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HIV-Related Neuronal Injury

Potential Therapeutic Intervention With Calcium Channel Antagonists and NMDA Antagonists

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Abstract

Perhaps as many as 25–50% of adult patients and children with acquired immunodeficiency syndrome (AIDS) eventually suffer from neurological manifestations, including dysfunction of cognition, movement, and sensation. How can human immunodeficiency virus type 1 (HIV-1) result in neuronal damage if neurons themselves are for all intents and purposes not infected by the virus? This article reviews a series of experiments leading to a hypothesis that accounts at least in part for the neurotoxicity observed in the brains of AIDS patients. There is growing support for the existence of HIV- or immune-related toxins that lead indirectly to the injury or demise of neurons via a potentially complex web of interactions among macrophages (or microglia), astrocytes, and neurons. HIV-infected monocytoid cells (macrophages, microglia, or monocytes), after interacting with astrocytes, secrete eicosanoids, i.e., arachidonic acid and its metabolites, including platelet-activating factor. Macrophages activated by HIV-1 envelope protein gp120 also appear to release arachidonic acid and its metabolites. In addition, interferon-γ (IFN-γ) stimulation of macrophages induces release of the glutamate-like agonist, quinolinate. Furthermore, HIV-infected macrophage production of cytokines, including TNF-α and IL1-β, contributes to astrogliosis. A final common pathway for neuronal susceptibility appears to be operative, similar to that observed in stroke, trauma, epilepsy, neuropathic pain, and several neurodegenerative diseases, possibly including Huntington's disease, Parkinson's disease, and amyotrophic lateral sclerosis. This mechanism involves the activation of voltage-dependent Ca^{2+} channels and *N*-methyl-D-aspartate (NMDA) receptor-operated channels, and, therefore, offers hope for future pharmacological intervention. This article focuses on clinically tolerated calcium channel antagonists and NMDA antagonists with the potential for trials in humans with AIDS dementia in the near future.

Index Entries: NMDA receptor; excitotoxicity; AIDS dementia; free radicals.

Introduction

Our laboratory has a long-standing interest in the relationship of neuronal viability/outgrowth to intracellular Ca^{2+} levels (reviewed in ref. *1*). Glutamate, or a related excitatory amino acid (EAA), is the major excitatory neurotransmitter that controls the level of intracellular neuronal Ca^{2+} ($[Ca^{2+}]_i$). Escalating concentrations of glutamate have been measured in vivo following focal stroke and head injury (reviewed in refs. *2,3*). As a result, there is a rapid rise in $[Ca^{2+}]_i$, which precedes neurotoxicity by ~24 h. Although the elevation in $[Ca^{2+}]_i$ may not account by itself for the ensuing neuronal injury, several laboratories have now reported that prevention of the increase in $[Ca^{2+}]_i$ leads to the amelioration of anticipated neuronal cell death (reviewed in refs. *2,3*). Excessive intracellular Ca^{2+} is thought to contribute to the triggering of a series of potentially neurotoxic events leading to cellular necrosis or apoptosis; these events include overactivation of the enzymes protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase II, phospholipases, proteases, protein phosphatases, xanthine oxidase, nitric oxide synthase, and endonucleases (for a recent review, *see* ref. *4*).

There are many mechanisms involved in intracellular calcium homeostasis, and this subject is beyond the scope of this article (but see the review in ref. *5*). This article will consider only two modes of Ca^{2+} entry into neurons during these pathological processes. These two routes of entry of Ca^{2+} occur via ion channels that are permeable to Ca^{2+} and can be summarized as follows: (1) glutamate or related EAAs trigger voltage-dependent calcium channels (VDCC) by depolarizing the cell membrane; the major VDCC subtype that is chronically activated by prolonged depolarizations is the L-type calcium channel (reviewed in ref. *6*). (2) glutamate or related EAAs activate ligand-gated ion channels directly; a predominant glutamate receptor-operated channel that is permeable to Ca^{2+} under these conditions is the *N*-methyl-D-aspartate (NMDA) subtype, but other non-NMDA types may also contribute (reviewed in refs. *2,4*).

We have shown that activation of these channel types can control neuronal plasticity during normal development, but, in excessive amounts, our laboratory and others have shown that this stimulation can lead to neuronal death, e.g., after a stroke (reviewed in ref. *1*). Similar mechanisms may obtain in various neurodegenerative conditions. In fact, this mechanism may represent a final common pathway of neuronal injury, although it is not involved in the primary pathophysiology of a neurologic disorder. Most importantly, this pathway makes the disease process amenable to pharmacotherapy. This line of reasoning led us to think that this mechanism might be involved in acquired immunodeficiency syndrome (AIDS)-related neuronal injury.

Neuronal Loss in the CNS of AIDS Patients

A substantial number of adults and children with AIDS eventually develop neurological manifestations of the disease, including dementia, myelopathy, and peripheral neuropathy; as many as 50% of infected children have neurological deficits presenting as delayed developmental milestones. These deficits often occur even in the absence of superinfection with opportunistic organisms or associated malignancies, and have been collectively grouped under the rubric HIV-associated cognitive-motor complex *(7)*.

An excellent review has recently considered the epidemiology of these cognitive and motor deficits associated with AIDS, as well as issues of neuroinvasion (entry of the virus into the CNS), neurotropism (predilection for macrophages of particular strains of HIV-1 that invade the CNS), and neurovirulence (invasion of the CNS by only a subset of macrophage-tropic virus) *(8)*. Therefore, these topics will not be reviewed here. In addition, any account of the pathogenesis of HIV-1 in the nervous system must attempt to explain the selective location of virus in monocytic, but not other cell types despite the neuropathological findings of widespread myelin pallor and reactive astrocytosis. However, for the purposes of this article, we will concentrate on the rather impressive injury to neurons that has only recently been described among the neuropathological manifestations of AIDS in the brain. Several groups have demonstrated the loss of 18–50% of cortical neurons and retinal ganglion cell neurons in the CNS of patients with AIDS *(9–12)*. In addition, in the neocortex, there is a loss in the complexity of dendritic arborization as well as presynaptic area *(13)*. The question remains, however, in at least this subset of patients with neurological manifestations: How can neurons be injured and yet not be infected to any significant degree?

