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# Antiretrovirals, Methamphetamine, and HIV-1 Envelope Protein gp120 Compromise Neuronal Energy Homeostasis in Association with Various Degrees of Synaptic and Neuritic Damage

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**HIV-1 infection frequently causes HIV-associated neurocognitive disorders (HAND) despite combination antiretroviral therapy (cART). Evidence is accumulating that components of cART can themselves be neurotoxic upon long-term exposure. In addition, abuse of psychostimulants, such as methamphetamine, seems to aggravate HAND and compromise antiretroviral therapy. However, the combined effect of virus and recreational and therapeutic drugs on the brain is poorly understood. Therefore, we exposed mixed neuronal-glial cerebrocortical cells to antiretrovirals (ARVs) (zidovudine [AZT], nevirapine [NVP], saquinavir [SQV], and 118-D-24) of four different pharmacological categories and to methamphetamine and, in some experiments, the HIV-1 gp120 protein for 24 h and 7 days. Subsequently, we assessed neuronal injury by fluorescence microscopy, using specific markers for neuronal dendrites and presynaptic terminals. We also analyzed the disturbance of neuronal ATP levels and assessed the involvement of autophagy by using immunofluorescence and Western blotting. ARVs caused alterations of neurites and presynaptic terminals primarily during the 7-day incubation and depending on the specific compounds and their combinations with and without methamphetamine. Similarly, the loss of neuronal ATP was context specific for each of the drugs or combinations thereof, with and without methamphetamine or viral gp120. Loss of ATP was associated with activation of AMP-activated protein kinase (AMPK) and autophagy, which, however, failed to restore normal levels of neuronal ATP. In contrast, boosting autophagy with rapamycin prevented the long-term drop of ATP during exposure to cART in combination with methamphetamine or gp120. Our findings indicate that the overall positive effect of cART on HIV infection is accompanied by detectable neurotoxicity, which in turn may be aggravated by methamphetamine.**

Infection with human immunodeficiency virus type 1 (HIV-1) and drug abuse continue to be significant public health concerns (1–3). Infection of the central nervous system (CNS) by HIV-1 often leads to several neurological complications known as HIV-associated neurocognitive disorders (HAND). The underlying neuropathology in humans is not completely understood and persists as a significant health problem despite the use of antiretrovirals (ARVs) since the mid-1990s (4–7). Although the incidence of HIV-associated dementia (HAD), the most severe manifestation of HAND, has declined with the use of ARVs, the prevalence of cognitive dysfunctions in patients following antiretroviral therapy (ART) remains high (8–10). ART regimens generally consist of two to five highly active ARV compounds exerting effects through different molecular mechanisms in order to avoid emergence of drug-resistant HIV mutants (http://aidsinfo.nih.gov/guidelines). The substances are categorized by their mechanisms of action and include (i) nucleoside/nucleotide reverse transcriptase inhibitors (NRTI), (ii) nonnucleoside reverse transcriptase inhibitors (NNRTI), (iii) protease inhibitors (PI), (iv) integrase strand transfer inhibitors (INSTI), (v) fusion inhibitors, and (vi) a chemokine receptor CCR5 blocker.

Several studies demonstrated toxicity of ARV compounds, associated with mitochondrial function and the capacity to generate ATP (11–13), with neuronal dysfunction (14), with peripheral neuropathy (15, 16), and with oxidative stress in the CNS (17). In addition, recent studies found that discontinuation of ARV use in HIV patients with controlled viral loads unexpectedly resulted in significant improvements in neurocognitive function (16, 18–20). These observations raised the possibility that ARVs may have neurotoxic effects that contribute to the development of HAND. Based on several lines of evidence, it is generally accepted that neuronal injury can be induced by neurotoxic viral proteins, such as Tat and gp120, which are generated by HIV-infected cells in the CNS. Moreover, while ARVs prevent new infections, the treatment does not affect proviral DNA in chronically infected cells, which may still produce viral proteins (21–25).

HIV infection is frequently associated with the use of recreational drugs, such as methamphetamine (METH) (26–28), and with a reduced adherence to ART regimens (29). METH abuse triggers behavioral symptoms, including agitation, anxiety, paranoia, psychosis, and aggression (26, 27, 30); a variety of cardiovas-

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cular problems (31, 32); reactive microgliosis (33); and hyperthermia and convulsions (34). METH users with HIV have also shown more neuronal injury and cognitive impairment due to HIV than individuals who do not abuse the drug (1, 35). Additionally, increased viral loads have been linked to METH use in ART-receiving HIV-positive individuals (29, 36). The combination of METH and HIV-1 seems to cause more neurocognitive deficits and neuropathology than either agent alone, but the potential mechanistic interactions of virus, antiviral treatment, and the psychostimulant drug is largely unknown (22, 35, 37). Abuse of METH seems to increase neuronal release of monoamine neurotransmitters, particularly dopamine (DA), in the synapse, which can be toxic to nerve terminals (38, 39). As a result of DA accumulation, levels of free radicals increase inside neurons and promote protein damage and/or dysfunction, resulting in upregulation of autophagy (40–42), a vital homeostatic mechanism required for healthy neurons. As a "recycling pathway," autophagy takes place in all eukaryotic cells, but it has also been connected with a wide range of human pathologies, including neurodegenerative diseases (43).

METH-using HIV patients are exposed to a combination of potential contributors to neurotoxicity, i.e., HIV and its components, ARVs, and psychostimulant drugs. However, the combined effects of these factors at the cellular level are unknown. Therefore, we investigated how ARVs, METH, and HIV gp120 or combinations thereof affected primary rat cerebrocortical neurons. We exposed mixed neuronal-glial cerebrocortical cells to ARVs of four different pharmacological categories and to METH for 1 and 7 days. Subsequently, we assessed neuronal damage by using fluorescence microscopy of fixed cells labeled for specific markers of neuronal dendrites and presynaptic terminals in combination with nuclear DNA staining. We also analyzed potential cellular and neuronal injury by combination ART (cART) compounds in the presence or absence of METH and the established viral neurotoxin gp120 by using the ATPlite assay, a well-established method used in large-scale drug screening for evaluation of cytotoxicity. Our findings indicate that different combinations of both therapeutic and recreational drugs, along with and similar to the established HIV-1-derived neurotoxin gp120, can all compromise cellular neuronal ATP homeostasis, even under conditions where neuronal structure and survival appear to be largely unaffected. Moreover, the loss of ATP was associated with an activation of AMP-activated protein kinase (AMPK) and autophagy that, however, failed to restore normal levels of cellular ATP. In contrast, activation of autophagy by rapamycin enabled neurons to restore ATP concentrations to nearly control levels in the presence of some drug combinations, suggesting that mitochondria remained functionally intact while mTOR activity limited autophagy in support of ATP production.

## MATERIALS AND METHODS

**Reagents.** Recombinant gp120 of HIV-1 BaL and antiretroviral compounds were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. HIV-1 gp120 was reconstituted in 0.1% bovine serum albumin (BSA), and controls received the BSA vehicle alone (0.001% final concentration). Stock solutions of zidovudine (AZT), nevirapine (NVP), saquinavir (SQV), and integrase inhibitor (IntInh) were prepared in dimethyl sulfoxide (DMSO) and diluted in culture medium to a 0.1% final concentration. Stock solutions of (+)-methamphetamine hydrochloride (Sigma-Aldrich, St. Louis, MO) and *N*-methyl-D-aspartate (NMDA; Abcam) were prepared in sterile purified water and filtered through a 0.2-μm membrane. The rapamycin stock was prepared to 1 mM in DMSO (Cayman Chemical, Ann Arbor, MI) and further diluted in culture medium.

