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Energy Dependency of Glucocorticoid Exacerbation of gp120 Neurotoxicity

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Abstract: The HIV envelope glycoprotein, gp120, a well documented neurotoxin, may be involved in AIDS-related dementia complex. gp120 works through an NMDA receptor- and calcium-dependent mechanism to damage neurons. We have previously demonstrated that both natural and synthetic glucocorticoids (GCs) exacerbate gp120-induced neurotoxicity and calcium mobilization in hippocampal mixed cultures. GCs, steroid hormones secreted during stress, are now shown to 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 calcium mobilization. A GC-induced reduction in glucose transport in hippocampal neurons, as previously documented, may contribute to this energetic dependency. These results may have clinical significance, considering the common treatment of severe cases of *Pneumocystis carinii* pneumonia, typical of HIV infection, with large doses of synthetic GCs. **Key Words:** Glucocorticoids—Stress—AIDS-related dementia—Neurotoxicity—Calcium—Hippocampus—gp120.
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Approximately 20% of AIDS patients develop AIDS-related dementia complex and exhibit a variety of neurologic and neuropsychologic impairments correlated with neuropathologic changes in cortical and subcortical regions (Masliah et al., 1992; Gendelman et al., 1994). The HIV envelope glycoprotein, gp120, has been associated with the neurotoxicity seen in this dementia through an as-yet-undetermined mechanism that is thought to include activation of NMDA receptors, an increase in cytosolic calcium levels, and an immune response (Lipton, 1994; Barks et al., 1995; Dubois-Dalcq et al., 1995). We have previously shown that corticosterone (CORT) exacerbates gp120 neurotoxicity and gp120-induced increases in cytosolic calcium in hippocampal mixed cultures (Brooke et al., 1997). CORT, the primary rodent glucocorticoid (GC), is secreted in response to stress and at physiological concentrations impairs the capacity of hippocampal and cortical neurons to survive a variety of neurological insults including seizure, ischemia, hypoglycemia, exposure to antimetabolite toxins, and excitatory amino acids (EAAs) (for review, see Sapolsky, 1996; Reagan and McEwen, 1997). Hippocampal neurons are especially susceptible to GC effects as they contain high levels of corticosteroid receptors (Jacobson et al., 1993). Our observations regarding GCs and gp120 are in close agreement with those of Limoges et al. (1997) who reported that the synthetic GC dexamethasone exacerbates the neuropathology of HIV infection in SCID mice.

GCs could endanger neurons by increasing voltage-gated calcium currents, contributing to the increase in cytosolic calcium seen in exacerbation of GC neurotoxicity (Joels and De Kloet, 1989; Kerr et al., 1989), or by decreasing the expression of neurotrophins and by inhibiting injury-induced sprouting (DeKosky et al., 1984; Smith et al., 1995). However, the mechanism most relevant to the present study is GCs' ability to decrease glucose transport into hippocampal neurons, most likely by decreasing the number of glucose transporters at the membrane (Kadekaro et al., 1988; Horner et al., 1990). This has been shown to be associated with a faster decline in mitochondrial potentials and ATP concentrations during insults (Tombaugh and Sapolsky, 1990; Lawrence and Sapolsky, 1994); as a measure of the important consequences of these disruptive energetic effects of GCs, the steroids no longer exacerbate the toxicity of neurological insults when neurons are supplemented with excess energy (Virgin et al., 1991; Tombaugh and Sapolsky, 1992; Elliott et al., 1993). Because of these findings, we test here whether the ability of GCs to worsen gp120 neurotoxicity is related to disruptive effects on energetics. Specifically, we examine whether gp120 or GCs (alone or in combination) alter energy profiles in hippocampal

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Abbreviations used: CORT, corticosterone; EAA, excitatory amino acid; GC, glucocorticoid; HBSSh, Hanks' balanced salt solution with HEPES buffer; MEM, minimal essential medium.

cultures and whether the GC exacerbation of the deleterious effects of gp120 can be prevented with energy supplementation.