gp120-Induced Neuronal Injury is Ameliorated by Calcium Channel Antagonists

As mentioned above, the major cell type infected with HIV-1 in the CNS is the macrophage or microglial cell. These cells act as a reservoir for the virus, and quite possibly release virus or viral proteins or protein fragments. Possibly accounting at least in part for the injury to neurons is the observation first made in vitro by Douglas Brenneman and colleagues that picomolar concentrations of the envelope protein of HIV-1, gp120, can induce neuronal loss in rodent hippocampal cultures *(14)*. Subsequently, our group demonstrated that in mixed cultures of neurons and glia, picomolar gp120 could increase $[Ca^{2+}]_i$ in rodent hippocampal neurons and retinal ganglion cells within a few minutes of application *(15)*. Recently, similar findings were reported by Thayer's group *(16)*, who were also able to resolve the increase in $[Ca^{2+}]_i$ into discrete oscillations by monitoring the calcium signal on a faster time scale. Within the next 24 h, neuronal injury ensued *(15)*. Several groups have now confirmed that picomolar concentrations of gp120 can cause injury in a variety of neuronal preparations, including rat cortical neurons *(17,18)* and cerebellar granule cells *(19)*. Both the early rise in $[Ca^{2+}]_i$ and the delayed neuronal injury can be largely prevented by antagonists of the L-type voltage-dependent calcium channel (VDCC), including nimodipine (100 n*M* in 5% serum or approx 4 n*M* free drug) *(15,18,19)*. Other antagonists of the L-type VDCC are also effective to some degree *(20)*. Not only are rat retinal ganglion cells and cortical neurons in vitro partially protected by nimodipine and other voltage-dependent Ca^{2+} channel antagonists, but also in a rat pup animal model, stereotactic injection of gp120 into the cortex produces a lesion consisting of cellular infiltrates of foamy macrophages and putative neuronal injury that is prevented by concomitant intraperitoneal administration of nimodipine *(21)*. Additional in vivo evidence that low concentrations of gp120 are associated with neuronal injury has come from experiments of Brenneman, Hill, Ruff, Pert, and coworkers, who have found that intraventricular injections of gp120 into rats result in dystrophic neurites in hippocampal pyramidal cells as well as behavioral deficits; moreover, cerebrospinal fluid of HIV-infected patients has gp120-like neurotoxic activity *(22–24)*. This evidence points to a potential role of gp120 in a neurodegenerative process. Because of these developments, the AIDS Clinical Trials Groups (ACTG) of the NIH Division of AIDS has asked us to begin a multicenter, randomized double-blind, placebo-controlled clinical trial to test the effects of nimodipine in adult patients with HIV-associated cognitive/motor complex (a subset of which has the more debilitating AIDS dementia complex), and this study is currently ongoing.

Nevertheless, these developments do not tell us the mechanism of action of gp120 on neurons, which more recent evidence has led us to believe is an indirect pathway via macrophages/microglia *(see below)*. For example, we noted that only neurons clustered in groups, presumably with synaptic contacts, were vulnerable to gp120, and this fact suggested that cellular interactions were necessary to produce injury. Moreover, the HIV envelope protein does not appear to act directly on calcium channels; in whole-cell and single-channel patch-clamp recordings, picomolar gp120 does not increase calcium current *per se* (H. S.-V. Chen, M. Plummer, P. Hess, S. A. Lipton, unpublished findings). It is possible that calcium channel antagonists ameliorate gp120-induced neuronal injury by reducing the overall intracellular Ca^{2+} burden of the neurons. After all, Ca^{2+} can accumulate in neurons during normal activity with each action potential fired, and nimodipine may be only indirectly beneficial by helping offset an increased calcium load owing to another mechanism.

Involvement of the NMDA Receptor in gp120-Induced Neuronal Injury

A outlined above, there is another prominent mode of Ca^{2+} entry via channels directly coupled to EAA/glutamate receptors. The type of glutamate receptor subtype that is primarily (but not exclusively) involved in this regard is the NMDA subtype; NMDA is a glutamate analog that is a selective agonist of this receptor, although NMDA does not occur naturally in the body. We reasoned that since gp120 causes an early rise in $[Ca^{2+}]_i$ and delayed neurotoxicity, similar to glutamate acting at the NMDA receptor, perhaps glutamate or a closely related molecule was involved in HIV-related neuronal injury. Furthermore, it was well known that VDCC antagonists, such as nimodipine, could block some forms of glutamate neurotoxicity *(25–27)*. Therefore, it was certainly possible that glutamate or a related NMDA agonist was somehow involved

in gp120-induced neuronal damage. In addition, Heyes and colleagues *(28,29)* had found that cerebrospinal fluid (CSF levels of quinolinate, a naturally-occurring (although weak) NMDA agonist, was correlated with the degree of dementia in AIDS patients. To test the possibility that EAAs were involved, the following experiments were undertaken. NMDA antagonists were assessed for their ability to prevent gp120-induced neuronal injury. We found that MK-801 (dizocilpine), an open-channel blocker of NMDA receptor-coupled ion channels, prevented gp120-induced neuronal injury *(30,31)*. D-amino 5-phosphonovalerate (APV), a competitive antagonist at the glutamate binding site of the NMDA receptor, was partially effective in ameliorating this form of neuronal injury. Recently, other groups have obtained similar results using NMDA antagonists or inhibitors of nitric oxide synthase (nitric oxide, or NO·, is believed to be involved in one of the toxic pathways activated by NMDA receptor stimulation) *(17–19)*. In contrast, CNQX, a non-NMDA antagonist, did not protect from gp120-induced neuronal damage, at least to retinal ganglion cells *(30,31)*.

Another possible link between the effects of gp120 and NMDA-receptor activation arises from the observation that one form of neuronal injury in both the brains of AIDS patients *(13)* and the brains of rats injected with gp120 *(22)* or transgenic gp120 mice *(96,134)* involves dystrophic neurites. These neurites are excessively tortuous and display a paucity of branches. Some of these neurites may be retracting, giving them a "bald" appearance. We and others have found a similar pattern of dystrophic neurites, including retraction of growth cones, in response to sublethal concentrations of NMDA or glutamate in cultured rat retinal ganglion cells and hippocampal neurons *(1,32,33)*. Furthermore, these effects are dependent on influx of Ca^{2+} into the neurons. These findings indicate that the end points for neuronal injury related to gp120 or excitotoxicity should include more subtle changes than death, and these alterations in neuronal cytoarchitecture could have important consequences for neuronal function and plasticity *(1)*.

The simplest potential explanation for all of these findings is that gp120 might simulate an NMDA-evoked current or somehow augment such currents. To examine this idea, we used the patch-clamp technique to determine if gp120 affected membrane currents. However, in whole-cell recordings, using both conventional and perforated-patch techniques, no effect of picomolar gp120 was observed, even in recordings lasting tens of minutes. Similarly, no enhancement of glutamate- or of NMDA-evoked currents was encountered *(31)*. Interestingly, nanomolar concentrations of gp120 (a 1000-fold excess over the levels used in the aforementioned experiments) have been reported to block NMDA receptor-operated ion channels, preventing NMDA-evoked increases in Ca^{2+} influx *(34)*. This finding may account, at least in part, for the dose–response curve of gp120 induced neuronal injury, which has an inverted "U" shape *(14)*; that is, at high nanomolar concentrations in contrast to picomolar concentrations, gp120 no longer induces neuronal cell injury. Nevertheless, during HIV-1 infection in the brain, it appears unlikely that nanomolar concentrations of gp120 actually occur because conventional ELISA analysis and Western blots are sensitive to these concentrations, but have failed to detect their presence.