**Preparation of mixed neuronal-glial cerebrocortical cell cultures.** Rat mixed neuronal-glial cerebrocortical cell cultures were prepared from embryos of Sprague-Dawley rats at days 15 to 17 of gestation, as previously described by our group (44–46). In brief, cells were cultured in 35-mm dishes with poly-L-lysine-coated glass coverslips ($1.87 \times 10^6$ cells per dish) or in poly-L-lysine-coated clear-bottom 96-well plates for imaging ($0.087 \times 10^6$ cells per well) (BD Falcon, BD Biosciences, San Jose, CA). Cells were cultured in D10C medium containing 70% Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco, Life Technologies), 12.5% heat-inactivated horse serum (Gibco, Life Technologies), 12.5% F-12 medium (HyClone, Thermo Scientific), 3.12% 1 M HEPES (Omega Scientific), 1.25% 200 mM L-glutamine (Gibco, Life Technologies), and 0.3% 100 U/ml penicillin with 100 mg/ml streptomycin (Gibco, Life Technologies). Cell cultures consisting of ~30% neurons, ~70% astrocytes, and ~0.1 to 1% microglia were used after 16 days *in vitro*, when the majority of neurons were considered fully differentiated and susceptible to NMDA toxicity (45). All experiments involving animals were approved by the Institutional Animal Care and Use Committee of the Sanford Burnham Prebys Medical Discovery Institute (formerly called the Sanford Burnham Medical Research Institute).

**Immunocytochemistry/immunofluorescence staining and microscopy.** After treatment and washing with phosphate-buffered saline (PBS), rat cerebrocortical cell cultures were fixed for 25 min with 4% paraformaldehyde (PFA) at 4°C and subsequently permeabilized with 0.2% Triton X-100 for 5 min at room temperature. Primary antibodies included mouse anti-microtubule-associated protein 2 (anti-MAP-2) (1:500) (clone HM2; Sigma-Aldrich) and rabbit anti-synaptophysin (anti-SYP) (1:200) (Dako). Secondary antibodies included goat anti-rabbit–Alexa Fluor 488 (1:400) (Invitrogen, Life Technologies) and anti-mouse IgG–Texas Red (1:150) (Vector Laboratories, Burlingame, CA). Controls were included in which primary antibodies were omitted or replaced with an irrelevant IgG of the same species and subclass. Nuclear DNA was stained with Hoechst H33342. Microscopy was performed using filters for 4′,6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC), and Cy3, using either an Axiovert 100M or 200M microscope (Zeiss). Images were acquired and fluorescence intensities for MAP-2, synaptophysin, and nuclear DNA were quantified using the Slidebook software package, versions 4 and 5 (Intelligent Imaging Innovations, Denver, CO), as described earlier (44, 45, 47). The two microscope setups used were both controlled by Slidebook software and equipped with the same objectives but had different light sources and cameras. Due to those hardware variables, the scales are different for the linear data reported by the two microscope setups (in arbitrary fluorescence units) and shown in Fig. 1A and B.

**Cell lysates.** After washing with PBS, cerebrocortical cells were harvested by adding 1× cell lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM $Na_3VO_4$, 1 mg/ml leupeptin; Cell Signaling Technology, Beverly, MA) and Complete protease inhibitor cocktail (Roche, Indianapolis, IN) on ice for 10 min. The cell lysates were transferred to microcentrifuge tubes, sonicated four times for 5 s each time, and then cleared by centrifugation (13,200 rpm, 10 min) at 4°C. Total protein concentrations in lysates were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Fisher Scientific, Rockford, IL) (45).

**Protein analysis by Western blotting.** Cell lysates were electrophoretically separated in SDS-PAGE gels (NuPAGE; Invitrogen, Life Technologies) (10% for p62, 12% for microtubule-associated protein 1 light chain 3 [LC3] and 4E-BP1, and 4 to 12% for AMPK) and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Life Technologies). After blocking with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20, membranes were incubated with primary antibodies against rabbit phospho-AMPK (1:1,000) (2535; Cell Sig-

naling), rabbit total AMPK (1:1,000) (2532; Cell Signaling), rabbit LC3 (1:500) (AP1802; Abgent), rabbit p62/SQSTM1 (1:3,000) (PM045; MBL), rabbit p4E-BP1(T37/46) (1:1,000) (2855; Cell Signaling), rabbit total 4E-BP1 (1:1,000) (9644; Cell Signaling), and mouse β-actin (1:10,000) (CP01; Millipore). Bound primary antibodies were detected with horseradish peroxidase (HRP)-conjugated antibodies specific for the different species, using anti-mouse–HRP (1:20,000) (AP128P; Millipore) and anti-rabbit–HRP (1:10,000) (31460; Pierce). Protein bands were visualized with an ECL SuperSignal West Pico chemiluminescence substrate kit (Thermo Scientific, Pierce), and chemiluminescence signals were detected using X-ray films (HyBlot autoradiography film; Denville Scientific Inc.). The exposure time to obtain the optimal density of the images varied from 3 s to 4 min. Protein amounts were estimated by densitometry analysis using ImageJ software (NIH, Bethesda, MD) (45, 48).

**Measurement of intracellular ATP.** The ATPlite assay is based on production of light caused by the reaction of ATP with added luciferase and D-luciferin. The amount of emitted light is proportional to the ATP concentration. The intracellular ATP level was measured using a luminescence-based ATP detection assay (ATPlite; PerkinElmer Inc., Waltham, MA) per the supplier's instructions. Briefly, 50 μl of mammalian cell lysis solution was added to each well of a 96-well plate to lyse the cells, allowing intracellular ATP to be released. After shaking of the plate for 5 min, 50 μl of ATPlite buffer (HEPES containing luciferase and D-luciferin) was added. The plate was then covered with an adhesive seal and dark adapted for 10 min, and luminescence was measured using a luminescence microplate reader (POLARstar Omega; BMG Labtech, Germany).

**Statistical analysis.** The data are expressed as mean values ± standard errors of the means (SEM) for three or more independent experiments. Values for arbitrary fluorescence units or luminescence units for ATP assays obtained for different 96-well plates or with different preparations of primary cerebrocortical cell cultures were normalized using the vehicle-treated controls of the respective replicate experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Fisher protected least significant difference (PLSD) *post hoc* test or the Student *t* test, using the StatView software package (version 5.0.1; SAS Institute, Cary, NC). The significance level was set at $P$ values of $<0.05$. Immunoblotting and immunofluorescence images are representative of at least three independent experiments.