MATERIALS AND METHODS

Culture preparation

Hippocampal mixed cultures were prepared from day 18 prenatal rats according to previously described methods (Brooke et al., 1997). Briefly, after dissection the tissue was treated with papain (Worthington Biochemical, Freehold, NJ, U.S.A.) according to the procedure recommended by the company. The cells were dissociated, filtered through an 80-μm cell strainer, and resuspended in a modified minimal essential medium [MEM; UCSF Tissue Culture Facility (San Francisco, CA, U.S.A.), based on the method of Yu et al. (1984)] and supplemented with 10% horse serum (Hyclone, Logan, UT, U.S.A.). Cells were plated at a density of 20,000/cm^2 on either 48-well tissue culture plates or on 12-mm glass coverslips (Carolina Biological, Burlington, NC, U.S.A.), both having been pretreated with poly-D-lysine (Sigma, St. Louis, MO, U.S.A.). Cells were maintained in this medium for 11–14 days before being used in experiments. At the time cultures were studied, 30–50% were neuronal. The results from all the experiments were obtained from at least 3 or 4 different weeks of cell cultures.

Solutions

gp120 (HIVSF2gp120) dissolved in a citrate/NaCl buffer was obtained from Ogden Biochemicals (Rockville, MD, U.S.A.) as part of the NIH AIDS Research Program. This form of gp120 is from a T-cell tropic isolate and not from a macrophage tropic isolate, which predominates in the brain. Aliquots of the stock solution were kept at −80°C until they were thawed and diluted in medium to a concentration of 200 p*M* for experiments; 200 p*M* was chosen based on our previous results (Brooke et al., 1997) and the fact that several other authors have also used this concentration effectively (Brenneman et al., 1988*b*; Dreyer and Lipton, 1995). A 10 m*M* stock solution of CORT (Steraloids, Wilton, NH, U.S.A.) was prepared in ethanol and diluted in medium to 100 n*M* for use in experiments. In experimental solutions not containing gp120 or CORT, an equivalent volume of the respective vehicle was used. This concentration of CORT, which is in the physiological range of activity, was chosen based on a CORT concentration curve from our previous article (Brooke et al., 1997).

Determination of ATP levels

The cells were grown in 48-well tissue culture plates for 11–13 days. The medium was then removed, and the cells were pretreated with and without 100 n*M* CORT in 5 m*M* glucose MEM (UCSF Tissue Culture Facility) without horse serum. After 24 h the wells were spiked with gp120 to obtain a final concentration of 200 p*M* or an equivalent amount of vehicle buffer. This resulted in four experimental treatments: control, CORT alone, gp120 alone, and gp120 + CORT.

After another 24 h, all the medium was removed and the cells were quickly frozen on dry ice and then placed in a −80°C freezer until analyzed for ATP levels using an ATP Bioluminescent Assay Kit from Sigma. Proteins were determined for standardization using a Pierce Protein Assay Kit (Pierce, Rockford, IL, U.S.A.). Experimental conditions on each plate were expressed as a percentage decline from control values (cells receiving neither CORT nor gp120). ATP concentrations in the control cells were of the order of 10 n*M*/mg of protein, in agreement with previously reported levels (Bridge and Henderson, 1983).

Determination of mitochondrial potential

Rhodamine is a fluorescent dye that readily crosses the cellular membrane and binds to the mitochondrial membrane in proportion to the amount of potential across the membrane (Duchen and Biscoe, 1992). Tetramethylrhodamine (Molecular Probes, Eugene, OR, U.S.A.), a nonquenching form of the dye (Loew, 1990), was used to measure mitochondrial potential in these experiments. The cells were grown on coverslips, and after 11 days in culture, the medium was completely changed to MEM without serum and with and without 100 n*M* CORT. Twenty-four hours later, the cells were treated for 10 min in tetramethylrhodamine dissolved in Hanks' balanced salt solution with HEPES buffer (HBSSh; 0.5 μg/ml) and then placed on a stage containing HBSSh (5 m*M* glucose) on an Olympus inverted microscope (Scientific Instruments, Sunnyvale, CA, U.S.A.). The cells were maintained in this medium throughout the experiment to prevent washout of the dye. Images were collected using MetaMorph software from Universal Imaging (West Chester, PA, U.S.A.). After 10 min of baseline reading, the cells were challenged by spiking with either gp120 to obtain a final concentration of 200 p*M* or an equivalent volume of vehicle buffer. Therefore, there were the same four experimental conditions as outlined in Determination of ATP levels. A series of images were taken during the initial 10 min and for 15 min after spiking. From these images, the intensity of the dye was determined at each time point in each cell in a given field. Using Excel (Microsoft, Seattle, WA, U.S.A.), we then calculated the slope of the change in fluorescence from the addition of the challenge to the end of the experiment. This would represent the rate of change in mitochondrial potential over this time.

