokine structure and effect indicate that they fall into four different
families, typified by the cysteine residues in their sequences (Baggiolini, 1998; Luster, 1998). In the α-chemokines, such as SDF-1α, one amino acid separates the first two cysteines (CXC), whereas in β-chemokines, such as RANTES, the first two cysteines are next to one another (CC). Two other emerging “families” of chemokines may be typified by fractalkine, in which the first two cysteines are separated by three amino acid residues (CXXXC), and lymphotactin, which has only two cysteines in total. Chemokines exert their effect by activating a family of G-protein-coupled receptors, named for the type of chemokine they bind. α-Chemokines activate the receptors CXCR1-5, β-chemokines activate the receptors CCR1-10, and fractalkine activates the receptor CX3CR1. However, there are many “orphan” chemokine receptors in the literature that await the identification of their appropriate ligand(s). It was shown that for infection of macrophages by M-tropic (i.e., macrophage-selective) strains of HIV-1, CD4 and the chemokine receptor CCR5 were required and that the infection of T lymphocytes by T-tropic (i.e., T lymphocyte-selective) strains of HIV-1 used the CXCR4 receptor (Bacon et al., 1995; Baggiolini, 1998; Broder and Collman, 1997; Broder and Dimitrov, 1996; Luster, 1998). The precise determinants of HIV-1 tropism in vivo are clearly very complex and continue to be defined. Thus, some strains of HIV-1 exhibit dual tropism, and others can use additional chemokine receptors as well as CCR5 and CXCR4.

These results raise the possibility that gp120 might bind directly to chemokine receptors on neurones and cause death. Binding of gp120 to neurones might initiate several events, including interference with the normal trophic effects of chemokines. These events may be directly neurotoxic or could enhance the sensitivity of neurones to other factors such as glutamate receptor activation (Lipton, 1994a; Meucci and Miller, 1996). Alternatively, gp120 might exert its action at some non-neuronal component of the culture that expresses chemokine receptors.
5.1.1. Role of Mitochondria in Cell Death

The potential across the inner mitochondrial membrane ($\Delta \psi_m$) is the central parameter that controls mitochondrial respiration, ATP synthesis, and Ca$^{2+}$ accumulation (for review see Duchen, 1999 and Nicholls and Budd, 2000), as well as the generation of reactive oxygen species (Boveris et al., 1972; van Belzen et al., 1997). Because each of these factors can influence the survival of the cell directly or indirectly, the monitoring of $\Delta \psi_m$ in glutamate-exposed neurones provides important information on the mechanism by which mitochondria influence the survival of glutamate-exposed neurones. The mitochondria load with Ca$^{2+}$ during glutamate exposure (Budd and Nicholls, 1996; Wang and Thayer, 1996; White and Reynolds, 1995; White and Reynolds, 1997), and there is general consensus that exposure of the neurones to glutamate results in a qualitative mitochondrial depolarization (Ankarcrona et al., 1995; Budd and Nicholls, 1996; Isaev et al., 1996; Keelan et al., 1999; Khodorov et al., 1996; Prehn, 1998; Scanlon and Reynolds, 1998; Schinder et al., 1996; Stout et al., 1998; Vergun et al., 1999). However, the cause, extent, and bioenergetic consequences of such depolarization remain unclear and need further clarification (Nicholls and Budd, 2000). Conversely, previous depolarization of mitochondria under conditions that prevent ATP depletion protects cultured neurones against delayed cell death (Budd and Nicholls, 1996; Castilho et al., 1998; Stout et al., 1998).

The high Ca$^{2+}$ permeability of the NMDA glutamate receptor seems central to this role, as the accumulation of $[\text{Ca}^{2+}]_c$ is clearly pivotal in triggering subsequent cell death (for review see Choi and Rothman, 1990). The downstream events that couple the rise in $[\text{Ca}^{2+}]_c$ to cell death are less well defined, and it is clear that a rise in $[\text{Ca}^{2+}]_c$ alone is insufficient to cause cell death.
(Tymianski et al., 1994), as not all Ca\(^{2+}\) loads produce pathology. Large sustained elevations of [Ca\(^{2+}\)]\(_{c}\) can be induced following kainate treatment of spinal neurones (Tymianski et al., 1993) without substantial cell death. The demonstration of a direct causal link between Ca\(^{2+}\) influx and neuronal death had been controversial (Michaels and Rothman, 1990), until Tymianski et al. (1993) and Sattler et al. (1998) showed that the source and duration of the Ca\(^{2+}\) influx were critical in determining the relationship between [Ca\(^{2+}\)]\(_{c}\) and neurotoxicity. This has been described in terms of a 'source specificity' of calcium-induced neurotoxicity, suggesting that cell fate is determined not only by the absolute Ca\(^{2+}\) load but also by the route of Ca\(^{2+}\) influx. Implicit in this hypothesis is the suggestion that NMDA-induced neurotoxicity results either from some specific attribute of the NMDA receptor-channel complex or from its spatial relationship to other cellular structures.

Nitric oxide synthase (NOS) has emerged as a plausible link between NMDA receptor activation and neurotoxicity. In cerebral cortical cultures, NMDA neurotoxicity is dramatically attenuated by inhibitors of NOS (Dawson and Dawson, 1996), and the use of neuronal NOS (nNOS) inhibitors in models of focal cerebral ischaemia produced dramatic reductions in infarct volume (Dalkara and Moskowitz, 1994). However, the mechanisms of NO toxicity remain uncertain and the involvement of NO has not been demonstrable in some in vitro models (Dawson et al., 1994; Garthwaite and Garthwaite, 1994).

Several recent studies have suggested that mitochondrial dysfunction may represent a key determinant of the outcome of glutamate neurotoxicity (Ankarcrona et al., 1996; Schinder et al., 1996; White and Reynolds, 1996) although the underlying mechanisms remain uncertain. Nevertheless, there are a number of possible mechanisms whereby NO-mediated pathways may
modify mitochondrial function (Schweizer and Richter, 1994), supporting a plausible link
between NO production and mitochondrial dysfunction. This is further supported by recent
observations showing that nNOS-knockout mice are dramatically resistant to mitochondrial
neurotoxins (Schulz et al., 1997).

In order to provide a clearer understanding of the mechanisms underlying glutamate-induced
mitochondrial dysfunction Duchen and coworkers have examined the relationship between
\([Ca^{2+}]_c\) and \(\Delta \psi_m\). More specifically, they have examined the hypothesis that NO serves as a link
between glutamate-induced \([Ca^{2+}]_c\) overload and mitochondrial dysfunction (Keelan et al., 1999).
They have suggested that \([Ca^{2+}]_c\) and NO act synergistically to cause mitochondrial dysfunction
and impaired \([Ca^{2+}]_c\) homeostasis during glutamate toxicity. They found that glutamate-induced
loss of \(\Delta \psi_m\) was dependent on \(Ca^{2+}\) influx. However, inhibition of nitric oxide synthase (NOS)
by L-NAME (N-nitro-L-arginine methyl ester) significantly attenuated the loss of \(\Delta \psi_m\).
Furthermore, photolysis of caged NO at levels that had no effect alone promoted a profound
mitochondrial depolarisation when combined with high \([Ca^{2+}]_c\), either in response to KCl or to
 glutamate in cultures at 7-10 DIV. Furthermore, in cells that showed only modest mitochondrial
responses to glutamate, induction of a mitochondrial depolarisation by the addition of NO was
followed by a secondary rise in \([Ca^{2+}]_c\). These data suggest that \([Ca^{2+}]_c\) and NO act
synergistically to cause mitochondrial dysfunction and impaired \([Ca^{2+}]_c\) homeostasis during
 glutamate toxicity.

To my knowledge, until now, little work has been done to study the effect of gp120 on
mitochondrial potential. It could be possible that gp120 produces mitochondrial depolarization
and hence, cell death. Brooke et al. (1998) showed that glucocorticoids (GCs), steroid hormones
secreted during stress, work in conjunction with gp120 to decrease ATP levels and to work synergistically with gp120 to decrease the mitochondrial potential in hippocampal cultures. Furthermore, energy supplementation blocked the ability of GCs to worsen gp120's effects on neuronal survival and Ca\(^{2+}\) mobilization.

5.1.2. G-Protein Activation by Gp120/Chemokines

Heterotrimeric \((\alpha,\beta,\gamma)\) G proteins serve as molecular switches to transduce information from occupied (activated) receptors to appropriate intracellular effectors (Gilman, 1987). Downstream targets regulated by heterotrimeric G proteins include adenylyl cyclase, phosphatidylinositol-specific phospholipases C, guanylate cyclase, cGMP phosphodiesterase, and ion channels, particularly those permeable to Ca\(^{2+}\) and K\(^+\). Other processes shown to be regulated by heterotrimeric G proteins include the mitogen activated protein (MAP) kinase cascade activated by growth factors (Cobb and Goldsmith, 1995) and vesicular trafficking (Nuoffer and Balch, 1994). These last pathways, as well as others, are also regulated by single monomeric G proteins that exhibit GTP binding and hydrolysis activities and are also considered part of the G-protein superfamily (Hall, 1994).