## RESULTS

**Short-term exposure to ARV drugs does not alter dendritic MAP-2 in cerebrocortical neurons.** In order to assess the potential of ART to cause neuronal damage, we exposed rat cerebrocortical cell cultures to representative compounds of four different pharmacological categories, separately and in combination, as follows: zidovudine (AZT) at 0.1 to 10 μM as an NRTI, nevirapine (NVP) at 0.1 to 1 μM as an NNRTI, saquinavir (SQV) at 5 to 50 nM as a PI, and INSTI 118-D-24 at 2 to 20 μM as an integrase inhibitor (IntInh). We chose these drug concentrations based on a range of reported therapeutic dosages for each ARV compound (49–53). A dose of 300 μM *N*-methyl-D-aspartate (NMDA) was used as an excitotoxicity positive control which causes massive neuronal loss via apoptosis and eventually leads to depletion of neurons (45). Reduced immunoreactivity for both microtubule-associated protein 2 (MAP-2) in neuronal dendrites and synaptophysin (SYP) in presynaptic terminals has been found to characterize neuronal injury in brain specimens from AIDS patients with HIV-associated dementia (HAD) (54), as well as in animal models of HIV-associated brain injury (47, 55). Twenty-four hours after treatment, cerebrocortical cell cultures were fixed, and neuronal injury was assessed using fluorescence microscopy for detection of MAP-2 and SYP. Even at the highest concentrations, none of the ARV compounds appeared to cause a significant reduction of neuronal MAP-2 when applied separately (Fig. 1A, left panel; note that data are shown only for the highest concentrations of ARVs) or in combination with one or two more therapeutic drugs (Fig. 1B, left panel). In addition, immunoreactivity levels for synaptophysin were very similar among all of the treatments, with the exception of IntInh (Fig. 1A, right panel). However, IntInh in combination with one or two of the other ARV compounds did not significantly damage presynaptic terminals (Fig. 1B, right panel). In fact, the combination of AZT plus IntInh increased synaptophysin immunoreactivity. Therefore, the highest concentration of each ART compound was used in all the following experiments. Note that NMDA treatment reproducibly caused a more pronounced loss of immunofluorescence reactivity for MAP-2 than for SYP within 24 h, which indicated that degradation of $SYP^+$ presynaptic terminals trails that of MAP-$2^+$ dendrites, at least after excitotoxic neuronal injury.

**Long-term exposure to ARV drugs and METH can injure neuronal dendrites and presynaptic terminals and alter intracellular ATP levels.** Since HIV-positive individuals on therapeutic treatment are frequently users of recreational drugs, such as METH, we next investigated the effect of this psychostimulant combined with different ARV compounds on neuronal-glial cultures after 24 h (referred to here as short-term exposure) and 7 days (referred to here as long-term exposure) of treatment. A concentration of 100 μM METH was chosen for our experiments, based on previous studies suggesting that a concentration in this range can be reached in the brain during METH binges (56, 57). Neuronal damage was assessed by quantifying the immunofluorescence signals of MAP-2-positive neurons and synaptophysin-stained presynaptic terminals (Fig. 2).

Overall, only 8 of the 19 conditions including METH, ART, or a combination thereof showed a significant change in MAP-2 or SYP immunofluorescence reactivity compared to that of a control without drugs or with NMDA (conditions 1, 2, 7, 8, 9, 17, 18, and 19 for MAP-2 and conditions 1, 2, 5, 8, 9, 14, 17, and 19 for SYP, as indicated in Fig. 2). Two of the eight conditions increased immunofluorescence reactivity for MAP-2 or SYP (conditions 7 and 17 for MAP-2 and 14 and 17 for SYP). No significant loss of immunofluorescence reactivity that would indicate neurotoxicity was detected, at both the dendritic and presynaptic levels, for any combination of three ARVs with or without METH in short- and long-term treatments (Fig. 2, conditions 10 to 17). Yet the combination of four ARV compounds with METH after 7 days of treatment decreased both MAP-2 and synaptophysin fluorescence, suggesting neuronal injury (Fig. 2). This turned into significant dendritic beading and neuronal pruning due to a loss of MAP-2 (Fig. 3A), in concert with a loss of presynaptic terminals (Fig. 3B). A different scenario was found when cerebrocortical cells were separately exposed to ARV compounds. In the short term, no neurotoxic effect was observed from individual compounds in the presence or absence of METH (Fig. 2, conditions 2 to 9), with the exception of IntInh at presynaptic terminals, as mentioned before (Fig. 2, condition 5). However, after long-term treatment, the presynaptic terminals appeared to have recovered from IntInh-induced neurotoxicity in the absence but not presence of METH (Fig. 2, condition 5 in synaptophysin panel). While treatment with AZT induced significant decreases of MAP-2 and SYP after 7 days, the combination of AZT and METH had the opposite effect, suggesting a preservation of neuronal dendrites and presynaptic terminals by this combination. On the other hand, while SQV did not

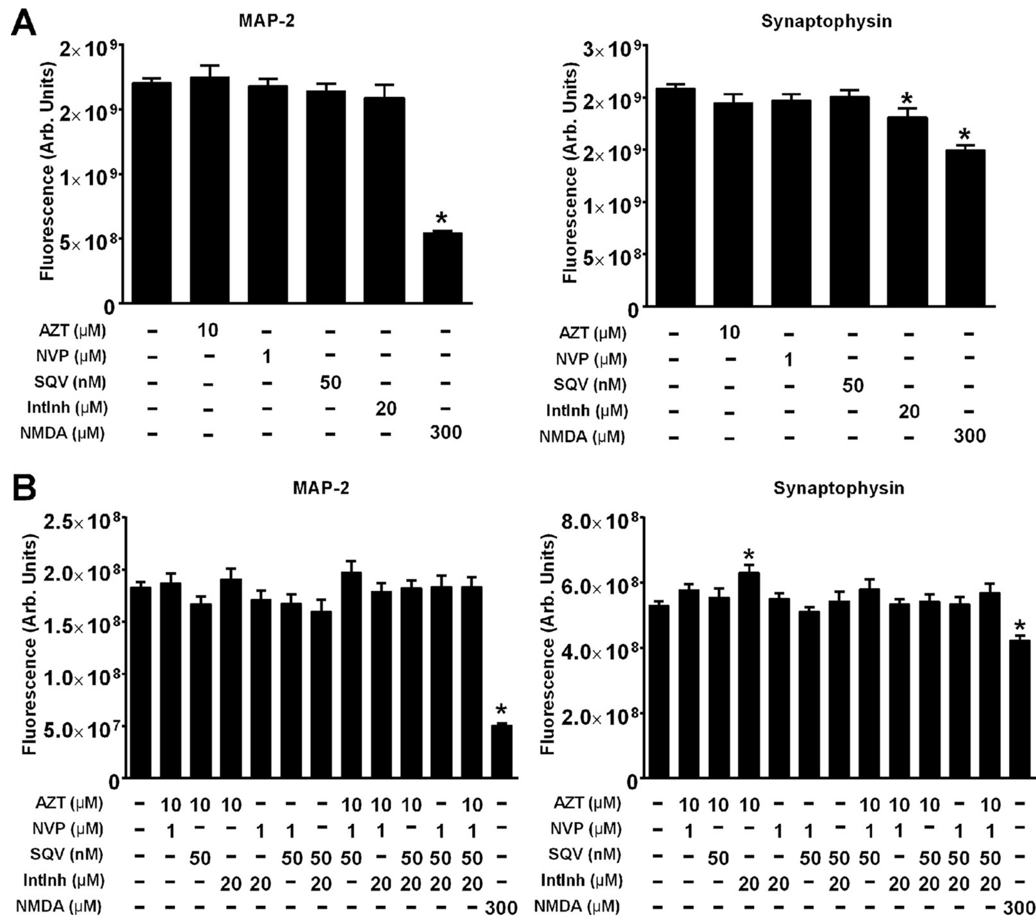


**FIG 1** Neurotoxicity induced by antiretroviral drugs in cerebrocortical neurons during 24 h of exposure. Mixed neuronal-glial cerebrocortical cell cultures were exposed for 24 h to different concentrations of zidovudine (AZT), nevirapine (NVP), saquinavir (SQV), and the integrase inhibitor 118-D-24 (IntInh) (A) or a combination of ARV drugs (B). NMDA (300 µM) was applied for 20 min only at the beginning of the incubation in order to induce excitotoxicity. Neuronal injury and death were analyzed using fluorescence staining for neuronal MAP-2, synaptophysin, and nuclear DNA as described in Materials and Methods. Samples were from at least three independent experiments with ≥15 replicates for each condition. Values are means ± SEM. *, $P < 0.05$ compared to control (ANOVA and Fisher's PLSD *post hoc* test).