Glucose supplementation

Neurotoxicity studies. Experiments were performed on cells grown in 48-well plates after 14 days in culture. At that time the medium in each well was completely changed to MEM without horse serum and one of six experimental conditions: CORT in 20 m*M* glucose, gp120 in 20 m*M* glucose, CORT and gp120 in 20 m*M* glucose, CORT in 30 m*M* glucose, gp120 in 30 m*M* glucose, and gp120 and CORT in 30 m*M* glucose.

After 3 days the medium was completely removed from the cells and replaced with cold methanol and fixed for at least 24 h. The neurons were stained immunohistochemically with MAP2 monoclonal antibody (Sigma) using Vectastain and DAB kits from Vector (Burlingame, CA, U.S.A.). A representative area of each well was counted for darkly stained large neurons with well developed processes that were not beaded. The results were expressed as a percent loss of neurons, setting the CORT alone as 0%. Our previous studies had shown that treatment with CORT alone gave similar neurotoxicity values as control wells that had received neither CORT nor gp120.

Calcium mobilization studies. After 11–12 days in culture, cells grown on glass coverslips were pretreated for 24 h with MEM without horse serum, with and without CORT (100 n*M*), and 5 or 20 m*M* glucose. Therefore, there were similar experimental conditions except for different glucose levels as in the neurotoxicity studies. Calcium levels in the cytoplasm of the cells were determined using standard im-

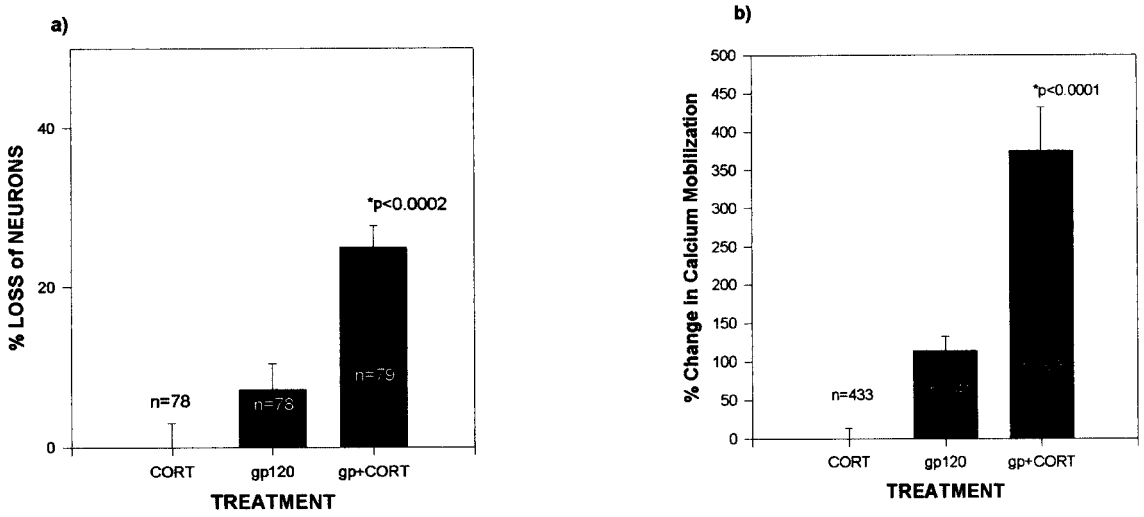

FIG. 1. Neurotoxicity (**a**) and calcium mobilization (**b**) in neurons from mixed hippocampal cultures treated with gp120 (200 p*M*) and CORT (100 n*M*) alone and in combination. Results (means ± SEM) are expressed as a percent of the CORT value as it had previously been demonstrated that CORT alone was not significantly different from no treatment (Brooke et al., 1997). gp120 + CORT caused a significant increase in both neurotoxicity and calcium mobilization as compared with CORT alone. n refers to the number of wells with each treatment in the neurotoxicity study and to the number of cells in the calcium mobilization study.