The heterotrimeric G proteins consist of three distinct subunits: \(\alpha\) (39-46 kDa), \(\beta\) (37 kDa), and \(\gamma\) (8 kDa). The \(\alpha\) subunit has a single, high affinity binding site for guanine nucleotides GTP and GDP and possesses intrinsic GTPase activity. The \(\beta\) and \(\gamma\) subunits exist as a tightly associated complex that functions as a unit. There is increasing evidence that the \(\beta\gamma\) complex and the free \(\alpha\) subunit as well as the heterotrimeric \((\alpha\beta\gamma)\) complex can all interact with and regulate effector proteins and may, in fact, act synergistically.
The initial identification of G proteins was defined by the function of the α subunit. There are currently four major subfamilies of α subunits classified on the basis of sequence relationships represented by Gαs, Gαi/Gαo, Gαq and the less defined Gα12 (Table 5.1). The Gs family was the first to be identified because of its ability to stimulate adenylyl cyclase to generate cAMP from ATP. More recently Gαs has been shown to stimulate dihydopyridine-sensitive voltage-gated Ca2+ channels in skeletal muscles and inhibit cardiac Na+ channels (Schubert et al., 1989). Similarly, Gi was initially classified by its ability to inhibit adenylyl cyclase, which may be a direct effect of βγ on adenylyl cyclase (Tang and Gilman, 1991). More recently Gi or related proteins (Go, Gr, etc.) have been shown to activate the rhodopsin-sensitive cGMP phosphodiesterase, and have been implicated in the stimulation of phospholipase C (PLC). The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by PLC generates two intracellular messengers: inositol 1,4,5-triphosphate (IP3), which increases [Ca2+]o and diacylglycerol (DAG), which stimulates protein kinase C (PKC).
<table>
<thead>
<tr>
<th>Family</th>
<th>Toxin</th>
<th>Receptors</th>
<th>Effector</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_s$</td>
<td>Cholera</td>
<td>Glucagon, TSH, LH, VIP, $\beta$-adrenergic</td>
<td>Adenylyl cyclase ($\uparrow$) Ca$^{2+}$ channels ($\uparrow$) Na$^+$ channels ($\downarrow$)</td>
</tr>
<tr>
<td>$G_i$</td>
<td>Pertussis</td>
<td>$\alpha$-Adrenergic, muscarinic, opiate, IGF-2, Rhodopsin</td>
<td>Adenylyl cyclase ($\downarrow$) Phospholipase C-$\beta$ ($\uparrow$) Ca$^{2+}$ channels ($\downarrow$)</td>
</tr>
<tr>
<td>$G_o$</td>
<td>Pertussis</td>
<td>$\alpha$-Adrenergic, muscarinic</td>
<td>Phospholipase C-$\beta$ ($\uparrow$) K$^+$ channels ($\uparrow$) Ca$^{2+}$ channels ($\downarrow$)</td>
</tr>
<tr>
<td>$G_q$</td>
<td>None</td>
<td>$\alpha$-Adrenergic, muscarinic</td>
<td>Phospholipase C-$\beta$ ($\uparrow$)</td>
</tr>
<tr>
<td>$G_{12}$</td>
<td>None</td>
<td>??</td>
<td>??</td>
</tr>
</tbody>
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Most evidence suggested that chemokines signalled to $G_i$ and therefore downregulated adenyllyl cyclase (Myers et al., 1995). In fact, studies with transfected cells confirmed that IL-8, fMLP and C5a signalled to members of both $G_i$ and $G_q$ families of $G$ proteins (Amatruda et al., 1993; Buhl et al., 1993; Wu et al., 1993). The involvement of the $G_i/G_o$ family of $G$ proteins in a particular response can be demonstrated by showing sensitivity to the IAP component of the toxin produced by Bordetella pertussis because this toxin ADP-ribosylates and hence deactivates $G_i/G_o$ family members. The signal transduction by the MCP-1 receptor both in a T-cell hybrid (Dubois et al., 1996) and in kidney cells transfected with the receptor (Myers et al., 1995) was blocked by pertussis toxin. Similarly, pertussis toxin abrogated the IL-8-mediated intracellular $Ca^{2+}$ mobilization and chemotaxis in Jurkat cells transfected with IL-8R1 and IL-8R2 receptors (Jones et al., 1995), fMLP-induced actin polymerization and $[Ca^{2+}]_c$ elevation in polymorphonuclear leukocytes (Omann et al., 1992), and IL-2-activated natural killer (NK) chemotaxis induced by RANTES and MCP-1 (Maghazachi et al., 1994). Recently, the $G_{\beta\gamma}$ subunits of $G$ proteins were also found to activate specific isoforms of PLC$\beta$. The $G_{\beta\gamma}$-linked pathway may account for the PTX-sensitive activation of PLC mediated by the IL-8 receptors in mature leukocytes (Wu et al., 1993).

In this chapter I also report the effect of the GABA-B agonist baclofen. GABA is the main inhibitory neurotransmitter in the adult brain. It acts via ionotropic $GABA_A$ and $GABA_C$ receptors and $G$ protein-coupled $GABA_B$ receptors (Barnard et al., 1998; Macdonald and Olsen, 1994; Mehta and Ticku, 1999). Due to its hyperpolarizing properties, GABA counteracts the excessive neuronal activity caused by excitatory amino acids that, if prolonged, leads to neurotoxicity (Choi, 1994a; Choi, 1994b; Macdonald and Olsen, 1994; Olsen and Avoli, 1997).
Accordingly, the enhancement of GABA function protects against glutamate-induced neuronal loss \textit{in vitro} and \textit{in vivo}. For example, enhancement of GABAergic transmission alleviates hippocampal and cortical neuronal damage in animal models of ischemia and epilepsy (Johansen and Diemer, 1991; Schwartz et al., 1994; Ylinen et al., 1991). Activation of GABA\textsubscript{A} receptors also protects against cell loss in models of glutamate-evoked toxicity in neuronal culture (Muir et al., 1996; Ohkuma et al., 1994). Drugs that increase the extracellular levels of brain GABA are widely used in the treatment of epilepsy in children and adults (Macdonald and Kelly, 1993).

5.2. Results

All the experiments in this chapter were done on cultures of hippocampal and cortical neurones that showed no acute $[\text{Ca}^{2+}]_{c}$ elevation in response to gp120.

5.2.1. Effect of Gp120 on Mitochondrial Potential

I examined the effect of gp120 upon mitochondrial membrane potential by recording fluorescence of the lipophilic cation Rh123. In the majority of experiments (13 fields of cells) no response was seen. However, in two fields addition of gp120 was followed by mitochondrial depolarization in one cell within the field of view, other cells showing no change (Figure 5.1). I never observed mitochondrial depolarization when vehicle alone was applied (15 fields of cells).
Figure 5.1

A culture of hippocampal neurones was loaded with fura-2 to measure $[Ca^{2+}]_c$ and Rh-123 to measure mitochondrial membrane potential. Before: Rh-123 image before addition of gp120. After: after addition of gp120, Rh-123 signal increased dramatically in one cell (blue arrow), indicating mitochondrial depolarization. FCCP: addition of FCCP to depolarize mitochondria caused an increase of Rh-123 signal from all neurons.

Graph: Fura-2 ratio as a measure of $[Ca^{2+}]_c$ (continuous blue line) and Rh-123 fluorescence as a measure of mitochondrial membrane potential (dashed red line) in the indicated cell. $[Ca^{2+}]_c$ transients associated with electrical activity are clear, but there is no $[Ca^{2+}]_c$ increase associated with the mitochondrial depolarization. (The slight fall in fura-2 ratio when Rh-123 signal rises is a crosstalk artefact).
5.2.2. Gp120 Toxicity on Hippocampal Neurones

The centre column of Figure 5.2A shows the effect of gp120 on hippocampal neurones. The cultures were maintained in NB medium + 2% B27 for 22 days, the medium was then changed to one containing 250 pM gp120 and the cultures incubated for a further 2 days. In control cultures, which also had the medium changed at 22 days, less than 5% of neurones died. In contrast, in cultures containing gp120 over 30% died. The staining method used distinguishes between apoptotic cells, in which the condensed nuclei show intense Hoechst fluorescence, and necrotic cells, which stain dimly with Hoescht but show bright propidium iodide stain. The neuronal death in my experiments was necrotic. VSLSYRCPCRFF, a peptide derived from SDF-1α, has been shown to protect HeLa cells against HIV-1 infection and to block the [Ca^{2+}]_c response to SDF-1α without triggering a [Ca^{2+}]_c response itself (Heveker et al., 1998). I examined the effect of including 50 μM VSLSYRCPCRFF in the bathing solution two hours before and during application of gp120. As shown in Figure 5.2A, the peptide protected against gp120 toxicity.