The next possible explanation that we considered is that endogenous levels of glutamate might become toxic in the presence of picomolar gp120. To test this hypothesis, the enzyme glutamate-pyruvate transaminase (GPI) was used to degrade the endogenous glutamate in our retinal cultures. High-performance liquid chromatography (HPLC) analysis of amino acids was used to verify glutamate degradation (~25 μM decreased to <5 μM). Under these conditions, the catabolism of endogenous glutamate in vitro protected neurons from gp120-induced injury to rat retinal ganglion cells *(30,31)*. Recently, Dawson et al. have also found that 25 μM glutamate were necessary in order for them to observe neurotoxicity in rat cortical cultures in the presence of 100 pM gp120 *(18)*. Taken together, these data argue that concurrent activation of NMDA receptors is needed for neuronal injury by gp120 in AIDS. These experiments do not tell us, however, if the action of gp120 is mediated directly on neurons or indirectly via an intervening cell type, such a astrocytes or macrophages/microglia.

Indirect Neuronal Injury Mediated by HIV-Infected or gp120-Stimulated Monocytic Cells

It is still not known definitively if the adverse effects of gp120 are mediated directly on neurons, via glial cells, such as microglia and astrocytes, or by a combination of these mechanisms. To deter-

mine at least some of the cell types involved in neurotoxicity, we performed the following experiment. L-Leucine methyl ester was used to deplete monocytoid cells from cultures of mixed glia and neurons. Under these conditions, gp120 no longer injured neurons, suggesting that at least under our culture conditions, macrophages/microglia were necessary to mediate the neurotoxic effects of gp120 *(35)*. Along these lines, it is also interesting to note that an increase in microglial cell density in the brains of AIDS patients is correlated with the degree of cerebral atrophy, as reflected by ventricular dilatation *(36)*.

It is well known that gp120 binds to CD4 on human monocytic as well as lymphocytic cells, and, in fact, this appears to be the major (but probably not exclusive) route of entry of the virus into these cells. Human macrophages, monocytes, and microglia, but apparently not rat or mouse cells, possess the proper CD4 molecule to bind gp120; however, lack of known receptors does not, of course, rule out alternative mechanisms of binding or toxicity. In several laboratories, for instance, it has not been necessary to have human macrophages present to observe gp120-induced neuronal injury in cultures. Along these same lines, in our laboratory's cultures of rat retinal cells, antirat CD4 antibodies do not block the neuronal injury to retinal ganglion cells engendered by gp120, which follows an EAA pathway, whereas anti-gp120 antiserum completely blocks this toxic effect *(37)*. On the other hand, gp120 incubation with human blood monocytes or the monocytic cell line THP-1 also produces the release of neurotoxins that follow an EAA pathway to cell injury; antibodies directed against the CD4-binding region of gp120, but not against the V3 loop of gp120, block this toxic effect *(38)*. Thus, there appear to be both CD4- and non-CD4-mediated mechanisms of gp120-induced neuronal injury, in a sense paralleling a similar situation concerning CD4- and non-CD4-mediated mechanisms for viral entry into cells.

In conjunction with the data of other laboratories, the aforementioned results suggest the following model of HIV-related neuronal injury (Fig. 1). HIV-infected macrophages *(39,40)* or gp120-stimulated macrophages *(35,38)* release neurotoxic products. These neurotoxins include relatively small, possibly heat-stable compounds that have recently begun to be characterized by Gendelman and colleagues *(41)*. They found that the products released by HIV-infected monocytes include a product of phospholipase A_2 activity, platelet-activating factor (PAF), as well as arachidonic acid and its metabolites, leukotriene B_4 (LTB_4), leukotriene D_4 (LTD_4), and lipoxin A_4 (LXA_4). Under their conditions, these substances are released only in the presence of astrocytes, implying the existence of a positive feedback loop between astrocytic and monocytoid cells. HIV-infected macrophages also release the cytokines TNF-α and IL-1β which have been shown to stimulate astrocyte proliferation *(42,43)*, another feature of HIV encephalitis. In addition, the cytokines present in conditioned medium from lipopolysaccharide (LPS)-treated astrocytes can stimulate HIV-1 gene expression in monocytic cells *(44)*. Under certain in vitro conditions, TNF-α and IL-1 can be associated with the death of oligodendrocytes and, by implication, demyelination (*see below* and ref. *45*).

Moreover, there are multiple, complex interactions and feedback loops affecting cytokine and arachidonic acid metabolite production by macrophages and astrocytes. For example, TNF-α enhances IL-1 production in macrophages *(46)*. Arachidonic acid metabolites can influence the production of TNF-α and IL-1β in macrophages, and, in turn, TNF can amplify arachidonic acid metabolism, including the release of LTB_4, in response to IL-1. PAF can enhance TNF and IL-1 production, and, in turn, PAF synthesis can be stimulated with TNF, IL-1β, or interferon-γ (IFN-γ) in human monocytes *(47–52)*. Finally, similar arachidonic acid metabolites and cytokines released by HIV-infected macrophages appear to be produced by gp120-stimulated monocytic cells. For example, this HIV glycoprotein induces the release of LTB_4, LTC_4, TNF-α, and IL-1β, as well as arachidonic acid itself from human monocytes *(53–55)*. It remains to be shown, however, if gp120-stimulated monocytic cells release PAF and LXA_4 in the presence of astrocytes, similar to their HIV-infected monocyte counterparts.