aging techniques (Grynkiewicz et al., 1985). The cells were pretreated for 15 min with the calcium-sensitive dye fura-2 AM (Molecular Probes, Eugene, OR, U.S.A.) at a concentration of 8.3 μg/ml. The cells were maintained in 5 or 20 m*M* glucose-HBSSh medium flowing over them on the stage of an Olympus inverted microscope. After baseline values were obtained for 5 min, the cells were challenged with gp120 (200 p*M*) dissolved in HBSSh or the vehicle for 20 min by switching the medium flowing over the cells. The calcium levels were determined during this 20 min using MetaFLUOR Software from Universal Imaging. The total calcium movement within the cytosol of the cells during the 20 min of challenge was determined by calculating the area under the curve of a plot of time versus calcium concentration. These values, referred to as calcium mobilization, were originally determined in nanomoles but were then expressed as a percent change, setting the values (on the order of 7,000 nmol) obtained with CORT alone as 0% change.

Statistical analysis

All results are expressed as means ± SEM. With use of SigmaStat (Jandel Scientific Software, San Rafael, CA, U.S.A.), ANOVAs, followed by the Student–Newman–Keuls test to determine significance between individual experimental conditions, were performed on the data.

RESULTS

As reported previously, neither gp120 nor CORT alone caused a significant loss of neurons or mobilization of calcium concentrations above control conditions. However, the combination of the two resulted in significant increase in neurotoxicity and calcium mobilization (Fig. 1).

Neither gp120 nor CORT alone caused a significant change in ATP levels, whereas a combination of the two produced a significant 19% decline in values (Fig. 2). Similarly, the combined treatment of gp120 and CORT created a significant decline in mitochondrial potential not observed with gp120 or CORT alone (Fig. 3).

With good evidence that the gp120 + CORT treatment disrupts the energy state of hippocampal neurons, we wanted to further investigate if this disruption is a crucial factor in explaining the neurotoxicity. We tested the importance of this energy disruption by sup-

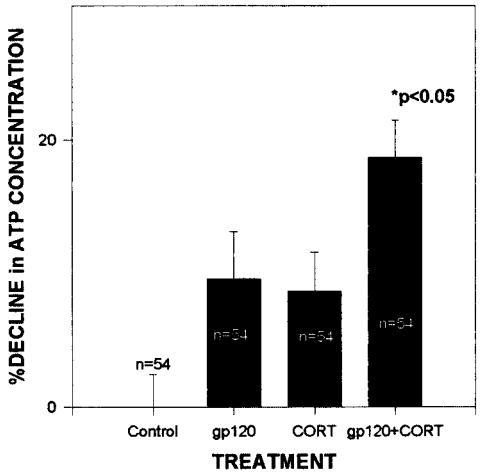

FIG. 2. Percent change in ATP levels in hippocampal mixed cultures pretreated with 100 n*M* CORT and 200 p*M* gp120 alone and in combination. The percentage (mean ± SEM) is expressed as a percent of control cells receiving neither treatment. Only the gp120 + CORT treatment showed a significant decrease (by ANOVA followed by Student–Newman–Keuls test) in ATP levels when compared with control wells.