Chemokine receptors are thought to signal to trimeric GTP binding proteins of the G_i/G_o family. To test for an involvement of G_i/G_o in gp120 toxicity, I preincubated hippocampal cultures with 500 ng/ml of the IAP component of pertussis toxin before applying gp120. This gave significant rescue, although not as dramatic as the rescue achieved with VSLSYRCPCRFF (Figure 5.3A).

Although these experiments were done on cultures of hippocampal neurones that showed no acute [Ca^{2+}]_c elevation in response to gp120, it could be argued that during the two days of culture in the presence of gp120 [Ca^{2+}]_c became elevated in the neurones. To test for this possibility, [Ca^{2+}]_c was measured in hippocampal neurones maintained in NB medium + 2% B27 alone for 22 days then incubated in 250 pM gp120 for a further 3 days. Under these conditions
about 30% of the neurones will have died, however, in those live neurones that loaded with fura-2 [Ca$^{2+}$]$_e$ (60.8 ± 4.2 nM, 50 cells) did not fluctuate and was not greater than [Ca$^{2+}$]$_e$ in untreated neurones.

5.2.3. Gp120 Toxicity on Cortical Neurones

As noted in chapter 3, I never saw acute [Ca$^{2+}$]$_e$ responses to gp120 in cortical cultures. Nevertheless, gp120 caused significant cell death in cortical cultures, very similar to that seen in the hippocampal cultures (Figure 5.2B). Once again, the chemokine inhibitory peptide VSLSYRCPCRFF gave considerable rescue (Figure 5.2B) while preincubating with the IAP component of pertussis toxin gave a significant but smaller rescue (Figure 5.3B).

5.2.4. Baclofen Kills Neonatal Hippocampal Neurones

I reasoned that if the toxicity was a direct effect of gp120 on neurones, then other agonists that signal to G$_i$/G$_o$ would kill. Activation of GABA receptors is normally thought of as neuroprotective (Johansen and Diemer, 1991; Schwartz et al., 1994; Ylinen et al., 1991), but the GABA-B receptor activates G$_o$ and therefore, on my hypothesis, would be neurotoxic. Figure 5.4 shows the results of culturing hippocampal neurones with 20 µM baclofen for 2 days. As predicted by my model, baclofen had a significant neurotoxic effect, although the fraction of neurones killed was only about half that seen with gp120.
**Figure 5.2.** A component of neurotoxicity is mediated by the CXCR4 chemokine receptor. Each histogram represents data from three independent platings, columns indicate % death in respectively control cultures, cultures with 250 pM gp120, and cultures with 250 pM gp120 and CXCR4 antagonist peptide VSLSYRCPCRFF at 50 μM in (A) hippocampal and (B) cortical cultures. Peptide was present for two hours before, and during, gp120 application. In both experiments, all values are significantly different (P < 0.001, Bonferroni corrected t test).
% death at 48 hours

A

control

gp120

+ peptide

B

control

gp120

+ peptide

Figure 5.2
Figure 5.3. A component of neurotoxicity is mediated by $G_i/G_o$. Each histogram represents data from three independent platings, columns indicate % death in respectively control cultures, cultures with 250 pM gp120, and cultures with 250 pM gp120 and IAP component of pertussis toxin at 500 ng/ml in (A) hippocampal and (B) cortical cultures. Toxin was present for two hours before, and during, gp120 application. In both experiments, all values are significantly different ($P < 0.001$, Bonferroni corrected t test).
Figure 5.3
**Figure 5.4.** Baclofen causes neuronal death: Hippocampal cultures 22 days old were incubated with NB medium + 2% B27 in control dishes and 20 μM Baclofen in NB medium + 2% B27 for 48 hours and then assayed for neurotoxicity. Death assay was performed on three independent cultures and then the average was calculated.
Figure 5.4

% death at 48 hours

control  Baclofen

18
16
14
12
10
8
6
4
2
0
5.3. Discussion

5.3.1. A Component of Gp120 Neurotoxicity is Independent of [Ca$^{2+}$]$_c$ Elevation

Lipton has proposed that [Ca$^{2+}$]$_c$ elevation represents a final common pathway by which HIV-1 virus, stroke, trauma and epilepsy trigger neuronal death. I observed gp120 neurotoxicity under conditions where I did not see [Ca$^{2+}$]$_c$ signals either immediately upon gp120 addition nor in cultures incubated with gp120 for days. These results indicate that [Ca$^{2+}$]$_c$ elevations are not an essential component of gp120 neurotoxicity.

5.3.2. Gp120 and Mitochondrial Depolarization

Only twice did I observe mitochondrial depolarization by gp120. Microglia are known to release NO in response to gp120. My working hypothesis is therefore that mitochondrial depolarization in response to gp120 is secondary to release of NO from microglia (Keelan et al., 1999).

An important point here is that these results are from those subset of cultures that did not show an acute rise in [Ca$^{2+}$]$_c$ by gp120. The fact that only limited cultures showed an acute rise in [Ca$^{2+}$]$_c$ on addition of gp120 did not allow me to test $\Delta\psi$ in those cultures that showed acute [Ca$^{2+}$]$_c$ responses to gp120.

5.3.3. Early Signalling Events in Gp120 Induced Cell Death

Two very distinct receptors have been proposed to mediate the toxic effects of gp120. As noted
above, gp120 could act at chemokine receptors, either on neurones or on non-neuronal cells. However, a number of groups have shown that gp120 will bind directly to the glycine site of the NMDA receptor and increase its open probability (Fontana et al., 1997; Pattarini et al., 1998; Pittaluga et al., 1996) and a number of authors have suggested that gp120 could be directly excitotoxic via this action. My finding that the CXCR4 blocking peptide VSLSYRCPFRR gave considerable rescue implies that this latter mechanism is relatively unimportant.

Chemokine receptors are thought to signal to trimeric GTP binding proteins of the Gα/Gβ family, and my finding that pertussis toxin pretreatment protects against gp120 toxicity is consistent with a role for Gα/Gβ downstream of CXCR4. Indeed, my results with baclofen suggest that any activation of Gα, by whatever route, tends to cause cell death. My results do not address the events downstream of Gα activation, however there is considerable literature on the general topic of G proteins and life/death decisions which I shall briefly discuss before speculating on how Gα might be operating in neurones.

5.3.4. Gp120, GPCR and MAP Kinase

Activation of the MAP kinase signalling pathways is a key event in the proliferation, differentiation and apoptosis of mammalian cells. In mammalian cells, three different MAPKs have been described, i.e., extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal protein kinase (JNK) which is also called stress activated protein kinase (SAPK), and p38 (Seger and Krebs, 1995). They belong to three different protein kinase cascades, Raf-MEK-ERK, SEK-JNK/SAPK, and MKK3/MKK6-p38. Classic hypotheses consider apoptosis to be associated with activation of the SEK-JNK/SAPK, and MKK3/MKK6-p38 pathway (Seger and Krebs,
1995). This view has been slightly amended, because a balance between activation of JNK/p38 and inhibition of the ERK pathway has been demonstrated to lead to apoptosis in PC12 cells (Minden et al., 1994). Thus, neuronal survival and induction of cell death may be controlled by the opposing actions of the ERK and JNK-p38 pathways. When the survival signalling pathway is activated through ERK, the cell death signalling pathways (JNK or p38) are suppressed. When the survival signalling pathway is deactivated, for example as in the case of nerve growth factor (NGF) withdrawal, the JNK-p38 pathways are activated (Xia et al., 1995).
Figure 5.5. Model for the roles of the ERK and JNK-p38 kinase pathways in apoptosis. Neuronal survival and induction of cell death may be controlled by the opposing actions of the ERK and JNK-p38 pathways. In the presence of NGF, the survival signalling pathway (through ERK) is activated, whereas the cell death signalling pathways (JNK or p38) are suppressed. When NGF is removed, the ERK signalling pathway is deactivated while the JNK-p38 signalling pathways are activated. Other signalling pathways, including the cAMP and Ca^{2+} signal transduction systems may also promote neuronal survival by stimulating the ERK or inhibiting the JNK-p38 signalling pathways.
5.3.5. Possible Pathways in Neurones