The cytokines TNF-α and IL-1β in the amounts produced by HIV-infected or gp120-stimulated macrophages do not appear to be neurotoxic in and of themselves *(41)*. Could, however, the arachidonic acid metabolites emanating from HIV-infected or gp120-stimulated macrophages be involved in neurotoxicity? In particular, PAF has recently been shown to increase intracellular neuronal Ca^{2+} *(56)* and, presumably via this mechanism, to increase glutamate release *(57)* and hence excitatory neurotransmission *(58)*. It is possible that TNF-α also contributes to this process by increasing voltage-

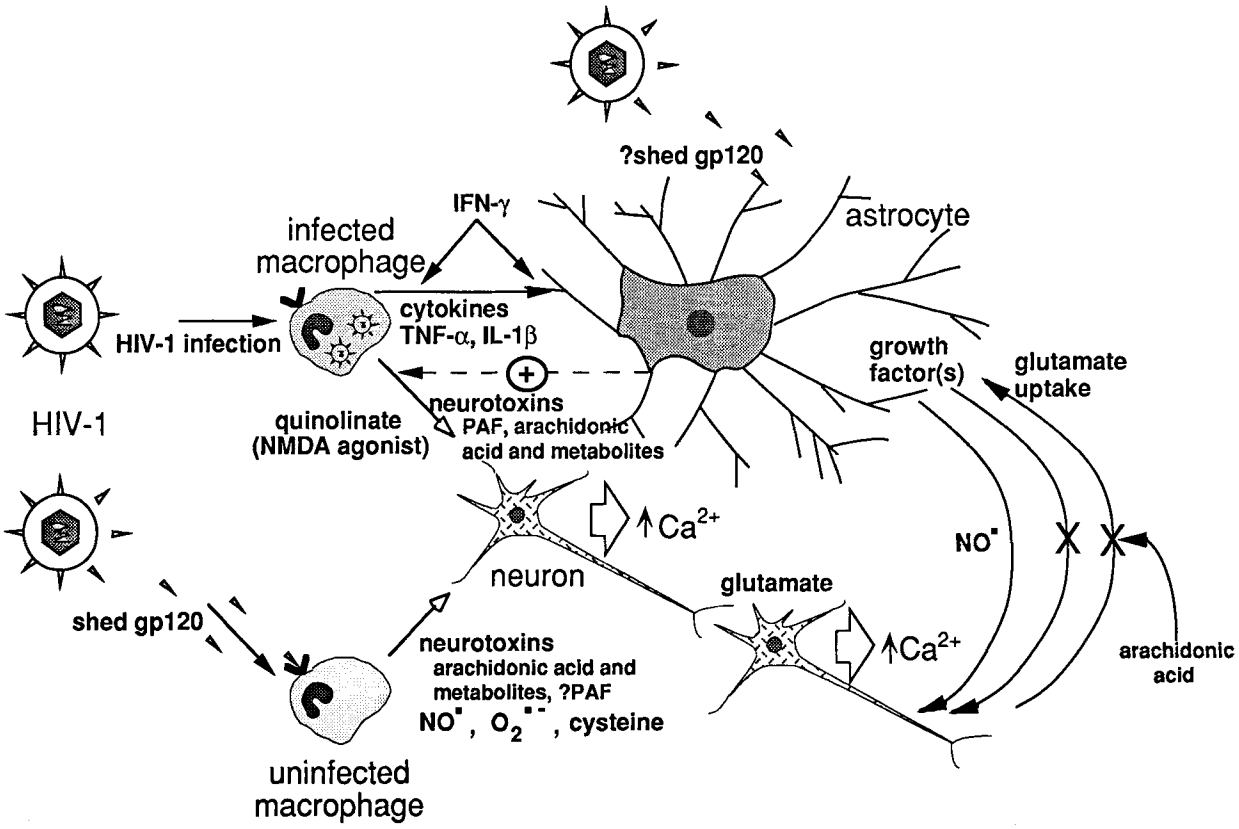

Fig. 1. Model summarizing evidence for at least one complete pathway of HIV-related neuronal injury. HIV-infected macrophages release factors that lead to neurotoxicity. These factors may include PAF (platelet-activating factor), arachidonic acid, and possibly its metabolites. Macrophages and astrocytes have mutual feedback loops in this system (signified by the reciprocal arrows). The excitatory action of the macrophage factors appears to lead to an increase in neuronal Ca^{2+} and the consequent release of glutamate. In turn, glutamate overexcites neighboring neurons leading to an increase in intracellular Ca^{2+}, neuronal injury, and subsequent further release of glutamate. This final common pathway of neurotoxic action can be blocked by NMDA antagonists. For certain neurons, this form of damage can also be ameliorated by some degree by calcium channel antagonists or non-NMDA antagonists.

The major pathway of entry of HIV-1 into monocytoid cells is via gp120 binding to the cells, and therefore, not surprisingly, gp120 (or a fragment thereof) appears capable of activating uninfected macrophages to release similar factors, at least including arachidonic acid. Cytokines participate in this cellular network in several ways. For example, HIV-infection or gp120-stimulation of macrophages enhances their production of TNF-α and IL-β (solid arrow).The TNF-α and IL-1β produced by macrophages stimulate astrogliosis. Astrocytes appear to feedback (dashed arrow) onto monocytic cells by an as yet unknown mechanism to increase the macrophage production of these cytokines. TNF-α may also injure oligodendrocytes and increase voltage-dependent calcium currents in neurons. Interferon-γ (IFN-γ) induces macrophage/microgliosis and macrophage production of quinolinate, PAF, β2-microglobulin, and neopterin; IFN-γ may also affect astrocytic cells in several ways, at least in vitro, e.g., by inducing expression of major histocompatibility (MHC) class II genes. Many other cytokine loops also exist. For example, TGF-β is produced by astrocytes and can recruit inflammatory macrophages into the brain. Moreover, TGF-β has been reported to enhance HIV replication in macrophages as well as their production of TNF and IL-1. Other cytokines, including TNF-β, IL-1α, IL-6, and IFN-α, may also be involved (not shown). The coat protein gp120 may also have direct effects on astrocytes, e.g., to decrease growth factor production or to inhibit glutamate reuptake. On the other hand, direct effects of picomolar concentrations gp120 on neurons have not been definitively established.

dependent Ca^{2+} currents *(59)*. In collaboration with Howard Gendelman's group, our laboratory has recently shown that the elevated levels of PAF that have been measured in cultures of HIV-infected monocytic cells, as well as in the CSF of patients with the AIDS dementia complex, are toxic to neu-

rons in vitro (60,61). Moreover, in these experiments, PAF-related neuronal injury is largely ameliorated by NMDA antagonists, similar to the pharmacology of neuroprotection observed in the face of HIV-infected or gp120-stimulated macrophages. These recent developments suggest that local release of excessive glutamate with resultant overstimulation of NMDA receptors contributes to neuronal damage in AIDS patients with high concentrations of PAF. On the other hand, at more physiological levels, PAF can increase glutamate release perhaps more modestly, and this may contribute to the observation that PAF can induce long-term potentiation (LTP, a cellular correlate of learning and memory) in the hippocampus (62). Thus, the spectrum of actions of PAF in exciting neurons may represent a bell curve with moderate levels of excitation enhancing normal physiological functions and excessive stimulation leading to neurotoxicity. The implications for such a mechanism are particularly important in the AIDS dementia complex, since extreme elevations in PAF may thus lead to disruption of memory and cognitive function prior to actual neuronal cell death.