show any neuronal injury in long-term treatment, its combination with METH led to significant decreases of both neuronal MAP-2 and presynaptic synaptophysin. The protease inhibitor SQV, the NNRTI NVP, and the combination of NVP and METH did not show neuronal injury either at the dendritic level or at presynaptic terminals, although NVP-METH significantly increased MAP-2 immunoreactivity over 7 days. The combination of four ART compounds induced a significant reduction only of MAP-2, not SYP, immunofluorescence reactivity. However, the four ARVs together with METH significantly diminished MAP-2 and SYP reactivity during long-term exposure. Altogether, significant alterations of immunofluorescence reactivity for neuronal MAP-2 and SYP occurred only during long-term exposure to AZT, METH, and distinct combinations of the various therapeutic ARVs and the psychostimulant.

Neurons are metabolically very active, with a high energy demand that makes the cells highly dependent on mitochondrial function (58–60). However, several ARVs have been linked to mitochondrial injury (61, 62). In addition, measurement of cellular ATP has been used to assess cytotoxicity in large-scale screening and neuroprotection studies (46, 63). Hence, we decided to measure cellular ATP levels of neurons by using an established assay (the ATPlite assay). In order to estimate the ATP levels in neurons, the amount of ATP produced by glial cells was measured in parallel in neuron-depleted glial cerebrocortical cell cultures and subtracted from the ATP levels obtained from mixed neuronal-glial cultures (Fig. 4). This approach was chosen because we found that none of the experimental conditions significantly affected ATP levels in neuron-depleted glial cell cultures (data not shown). In short- and long-term exposures, METH, AZT-SQV-IntInh, AZT-NVP-IntInh-METH, and the combination of four ARVs caused significant decreases of neuronal ATP levels. METH plus SQV or IntInh, AZT-NVP-IntInh, METH-AZT-NVP-SQV, METH-AZT-SQV-IntInh, and METH plus the four ARVs diminished neuronal ATP only upon long-term exposure. Additionally, NVP ($P = 0.0933$), NVP-METH ($P = 0.07$), IntInh ($P = 0.0598$), and short-term exposure to four ARVs plus METH ($P = 0.1681$) showed a trend of reduced ATP levels. During long-term exposure, the four ARV compounds, with or without METH, presented significantly lower levels of ATP than those in neuronal cells treated with vehicle (DMSO), which were used as a control. Interestingly, not all ARVs used individually showed a significant

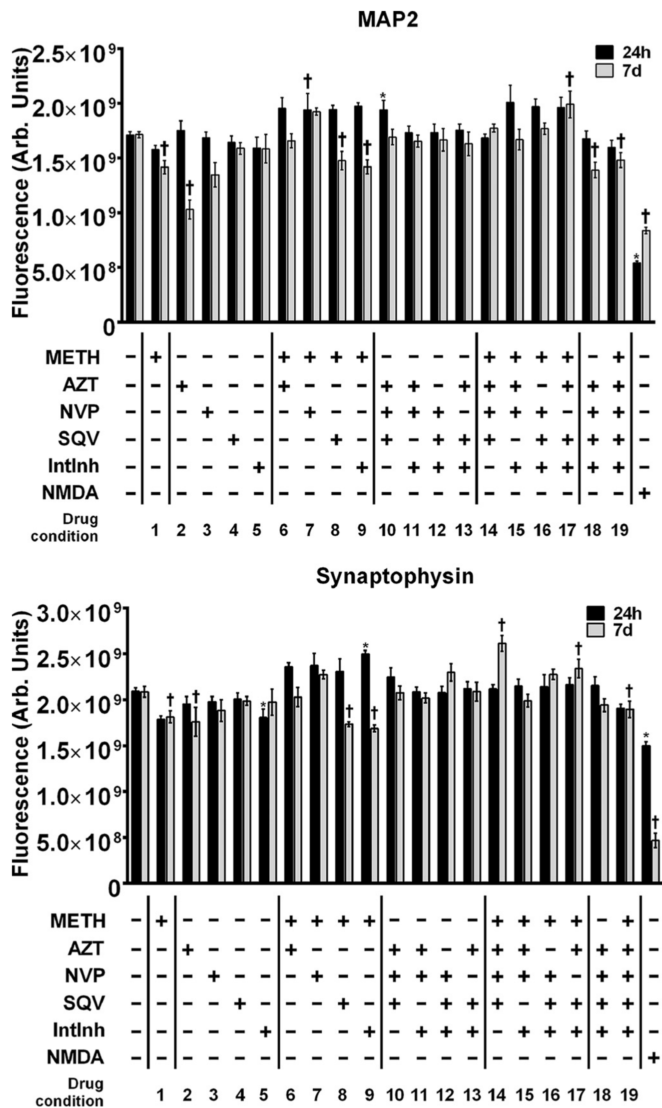


FIG 2 Neuronal damage in cerebrocortical cells in the presence of METH and ARV drugs for 24 h and 7 days. Cerebrocortical cell cultures were incubated for 24 h or 7 days with a single drug or a combination of different antiretroviral drugs (10 μM AZT, 1 μM NVP, 50 nM SQV, and/or 20 μM IntInh) and METH (100 μM). Neuronal injury was analyzed using fluorescence staining for neuronal MAP-2 (top) and SYP (bottom) and microscopy as described in Materials and Methods. Samples were from at least three independent experiments with seven replicates for each condition. *, $P < 0.05$ compared to the control (24 h); †, $P < 0.05$ compared to the control (7 days) (ANOVA and Fisher's PLSD *post hoc* test).

loss of ATP. However, when the drugs were combined with METH, significant decreases of ATP were observed with the SQV-METH and IntInh-METH combinations. Interestingly, ATP levels were not affected by AZT or the combination of AZT and METH. The AZT-NVP-SQV combination (drug combination 10) transiently increased MAP-2 immunoreactivity but otherwise did not significantly alter presynaptic SYP or ATP levels. However, only the NVP-SQV-IntInh combination, with or without METH (drug combinations 12 and 16), did not significantly affect levels of MAP-2, SYP, or neuronal ATP at any time point. The combination of four ARVs showed the most pronounced loss of neuronal ATP compared to the level in untreated cells in the short term, with the ATP levels recovering to about 50% during long-term exposure. Strikingly, METH prevented the significant short-term drop of ATP levels in the combined presence of four ARVs. Moreover, in several instances the ATP levels were significantly reduced under conditions where MAP-2 and SYP immunoreactivities remained unchanged, most strikingly upon short-term exposure to METH, AZT-SQV-IntInh, and the combination of four ARVs. Levels of MAP-2, SYP, and neuronal ATP all dropped significantly only upon long-term exposure to METH, METH plus SQV or IntInh, or METH plus the four ARVs or short- and long-term exposures to the excitotoxic concentration of NMDA. Taking all the data together, the assessment of cellular ATP levels in our experiments appeared to be a more sensitive indicator of a drug effect on neurons than changes in immunoreactivity of MAP-2 and SYP, while at the same time a significant drop of ATP levels did not necessarily indicate neuronal injury and loss as judged by quantification of MAP-2 and SYP.