In this light of the possible involvement of chemokine receptors in neuronal degeneration or survival much research is going in this field at the moment. In fact activation of neuronal chemokine receptors produces increases in ERK activity, without corresponding changes in JNK and p38 activity (Meucci et al., 1998). Conversely, gp120 activated both ERK and JNK (Lannuzel et al., 1997). It has also been demonstrated that activation of neuronal receptors produces increases in the activated form of the transcription factor cAMP-response-element-binding protein (CREB) (Meucci et al., 1998). Such data suggest that, in addition to mediating rapid neuronal signalling events that regulate synaptic transmission, activation of neuronal chemokine receptors also produces signals that influence neuronal survival and viability (Finkbeiner et al., 1997). Kaul and Lipton’s recent finding has shed some more light on gp120-induced neuronal death via CXCR4 receptor (Kaul and Lipton, 1999). They showed that in rat cerebrospinal cultures the β-chemokines RANTES (regulated on activation, normal T cell expressed and secreted) and macrophage inflammatory protein (MIP-1β) protect neurones from gp120-induced apoptosis. MIP-1β and RANTES presumably inhibit the neurotoxic effect of gp120 in an indirect manner, because RANTES binds to the β-chemokine receptors CCR1, CCR3 and CCR5, and MIP-1β binds CCR5 (or a functional rat homologue) (Bonini et al., 1997; Luster, 1998; Spleiss et al., 1998) whereas gp120 (and SDF-1α/β) interact with the α-chemokine receptor (Davis et al., 1997). The gp120 prefers binding to CXCR4 receptors, similar to physiological α-chemokine ligands, SDF-1α/β. SDF-1α/β failed to prevent gp120 neurotoxicity, and in fact also induced neuronal apoptosis. They found that gp120-induced neuronal apoptosis was completely abrogated with the tripeptide TKP, which inhibits activation of macrophage/microglia. TKP is comprised of three of the four amino acid residues of tuftsin, a well characterized peptide known to display the opposite effect, i.e., activation of macrophages.
(Najjar, 1979). In contrast, TKP or depletion of macrophages/microglia did not prevent SDF-1 neurotoxicity and also occurred in cultures depleted of macrophages/microglia. This fact does not exclude a direct interaction of gp120 with neuronal or astroglial CXCR4 or other chemokine receptors. But, if this interaction occurs, in contrast to the effect of SDF-1 it is apparently not sufficient to trigger neuronal apoptosis in these mixed neuronal/glial cultures. Also, inhibition of p38 mitogen-activated protein kinase ameliorated both gp120- and SDF-1-induced apoptosis. Taken together, these results suggest that gp120 and SDF-1 differ in the cell type on which they stimulate CXCR4 to induce neuronal apoptosis, but both ligands use the p38 mitogen activated protein kinase pathway for the death signalling. Moreover, gp120-induced apoptosis depends predominantly on an indirect pathway via activation of chemokine receptors on macrophages/microglia, whereas SDF-1 may act directly on neurones or astrocytes.

On the other hand, interleukin 8 (IL8) (Araujo and Cotman, 1993) and several other chemokines (Meucci et al., 1998) can act as survival factors for hippocampal neurones under a variety of conditions, and can inhibit gp120-induced neuronal death (Meucci et al., 1998). Leukocyte responses to IL-8 are mediated via specific cell surface receptors for IL-8. Two subtypes of IL-8 receptors have been described in neutrophils, IL-8RA (CXCR1) and IL-8RB (CXCR2) (Holmes et al., 1991; Murphy and Tiffany, 1991). CXCR1 and CXCR2 couple to a pertussis toxin (Ptx)-sensitive G protein to mediate leukocyte functions (Horuk, 1994). It is possible that IL-8 by binding to CXCR1 and/CXCR2 is neurotrophic. Indeed, chemokines, by stimulating IL-8RB/CXCR2, can also activate the phosphatidylinositol 3-kinase/Akt signaling pathway (Tilton et al., 1997), which plays an important role in suppressing the development of apoptotic processes (Crowder and Freeman, 1998; Dudek et al., 1997).
More data is accumulating regarding the role of various chemokine receptors in neuronal death/survival. Boehme et al. (2000) have found that Fractalkine is a CXXXC-family chemokine, highly and constitutively expressed on the neuronal cell surface, and its interaction with its receptor CX3CR-1 may play an important role in promoting and preserving microglial cell survival in the CNS. They have shown that treatment of microglia with fractalkine maintains cell survival and inhibits Fas ligand induced cell death in vitro. Biochemical interaction indicates that this occurs via mechanisms that may include 1) activation of the phosphatidylinositol-3 kinase/protein kinase B pathway, resulting in the phosphorylation and blockade of the proapoptotic functions of BAD; 2) up-regulation of the antiapoptotic protein Bcl-xL; and 3) inhibition of the cleavage of the BH3-interacting domain death agonist (BID). The observation that fractalkine serves as a survival factor for primary microglia in part by modulating the protein levels and the phosphorylation status of Bcl-2 family proteins reveals a novel physiological role for chemokines.

Taken together, these data suggest that activation of the CXCR1, CXCR2, and CXXXC receptors leads to survival of cells, whereas the activation of the CXCR4 receptor leads to neuronal death. Thus, different downstream events are involved resulting in survival/death signal.

5.3.6. \(G_o\) Activation has Antisurvival Effects

I hypothesize that other receptor systems that activate \(G_o\), such as gp120 acting at CXCR4 or indeed GABA-B agonists cause similar toxicity. Activation of the chemokine receptor or the GABA-B receptor each activate the \(G_o\) pathway, and can initiate aberrant signalling events leading to neuronal death.
The exact nature of the downstream pathway is not addressed by my present study. Gp120 has been reported to activate JNK in cortical cultures (Lannuzel et al., 1997). It is possible that Go activates JNK to induce cell death. The hypothesis that Go activation is anti-survival ties in with results from developing axons. When these contact a non-permissive substrate the distal tip collapses, causing the growing axon to retract before recommencing growth. This effect is also blocked by pertussis toxin pretreatment (Igarashi et al., 1993).

In agreement with the neurotoxicity that I observed in primary hippocampal culture with baclofen, Lafon-Cazal et al. (1999) found that mGluR7-like receptor and GABA-B receptor activation enhance the neurotoxic effects of N-methyl-D-aspartate in cultured mouse striatal GABAergic neurones. The neurotoxic effects of both L-AP4 and baclofen were mediated via G\textsubscript{i}/G\textsubscript{o}-coupled mGluRs. They found that L-AP4 and baclofen inhibited both basal and NMDA-stimulated GABA release. These inhibitions of GABA release may be responsible for the increase in basal and NMDA-stimulated neuronal death. Indeed blockade of GABA(A) receptors with bicuculline increased neuronal death of control and NMDA-treated cultures. These results, therefore suggest that L-AP4 and baclofen, via mGluR7 and GABA(B)R, reduced the neuroprotective effect of GABA present in striatal cultures acting via GABA(A) receptors. Although caution must be taken when extrapolating from in vitro to in vivo situations, the present experiments and the recent observations that mGluR7 and GABA(B)R are expressed in heterologous synapses, should be taken into consideration when evaluating the neuroprotective action of future mGluR7 specific agonists or GABA(B)R specific antagonists.
Chapter 6. Neuronal Calcium Transients Evoked by HIV-1 Coat Protein Gp120 Require the Presence of Microglia

6.1. Introduction

Microglia, which may constitute as many as 12% of the cells in the CNS, were described by del Rio-Hortega as a distinct cell type within the CNS with characteristic morphology and specialized staining characteristics that differentiated them from other glial cells and neurones (del Rio Hortega, 1932). Hickey and collaborators (Lassmann et al., 1993; Rinner et al., 1995) and others have proposed that microgliamacrophages in the CNS can be divided into several subtypes based on their localization and morphology. Parenchymal microglia differentiate from monocytes that migrate into the CNS early during embryonic development (Richardson et al., 1993) and are maintained as a pool with a low turnover rate and limited, if any, replication during adulthood. This later point is the most controversial, since microglia increase in numbers at sites of CNS reaction after trauma or other injury. However, the origin of reactive cells could be as either migrating parenchyma microglia or monocytes induced to differentiate into microglia-like cells by the CNS environment. Perivascular microglia constitute a somewhat separate group with a higher turn over rate than parenchymal microglia. This pool is generally thought to be replenished by monocytes from the peripheral circulation, which have infiltrated the CNS and may be responsible for the initial contact with an invading microorganism. An occasional perivascular microglial cell may penetrate the CNS and differentiate into parenchyma microglia, but the use of bone marrow-chimeric animals has shown that this sequence is a rare event, at least in experimental animals.

Although there are quantitative differences in the expression of some cell surface proteins
common to both cell types, no single antibody can differentiate between monocytes-macrophages and parenchymal microglia (Williams et al., 1992a). Among the differences that have been described are differential expression of antigenic markers like CD14 and CD68 and major histocompatibility complex (MHC) class 2 expression (Peudenier et al., 1991; Williams et al., 1992a), and morphology, microglia adopting an extended, ramified morphology unlike the compact shape of monocytes and macrophages (Williams et al., 1992a). Some investigators have also found differences in potassium channel activity in microglia in comparison with that in peritoneal macrophages (Banati et al., 1991; Ilschner et al., 1995; Kettenmann et al., 1990) and in high affinity neurotransmitter transport (Kolson et al., 1993).

