

HIV protease inhibitors disrupt astrocytic glutamate transporter function and neurobehavioral performance

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Objective: The neurotoxic actions of the HIV protease inhibitors, amprenavir (APV) and lopinavir (LPV) were investigated.

Design: With combination antiretroviral therapy (cART), HIV-infected persons exhibit neurocognitive impairments, raising the possibility that cART might exert adverse central nervous system (CNS) effects. We examined the effects of LPV and APV using in-vitro and in-vivo assays of CNS function.

Methods: Gene expression, cell viability and amino-acid levels were measured in human astrocytes, following exposure to APV or LPV. Neurobehavioral performance, amino-acid levels and neuropathology were examined in HIV-1 Vpr transgenic mice after treatment with APV or LPV.

Results: Excitatory amino-acid transporter-2 (EAAT2) expression was reduced in astrocytes treated with LPV or APV, especially LPV ($P < 0.05$), which was accompanied by reduced intracellular L-glutamate levels in LPV-treated cells ($P < 0.05$). Treatment of astrocytes with APV or LPV reduced the expression of proliferating cell nuclear antigen (PCNA) and *Ki-67* ($P < 0.05$) although cell survival was unaffected. Exposure of LPV to astrocytes augmented glutamate-evoked transient rises in $[Ca_i]$ ($P < 0.05$). Vpr mice treated with LPV showed lower concentrations of L-glutamate, L-aspartate and L-serine in cortex compared with vehicle-treated mice ($P < 0.05$). Total errors in T-maze assessment were increased in LPV and APV-treated animals ($P < 0.05$). EAAT2 expression was reduced in the brains of protease inhibitor-treated animals, which was associated with gliosis ($P < 0.05$).

Conclusion: These results indicated that contemporary protease inhibitors disrupt astrocyte functions at therapeutic concentrations with enhanced sensitivity to glutamate, which can lead to neurobehavioral impairments. ART neurotoxicity should be considered in future therapeutic regimens for HIV/AIDS.

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Introduction

HIV-1 enters the central nervous system (CNS) soon after primary infection with subsequent infection of glial cells and eventual injury and death of neurons in susceptible

persons leading to neurocognitive impairment, termed HIV-associated neurocognitive disorder [1,2]. Among combination antiretroviral therapy (cART)-exposed patients with HIV/AIDS, a subset will develop neurocognitive impairment despite suppression of viral levels in

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blood and CSF; in some instances, these patients will show detectable viral RNA with associated drug resistance mutations in viral genomes in cerebrospinal fluid (CSF) [3]. There are also reports of HIV/AIDS patients with neurocognitive impairment linked to specific antiretroviral drugs, including efavirenz, protease inhibitors and nucleoside reverse transcriptase inhibitors [4–7]. The effects of cART on HIV infection within the brain parenchyma and accompanying cellular functions remain uncertain, particularly in terms of its effects on viral replication but also its actions on neural cellular viability (reviewed [8]). Individual drug levels in CNS parenchyma as well as their efficacy are unclear although drug levels in CSF are well defined [9]. Lumbar CSF represents an admixture of blood and brain-derived components including cells, virus, solutes, drugs, etc., which calls into question whether the brain tissue levels of these constituents are represented in lumbar-derived CSF analyses.

The HIV protease inhibitors have been associated with a range of off-target effects, leading to substantial morbidity [10]. Several generations of protease inhibitors have been developed with improved side-effect profiles. Nonetheless, contemporary protease inhibitors have recognized side-effects on multiple organs. Recent studies indicate that patients receiving ritonavir-boosted lopinavir (LPV) exhibited increased levels of S100B in CSF, implying astrocytosis [11]. Astrocytes represent the most abundant cell type in the brain and one of their chief functions is to ensure the extracellular glutamate levels are maintained at homeostatic concentrations that are below excitotoxic levels to neurons, enabling preserved neuronal function without risk of cell death [12]. The excitatory amino-acid transporters (EAATs) expressed on glia, particularly astrocytes, are critical for sustaining this intracellular-extracellular equilibrium in glutamate concentrations in the synaptic cleft; with reduced EAAT expression the likelihood of glutamate-mediated excitotoxicity and eventual neuronal injury or death increases substantially [13].

In the current study, we investigated the neurotoxic effects of two protease inhibitors, LPV and amprenavir (APV) because of their wide use globally, established CSF levels (LPV: 5–74 ng/ml; APV: 10–123 ng/ml), and lack of reported adverse effects [14]. LPV has been linked to neurobehavioral impairments in treated mice [15,16]. The present studies indicated that LPV in particular appears to exert cytotoxic actions on astrocytes with a capacity to disrupt glutamatergic signaling and uptake.