**Long-term exposure to ARV drugs, METH, and BaL gp120 alters intracellular ATP levels and injures presynaptic terminals.** In previous studies, we determined that HIV-1 gp120 proteins from CCR5-preferring, CXCR4-preferring, and dual-tropic HIV-1 strains are all neurotoxic (44). To investigate how therapeutic and recreational drugs can affect this neuronal injury during long-term exposure, we exposed neuronal-glial cerebrocortical and, in parallel, neuron-depleted cell cultures to HIV-1 BaL gp120, ARVs (combination of four therapeutic drugs), METH, and combinations thereof and assessed neuronal ATP levels as described above. Compared to the vehicle control, all treatment conditions caused a statistically significant loss of neuronal ATP (Fig. 5). Interestingly, the exposure to ARV, METH, and BaL gp120 together did not show additive neurotoxic effects (Fig. 5). The loss of neuronal ATP corresponded with decreased MAP-2 and synaptophysin reactivities observed by immunofluorescence microscopy.

**Long-term exposure to ARV drugs, METH, and HIV-1 gp120 changes expression of neuronal autophagic markers.** One way for neurons, like other cell types, to support ATP production is through autophagy. This mechanism serves to maintain cellular homeostasis and to turn over organelles (64). AMP-activated protein kinase (AMPK) is one of the sensors for energy status in the CNS and is a positive regulator that stimulates autophagy when cellular ATP concentrations decline. Hence, activation of AMPK was examined by Western blotting for cerebrocortical cell cultures exposed to METH, ARV drugs, and BaL gp120 for 7 days. Increased phosphorylation of AMPK was detected for all treatments, to various degrees (Fig. 6A), suggesting an activation of autophagy. To confirm the alterations in autophagy, microtubule-associated protein 1 light chain 3 (LC3), an established marker for monitoring autophagy, was analyzed by immunoblotting. The ratio of proteolytically processed LC3-II to full-length LC3-I was significantly increased for all treatments (Fig. 6B), indicating an increased formation of autophagosomes. This finding was confirmed by immunocytochemistry, as the presence of granular structures containing the autophagy marker LC3 was higher than that in untreated neuronal cultures but similar to that in cells treated with 20 nM rapamycin (65), which served as a positive control for induction of autophagy (Fig. 7). Yet autophagosome accumulation may represent either autophagy induction or suppression of downstream steps of autophagosome processing. To

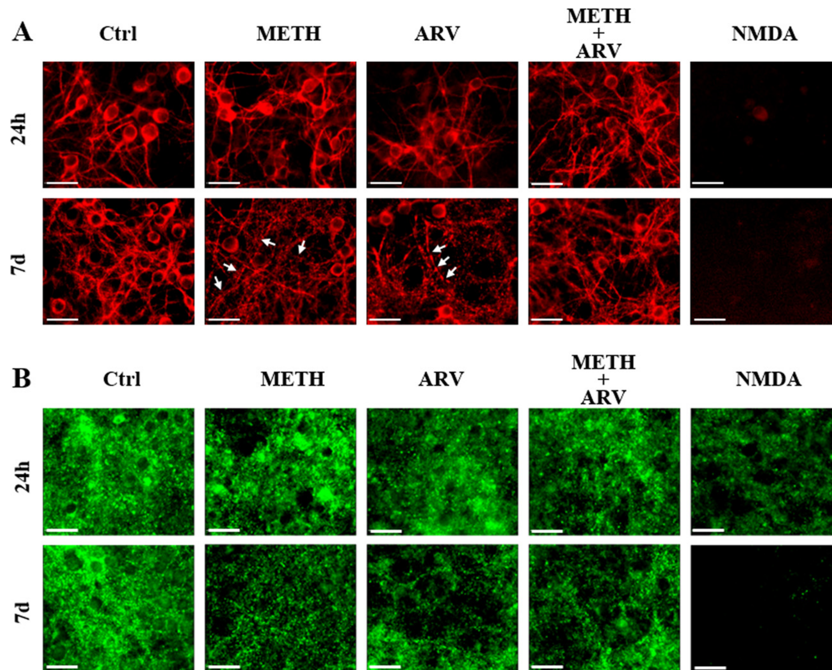


**FIG 3** Immunofluorescence staining of cerebrocortical neurons exposed to METH and ARV drugs for 24 h and 7 days. Cerebrocortical cell cultures were incubated with a combination of antiretroviral drugs (10 μM AZT, 1 μM NVP, 50 nM SQV, and 20 μM IntInh) and METH (100 μM). After 24 h and 7 days of treatment, cells were fixed, permeabilized, and stained for MAP-2 (A) and synaptophysin (B). Images were analyzed using microscopy as described in Materials and Methods. Bars, 20 μm. Arrows indicate examples of fragmented processes.

verify that the LC3-II/LC3-I ratio increased as a result of upstream induction of autophagy, expression levels of p62/SQSTM1, a selective protein incorporated into autophagosomes through direct binding to LC3 (66), were examined. Although the reduction was not statistically significant when cells were treated individually with gp120, METH, and the ARV cocktail, significantly decreased levels of p62 were observed after treatment with various combinations of ARVs, METH, and gp120 (Fig. 6C). These data indicate that p62 was efficiently degraded in functional autolysosomes, which indicated induction and flux of autophagy. An additional cellular energy protein sensor is the mammalian target of rapamycin (mTOR), which acts as a negative regulator of autophagy. Activation of mTOR inhibits autophagy (67). To investigate a possible contribution of mTOR activity in association with our

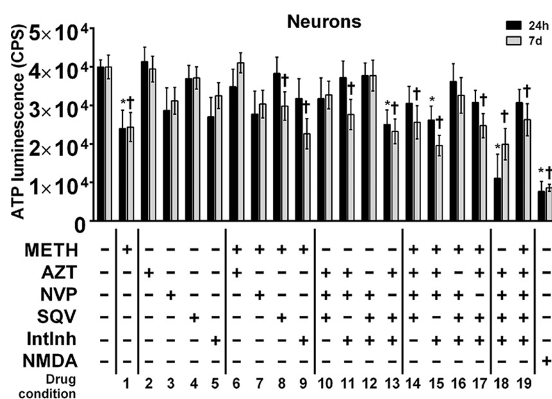


**FIG 4** Neurotoxicity in the presence of METH and ARV drugs for 24 h and 7 days, as measured by levels of ATP in cerebrocortical cells. Cerebrocortical cell cultures were incubated for 24 h or 7 days with a single drug or a combination of antiretrovirals (10 μM AZT, 1 μM NVP, 50 nM SQV, and/or 20 μM IntInh) and METH (100 μM). ATP levels of mixed glial and neuronal-glial cultures were measured using an ATPlite kit. The assays were run in four replicates, and the experiments were repeated three times. *, $P < 0.05$ compared to the control (24 h); †, $P < 0.05$ compared to the control (7 days) (ANOVA and Fisher's PLSD *post hoc* test).