As the primary immune effector cells of the CNS, microglia respond to traumatic injury or the presence of pathogens by migrating to the site of injury, where they (perhaps) proliferate. Activated microglia at the site of inflammation express increased levels of MHC antigens and become phagocytic. Like other tissue macrophages, microglia release inflammatory cytokines that amplify the inflammatory response by recruiting cells to the site of injury. In addition, microglia (like macrophages) can release potential neurotoxins like TNF-α and others, which may potentiate damage to nervous system cells. It is this type of secretory activity that has become associated with diseases like AIDS and MS, in which the microglial response is prominent: this activity may be somewhat deleterious to the CNS and, ultimately, the organism. Additionally, recent studies have implicated this type of secretory activity as potentially increasing neuronal destruction in AD (for review see Gonzalez Scarano and Baltuch, 1999).
6.1.1. Microglia and HIV-1 Infection of the CNS

Neurological impairment in HAD is believed to be caused by HIV-1 infection, because (a) it is associated with HIV-1 replication within the CNS, which has been detected even during the primary viremia (i.e. long before the onset of immunodeficiency), (b) HIV-1 infection results in the presence of HIV-1-specific antibodies within the neuraxis, which is usually taken as evidence of an active infection, and (c) neuropathological studies have demonstrated the presence of HIV-1-infected cells in the brain and spinal cord. Unlike the other opportunistic infections characteristic of HIV-1, this disorder is not seen in other immunodeficiencies, providing additional support for the concept that it is caused by the virus itself. HAD is distinguished from virtually all other viral diseases of the nervous system by its chronicity, but most importantly because in spite of severe cognitive and motor deterioration, there is scant evidence of infection of neurones. Instead virus is concentrated in microglia and macrophages, with a very limited infection of astrocytes, endothelial cells, or even more rarely (if at all) neurones (Bagasra et al., 1996; Gabuzda et al., 1986; Nuovo et al., 1994; Wiley et al., 1986). Microglia are the cells primarily responsible for viral load in the CNS, where they probably play a role in the development of HAD, and because of poor penetration of antiretrovirals into the CNS, they may serve as a potential "sanctuary" site for the virus (Kolson et al., 1998). In summary HIV-1 in the CNS is characterised by the relative paucity of the infected cells and the overwhelming preponderance of infection in microglial cells.

6.1.2. Role of Microglia in Entry of HIV-1 into the CNS

Macrophages are probably involved in neuropermisiveness, that is, penetration of the CNS by the virus. The potential routes for HIV-1 neuroinvasion are hematogenous (either cell free or
within infected cells) or retroaxonal. There is little evidence for the latter, whereas there is a very high turnover of HIV-1 within the peripheral circulation estimated to be around $10^9$ virions/day (Ho et al., 1995), providing a rich source of potential infecting particles, either within cells or in the plasma itself. Cell free virus could penetrate the BBB by infecting endothelial cells and then cells in contact with endothelium, perhaps astrocytes. For HIV-1, either lymphocytes or monocytes may subsume this role, since there is constant trafficking of these into the perivascular region. In contrast, later in the course of the CNS infection, HIV-1 antigens are concentrated in the parenchymal microglia (Lane et al., 1996), supporting a role for these cells in the chronic persistence of virus within the brain.

6.1.3. Role of Microglia in Viral Persistence in the CNS

HIV-1 strains have been traditionally divided based on their infectious phenotype in cultured cells. T-tropic viruses (i.e., T lymphocyte-selective) replicate in primary T-cells and in some T-cell lines, induce syncytia, and are sometimes referred to as SI (syncytia-inducing) isolates. M-tropic isolates (i.e., macrophage selective) replicate in primary T-cells and in monocyte derived macrophages, are less likely to induce syncytia in tissue culture, and are sometimes referred to as nonsyncytia-inducing or NSI isolates. These phenotypic differences are now recognized to result from the utilization of specific chemokine receptors by HIV-1 for entry into cells. The majority of CNS isolates are M-tropic and NSI, and it is these isolates that replicate best in cultured human microglia (Strizki et al., 1996; Watkins et al., 1990). In the simian immunodeficiency virus (SIV) model, an animal inoculated with some SIV strains may develop immunodeficiency without replication of virus in the CNS. However, if during the course of infection some strains become adapted for rapid growth in macrophages and microglia, the animal is more likely to demonstrate replication within the brain (Lane et al., 1995). Thus, there is considerable
circumstantial evidence that M-tropism is associated with the establishment of a CNS infection by HIV-1.

Together, these data can be interpreted as consistent with a model in which HIV-1 entry into the CNS is mediated by circulating lymphocytes or monocytes, which in turn transmit virus to perivascular macrophages-microglia. In time parenchymal microglia also become infected by virus produced within the CNS. Infected microglia may survive for relatively long periods of time, produce enough virus to maintain a cycle of new infections, and by the process of sequential mutation characteristic of HIV-1, virus may become increasingly divergent from the species in the peripheral circulation.

6.1.4. HIV-1 Infection of Cultured Microglia

Human microglia isolated from adult or fetal tissue can be cultured for several weeks and infected with many HIV-1 strains (He et al., 1997; Strizki et al., 1996; Watkins et al., 1990).

HIV-1 enters a cell through a multistep process that requires at least two receptors. CD4, the first receptor identified, is a cellular determinant involved in immune recognition that is present in a subset of T-lymphocytes as well as in microglia and macrophages. Although the levels of CD4 in microglia are too low for detection with immunohistochemical methods (Williams et al., 1992a), HIV-1 infection of microglia can be easily blocked with monoclonal antibodies that react with an epitope close to the known HIV-1 binding site on CD4 (Jordan et al., 1991), confirming its role in infection of these cells. Feng et al., (1996) first identified a G-protein coupled, seven transmembrane-domain receptor (GPCR) called fusin and now known as CXCR4, which renders non primate cells susceptible to HIV-1 infection when expressed in conjunction with CD4. This
GPCR had originally been cloned from both brain and peripheral leukocytes, and is also present in microglia (Lavi et al., 1997). CXCR4 is a member of the chemokine receptor family. Microglia also express another member, CCR5, at levels easily detectable by immunohistochemical and other methods (He et al., 1997; Lavi et al., 1997), and indeed CCR5-using strains of HIV-1 infect microglia in vitro (Sheih et al., 1998). A third member, CCR3, has also been reported in microglia (He et al., 1997). Furthermore, antibodies against either protein or their natural ligands can inhibit infection of cultured microglia (He et al., 1997). More surprisingly, both CXCR4 and CCR3 are present in subpopulations of neurones, particularly in the hippocampus, and CCR5 has been detected in CNS neurones in humans (Rottman et al., 1997) and other primates (Westmoreland et al., 1998). Although it is unlikely that either molecule by itself could mediate HIV-1 entry into neurones, the presence of a receptor primarily intended to attract lymphocytes to sites of inflammation on the surface of neuronal cells provides a potential site for interaction between the immune and the nervous systems. Mice that have had the murine homologue of CXCR4 deleted (knocked out) are not viable and have development anomalies in the cerebellum (Zou et al., 1998).

6.1.5. Role of Microglia in Mediating Neuronal Injury in HIV-1 Infection

Since microglia and other macrophage-like cells are the significant focus of productive HIV-1 infection within the brain many investigators have focused their efforts toward the identification of potential secretory products that are either produced or induced by microglia and that could mediate neurodegeneration (Bruce Keller, 1999; Kingham et al., 1999; Lipton, 1998). As I have already discussed these microglial secretory products in detail in the Introduction chapter, I will
To what extent these putative neurotoxins can induce neuronal degeneration at the concentrations likely to be present in the CNS remains an open question. PAF appears to be effective at the lowest concentration \textit{in vitro}, whether the target neurones are obtained from human fetuses (Gelbard et al., 1994) or are differentiated cell lines (Westmoreland et al., 1996).

For other toxins like TNF-\(\alpha\), the concentrations required to mediate neurodegeneration are higher than one would expect to be present in brain, except perhaps in the microenvironment immediately surrounding microglia or MDM (Gonzalez Scarano and Baltuch, 1999). However, lower levels of TNF-\(\alpha\) could mediate increases in other toxins by increasing the activity of phospholipase A\(_2\), which in turn cleaves phosphoglycerocholine to release PAF and arachidonic acid (Bachwich et al., 1986).

The CSF has been widely sampled in many studies of HAD. In HAD, elevations of IL-6 (Gallo et al., 1989), IL-1\(\alpha\) (Perrella et al., 1992) and TNF-\(\alpha\) (Perrella et al., 1992; Tyor et al., 1992) have been demonstrated. Whether these arise from macrophages or lymphocytes is unknown.

HIV-1 coat protein causes a rise in the \([\text{Ca}^{2+}]_c\) in neurones. This could be either due to the interaction of gp120 with neurones (direct effect) or due to an interaction of gp120 with microglia which in turn release neurotoxins that then cause an influx of \(\text{Ca}^{2+}\) into neurones (indirect effect) (Lipton 1994a,b). Thus, a major question addressed in the present study is whether the gp120-induced \([\text{Ca}^{2+}]_c\) rise in neurones is due to the direct interaction of neurones with gp120 via chemokine receptors or due to an indirect route involving the release of
neurotoxins from activated microglia/macrophages.

6.2. Results

As described in Chapter 3, there was a marked variability between cultures in the degree to which they showed a neuronal \([\text{Ca}^{2+}]_c\) elevation in response to gp120. The biggest puzzle in my 3 year project was why some cultures showed a neuronal \([\text{Ca}^{2+}]_c\) response to gp120 while others did not. In contrast all cultures did show a neuronal \([\text{Ca}^{2+}]_c\) response to glutamate as well as to NMDA, only the neuronal \([\text{Ca}^{2+}]_c\) response to gp120 was variable.