Materials and methods

Reagents

APV and LPV for in-vitro experiments were purchased from Toronto Research Chemicals Inc (Ontario,

Canada). Both antiretroviral drugs were dissolved in dimethyl sulfoxide (DMSO Hybri-Max; Sigma, Oakville, Ontario, Canada). FosAPV (oral Lexiva; ViiV Healthcare, Laval, Quebec, Canada), ritonavir (oral Norvir; Abbott Laboratories, Edmonton, Alberta, Canada) and LPV/ritonavir (oral Kaletra; Abbott Laboratories) were used for in-vivo studies.

Cell cultures

Primary human fetal astrocytes (HFAs) were prepared (95% pure), as previously described [17]. Briefly, human fetal brain tissues were obtained from 15 to 19 week aborted fetuses with written consent approved under the protocol 1420 by the University of Alberta Human Research Ethics Board (Biomedical Ethics Board). All experiments were performed with cells from the fifth to the 10th passage.

In-vitro cytotoxicity assay

HFAs were plated at 2×10^4 cells in 96-well flat bottom plates. At 50–70% confluences, cells were exposed to APV, LPV or DMSO 0.1%. After 48 h of exposure, cells were fixed, permeabilized and stained with mouse anti- β -tubulin antibodies (Sigma) followed by antimouse antibodies conjugated with Alexa Flour 680 (Life Technologies, Burlington, Ontario, Canada) [17,18]. The immunoreactivity of β -III-tubulin was quantified using Odyssey Imager (LI-COR, Lincoln, Nebraska, USA) and was normalized to the immunoreactivity of untreated cells. Data from four separate experiments were presented as the mean \pm SEM.

Real-time PCR

HFAs in 6-well plates were exposed to APV, LPV or DMSO for 8 h. RNA extraction, first-strand complementary DNA (cDNA) synthesis and semi-quantitative real-time polymerase chain reaction (real-time PCR) were performed as previously described [17]. All PCR primers are listed in Supplementary Table 1, <http://links.lww.com/QAD/A826>. Data were normalized to GAPDH mRNA levels and expressed as relative fold increases compared with controls \pm SEM. All experiments were performed at least four times.

Western blotting

HFAs were exposed to APV, LPV or DMSO and 12 h after the initial treatment, supernatants were replaced with APV, LPV or DMSO in fresh media. 24 h after exposure, HFAs were lysed in lysis buffer [20 mmol/l Tris, 1% NP-40, 50 mmol/l NaCl, protease inhibitor cocktail set III (1 : 1000, Calbiochem, La Jolla, California, USA)]. Crude protein lysates were separated by 12% SDS-PAGE gel and electro-transferred to nitrocellulose membrane. Membranes were exposed to anti-EAAT2 (Millipore, BilleStrica, Massachusetts, USA) or β -actin (Santa Cruz Biotechnology, Santa Cruz, California, USA) antibodies. Immunoreactive bands were visualized on films using HRP-conjugated secondary antibodies

(Jackson Laboratory, Bar Harbor, Maine, USA) and enhanced chemiluminescence substrate (Life Technologies, Carlsbad, California, USA) and quantified using ImageJ (National Institutes of Health). Data from four separate experiments were presented as the mean \pm SEM.

Calcium imaging

HFAs were plated in 35 mm Nunclon tissue culture dishes (Nunc, Rochester, New York, USA). Next day, cells were exposed to APV, LPV or DMSO, and every 12 h cultured media were replaced with APV, LPV or DMSO in fresh media. 48 h after exposure, cells were treated with 5 μ mol/l Fluo-8 acetoxymethyl ester (Fluo-8 AM) (ABD Bioquest, Sunnyvale, California, USA) for 45 min. Changes in Fluo-8 AM fluorescence intensity evoked by glutamate (1 mmol/l 2 min) were measured using an inverted microscope (IX81 microscope system; Olympus, Richmond Hill, Ontario, Canada) with a xenon lamp (Lambda DG-4; Sutter Instruments, Novato, California, USA) and a filter (Ex: 482/35, Em: 536/40) [19]. During recording, the cells were perfused with a solution containing 127 mmol/l NaCl, 2.5 mmol/l KCl, 1.2 mmol/l NaH₂PO₄, 1.3 mmol/l MgSO₄, 26 mmol/l NaHCO₃, 25 mmol/l D-glucose and 2.5 mmol/l CaCl₂. Images were recorded using a CCD camera (Roleta-XR F-M-12-C; QImaging, British Columbia, Canada) at 3 s/frame. Selected regions of interest were drawn around distinct cells and traces of time course of average changes of fluorescence intensity were generated with Metamorph software (Olympus) and were measured in arbitrary units (a.u.). Photo-bleaching was evident during the course of recording. To adjust for this problem, the difference between the peak value and the lowest value before the peak was calculated. For uniformity in measurement, only the first evoked response was measured from each cell [20].