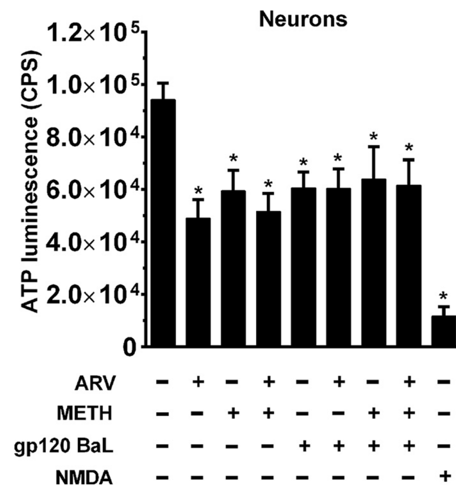


**FIG 5** Neurotoxic effects of long-term exposure to ARV drugs, METH, and HIV-1 gp120. Cerebrocortical cell cultures from rats were incubated for 7 days with a combination of antiretroviral drugs (ARV; 10 μM AZT, 1 μM NVP, 50 nM SQV, and 20 μM IntInh), METH (100 μM), and BaL gp120 (200 pM). ATP levels of mixed glial and neuronal-glial cerebrocortical cell cultures were measured using an ATPlite kit. The assays were run in eight replicates, and the experiments were repeated three times. *, $P < 0.05$ compared to the control (ANOVA and Fisher's PLSD *post hoc* test).

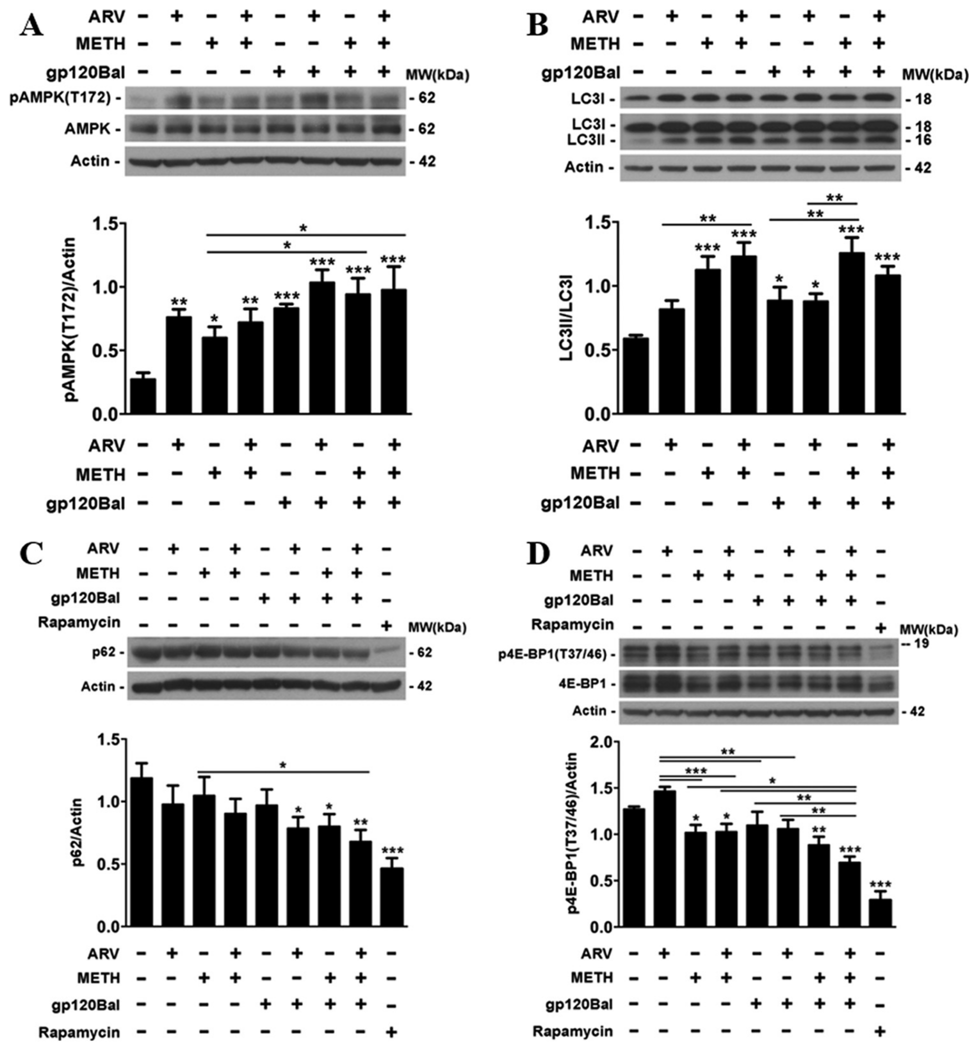


**FIG 6** METH, gp120, and ARV drugs alter autophagy in rat cerebrocortical cells. Rat cerebrocortical cells were treated with a combination of antiretroviral drugs (ARV; 10 μM AZT, 1 μM NVP, 50 nM SQV, and 20 μM IntInh), METH (100 μM), and BaL gp120 (200 pM). Seven days later, cytosolic extracts were prepared and analyzed by Western blotting using antibodies for the following proteins: phospho- and total AMPK (A), LC3 (B), p62/SQSTM1 (C), and phospho- and total 4E-BP1 (D). Total β-actin was used as a loading control. Histograms show the results of densitometric analysis for four to eight independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (ANOVA and Fisher's PLSD *post hoc* test).

experimental treatments, we quantified the activation of 4E-BP1, which is a substrate for phosphorylation by mTOR, using Western blotting and densitometry (Fig. 6D). All treatments, except for ARVs, inhibited phosphorylation of 4E-BP1. Overall, our observations are consistent with the fact that autophagy is induced to various degrees in the presence of METH, ARVs, gp120, or combinations thereof. However, the effect of rapamycin on p62 and p4E-BP1 suggested that cerebrocortical cells did not activate autophagy in the presence of METH, ARVs, gp120, or combinations thereof to the same extent that could be achieved by blocking mTOR activity, despite the fact that ATP concentrations remained below control levels.

**Rapamycin treatment increases ATP levels of drug-viral gp120-exposed neurons.** To investigate if further activation of autophagy would allow recovery of neuronal ATP levels, we assessed ATP levels in our cerebrocortical cells treated with ARV compounds, METH, and HIV-1 gp120 in the presence and absence of 20 nM rapamycin. This concentration of rapamycin has been shown to induce autophagy by directly blocking mTOR (65). In the presence of rapamycin, ATP levels were increased under all conditions after 24 h (Fig. 8A). Interestingly, 7 days after application of ART, METH, or gp120, we found that ATP levels improved if rapamycin had been present, reaching almost control levels for exposure to METH, ARV-gp120, METH-gp120, and the ARV-METH-gp120 combination (Fig. 8B). This observation indicates that cells are principally able to recover in the presence of certain drug combinations, with their normal ATP levels suggesting that their mitochondria are functional. In contrast, rapamycin was unable to elevate neuronal ATP levels to nearly control levels during exposure to the ARV cocktail or gp120 alone. On the other hand, in cerebrocortical cell cultures exposed to rapamycin alone, neuronal ATP levels were only temporarily elevated. After 7 days of exposure, the levels of p62 and p4E-BP1 indicated continued inhibition of mTOR and increased autophagy compared to those of the vehicle control, but neuronal ATP was present at control levels (Fig. 8B).