6.2.1. Microglia Staining of Successive Hippocampal Cultures Suggests a Possible Mechanism Responsible for a Neuronal \([\text{Ca}^{2+}]_c\) Response to Gp120

One factor that seemed to increase the likelihood of observing the \([\text{Ca}^{2+}]_c\) response was culture of the cells in medium containing HS, rather than the defined medium of Gibco NB plus 2% B27 supplement. Since neurones were supported by both media, I hypothesized that the HS was supporting the survival of another cell type necessary for the neuronal \([\text{Ca}^{2+}]_c\) response to gp120. Microglia have been proposed to mediate the gp120 response (Lipton, 1992c), and I therefore stained the cultures for microglia. Initially I used the antibody OX-42, a monoclonal antibody for the CR3bi complement receptor. This marker binds to both resting and activated microglial cells (Flaris et al., 1993). Later I used the ED1 monoclonal antibody, which recognises an antigen in the lysosomal membranes of phagocytes. The expression of this antigen in cells increases during phagocytic activity (Bauer et al., 1994). This latter protein is expressed at low levels by all
microglia but at much higher levels on activated microglia.

To my surprise when I compared matched cultures grown with or without HS I found microglia in both cultures. Figure 6.1 shows low power views of cover glasses from the same dissection grown in NB medium + 2% B27 (Figure 6.1A) and NB medium + 2% B27 + 5% HS (Figure 6.1B). Microglia were present in both cultures. On average microglia constituted 19.94 ± 6.43% of total cell number in the absence of HS (10 cover glasses from 5 dissections) and 14.47 ± 7.66% in the presence of HS (6 cover glasses from 3 dissections). These values are not significantly different (p = 0.121). Among the earliest changes in microglial cell activation are an upregulation of CR3bi (Graeber et al., 1988) and a change in morphology, whereby the cells retract their delicate and highly branched processes to form stout, intensely staining, and short cytoplasmic extensions (Finsen, 1993; Streit and Kincaid Colton, 1995). By this criterion, most of the microglia in these cultures were activated.

While performing these measurements I noticed enormous variation between dissections in the density of the microglia. Figure 6.2 and 6.3 shows low power views of cover glasses from dissections performed 5 weeks apart. The culture of Figure 6.2A has only sparse microglia, while the culture of Figure 6.3A has a high density of microglia. It seemed possible that this variability might underlie the variable appearance of the gp120-induced $[Ca^{2+}]_c$ signals. I therefore examined gp120 induced $[Ca^{2+}]_c$ in successive cultures. The results are shown in Table 6.1, while Figure 6.2C and 6.3C show $[Ca^{2+}]_c$ responses corresponding to the cultures of Figure 6.2A and 6.3A. On average, the maximum $[Ca^{2+}]_c$ during vehicle application was 63.64 ± 1.80 nM, n = 60 in these cultures with high microglia density (Figure 6.3 and 6.5).
<table>
<thead>
<tr>
<th>Date of dissection</th>
<th>Culture medium (with or without serum, with or without leucine methyl ester)</th>
<th>Unstimulated microglia (Dim brown stain)</th>
<th>Activated microglia (Intense brown stain)</th>
<th>([\text{Ca}^{2+}]_c) response to gp120 as responding cells/total cells Studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>20/03</td>
<td>NB - HS</td>
<td>0.8 ± 0.5</td>
<td>35.41 ± 2.88</td>
<td>23/25</td>
</tr>
<tr>
<td></td>
<td>NB + 5% HS</td>
<td>2.22 ± 0.58</td>
<td>29.8 ± 2.4</td>
<td>21/23</td>
</tr>
<tr>
<td>5/04</td>
<td>NB - HS</td>
<td>1.02 ± 0.36</td>
<td>8.43 ± 0.36</td>
<td>0/25</td>
</tr>
<tr>
<td>17/04</td>
<td>NB - HS, L-methyl-ester</td>
<td>1.77 ± 0.31</td>
<td>35.96 ± 1.24</td>
<td>26/26</td>
</tr>
<tr>
<td></td>
<td>NB + 5% HS</td>
<td>1.405 ± 0.28</td>
<td>9.29 ± 0.59</td>
<td>0/22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.644 ± 0.446</td>
<td>7.27 ± 0.07</td>
<td>0/26</td>
</tr>
<tr>
<td>2/05</td>
<td>NB - HS</td>
<td>3.36 ± 0.64</td>
<td>10.62 ± 0.42</td>
<td>0/20</td>
</tr>
<tr>
<td>8/05</td>
<td>NB + 5% HS</td>
<td>1.4 ± 0.23</td>
<td>6.34 ± 0.19</td>
<td>0/27</td>
</tr>
</tbody>
</table>

**Table 6.1** Hippocampal cultures were stained for immunoreactivity to ED1 (a marker for activated microglia). Data are mean ± SEM values, microglial number being percentage of the total cell number in 10 discrete fields from 2 independent coverslips. There is a clear tendency for \([\text{Ca}^{2+}]_c\) responses to appear only when the microglial density is high. However, while performing these experiments I never observed the massive, sudden gp-120 induced \([\text{Ca}^{2+}]_c\) elevations that I had observed earlier.
6.2.2. Killing of Microglia Eliminates the Neuronal $[Ca^{2+}]_c$

Response to Gp120

To test the hypothesis that microglia are necessary for the gp-120 induced $[Ca^{2+}]_c$ responses, I treated cultures with leucine methyl ester for seven days from day 1 to day 7 of culture. Leucine methyl ester has been used to selectively kill microglia (Lipton, 1992c). When assayed on day 22, cultures still contained ED1 positive cells, but these were mis-shapen and appeared to be lysed 'ghosts' and some had pyknotic nuclei characteristic of apoptosis (Kerr et al., 1972). In agreement with the model, cultures treated with leucine methyl ester did not show a $[Ca^{2+}]_c$ response to gp120 even though cultures from the same dissection that were not treated with leucine methyl ester did show a $[Ca^{2+}]_c$ response to gp120 (Figure 6.4 and 6.5, Table 6.1). To check that the neurones were able to generate $[Ca^{2+}]_c$ responses to agonist, I tested leucine-methyl-ester treated dishes with NMDA and observed a $[Ca^{2+}]_c$ response (resting $[Ca^{2+}]_c = 57.01 \pm 1.69$ nM, rose to $249.57 \pm 32.79$ nM, $n = 17$).
Figure 6.1. Immunocytochemical identification of microglia in matched cultures grown with or without HS. A: Primary hippocampal culture grown in NB medium + 2% B27 with no added HS. Activated microglia are recognised with ED1 (a marker for activated microglia), activated microglia display dark brown color whereas the neurones and astrocytes nuclei stain purple with haematoxylin. B: Primary hippocampal culture grown in NB medium + 2% B27 + 5% HS. Scale bar = 100 μm.
Figure 6.2. Variation in the number of microglia in primary hippocampal cultures grown in NB medium + 2% B27 + 5% HS. A: Culture of (25/04/00). In this culture few activated microglia were present. B: A magnified portion of image (A) shows activated microglia with partially retracted processes. C: gp120 evoked no significant neuronal $[\text{Ca}^{2+}]_{\text{e}}$ response in these cultures. Scale bar = 100 $\mu$m in panel A and 50 $\mu$m in panel B (magnified image).
Figure 6.2

A

B

C

\[ [Ca^{2+}]_c \text{ (nM)} \]

\( \text{gp120} \)

Time (min)
**Figure 6.3.** Variation in the number of microglia in primary hippocampal cultures grown in NB medium + 2% B27 + 5% HS. **A:** Culture of (20/03/00), 5 weeks apart from the culture of Figure 6.3. A high density of microglia was present. **B:** A magnified portion of image (A) is again shown, the microglia have retracted their processes and have become macrophage like. **C:** gp120 evoked a significant neuronal $[\text{Ca}^{2+}]_c$ response in the cultures of 20/03/00. **D:** Control vehicle evoked no significant neuronal $[\text{Ca}^{2+}]_c$ response in these cultures. Scale bar = 100 μm in panel A and 50 μm in panel B.
**Figure 6.4.** Artificial reduction in microglial number eliminates the $[Ca^{2+}]_c$ response to gp120 in neurones. **A:** Image of a dish from the same culture as in Figure 6.5 treated with leucine methyl ester (7.5 mM) for 7 days. Treatment with leucine methyl ester selectively kills microglia **B:** A magnified portion of the image shows a condensed pyknotic microglia with a solid arrow, and a damaged microglia cell resembling a lysed ghost shown with a broken arrow. **C:** gp120 evoked no significant neuronal $[Ca^{2+}]_c$ response in these cultures. **D:** $[Ca^{2+}]_c$ from a second leucine methyl ester treated dish showed a rise in neuronal $[Ca^{2+}]_c$ on addition of NMDA. Scale bar = 100 $\mu$m in panel A and 50 $\mu$m in panel B.
Figure 6.4
Figure 6.5. A: Sister culture with high density of microglia that did not receive leucine methyl ester as compared with Figure 6.4. B: Magnified portion of the image shows a microglia with partially retracted processes (solid arrow) and another microglia which has retracted its processes and has become macrophage like (broken arrow). C: Neurones of this culture show a rise in $[\text{Ca}^{2+}]_c$ as gp120 is added. D: $[\text{Ca}^{2+}]_c$ from a second dish showed no rise in the level of neuronal $[\text{Ca}^{2+}]_c$ on addition of vehicle. Scale bar = 100 \mu m in panel A and 50 \mu m in panel B.
6.2.3. Addition of Purified Microglia to Hippocampal Cultures