In-vivo studies

HIV-1 Vpr transgenic mice (males and females, 9–14 months, evenly distributed across studies) were bred and housed in standard cages (4–5 animals per cage) with 12:12 light:dark cycle [21] and maintained in the Health Sciences Laboratory Animal Services facility of the University of Alberta under conventional housing conditions. All experiments were approved by the University of Alberta Animal Care & Use Committee for Health Sciences (AUP00000452). The Vpr encoding gene is expressed under the control of the *c-fms* (CSF-1 receptor) promoter, driving the transgene expression chiefly in myeloid cells [22]. Using adult human daily dosing and body surface area normalization, as previously described [15], daily oral doses of fosAPV and LPV were 300 and 200 mg/kg, respectively, for 3 weeks in groups of 4–6 animals. Drugs were suspended in the solvent, propylene glycol (15%) as a vehicle; LPV suspension from Kaletra tablet contains 50 mg/kg ritonavir whereas ritonavir was added to the fosAPV suspension with a

final concentration at 75 mg/kg. Fifteen percent propylene glycol was used as a vehicle control.

Tissue preparation and staining

Paraffin-embedded sections (6 μ m) of coronal brain sections from each group (vehicle, $n = 3$; APV/R, $n = 3$, LPV/R, $n = 3$) were deparaffinized and hydrated using decreasing concentrations of ethanol. Antigen retrieval was performed by boiling deparaffinized slides in 0.01 mol/l trisodium citrate buffer, pH 6.0, for 10 min. For diaminobenzidine staining, slides were treated with 0.3% hydrogen peroxide solution for 20 min and then blocked with 10% phosphate-buffered saline containing 10% normal goat serum, 2% bovine serum albumin and 0.1% Triton X-100 for 2 h at room temperature. Sections were incubated overnight at 4°C with mouse anti-EAAT2 (Millipore), mouse anti-proliferating cell nuclear antigen (PCNA; Millipore, Etobicoke, Ontario, Canada) rabbit anti-glial fibrillary acidic protein (GFAP, DAKO, Denmark) or rabbit anti-ionized calcium binding adaptor molecule 1 (Iba-1, Wako Chemicals, Neuss, Germany). Immunoreactivity was visualized using secondary biotinylated goat antibodies followed by avidin-biotin-peroxidase amplification (Vector Laboratories, Burlington, Ontario, Canada) and 3,3'-diaminobenzidine tetrahydrochloride staining (Vector Laboratories). Images were taken using a Zeiss Axioskop 2 upright microscope (Oberkochen, Germany). Brain sections were deparaffinized and hydrated followed by staining with 0.1% cresyl violet solution (Sigma) for 30 min [23]. Labeled cell counts were performed in EAAT2 (astrocytes) immunostained and Nissl (neuron) stained brain sections. Cells per field (EAAT2, 1200 μ m²; Nissl 300 μ m²) were counted for each animal in serial sections by an examiner unaware of the slide identity.

Amino-acid level analyses

Astrocytes or brain tissues were weighed and homogenized in five volumes of ice-cold double-distilled water. HPLC using a symmetry C18 column and fluorescence detector (Waters Corporation, Mississauga, Ontario, Canada) was used to determine the levels of amino acids (\pm isomers of those amino acids with chiral centers) per gram of brain tissue, as previously described [24].

T-maze test

The spatial memory of Vpr transgenic mice was measured using a T-maze. During the light period, animals were trained to turn to the goal arm using the food reward alteration protocol, as previously described [25]. Briefly, the preference arm of each animal was determined at the beginning of the experiment by placing the animal in the start area and allowing it to choose the side freely without any blocking doors. Twelve days after the initial treatment with fosAPV/ritonavir, LPV/ritonavir or vehicle, lab chow was reduced to 1.5 g/animal. The food reward, 1:1 (vol/vol) water/sweet condensed milk, was fed in the home cages at 1 h prior to the dark period for

3 consecutive days. After habituation, animals were trained to go to the goal/rewarded arm (with food) or to the nonrewarded arm (no food) with the total of eight runs per day for 4 consecutive days. The day after the training session (test day), the blocking doors were removed and animals were allowed to turn left or right freely. If all four paws of animals stepped into the nonrewarded arm, it was counted as an error. The cumulative number of wrong turns or errors was counted and the duration from the start to eating the food reward was recorded. Nine trials were performed for each animal.

Statistical analysis

Data were tested by one-way analysis of variance (ANOVA) with Dunnett's or Bonferroni *post hoc* test. Behavioral data were tested by Kruskal–Wallis nonparametric ANOVA. The level of significance was defined as $P < 0.05$.