In addition, arachidonic acid is released from HIV-infected and gp120-stimulated monocytic cells (41,53). Recently, arachidonic acid has been shown to inhibit high-affinity uptake of glutamate into synaptosomes and astrocytes (63), and to potentiate NMDA receptor-activated currents by increasing open-channel probability (64). In conjunction with PAF, arachidonic acid may therefore contribute to excessive NMDA receptor stimulation by increasing the release of glutamate, inhibiting its reuptake, and enhancing its action at the NMDA receptor. Additional glutamate receptor activation may occur as a further consequence of these events, as neurons are excited or injured and release their stores of glutamate onto neighboring neurons (16,30,31,65).

One line of evidence for this supposition lies in the finding, as detailed above, that NMDA antagonists or enzymatic degradation of glutamate ameliorates gp120 induced neuronal injury in mixed neuronal-glial cultures (30,31). Intensive investigation in several laboratories is currently underway to study the potential pathway for neuronal injury that is triggered by HIV-infected or gp120-stimulated macrophages involving PAF, arachidonic acid, and potentially other metabolites.

Also, as alluded to above, another possible link between HIV-1 infection and EAA-induced neurotoxicity involves quinolinate, an endogenous NMDA agonist that is increased in the CSF of patients with the AIDS dementia complex (29). Quinolinate levels are known to be influenced by cytokines that are increased after HIV-1 infection. For example, it is known that IFN-γ is present in the brains of patients with AIDS (66), and human macrophages activated by IFN-γ release substantial amounts of quinolinate (67). In addition, under some conditions, e.g., following neuronal loss, quinolinate can also be produced by astrocytes (68,69). Quinolinate, therefore, may also contribute to neuronal injury by activating NMDA receptors during HIV infection, although this scenario appears to also be true for a variety of other CNS infections.

Possible Involvement of Astrocytes, Oligodendrocytes, and Other HIV-1 Proteins in Neuronal Injury—Astrocytes and HIV Related Neuronal Damage

In at least some model systems, the presence of astrocytes is necessary for HIV-infected macrophages to release substantial amounts of their neurotoxic factors (41). In addition, astrocytes may be important in mediating HIV-related neuronal injury in other ways. For example, in murine hippocampal cultures, Brenneman et al. (14) found that gp120-induced neurotoxicity can be prevented by the presence of vasoactive intestinal polypeptide (VIP) or by a five amino-acid substance with sequence homology to VIP, peptide T. These workers also found that VIP acts on astrocytes to increase oscillations in intracellular Ca^{2+} and to release factors necessary for normal neuronal outgrowth and survival (70). Thus, these results raise the possibility that gp120 may compete with endogenous VIP for a receptor, most likely on astrocytes, that is critical to normal neuronal function. The receptor may bear some resemblance to mouse CD4 because mouse anti-CD4 antibodies blocked the toxic effects of gp120 in this system (14). This effect of gp120 is hypothesized to prevent the release of such astrocyte factors that are necessary to prevent neuronal injury and suggests that one pathway for neuronal damage is an indirect one that is mediated via astrocytes. Therefore, gp120 might interact with a receptor on astrocytes (Fig. 1); neurotoxicity may in part be realized by interfering with the normal function of astrocytes and their release of neuronal growth factors) (71). Our laboratory has gathered

preliminary data suggesting that gp120 might also affect astrocyte function, either directly or indirectly, in another manner: gp120 inhibits the ability of cultured astrocytes (and possibly neurons) to take up glutamate, thus possibly contributing to EAA-induced neurotoxicity (E. B. Dreyer and S. A. Lipton, in preparation). Such an effect might account for the apparent increase in sensitivity of neurons to glutamate toxicity in the presence of gp120 and would also help explain the requirement for some glutamate (~25 μ*M*) to be present in the culture medium in order to observe gp120-induced neurotoxicity *(18,30,31)*. Ongoing experiments are addressing these possibilities.

gp120 Binding to the Oligodendrocyte Surface Molecule GalC

The envelope protein gp120 has also been shown to bind to galactosyl ceramide (GalC), a molecule on the surface of the oligodendrocyte, which represents the cell type responsible for myelination in the CNS *(72,73)*. However, relatively high concentrations of gp120 (nanomolar) were necessary to observe this binding compared to the low (picomolar) concentrations of the coat protein that have been found to lead to neuronal injury. Nonetheless, the findings concerning binding to GalC raise the possibility of participation of gp120 in myelin disruption representing a further indirect influence on the welfare of neurons. Future studies will be necessary to determine the significance of this potential pathway for cellular injury.

gp120 Binding to Sulfatide and Myelin-Associated Glycoprotein

Recently, in addition to GalC, gp120 has been shown to bind to sulfatide (GalS), a sulfated glycoprotein implicated in sensory neuritis, and to myelin-associated glycoprotein (MAG), an autoantigen observed in demyelinating neuropathy *(74)*. Similar to GalC, binding became significant in the nanomolar range of gp120. The authors speculate that this binding could have implications for the peripheral nervous system, e.g., in an acute or chronic demyelinating neuropathy or a painful sensory axonal neuropathy, such as that frequently observed during HIV-infection. However, as alluded to above, the significance of nanomolar levels of gp120 binding in the nervous system remains uncertain. The same group of workers has recently published a preliminary report that gp120 may also bind to neurons in immunofluorescence assays *(75)*, but it is not yet clear if the concentration of gp120 required to see this effect is attained in the CNS during HIV-1 infection. Future studies along these lines will be important.

gp120 Attenuates β-Adrenergic Stimulation of Astrocytes and Microglia

Additional effects of gp120 have been reported. Levi's group have found that picomolar gp120 can attenuate β-adrenergic stimulation of cAMP in astrocytes and microglia *(76)*. When added alone, gp120 modestly enhanced the basal levels of cAMP. These effects of gp120 could also interfere with β-adrenergic modulation of cytokine production, e.g., of TNF-α. Thus, gp120 may have other, complex effects on glial cells in the CNS.

Neurotoxicity of Other HIV-1 Proteins

Besides gp120, two other HIV-1 proteins have been reported to affect neurons or neuronal-like cells, raising the possibility of their involvement in HIV-related neuronal injury. The nuclear protein tat was shown to be toxic to glioma and neuroblastoma cell lines in vitro and to mice in vivo *(77,78)*. The basic region of the peptide (amino acid residues 49–57) appears to act nonspecifically to increase the leakage conductance of the membrane, thus altering cell permeability. Moreover, neurotoxicity of the related Maedi-Visna virus peptide was ameliorated by NMDA antagonists or by inhibitors of nitric oxide synthase *(79)*, reminiscent of the pharmacology of antagonism of the neurotoxic effects of gp120 and HIV-infected macrophages. Further work will be necessary to attempt to relate these findings with the tat peptide to the neuropathology encountered in the brains of patients with HIV-1-associated cognitive/motor complex.