As a further test of the hypothesis that a neuronal \( [Ca^{2+}]_c \) response to gp120 is only seen in cultures with a relatively high density of microglia I examined the effect of adding purified microglia to hippocampal cultures. Purified microglia were a kind gift of Dr Jennifer Pocock and were prepared as described in publications from the Pocock laboratory (Kingham and Pocock, 2000). Microglia, suspended in MEM, were added to hippocampal cultures that had been maintained without HS for at least 22 days since dissection, 10,000 microglia being added to a 22 mm diameter area on each cover slip. In a subset of cultures from each dissection, IFN \( \gamma \) (10 ng/ml) was added to activate the microglia. The microglia were allowed to settle overnight (approx. 20 hours) and \( [Ca^{2+}]_c \) imaging was performed on the next day.

In 24 dishes from eight sister cultures to which microglia were not added, gp120 never evoked a \( [Ca^{2+}]_c \) signal in neurones. The average maximum value of \( [Ca^{2+}]_c \) during gp120 application was 62.34 ± 3.2 nM, \( n = 21 \), not significantly different from the value before gp120 application (Figure 6.6C). In contrast, one set of cultures to which microglia was added generated marked \( [Ca^{2+}]_c \) responses to gp120 in neurones. These responses are illustrated in Figure 6.6. In two dishes to which both microglia and IFN \( \gamma \) had been added every neurone generated a robust \( [Ca^{2+}]_c \) response to gp120 (resting \( [Ca^{2+}]_c = 80.05 \pm 1.7 \text{ nM} \), peak \( [Ca^{2+}]_c = 969 \pm 38 \text{ nM} \), \( n = 19 \); Figure 6.6A) while every neurone in two dishes to which microglia only had been added generated distinct \( [Ca^{2+}]_c \) responses, although these were on average delayed and smaller than those seen in the IFN \( \gamma \) treated cultures (resting \( [Ca^{2+}]_c = 74.33 \pm 1.56 \text{ nM} \), peak \( [Ca^{2+}]_c = 371.89 \pm 32.67 \text{ nM} \), \( n = 18 \); Figure 6.6B). Figure 6.6C shows a sister culture without added microglia, in which gp120 evoked no \( [Ca^{2+}]_c \) signal in neurones. These results appeared to be strong evidence
for the model that microglia were a critical component of the $[\text{Ca}^{2+}]_c$ response to gp120 in neurones. However, in no dish from any of the seven other cultures to which microglia were added was a $[\text{Ca}^{2+}]_c$ response to gp120 observed, 357 neurones in all being monitored. Figure 6.6D is one example of a culture to which microglia plus IFN $\gamma$ had been added; gp120 evoked no $[\text{Ca}^{2+}]_c$ signal.

These experiments were performed before I found a reliable method of staining microglia, and therefore I did not have an estimate for the numbers of microglia already present in the cultures. In fact the applied density corresponded to 26 extra microglia/mm$^2$, a value intermediate between the values seen in the unmanipulated cultures. For example the culture of Figure 6.2, in which gp120 did not elicit a $[\text{Ca}^{2+}]_c$ response, had a density of about 12 microglia/mm$^2$ while the culture of Figure 6.3, where gp120 evoked a significant $[\text{Ca}^{2+}]_c$ increase, had a density of about 500 microglia/mm$^2$. It is therefore possible that the added microglia were enough to push one of the cultures over a density threshold, but that the other cultures had too low a microglia density to generate neuronal $[\text{Ca}^{2+}]_c$ signals to gp120 even with additional microglia at 26/mm$^2$. I intend to repeat these experiments with higher applied microglia concentrations together with post-experiment microglial counting.
Figure 6.6. Addition of purified microglia to hippocampal cultures. In cultures from one dissection, added microglia appeared to reveal a $[Ca^{2+}]_c$ response to gp120 in neurones. Purified microglia were added at a final density of 26 cell/mm$^2$ to cultures that had been maintained without HS for at least 22 days since dissection. Each line is a plot of $[Ca^{2+}]_c$ in one neurone, all neurones in the field of view being plotted. 

A: gp120 evoked a marked $[Ca^{2+}]_c$ response in a culture supplemented with microglia and treated with IFN $\gamma$. B: gp120 evoked smaller but distinct $[Ca^{2+}]_c$ responses in a sister culture supplemented with microglia without added IFN $\gamma$. C: gp120 evoked no $[Ca^{2+}]_c$ response in a sister culture to which no microglia had been added. D: In a culture from a different dissection, gp120 evoked no $[Ca^{2+}]_c$ response in a culture supplemented with microglia and treated with IFN $\gamma$. 
6.3. Discussion

6.3.1. Data are Consistent with the Hypothesis that it is the Presence of Microglia that is Critical to Seeing the Neuronal $[\text{Ca}^{2+}]_c$ Elevation to Gp120

Lipton and coworkers have proposed that HIV-1-infected brain mononuclear phagocytes, especially after immune activation, secrete substances that contribute to neurotoxicity (Genis et al., 1992; Giulian et al., 1990; Nottet and Gendelman, 1995; Pulliam et al., 1991).

I found that in primary hippocampal cultures there is a clear tendency for the neuronal $[\text{Ca}^{2+}]_c$ responses to appear only when the activated microglial density is high. Thus, my results are consistent with the model that gp120 stimulates the activated microglia to release a neurotoxic substance that in turn causes a rise in $[\text{Ca}^{2+}]_c$ in neurones. When the density of activated microglia is lower, they are unable to cause neuronal $[\text{Ca}^{2+}]_c$ changes to be observed on addition of gp120. To confirm that microglia are necessary for the gp120 induced neuronal $[\text{Ca}^{2+}]_c$ responses, I treated cultures with leucine methyl ester for seven days to selectively kill microglia. In agreement with the model, cultures treated with leucine methyl ester did not show a neuronal $[\text{Ca}^{2+}]_c$ response to gp120 even though cultures from the same dissection that were not treated with leucine methyl ester showed a neuronal $[\text{Ca}^{2+}]_c$ response. Hence, my data are consistent with the hypothesis that it is the presence of activated microglia that is critical to seeing the $[\text{Ca}^{2+}]_c$ elevations to gp120 in neurones.
6.3.2. Importance of Activated vs. Non-Activated Microglia

With respect to microglia, where intrinsic "resting" cells are highly ramified and apparently limited in their capabilities, the activated phenotype reflects a cytologically less differentiated state, associated with a less ramified or ameboid shape, and probably a wider range of functional attributes. Microglia are activated in a number of CNS disorders, especially infectious, immunological, and neurodegenerative diseases. As the primary effector cells of the CNS, microglia respond to traumatic injury or the presence of pathogens by migrating to the site of injury, where they may proliferate. Activated microglia at the site of inflammation express increased levels of MHC antigens and become phagocytic. Like other tissue macrophages, microglia release inflammatory cytokines that amplify the inflammatory response by recruiting cells to the site of injury. In addition microglia can release neurotoxins such as TNF-α and others, which may potentiate damage to nervous system cells. It is this type of secretory activity that has become associated with diseases like AIDS and MS, in which the microglial response is prominent; this activity may be somewhat deleterious to the CNS and, ultimately the organism. Additionally, recent studies have implicated this type of secretory activity as potentially increasing neuronal destruction in Alzheimer's disease. Although I have no specific evidence on the point, it is likely that it is the activated microglia in my cultures that mediated the neuronal [Ca^{2+}]_i responses to gp120. Whether microglial density was low or high, the majority of the microglia were activated (Table 6.1).

6.3.3 The Numbers of Microglia Vary between Dissections

Although I strictly followed the same protocol for hippocampal dissection the number of microglia was different each time. Even a gap of couple of weeks meant that the culture could
have few microglia or lots of microglia. I used 1 day old rat pups, therefore, a simple explanation for this variability could be that the mother rat sometimes suffered from stress, trauma or an infection. The resultant increase of cytokines in the maternal circulation could perhaps result in an increased microglial load in the embryos.