Results

Protease inhibitors suppress excitatory amino-acid transporter-2 expression in astrocytes

The effects of the protease inhibitors were assessed in human astrocytes at a range of concentrations encompassing those reported clinically and compared with the vehicle, dimethyl sulfoxide (DMSO, 0.1%) as a control. LPV and APV treatment resulted in significant suppression of the glutamate transporter *EAAT2* transcript levels in astrocytes (Fig. 1a) although another amino-acid transporter, *ASCT1* (Fig. 1b) was unaffected by exposure to either drug. Additionally, EAAT2 immunoreactivity on western blot was reduced by each drug (Fig. 1c); graphic analyses demonstrated that LPV, but not APV, significantly decreased EAAT2 protein expression (Fig. 1d). Of note, the transcript levels of *Ki67* (Fig. 1e) and *PCNA* (Fig. 1f), which are the markers for cell proliferation, were also significantly reduced by exposure to each drug. Neither drug affected the expression of *BDNF* in astrocytes (Supplementary Fig. 1B, <http://links.lww.com/QAD/A827>) but EAAT1 was reduced at the highest concentration only of LPV (Supplementary Fig. 1A, <http://links.lww.com/QAD/A827>). Neither drug affected astrocyte overall viability, measured by β -III-tubulin immunoreactivity (Supplementary Fig. 1C, <http://links.lww.com/QAD/A827>). Because of the absence of cytotoxicity despite altered gene expression in astrocytes exposed to each protease inhibitor, subsequent studies concentrated on altered functions in astrocytes.

Lopinavir exposure alters neurotransmitter levels in astrocytes

Treatment of astrocytes with LPV resulted in significantly reduced intracellular L-glutamate (Supplementary Fig. 2A,

<http://links.lww.com/QAD/A827>) with significantly increased extracellular levels in corresponding medium (Supplementary Fig. 2B, <http://links.lww.com/QAD/A827>) although the effects of APV on amino-acid levels were less apparent (Supplementary Fig. 2C and D, <http://links.lww.com/QAD/A827>). Notably, there was also an increase in intracellular gamma-aminobutyric acid (GABA) levels with LPV treatment, which was associated with reduced extracellular concentrations (Supplementary Fig. 3C and D, respectively, <http://links.lww.com/QAD/A827>). Extracellular glutamine levels were also significantly reduced by LPV exposure (Supplementary Fig. 4D, <http://links.lww.com/QAD/A827>). These data indicated that exposure to protease inhibitors could interfere with astrocyte functions without direct cytotoxic effects.

Lopinavir exposure increases excitability of astrocytes to glutamate

In addition to glutamate transporters, astrocytes express ionotropic and metabotropic glutamate receptors [26]. Activation of these receptors can result in increase in cytoplasmic calcium, either through calcium influx via ionotropic receptors or triggering calcium release from endoplasmic reticulum through the actions of metabotropic glutamate receptors. Changes in cytosolic calcium can result in several downstream cellular signaling. To investigate whether protease inhibitors affected the glutamate-mediated calcium signaling in astrocytes, primary human astrocytes were loaded with Fluo-8 and fluorescence images were captured with or without the presence of glutamate (1 mmol/l). Acute application of APV or LPV did not affect cytosolic calcium flux $[Ca_i]$ (data not shown). Conversely, LPV exposure (100 ng/ml added every 12 h for 48 h) increased glutamate-evoked transient rises in $[Ca_i]$ but did not alter spontaneous transient rises in $[Ca_i]$ in astrocytes (Fig. 2a–d). LPV-exposed astrocytes displayed higher numbers and larger peak amplitude of glutamate-evoked transient rises (Fig. 2c and d) ($P < 0.01$), indicating that the excitability of these cells was higher than that of unexposed astrocytes. Chronic exposure of APV had no effects on either spontaneous or glutamate-evoked transient rises in $[Ca_i]$ (Fig. 2c and d). These studies highlighted the selective effects of protease inhibitors on astrocyte function.

Lopinavir exposure alters amino-acid levels in brain

The in-vitro studies described above suggested an alteration in the glutamate and GABA levels, which are often associated with neurodegenerative diseases [27], schizophrenia and seizures [28]. We wanted to test if similar changes were apparent *in vivo*, using Vpr transgenic (Tg) mice. These animals express the HIV-1 Vpr protein selectively in myeloid cells (microglia and macrophages), display neurocognitive dysfunction and to a limited extent, recapitulate the neuropathological changes in HIV-infected brains [22]. Total amino-acid levels were measured in cortices from animals treated with