Another HIV-1 protein, Nef, has also been shown to affect neuronal cell function. Nef shares sequence and structural features with scorpion toxin peptides; both recombinant Nef protein and a synthetic portion of scorpion peptide increase total K^+ current in chick dorsal root ganglion cells *(80)*.

Direct Effect of HIV-Infected Macrophages on Neurons

Finally, it is possible that HIV-infected monocytoid cells may have a cytopathic effect on neurons by direct contact *(81)*. This mechanism does not preclude, however, an additional mechanism of neuronal injury mediated by soluble factors leading to excessive stimulation of NMDA receptors *(82)*.

Excessive Stimulation of NMDA Receptor—a Final Common Pathway

From the foregoing, there appear to be at least two sites of potential interaction of HIV-related neurotoxins with NMDA receptors (Fig. 1). First, quinolinate emanating from macrophages may directly stimulate neurons. Second, after excitation contributed to by quinolinate, PAF, and possibly arachidonic acid or its metabolites, or after injury resulting from other toxic pathways, neurons would release glutamate onto second-order neurons. Additionally, astrocytes might fail to take up the glutamate. This hypothesis, in which one neuron acts as a "bad neighbor" by releasing excessive glutamate, is in some ways similar to the damage thought to occur in the penumbra of a stroke—glutamate released by injured neurons contributes to further injury to neighboring neurons.

Moreover, NMDA antagonists ameliorate HIV-related neuronal injury induced by either HIV-infected macrophages *([39]* and H. E. Gendelman, personal communication), or, as mentioned earlier, gp120 activated macrophages *(17–19,30,31,83)*. Furthermore, in some cases, calcium channel antagonists can attenuate this form of damage *([15,20]* and L. Pulliam, personal communication). In general, the pharmacology of neuroprotection from noxious agents depends on the repertoire and diversity of ion channel types in a particular class of neurons *(6)*. For example, neurons lacking NMDA receptors will obviously not be protected by NMDA antagonists. Conversely, if NMDA receptor-associated channels are the predominant channel in a specific neuronal cell type whereby Ca^{2+} enters the cell, then the lethal effects of excessive stimulation by glutamate may be ameliorated with NMDA antagonists. Some non-NMDA receptor-associated channels are directly permeable to Ca^{2+}, but most appear not to be (e.g., those containing the GluR2 receptor subunit). However, depolarization of neurons by stimulation of non-NMDA receptors will trigger voltage-dependent Ca^{2+} channels (VDCCs). If sufficient L-type calcium channels exist on a particular neuronal cell type, then the excessive influx of Ca^{2+} via these channels could lead to toxic consequences. Hence, in some cell types, such as hippocampal pyramidal cells, cortical neurons, and retinal ganglion cells, there is evidence that calcium channel antagonists may attenuate damage because of activation of either NMDA or non-NMDA receptors *(25–27)*.

As outlined above, glutamate may be involved in the final common pathway of neuronal injury by HIV-infected macrophages or by gp120 stimulated macrophages. Thus, either NMDA or non-NMDA receptor activation may play a role in this form of toxicity depending on the exact repertoire of ion channels in a particular cell type. In fact, it has been suggested that non-NMDA receptors could also be important in contributing in the neurotoxic events triggered by gp120 *(18,84)*. Nevertheless, the majority of findings to date suggest that NMDA receptor-mediated neuronal injury plays a predominant role in the pathogenesis of the neurological manifestations of AIDS in the CNS *(21,65)*.

Development of Clinically Tolerated NMDA Antagonists for HIV-Related Neuronal Injury

NMDA receptors may be involved in HIV-related neurotoxicity at two separate sites, located (1) on the primary neuron injured by factors released from glial cells, and (2) on neurons that are secondarily affected (*see above* and Fig. 1). This fact has provided an impetus for our laboratory to begin a drug-development program for clinically tolerated NMDA antagonists. To understand our approach, we must first entertain a brief review of the possible mechanisms and sites of action of NMDA receptor antagonists.

Sites of Action of Potential Clinically Tolerated NMDA Antagonists

Given the growing number of genes (at least 20) that have been cloned for various glutamate receptors, the complexity of EAA receptor pharmacology is quite great. Despite these concerns, we can consider currently available agents that appear to work on broad classes of these receptors. For the purposes of this article, the author will concentrate on NMDA

antagonists that appear to be clinically tolerated and therefore can be considered for human trials.

There are several modulatory sites on the NMDA receptor–channel complex that could potentially be used to modify the activity of the receptor-operated ion channels and thus to prevent the excessive influx of Ca^{2+} (Fig. 2). The first site is the glutamate or NMDA binding site. An antagonist acting here would be competitive in nature, i.e., competing for the site with an EAA. For both theoretical and practical reasons, a competitive inhibitor might not be as desirable an antagonist as one that is not competitive for the glutamate binding site. A competitive antagonist would perforce eliminate the normal, physiological activity of the NMDA receptor even before it would affect potentially excessive levels of glutamate. Thus, cognition and memory, thought to be related to long-term potentiation (LTP), might be compromised as well as other important functions mediated by excitatory transmission in the brain. Even putting aside these concerns, as part of the disease process, escalating levels of glutamate might be able to overcome or "out-compete" such an antagonist.

NMDA Open-Channel Blockers

In contrast, other modulatory sites of the NMDA receptor–channel complex should be able to inhibit the effects of high levels of glutamate in compromised areas of the brain while leaving relatively spared the effects of normal neurotransmission in other regions of the brain *(85–87)*. For example, one site that appears to have this advantageous effect is located in the ion channel itself. There are drugs that only block the channel when it is open, i.e., the antagonist can only gain access to the channel in the open state. On average, escalating levels of glutamate result in the channels remaining open for a greater fraction of time. Under these conditions, there is a better chance for an open-channel blocking drug to enter the channel and block it. The result of such a mechanism of action is that the untoward effects of greater (pathological) concentrations of glutamate are inhibited to a greater extent than lower (physiological) concentrations *(87)*. Unfortunately, some of these open-channel blockers, which include phencyclidine (angel dust) and MK-801 (dizocilpine), have neuropsychiatric side effects and probably cannot be administered safely *(88)*. Another concern with NMDA antagonists, such as phencyclidine and MK-801, is the development of neuronal vacuolization, although it is reversible *(89)*. Finally, a very important problem with MK-801 is that once it enters an open channel, it leaves the channel only very slowly (half-time >1 h). In practical terms, this means that the degree of blockade builds up after MK-801 administration because each molecule of the antagonist entering a channel effectively does not leave.