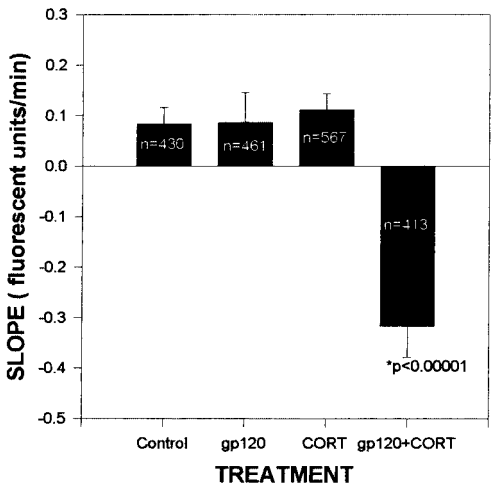

FIG. 3. Slope (mean ± SEM) of rhodamine fluorescence versus time (a measure of mitochondrial potential) in hippocampal cultures. Incubation with 100 n*M* CORT for 24 h followed by spiking with 200 p*M* gp120 + CORT, but not gp120 or CORT alone, reduced mitochondrial potentials significantly (determined by ANOVA followed by Student–Newman–Keuls test) when compared with control wells.

plementing the cells with excess energy under the same conditions. As in Fig. 1, the combination of gp120 + CORT was neurotoxic, whereas neither alone had this effect (Fig. 4a); increasing the glucose in the medium from 20 to 30 m*M* reversed the neurotoxicity. gp120 + CORT wells incubated in 30 m*M* glucose did not show a significant loss, in contrast to gp120 + CORT wells incubated in 20 m*M* glucose. A similar effect was shown with the calcium mobilization. The increase in calcium flux into the cytoplasm with CORT and gp120 in 5 m*M* glucose was eliminated with the supplementation of 20 m*M* glucose (Fig. 4b).

DISCUSSION

In the present report, we observe an energetic component to the exacerbation of gp120 neurotoxicity by GCs. This represents an extension of prior work with GCs and gp120 (Brooke et al., 1997; Limoges et al., 1997) as well as of reports that the GC exacerbation of other neurotoxic insults is energy dependent and can be reversed by supplementation with glucose, mannose, or ketones (Tombaugh and Sapolsky, 1992; Elliott and Sapolsky, 1993; Flavin, 1996; Goodman et al., 1996). Before discussing the present findings, we would like to clarify several technical points. The use of 20 m*M* glucose and supplementation with 30 m*M* in the neurotoxicity studies (Fig. 4a), but only 5 and 20 m*M* for the calcium mobilization studies (Fig. 4b), may seem incongruous. However, two very different experimental conditions were employed: Incubation for 3 days without horse serum necessitated 20 m*M* glucose for sufficient survival of control cultures, whereas overnight incubation allowed for use of the normal glucose level of 5 m*M*. There may also be some question as to why the ATP levels took a day to decline (Fig. 2) after gp120 treatment but the mitochondrial potential (Fig. 3) declined within 15 min. The cells in the mitochondrial potential experiments show more rapid deterioration because of less favorable experimental conditions (HEPES buffer at room temperature and periodic bursts of UV light).

The present results, focusing on hippocampal mixed cultures where corticosteroid receptors are most concentrated (Jacobson et al., 1993), confirmed our previous findings with respect to GC exacerbation of gp120 neurotoxicity. A similar trend of energy involvement in the CORT exacerbation of gp120 neurotoxicity in cortical cultures was also seen (data not presented).

In the course of these studies, we found that 200 p*M* gp120 alone was not always neurotoxic in contrast to our prior report, making use of cultures at a different time of year (Brooke et al., 1997). This variability is consistent with the existing literature on gp120 neurotoxicity; to obtain consistent toxicity with gp120, other researchers have conducted studies in the presence of glutamate (Dawson et al., 1993), NMDA (Barks et al., 1997), or CORT (Brenneman et al., 1988*a*) (as part of a defined medium or in horse serum) or by culturing with a high glia-to-neuron ratio (Brenneman et al., 1988*b*; Meucci and Miller, 1996; Scorziello et al., 1997). Sufficient glia, including microglia, may be necessary for gp120 neurotoxicity as a factor in the production of an intermediate toxin. Also, gp120 has been shown to interfere with glial uptake of glutamate (Lipton, 1994; Vesce et al., 1997). Because the amount of glia varies in our mixed cultures, we are now establishing glial feeder layers in an attempt to obtain more consistent results. Therefore, in this article we are concentrating only on the energy involvement in the synergistic effect of gp120 and CORT.