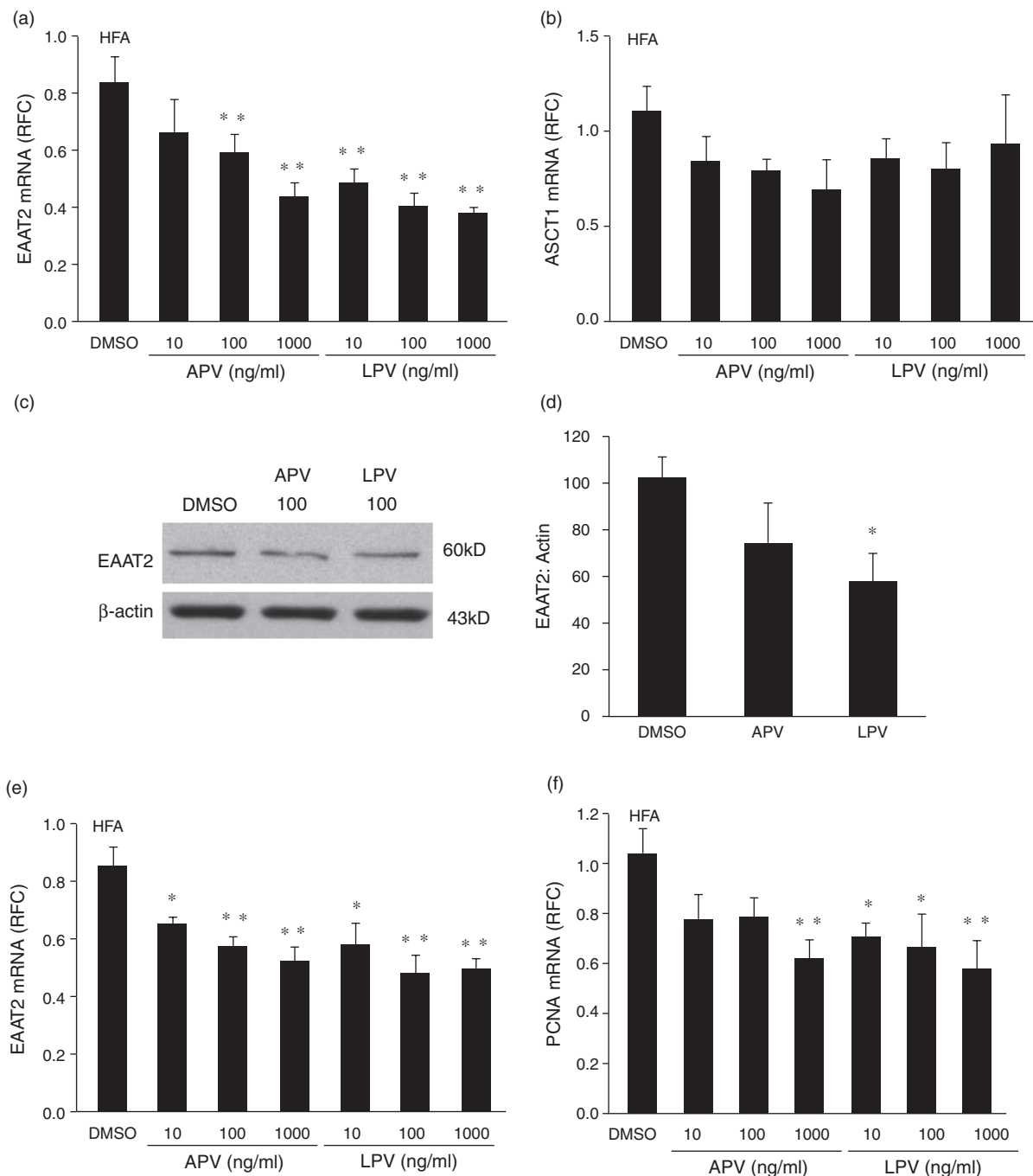


Fig. 1. Astrocytes exposed to protease inhibitors show reduced EAAT2 expression. (a) Human astrocytes exposed to either LPV or APV showed reduced *EAAT2* transcript abundance but (b) another amino-acid transporter, expressed on astrocytes, *ASCT1*, did not display reduced expression in protease inhibitor-exposed cells. (c) *EAAT2* immunoreactivity was reduced in astrocytes exposed to LPV and APV although (d) this effect was significant for LPV only. The markers of cellular proliferation, *Ki-67* (e) and *PCNA* (f) displayed reduced transcript abundance in protease inhibitor-exposed cells. (Bars represent mean \pm SEM; ANOVA with Dunnett *post hoc* comparisons; * $P < 0.05$, ** $P < 0.01$.)

either the vehicle control (solvent, 15% propylene glycol), or APV or LPV daily for 3 weeks. L-Glutamate levels in cortex from LPV-treated animals were reduced significantly compared with solvent-treated (control) animals (Fig. 3a) with a trend toward lower L-glutamate levels in APV-treated animals mice. Similar to glutamate

concentration changes, animals treated with LPV showed a significant reduction in L-aspartate levels (Fig. 3b) and APV-treated animals exhibited slightly lower reduction of the L-aspartate levels relative to the solvent-treated group. Glutamate is converted to GABA by glutamate decarboxylase but there was no change in the levels of GABA