Several members of this open-channel blocking class of agents, however, are tolerated, such as ketamine and dextromethorphan or the related molecule dextrorphan *(90–94)*. Unfortunately, it is not clear whether these particular drugs are sufficiently potent NMDA antagonists at clinically tolerated doses. Nevertheless, the fact that certain members of this open-channel blocker family are clinically tolerated appears to be associated with their rapid kinetics of action with the channel (the kinetic parameters are composed of the on-rate and off-rate for channel blockade) *(87,95)* Most importantly, the safe drugs, such as memantine (see ref. *97* and the discussion below) leave the channel promptly, with an off-rate ~5 s *(87)*.

Mg^{2+} also blocks open NMDA channels, and this may be the basis for its antiepileptic and neuroprotective effects *(98–100)*. These beneficial effects, however, may not be robust, probably because Mg^{2+} leaves the channel so quickly that it may not act effectively to offset toxic levels of glutamate. In addition, these charged channel-blocking drugs act to a lesser degree when neurons are depolarized, e.g., under conditions of energy compromise *(101)*.

In summary, an agent that remains in the channel for at least some period of time is necessary to block the effects of glutamate overstimulation. Of the known NMDA open-channel blockers, memantine is one candidate for clinical trials to combat neurological disorders, such as HIV-associated cognitive/motor complex, that have a component of NMDA receptor-mediated neurotoxicity. Memantine has been used clinically with considerable safety in Germany for over a dozen years in the treatment of Parkinson's disease and spasticity. Memantine is a congener of amantadine, the well-known antiviral and antiparkinsonian drug used in the United States. Amantadine, however, is considerably less potent on NMDA receptor-operated ion channels at clinically tolerated doses *(87)*, probably precluding its use for these other neurological diseases. It may be no accident that memantine both inhibits NMDA receptor responses and alleviates parkinsonian symptoms; one theory of Parkinson's disease is that neurons die, at least in part, because of a form of NMDA receptor-mediated toxicity.

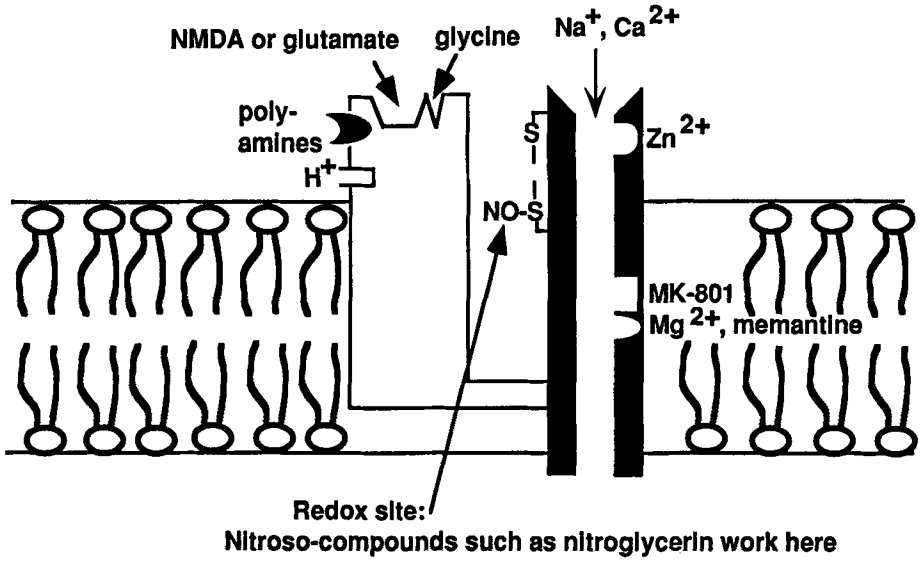

Fig. 2. Sites of potential antagonist action on the NMDA receptor–channel complex. Competitive antagonists can compete with NMDA or glutamate for binding to the agonist site. Several antagonists to the glycine coagonist site have been described that are chlorinated and sulfated derivatives of kynurenic acid. It is not yet known if any will prove to be tolerated clinically although felbamate, a putative glycine-site antagonist, was recently approved by the FDA as an anti-epileptic. H^+ effects are transmitted through a modulatory site; decreasing pH acts to downregulate channel activity. Other sites for polyamines and Zn^{2+} can also be used to affect receptor–channel function. Sites that inhibit channel activity by binding Mg^{2+} or drugs, such as MK-801, phencyclidine, and memantine, are within the electric field of the channel and are only exposed when the channel is previously opened by agonist (termed uncompetitive antagonism). Finally, a redox modulatory site(s) (probably a disulfide bond, or at least a long-lasting covalent modification of a thiol group, that can be converted to free sulfhydryl groups [S—S ⇄ 2-SH]) is affected by chemical reducing and oxidizing agents. Oxidation can favor the disulfide conformation (S—S) over free thiol (—SH groups and, thus, downregulate channel activity. In addition, several nitroso-compounds can downregulate channel activity by transferring an NO group to the thiol(s) of the NMDA receptor's redox site, producing RS-NO, a nitrosonium ion (NO^+) equivalent, which may lead to disulfide bond formation.

NMDA Receptor Redox Modulatory Site

Another modulatory site on the NMDA receptor-channel complex of possible clinical utility in the near future was discovered several years ago in our laboratory and has been termed the redox modulatory site(s) *(102)*. This site(s) consists of one or more sulfhydryl groups; these sulfhydryl groups may possibly be in close approximation and form a disulfide bond under oxidizing conditions. Under chemical reducing conditions that favor the formation of free thiol (—SH) groups over a disulfide, the opening frequency of NMDA receptor-associated channels increases *(102,103)*, and thus, there is a net increase in Ca^{2+} influx through the channels *(104,105)* and an increase in the extent of NMDA receptor-mediated neurotoxicity *(106,107)*. Conversely, redox reagents that mildly oxidize the NMDA receptor, for example, to reform disulfide bonds or form ligands on the free thiol groups, downregulate receptor-mediated neurotoxicity. Thus, these agents might prove useful in combating the myriad of neurological maladies resulting, at least in part, from a final common pathway of NMDA receptor-mediated neuronal damage *(108)*.