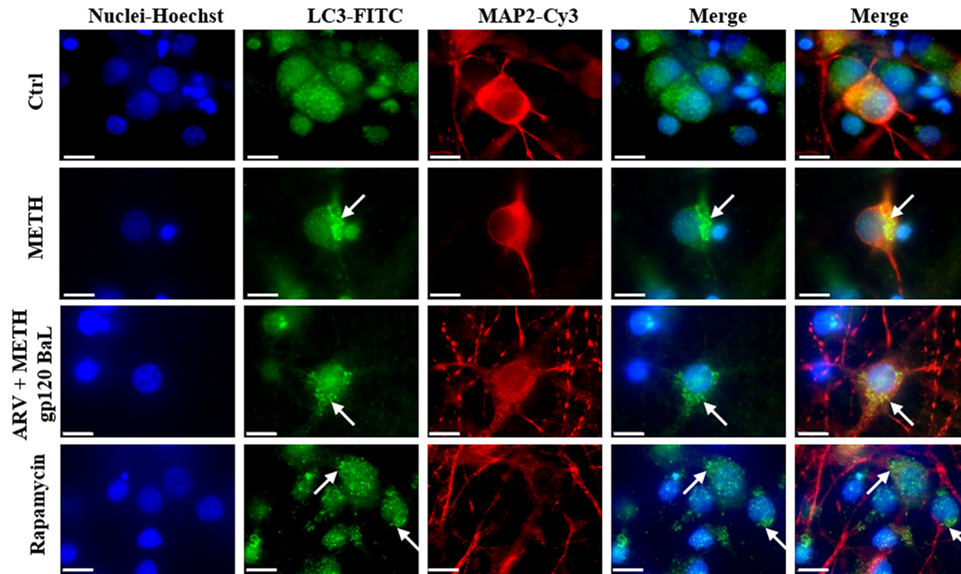


**FIG 7** Localization of LC3 and autophagic granular structures in neurons exposed to METH, gp120, and ARV drugs. Cerebrocortical cell cultures from rats were incubated for 7 days with a combination of antiretroviral drugs (ARV; 10 μM AZT, 1 μM NVP, 50 nM SQV, and 20 μM IntInh), HIV-1 BaL gp120 (200 pM), and METH (100 μM). Rapamycin (20 nM) was used as a positive control for induction of autophagy. Cells were fixed, permeabilized, and fluorescence stained for LC3 (FITC) and MAP-2 (rhodamine red), as a neuronal marker, as described in Materials and Methods. The overlap of red and green signals appears yellow in the merged images. Bars, 20 μm. Arrows indicate examples of autophagosomes.

## DISCUSSION

One of the major problems in the era of ART is the still-growing prevalence of HAND (9, 10). Neuronal damage found in neuro-AIDS can have different origins, ranging from neurotoxins and viral proteins produced by HIV-infected cells in the CNS (44, 68–71) to toxicity induced by the use of both therapeutic and recreational drugs (14, 17, 37, 38, 72). In this study, we assessed for the first time experimentally the contribution to neuronal injury of METH, ARVs, and an established viral neurotoxin, HIV-1 gp120, in combination.

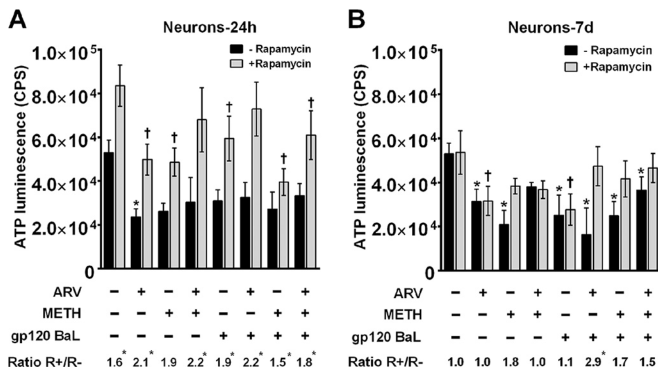


**FIG 8** Rescue of neuronal ATP levels during long-term exposure to ARV drugs, HIV-1 gp120, and METH in the presence of rapamycin. Cerebrocortical cell cultures were incubated for 24 h (A) and 7 days (B) after treatment initiation with a combination of antiretroviral drugs (ARV; 10 μM AZT, 1 μM NVP, 50 nM SQV, and 20 μM IntInh), BaL gp120 (200 pM), and METH (100 μM) in the presence or absence of rapamycin (20 nM). ATP levels of mixed glial and neuronal-glial cerebrocortical cell cultures were measured using an ATPlite kit. Samples were from five to seven replicates for each condition. *, $P < 0.05$ compared to the control (without rapamycin [R−]); †, $P < 0.05$ compared to the control (with rapamycin [R+]) (ANOVA and Fisher's PLSD *post hoc* test; $n = 3$ each for 24 h and 7 days). Note that ATP levels remained significantly below control levels, despite rapamycin addition, in neurons of cultures exposed to the ARV cocktail or gp120 alone.

Several other studies have provided direct evidence that antiretroviral drugs can exert neurotoxic effects in connection with oxidative stress, dysregulation of $Ca^{2+}$ homeostasis, and alteration of mitochondrial respiration (14, 17, 62, 73–78). Furthermore, similar to HIV-1 gp120, the earliest antiretroviral drug, AZT, has been shown to impair neurogenesis (79–81), besides affecting cerebrocortical mitochondria (82). Efavirenz (EFV) and NVP, which are currently FDA approved and used in NNRTI regimens (http://aidsinfo.nih.gov/guidelines), have been suggested to exert neurotoxicity (14, 62, 83). One study found that EFV caused a loss of ATP, depolarization and fragmentation of mitochondria, and increased mitophagy and autophagy, in general, in neuron-like SHSY-5Y cells and primary rat striatal neurons, suggesting a disturbance of energy homeostasis as a mechanism of toxicity (84). EFV was also found to cause endoplasmic reticulum (ER) stress in human brain endothelial cells and in microvessels of the CNS in HIV-transgenic mice (85). However, the latter study found that EFV inhibited autophagy by binding to a complex comprising Beclin 1, ATG14, and phosphatidylinositol 3-kinase III (PI3KIII), which is required for formation of an autophagosome.

Two recent studies investigated drug combinations and cell culture models different from those in the present study (17, 62), and one of the studies assessed only the effects of cART for up to 48 h (17). However, HIV treatment regimens commonly consist of combinations of three and sometimes more drugs (8–10). In addition, drug abuse is a frequent comorbidity of HIV infection (26–28). Hence, the brains of many HIV patients are exposed to HIV-1, combinations of ARVs, and psychostimulants, such as METH, at the same time. Therefore, in the present study, we investigated the effects on neurons of ARV combinations in the

presence and absence of METH and the neurotoxic HIV envelope protein gp120.

The therapeutic compounds used in our study represent the four most commonly used pharmacological classes of ARVs. Even though some of the drugs we studied here are no longer first choices for HIV therapy, three of the compounds have been used for many years: AZT since 1987, NVP since 1996, and SQV since 1995 (http://www.avert.org/antiretroviral-drugs.htm). Thus, many individuals living with HIV infection today are likely to have been treated with one or more of these drugs or combinations thereof.