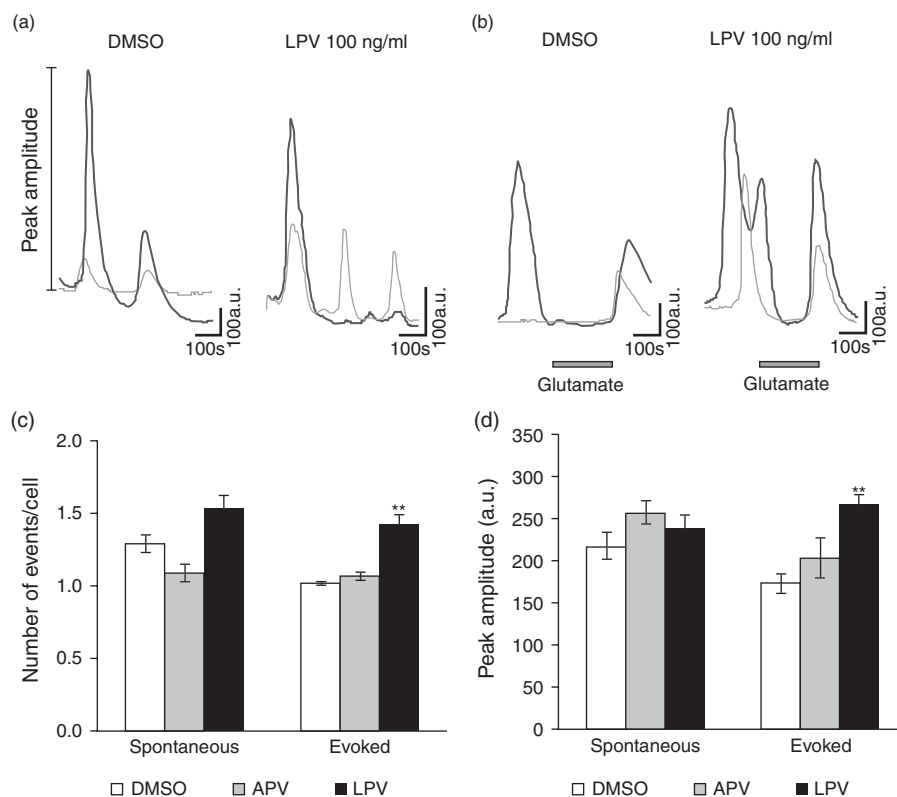


Fig. 2. LPV modulates evoked calcium fluxes in astrocytes. (a) LPV exposure increased the number and (b) the peak amplitude of glutamate-evoked events. Both (c) the number of evoked events and (d) peak amplitudes were significantly increased by LPV exposure. (ANOVA with Dunnett *post hoc* comparisons; * $P < 0.05$.)

in either treated group (Fig. 3c) whereas L-serine levels were significantly reduced in the LPV-treated group (Fig. 3d). Levels of L-glutamine, L-alanine, D-serine,

L-arginine, taurine and glycine in the mice treated with either protease inhibitor were similar to the solvent-treated animals (Supplementary Fig. 5, <http://links.lww.com/>

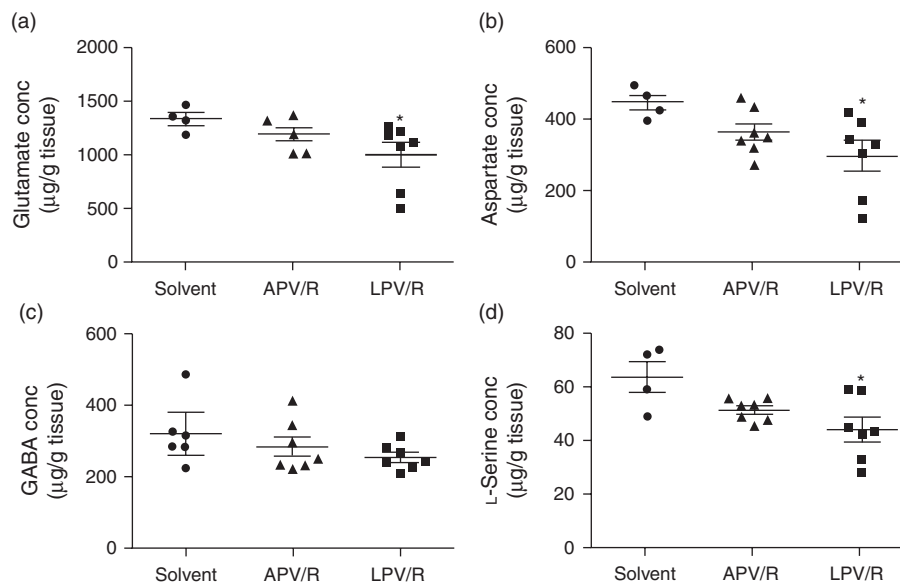


Fig. 3. LPV suppresses cortical amino acid levels in HIV-1 Vpr transgenic animals. (a) L-Glutamate, (b) L-aspartate and (d) L-serine levels in cerebral cortex were diminished after LPV/R treatment of Vpr animals although (c) GABA levels were unaffected. (ANOVA with Dunnett *post hoc* comparisons; * $P < 0.05$.)

QAD/A827). These data implied that LPV treatment reduced total L-glutamate and L-aspartate levels in brain, which reflected similar findings in the current in-vitro data, which could be linked to reduced EAAT2 function. At the same time the reduction of L-serine levels, a neurotrophic factor released by astrocytes [29], might interfere with neuronal homeostasis and function.