Indeed, several such redox reagents have recently been reported, including quite surprisingly the common nitroso-compound, nitroglycerin *(109)*. One mechanism of nitroglycerin's action in this regard is mediated by a substance related to nitric oxide (NO·), but in a different redox state, for example, in the form of RS-NO (nitrosonium ion equivalents, NO^+, having one less electron than NO·) *(110)*. Nitric oxide (NO·) itself can participate in reactions to form products that are toxic to nerve cells, such as peroxynitrite ($ONOO^-$) and its breakdown products including hydroxyl radical (HO·)-like compounds *(109,111–115)*. In other redox states, however, monoxides of nitrogen can interact with

thiol groups, such as those comprising the redox modulatory site of the NMDA receptor, by an *S*-nitrosylation reaction, resulting in transfer of the NO group to a thiol *(110,115)*. This action results in downregulation of NMDA receptor activity and protects neurons from excessive stimulation of the receptor *(109)*. Patients can be made tolerant to the cardiovascular effects of nitroglycerin within hours of continuous therapy. Under these conditions, our laboratory has shown in animal models that the extent of NMDA receptor-mediated neurotoxicity can be markedly attenuated in the absence of behavioral or systemic side effects of the drug *(116)*. Nevertheless, the exact dosing regimen must be carefully worked our before attempting to apply this technique to humans. In addition, nitroso-compounds, such as nitroglycerin, can be administered acutely to affect NMDA receptor activity if the blood pressure is maintained with a pressor agent, such as phenylephrine. Other promising reagents that appear to act either directly or indirectly on the NMDA redox modulatory site include oxidized glutathione *(117–119)* and the putative essential nutrient and redox cofactor, pyrroloquinoline quinone (PQQ) *(120)*.

In addition, there are other important modulatory sites of the NMDA receptor, several of which are illustrated in Fig. 2. Antagonists of each of these sites could possibly be useful in the treatment or prevention of NMDA receptor-mediated neurotoxicity. For the purposes of this relatively brief article, the author has chosen to highlight only two of these, the ion channel and redox modulatory sites. The other modulatory sites may become therapeutically relevant, however, if clinically tolerated antagonists can be developed to interact with them. Intensive research efforts along these lines are now under way in both academic institutions and the pharmaceutical industry, which are exploring, for example, antagonists of the glycine coagonist site of the NMDA receptor.

Since NMDA and non-NMDA receptor stimulation alike lead to neuronal depolarization and consequent activation of VDCCs, blockade of VDCCs might also ameliorate neurotoxicity, as discussed above. It has become apparent that different subpopulations of neurons have different repertoires of VDCCs, so it might be anticipated that an antagonist specific for a particular type of calcium channel may be effective only in certain regions of the brain or for certain cell types *(vide supra) (6,121)*. Therefore, it will be important to develop antagonists specific for these various types of calcium channels, and many investigators are working in this area. Currently available in the clinics are CNS-permeable antagonists of the L-type calcium channel, such as nimodipine. Other calcium channel antagonists that are permeable to the blood–brain barrier are also being tested in multicenter trials for entities other than the AIDS dementia complex (for a review, *see* ref. *6*).

Excitatory Amino Acid Antagonist Treatments on the Horizon

Among the aforementioned classes of NMDA antagonists, the pharmaceutical industry is currently sponsoring in humans Phase II (efficacy) clinical trials for stroke using the putative open-channel blockers CNS 1102 (Cambridge Neuroscience Inc., Cambridge, MA) and dextrorphan (Roche, Nutley, NJ). There is some evidence that the dextrorphan and the related drug dextromethorphan also antagonize VDCCs as well as NMDA receptor-operated channels, which might be a helpful dual property *(122)*.

A Phase II (efficacy) clinical stroke trial was recently completed for the NMDA competitive antagonist CGS19755 (Ciba-Geigy, Summit, NJ), and the results should be available shortly. Other companies are currently investigating both NMDA and non-NMDA antagonists, but for proprietary reasons, information is scanty, and the indications do not as yet include the AIDS dementia complex. Based on animal testing, it is quite possible that for various forms of glutamate-related neurotoxicity, a combination of agents may be the most effective, e.g., combining calcium channel antagonists with NMDA antagonists *(123–125)*.

Human clinical trials for indications other than the AIDS dementia complex are also in progress using agents that work downstream from EAA receptors. These include monosialogangliosides (GM1), which are being tested for improvement of outcome after stroke *(126)*, and the 21-aminosteroid, tirilazad mesylate, which is entering a Phase III multicenter efficacy trial. Recent reports that gangliosides can result in a polyneuropathy resembling Guillain-Barré syndrome have caused several authorities to conclude that clinical studies with GM1 in humans should be suspended pending further assessment of this problem *(127,128)*.

Finally, a case can be made that the proven NMDA open-channel blocker, memantine (as well as its less potent congener, amantadine), has already been in clinical use for years because it is known to

ameliorate some of the symptoms of Parkinson's disease. Furthermore, it is now known that the level of memantine (2–12 μ*M*) achieved in the human brain during this form of treatment *(129)* can afford protection from NMDA receptor-mediated neurotoxicity both in vitro and in vivo *(87,130–133)*. Recently, our laboratory as well as another group reported that low micromolar levels of memantine can also protect neurons from damage induced by gp120 in vitro *(17,83)* and in vivo in an animal model *(21)*. These preliminary findings raise the possibility that a clinically tolerated NMDA antagonist, memantine, might be useful in the treatment or prevention of the AIDS dementia complex. Therefore, it has been proposed to study the use of memantine as an adjunctive therapy with antiretroviral drugs, such as zidovudine or didanosine, and the AIDS Clinical Trials Group of the NIH is currently considering this option.

Conclusions

Although it is likely that a complex web of cell interactions leads to neuronal loss in AIDS, HIV-infected macrophages or gp120-stimulated macrophages release toxins whose action appears to be mediated by a final common pathway involving excessive stimulation of neurons by EAAs, such as glutamate and quinolinate. The macrophage toxins are released in increased amounts in the presence of astrocytes, and appear to include PAF, arachidonic acid, and possibly its metabolites. This represents at least one complete pathway to neuronal injury that is amenable to pharmacotherapy, although other pathways may also exist. A strong body of scientific evidence supports the premise that the mechanism for this form of HIV-related neuronal injury is similar to that currently thought to be responsible for a wide variety of acute and chronic neurological diseases *(2,3,65)*. EAAs apparently exert this excitotoxic effect by engendering an excessive influx of Ca^{2+} into neurons. Currently, there is intensive investigation to discover clinically tolerated drugs to combat the neurotoxic effects associated with the excessive stimulation of glutamate receptors or the events triggered downstream to receptor activation. One therapeutic approach has been to use glutamate receptor antagonists, and although several promising drugs are already in hand, additional agents are needed. With the possibility of a final common pathophysiology involving EAA receptors for many disorders of the CNS, including the AIDS dementia complex, the future development and testing of safe and effective EAA antagonists should become a high priority.

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