Here we investigate for the first time whether GCs' ability to worsen gp120 neurotoxicity is energy dependent. Previously, gp120 has been linked to energy in vivo, disrupting glucose metabolism in various brain regions (Kimes et al., 1991) and exacerbating hypoglycemia-induced damage (Barks et al., 1995). There are two categories of energetic neuronal insults, namely, those that actually disrupt energy formation (such as hypoglycemia, ischemia, and antimetabolite exposure) and those that put a high energy demand on the cells (such as EAA exposure and seizure). GCs exacerbate both types of insults (Sapolsky, 1996). The GC-induced mobilization of energy to muscle and away from other tissues, including the brain, is well understood as part of the adaptation to an acute, stressful emergency. This means, however, that corticosteroid receptor-rich regions such as the hippocampus (Jacobson et al., 1993) experience a decrease in glucose transport when GC levels are high (Kadekaro et al., 1988; Horner et al., 1990). To test the hypothesis that GCs exacerbate gp120 neurotoxicity by reducing available energy, we measured the energy levels of

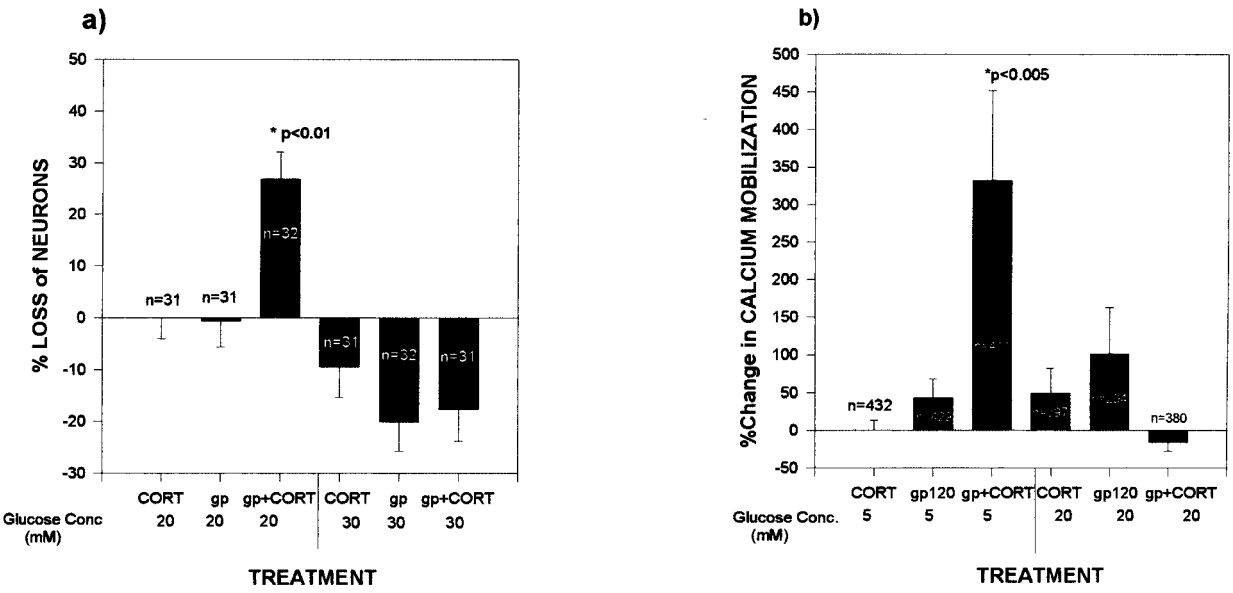

FIG. 4. Neurotoxicity (**a**) and calcium mobilization (**b**) in neurons from mixed hippocampal cultures treated with gp120 (200 p*M*) and CORT (100 n*M*) alone and in combination with varying levels of glucose. a: Glucose supplementation reversed GC exacerbation of gp120 neurotoxicity. Neurotoxicity in hippocampal cultures was represented as percent loss of neurons (means ± SEM) relative to CORT treatment alone. Cells were treated for 3 days with 100 n*M* CORT, 200 p*M* gp120, or gp120 + CORT in either 20 or 30 m*M* glucose medium. Significance was determined by ANOVA followed by Student–Newman–Keuls test when compared with CORT alone with the same glucose concentration. n refers to the number of wells with each treatment. b: Glucose supplementation reversed gp120 + CORT calcium mobilization. Hippocampal mixed cultures were incubated for 24 h in 5 or 20 m*M* glucose medium with 100 n*M* CORT or vector and then spiked with gp120 to a final concentration of 200 p*M* or with an equivalent volume of vector. Calcium mobilization (mean ± SEM) is expressed as a percent of CORT treatment alone. Significance was determined by ANOVA followed by Student–Newman–Keuls test. n refers to number of cells.

treated cells. Our results, namely, that decreases in mitochondrial potential and ATP levels in gp120-challenged neurons occur only when GCs are present, support the hypothesis. Although the ATP and mitochondrial potential assays do not distinguish between neurons and glia, the results are still highly relevant. Both cell types carry GC receptors (Vielkind et al., 1990), and a reduction in the amount of energy in both types of cells could contribute to enhanced neurotoxicity. The NMDA/calcium cascade activated by gp120 puts a high-energy demand on neurons to maintain the activity of calcium extrusion