Protease inhibitor treatment impairs neurobehavioral performance

Previous studies from our group have shown that HIV-1 Vpr transgenic mice displayed impaired function on a battery of neurobehavioral tasks compared with wild-type littermates [22]. The effects of LPV and APV on spatial memory were assessed in animals using a simple T-maze with a food reward alteration protocol. The mean total completion time, which started from the beginning of the trial until animals began to eat the food reward, was significantly higher in APV-treated group (Fig. 4a). The mean number of errors (incorrect turns) per animal was recorded; both APV and LPV-treated groups displayed significantly higher errors compared with vehicle-treated animals (Fig. 4b). In a sub-analysis, animals treated with solvent only made fewer total errors during repeated testing whereas total errors were generated from the start of experiments in mice receiving either protease inhibitor (Fig. 4c). These findings showed that protease inhibitor treatment impaired learning and memory.

Lopinavir treatment reduces in-vivo excitatory amino-acid transporter-2 expression

To investigate the neuropathological consequences of in-vivo protease inhibitor treatment, coronal brain sections were examined. EAAT2 immunoreactivity was evident with a particulate appearance in solvent-treated animals' cortex (Fig. 5a), but was also apparent on glial cell bodies and processes in adjacent corpus callosum (cc) white matter (Fig. 5a, inset). In contrast, EAAT2 immunoreactivity was reduced in APV-treated animals' cortex and white matter (Fig. 5b); a similar effect was observed in LPV-treated animals' cortex and white matter (Fig. 5c). PCNA immunoreactivity was detected in nuclei of cells resembling glia in the vehicle-treated animals' white matter (Fig. 5d) but was reduced in APV (Fig. 5e) and LPV-treated (Fig. 5f) animals' brains. Iba-1 is expressed in brain myeloid-derived cells (macrophages and microglia) and its immunoreactivity was detected in quiescent microglia (Fig. 5g) but was increased in sections from APV (Fig. 5h) and LPV-treated (Fig. 5i) animal cortices. GFAP immunoreactivity in astrocytes was minimal in solvent-treated animals (Fig. 5j) but was more apparent in APV (Fig. 5k) and particularly in LPV-treated (Fig. 5l) animals' cortices. In Nissl-stained sections, neuronal densities in cortex appeared similar in solvent (Fig. 5m), APV (Fig. 5n) and LPV-treated (Fig. 5o) animals. Analyses of mean counts/field of Nissl-positive cortical neurons showed that solvent (8.6 ± 1.14), APV (8.4 ± 1.40) and LPV (8.0 ± 1.22) groups did not differ

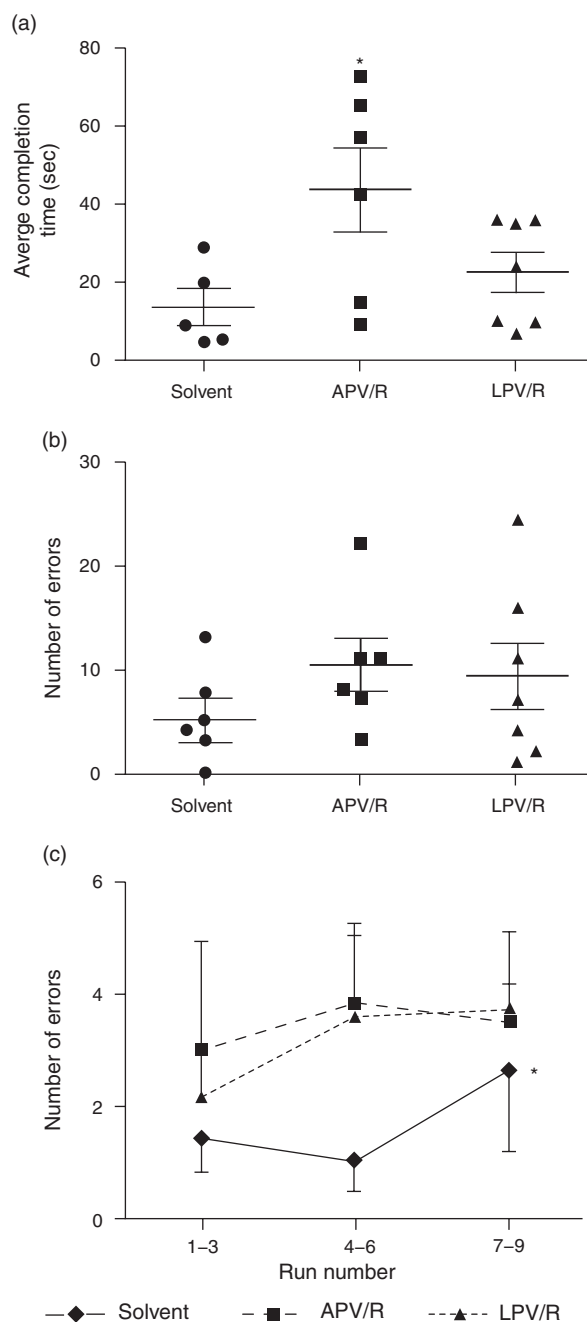


Fig. 4. Protease inhibitor treatment affects neurobehavioral performance in HIV-1 Vpr transgenic animals. (a) APV/R treatment delayed maze completion time. (b) Neither protease inhibitor affected the total number of errors made by Vpr animals. (c) However, the cumulative error rate among animals exposed to each protease inhibitor was significantly greater than the vehicle control-treated group. (Kruskal-Wallis nonparametric ANOVA; * $P < 0.05$.)