The tested ARVs caused no or limited detectable alterations to neuronal dendrites and presynaptic terminals over 24 h when applied as single drugs or in combinations of two or three. Any alterations to neuronal MAP-2 or synaptophysin immunoreactivity occurred with the drugs alone or in specific combinations after 7 days, except for IntInh and AZT-NVP-SQV. The same was true overall for neuronal ATP levels. Most strikingly, ATP levels could be diminished significantly under conditions where immunoreactivities of dendritic MAP-2 and presynaptic synaptophysin were minimally or not detectably changed (AZT-SQV-IntInh, with and without METH, or AZT-NVP-SQV-IntInh). These observations are in line with reports that ARVs can compromise mitochondria at various levels by interfering with (i) mitochondrial membrane components, such as transporters; (ii) mitochondrial kinases, such as thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) (77); (iii) mitochondrial bioenergetics, such as the membrane potential; and (iv) mitochondrial DNA (mtDNA) homeostasis, such as blocking polymerase $\gamma$ (74–76) or inducing major deletions in neuronal mtDNA (73, 78). Our findings also suggest that neurons are capable of maintaining neurites and presynaptic terminals when cellular ATP drops below normal levels, through regulatory mechanisms that remain to be explored.

Also striking is the fact that METH, which itself is toxic to neurons (37), lacked any apparent toxic effect in combination with several ARVs and limited a significant drop of neuronal ATP levels when combined with a cocktail of four ARVs. Also surprising was the finding that METH, cART, and HIV gp120, each alone and in combination, caused similar losses of neuronal ATP but lacked an additive effect. Thus, the toxic effects of METH, cART, and HIV gp120 were context dependent in that METH, gp120, and AZT as an ARV each exerted neurotoxicity in terms of a loss of MAP-2 or synaptophysin immunoreactivity or a loss of neuronal ATP or, in the case of gp120, of neurons themselves when applied alone, but not necessarily when combined. Thus, therapeutic and psychostimulant drugs and neurotoxicity-inducing viral components may interact in unexpected ways that remain to be elucidated.

Our data indicated that combinations of AZT, NVP, SQV, and IntInh in any cocktail of three of the four drugs in the presence or absence of METH did not affect expression of either MAP-2 or SYP. Nevertheless, when cellular ATP concentrations were analyzed under the various experimental conditions, reduced levels of ATP were found for the following seven conditions: AZT-SQV-IntInh and AZT-NVP-IntInh-METH (short-term treatment) and AZT-NVP-IntInh, AZT-SQV-IntInh, AZT-NVP-SQV-METH, AZT-NVP-IntInh-METH, and AZT-SQV-IntInh-METH (long-term treatment). These data suggest that although neurons maintain their overall dendritic and presynaptic structures, a disruption of neuronal energy homeostasis occurs which may contribute to progressive neurological diseases, such as HAND. However, we did not observe additive effects on ATP levels when three different ARV drugs were combined with METH. To the contrary, METH mitigated the reduction of ATP levels when it was combined with four ARV drugs. However, a reduction of ATP levels was also observed when ARVs and METH were combined with HIV-1 gp120, an established neurotoxic viral protein. In addition, the mixture of therapeutic compounds and psychostimulant drug compromised neuronal MAP-2-positive dendrites, while apparently sparing SYP-reactive presynaptic terminals, during long-term exposure. MAP-2 is enriched and involved in stabilizing neuronal dendrites, and beading and pruning were observed in dendrites (Fig. 7), as other authors have also reported (14), as a result of ARV treatment.

In order to maintain homeostasis, membrane potentials, and neurotransmission, neurons require significant amounts of energy in the form of ATP (59). The lack of ATP as an energy source could be one of the factors that compromise neuronal function before the loss of $SYP^+$ presynaptic terminals and $MAP\text{-}2^+$ dendrites ensues. ATP depletion is usually an indication of impairment or failure of cellular respiration (mitochondrial malfunction). In the brain, astrocytes stimulate glycolysis by activation of 6-phosphofructo-2-kinase (PFK2) through AMPK in order to maintain ATP levels. However, low expression of PFK2 in neurons renders this pathway insufficient (60, 86). Nevertheless, neurons can activate AMPK in response to low intracellular ATP levels, thus inducing autophagy that can recycle cellular, possibly damaged components and contribute to the reestablishment of baseline cellular ATP levels (87, 88). Interestingly, we found that although autophagy was induced in the presence of ARV compounds, METH, and HIV-1 gp120 (Fig. 6), ATP levels were not restored to baseline levels (Fig. 5 and 8). The underlying mechanism of ATP dysregulation remains to be explored. However, the two apparently contradictory reports of EFV inhibiting autophagy in microvessels and endothelial cells in the presence of ER stress but increasing mitochondrial fragmentation and autophagy in neurons suggest that ARVs could shift the cellular threshold for activation of autophagy to lower ATP levels.

Autophagy is induced when mitochondria, which are the cellular organelles responsible for generation of ATP, are impaired. As mentioned before, this process is regulated by AMPK and mTOR signaling pathways (89). Based on the failure to recover homeostatic levels of ATP, we further triggered autophagy under neurotoxic conditions by blocking mTOR, which is an inhibitor of autophagy, with rapamycin. We observed that in the presence of combinations of ARV compounds, METH, and gp120, rapamycin can restore neuronal ATP to levels close to control levels (Fig. 8B). These observations indicate that neurons can, in principle, restore ATP levels by autophagy but fail to do so when challenged with gp120 alone or the ARV cocktail or in the absence of rapamycin, for reasons that remain to be elucidated. Autophagy can also be activated by oxidative stress, which can be induced in neurons by ARV drugs or METH (17, 40). In fact, it has been suggested that autophagy can be responsible for neurite degeneration in the presence of METH (40). Thus, autophagy may not always be protective. However, while the induction of endogenous antioxidant pathways has been reported to block the toxicity of ARV drugs, it is not known if this protective effect is associated with normal neuronal ATP levels (17).

Interestingly, neuronal ATP levels were elevated only tempo-

rarily in cerebrocortical cell cultures exposed to rapamycin alone. After 7 days of exposure, the levels of p62 and p4E-BP1 indicated continued inhibition of mTOR and increased autophagy compared to those of the vehicle control, but neuronal ATP was at control levels. Thus, in the absence of HIV gp120, METH, or ARVs, continued induction of autophagy, at least by rapamycin, seemed not to injure neurons that normalized their ATP levels. On the other hand, we found that ARVs and gp120 each alone, in contrast to their combination, prevented rapamycin from restoring neuronal ATP levels over 7 days. That situation could be explained if ARVs and gp120 were pushing neuronal ATP homeostasis off balance by different disturbance mechanisms which, however, neutralized each other when ARVs and gp120 were combined.

In summary, we evaluated alterations of two neuronal markers that have been used widely to characterize neuronal injury, in addition to ATP levels and autophagy, in primary neural cells exposed to HIV-1 gp120 and recreational and therapeutic drugs. Strikingly, neuronal ATP levels may drop significantly during exposure to some drugs or combinations thereof without necessarily destructing MAP-$2^+$ neuronal structures and presynaptic terminals. Yet reduced ATP levels suggest that neurons lose significant parts of their energy reserve, which is vital to keeping up their normal membrane potential and physiological function. Altogether, our findings indicate that the overall positive effect of cART on HIV infection can be accompanied by detectable neurotoxicity, which in turn may be aggravated in the presence of psychostimulant drugs, such as methamphetamine.

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