6.3.4. Direct Effect of Gp120 on Neurones to Cause \([Ca^{2+}]_c\) Elevations

According to the Meucci and Miller model for the rise of neuronal \([Ca^{2+}]_c\), the effect of gp120 is direct. The hippocampal neurones possess a wide variety of chemokine receptors and activation of these receptors can lead directly to \([Ca^{2+}]_c\) transients (Meucci et al., 1998). They argue that the effects of chemokines and gp120 could not have resulted from the secondary release of substances from any contaminating microglia in neuronal cultures as they had selectively depleted neuronal cultures of any microglia by immunopanning by using anti-CD11b/c. My results clearly indicate that although neuronal CXCR4 may be involved in gp120 neurotoxicity, a direct action of gp120 on neurones is insufficient to generate \([Ca^{2+}]_c\) signals.

6.3.5. The Neuronal \([Ca^{2+}]_c\) Response to Gp120 Requires Activated Microglia.

The phenomenon that the addition of gp120 to neurones in culture causes an elevation of \([Ca^{2+}]_c\) has proved variable and elusive, with some authors reporting no such \([Ca^{2+}]_c\) rise with gp120 alone, some a rise in a few percent of the cells, and others a \([Ca^{2+}]_c\) effect in most cells (Dreyer et al., 1990; Holden et al., 1999; Kaul and Lipton, 1999; Lannuzel et al., 1995; Lo et al., 1992; Medina et al., 1999; Meucci and Miller, 1996). I found that neurones generate a \([Ca^{2+}]_c\) response
to gp120 only when microglia are present in high density in the primary hippocampal cultures.

My results therefore support the hypothesis that gp120 triggers release of one or more neuroactive substances from microglia, and it is these substances that trigger the neuronal \([\text{Ca}^{2+}]_e\) rise (Holden et al., 1999; Kaul and Lipton, 1999; Lo et al., 1992).
Chapter 7. Conclusions

7.1. A Component of Gp120 Neurotoxicity is Independent of Neuronal $[\text{Ca}^{2+}]_c$ Elevation

I began by studying the role of HIV-1 coat protein on the rise of $[\text{Ca}^{2+}]_c$ in neurones. This was highly variable and confusing where for months I saw an acute neuronal $[\text{Ca}^{2+}]_c$ rise on the addition of gp120 to hippocampal cultures grown in NB medium as well as NB medium + 5% HS followed by months where I applied gp120 for hours and days and still the neuronal level of $[\text{Ca}^{2+}]_c$ would not rise. This was all the more confusing for the fact that a common theme in the literature is the idea that gp120 kills neurones by raising the level of $[\text{Ca}^{2+}]_c$ and that this $[\text{Ca}^{2+}]_c$ elevation represents a final common pathway by which HIV-1 virus, stroke, trauma and epilepsy trigger neuronal death (Lipton, 1998). So, a major question arises, in my cultures where gp120 does not cause a rise in the $[\text{Ca}^{2+}]_c$, are neurones still dying?

I incubated both hippocampal and cortical cultures for 48 hours in the presence of gp120. Sister cultures were tested again to make sure whether they do show a rise in neuronal $[\text{Ca}^{2+}]_c$ or not by $\text{Ca}^{2+}$ imaging. There was significant neuronal death in both primary hippocampal as well as cortical cultures when incubated with gp120 for 48 hours, even in cultures in which gp120 evoked neither rapid nor slow neuronal $[\text{Ca}^{2+}]_c$ elevation. Thus, $[\text{Ca}^{2+}]_c$ elevation is not a necessary component of gp120 neurotoxicity.

The effects of gp120 have been variously assigned to an effect of gp120 at NMDA receptor, at
chemokine receptors, or on glial pH homeostasis. I examined the effect of VSLSYRCPCRFF, a peptide antagonist of CXCR4 (Heveker et al., 1998) and found that when both hippocampal and cortical cultures were preincubated for 2 hours with this peptide, neuronal death was largely rescued suggesting that it is the activation of this chemokine receptor which is involved in neuronal death. Chemokine receptors are seven transmembrane proteins signalling to trimeric G proteins of the G\textsubscript{i}/G\textsubscript{o} family (Baggiolini et al., 1997). In confirmation of this, I found that pretreatment with pertussis toxin to inactivate G\textsubscript{i}/G\textsubscript{o} protected neurones from gp120 toxicity. These results indicate that CXCR4, signalling via G\textsubscript{i}/G\textsubscript{o}, is responsible for a component of gp120 neurotoxicity.

Thus, a key finding of my research is that gp120 does not always cause a rise in the [Ca\textsuperscript{2+}]	extsubscript{c} in neurones. One should not focus entirely on [Ca\textsuperscript{2+}]	extsubscript{c} and should explore other mechanisms responsible for neuronal death.

7.2. Microglia are Responsible for the Neuronal [Ca\textsuperscript{2+}]	extsubscript{c} Transients Caused by Gp120 in Primary Hippocampal Cultures

I further investigated the phenomenon that certain cultures responded to gp120 with an acute neuronal [Ca\textsuperscript{2+}]	extsubscript{c} rise and others did not. If only NB medium + 5% HS cultures showed robust responses on addition of gp120, this would have suggested a simple hypothesis: that HS contained survival factors for microglia, which are therefore present in the + HS cultures and absent in the HS-free cultures. However, I found that some cultures grown even in NB medium HS free condition showed a neuronal [Ca\textsuperscript{2+}]	extsubscript{c} response to gp120 although I never observed
massive, synchronous neuronal $[Ca^{2+}]_c$ rise in these cultures. I then followed these cultures closely and stained for microglia and found that in both NB medium as well as NB medium + 5% HS cultures the amount of microglia was highly variable. Furthermore, those cultures which showed less microglia did not show a neuronal $[Ca^{2+}]_c$ response to gp120 and those cultures (both NB medium, and NB medium + 5% HS) which showed lots of microglia responded to gp120 with a neuronal $[Ca^{2+}]_c$ rise. However, by the time I worked out the protocol for microglia staining the hippocampal cultures in NB medium + 5% HS had stopped showing the massive, synchronous neuronal $[Ca^{2+}]_c$ response on addition of gp120. As a result, I do not know the status of microglia in those cultures that showed this type of neuronal $[Ca^{2+}]_c$ response to gp120.

I concluded that variability in the number of microglia in the hippocampal cultures underlies the variable appearance of the gp120-induced neuronal $[Ca^{2+}]_c$ rise. To test this hypothesis I treated cultures with leucine methyl ester which specifically kills microglia (Lipton, 1992c). I then again measured the level of neuronal $[Ca^{2+}]_c$ by fura imaging. In one culture, with an unusually high microglia density, there was a robust though slow rise in neuronal $[Ca^{2+}]_c$. In contrast sister dishes treated with leucine methyl ester did not show a neuronal $[Ca^{2+}]_c$ response to gp120. I confirmed by immunostaining that most microglia had died in the leucine methyl ester treated dishes. In conclusion my hypothesis is that gp120 acts on CXCR4 on microglia, causing them to release a number of neurotoxic substances that lead to the rise in $[Ca^{2+}]_c$ observed in neurones.

7.3. **CXCR4 lies upstream of NMDA receptors**

I found that the neuronal $[Ca^{2+}]_c$ response of hippocampal cultures to gp120 is blocked both by a cocktail of glutamate receptor blockers (20 μM MK-801, 20 μM CNQX, 500 μM MCPG) and
independently by VSLSYRCPCRFF, a peptide antagonist of CXCR4 (Heveker et al., 1998). How can a neuronal $[Ca^{2+}]_e$ response be mediated both through CXCR4 and NMDA receptors?

I observed a strange phenomenon that is strongly suggestive that CXCR4 is upstream of the NMDA receptor. When hippocampal cultures are treated with high potassium saline in the presence of cocktail of glutamate receptor blockers, the neuronal membranes depolarize allowing $Ca^{2+}$ to enter through voltage gated calcium channels. When gp120 is applied to hippocampal cultures in the presence of MK-801, CNQX and MCPG there is no neuronal $[Ca^{2+}]_e$ response. However, a subsequent application of high potassium saline to these cultures in the continued presence of MK-801, CNQX and MCPG evokes no depolarization and no neuronal $[Ca^{2+}]_e$ rise. My explanation for this surprising result is based on the fact that hippocampal cultures contain both glutaminergic and GABAergic neurones (Hoch and Dingledine, 1986). I propose that gp120 acts either directly or indirectly to cause the release of all transmitters from all the neurones. When glutamate receptors are blocked, the GABA effect, an opening of chloride channels, is unmasked. The membrane voltage is clamped close to its resting value, so that when high potassium saline is applied, the neurones do not depolarize. This result is therefore strong evidence that gp120 is still acting on hippocampal cultures even when NMDA receptors are blocked, and therefore that the other potential receptor, CXCR4, lies upstream of NMDA receptors.
Working model for gp120 toxicity in hippocampal and cortical cultures. Green indicates a direct action of gp120 on CXCR4 on neurons, activating downstream pathways that lead to death. Blue indicates an indirect pathway in which gp120 acts on CXCR4 on microglia, which in turn release agents that trigger concerted, self-amplifying electrical activity in the neurons leading to excessive calcium influx and excitotoxic cell death.
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