pumps, Na^+,K^+-ATPase, and reuptake systems for EAAs (Lipton, 1994; Schinder et al., 1996). Glia have the energy-expensive task of maintaining the extracellular environment, which GCs further disrupt by inhibiting the glial uptake of glutamate (Virgin et al., 1991; Tombaugh et al., 1992). Our results may demonstrate the scenario in which GCs, which disrupt energy stores, and gp120, which puts a demand on energy reserves, each on its own, do not cause perceptible changes in energy levels but in combination can push neurons into an energy crisis that can lead to increased neurotoxicity.

To determine whether the decrease in energy contributes to the exacerbated neurotoxicity, we supplemented the cells challenged with gp120 and GCs with glucose. The reversals seen in both neurotoxicity and calcium mobilization indicate that the failure of the cell to maintain its energy supply during the combined GC and gp120 insult is a crucial factor in this form of neurotoxicity. This agrees with findings that energy supplementation can reverse GC exacerbation of neurotoxicity induced by kainate (Elliott and Sapolsky, 1993) and ischemia (Tombaugh and Sapolsky, 1992) in the hippocampus. In these latter articles, the energy supplementation took the form of mannose and ketones as well as glucose (Sapolsky, 1986). The generality of these interventions suggests that the energetic features of glucose supplementation ultimately help maintain ATP levels.

The exact mechanism(s) for neuron death in AIDS patients is still controversial and awaits further study. One theory is that detachment of gp120 from the virus that enters the brain via infected blood monocytes (Lipton, 1994) sets up a neurotoxic cascade involving NMDA receptors and waves of calcium. The gp120 theory is given more credibility by the fact that transgenic overexpression of gp120 in mice results in neuropathological changes reminiscent of HIV infection (Limoges et al., 1997). A second theory involves the inflammatory response and corresponding rise in neurotoxic cytokines (Genis et al., 1992) that may be mediated by gp41, the membrane-bound portion of gp160 (the combined form of gp41 and gp120) (Adamson et al., 1996).

Our finding of GC exacerbation of gp120 neurotox-

icity is potentially clinically relevant, considering that at least 20% of AIDS patients develop AIDS-related dementia and that current clinical practices often employ large doses of artificial GCs (e.g., prednisone) to treat severe cases of *Pneumocystis carinii* in AIDS patients. The present data suggest that GCs can exacerbate gp120 neurotoxicity through an energetic route. Since the nature of HIV neurotoxicity is still controversial and the mechanism of gp120 neurotoxicity is not completely elucidated, this research may direct further studies to answer both questions. Whether the information presented here would be useful in preventing or blunting the development of AIDS dementia also requires further investigation.

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