significantly. In contrast, mean counts/field of EAAT-2 immunopositive astrocytes revealed that the solvent group (3.4 ± 0.89) were higher than both APV (2.0 ± 0.71) and LPV (1.4 ± 0.55) groups ($P < 0.05$). These data indicated that exposure to protease inhibitors

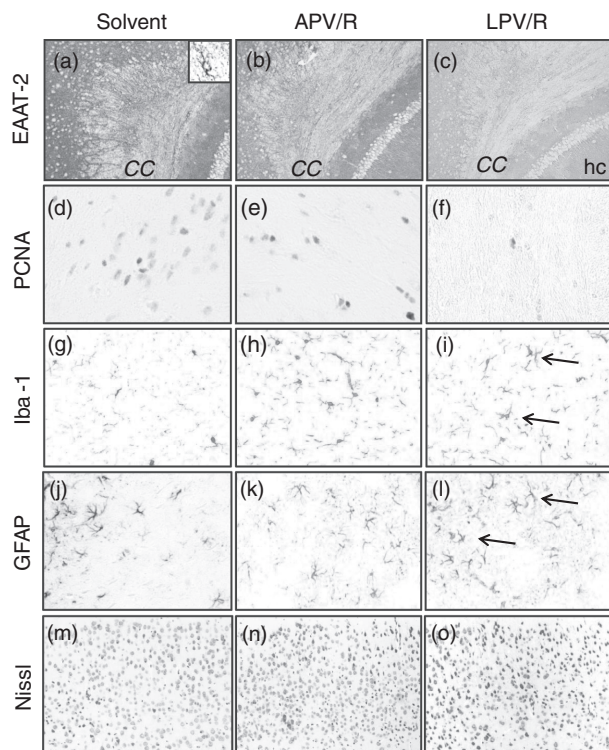


Fig. 5. Protease inhibitors cause neuropathological changes in HIV-1 Vpr transgenic animals. (a) EAAT2 immunoreactivity was evident in solvent (vehicle)-treated animals in both corpus callosum (cc) and hippocampus (hc) but was reduced in (b) APV/R-treated animals and (c) LPV/R-treated animals in both white matter and cortex. (d) White matter PCNA immunolabeling was apparent in the solvent-treated group but diminished in both (e) APV/R-treated and (f) LPV/R-treated animals. (g) Cortical Iba-1 immunopositive microglia were apparent in the solvent-treated group but increased in number with hypertrophy in both (h) APV/R-treated and (i) LPV/R-treated animals (arrows indicate glial cells). (j) Cortical GFAP immunostaining on astrocytes was detected in solvent-treated animals (l) but was increased in LPV-treated animals (arrows indicate glial cells). Neuronal staining did not differ among (m) solvent-treated, (n) APV/R-treated animals or (o) LPV/R-treated animals. [Original magnification: (a)–(c), 50 \times ; (d)–(o), 200 \times .]

caused microglia and astrocyte activation, which was associated with reduced EAAT2 expression without neuronal loss.

Discussion

In the present study, exposure to two widely prescribed protease inhibitors caused reduced EAAT2 expression together with diminished intracellular abundance of glutamate that was accompanied by reduced expression of cell proliferation markers in human astrocytes. In addition, LPV enhanced the sensitivity to glutamate-

induced calcium fluxes in astrocytes. In-vivo studies showed that treatment with these same protease inhibitors, particularly LPV, resulted in suppression of total L-glutamate and L-aspartate levels in cortex in conjunction with reduced L-serine, which was associated with neurobehavioral deficits and neuropathological features indicative of reduced EAAT2 and increased gliosis. Although these neuropathogenic outcomes were more apparent for LPV, APV also exerted potentially neurotoxic effects. Indeed, these changes were largely functional with limited effects on cell viability based on both in-vitro and in-vivo studies. Nonetheless, both protease inhibitors produced these effects at concentrations or dosages used to achieve HIV-1 control in contemporary clinical care [14].

The potential neurotoxic effects of protease inhibitors have received attention in the literature with studies showing that APV and atazanavir, at concentrations similar to those in CSF levels, were toxic to primary rat cortical neurons [6]. Additionally, studies using a Stone T-maze proposed that lipodystrophy and insulin resistance might contribute to impaired neurological performance induced by LPV [15,16]. Of interest, HIV-1 Vpr is neurotoxic to human neuronal cells but we did not find additive neurotoxicity when primary human neurons were co-exposed to Vpr with protease inhibitors (data not shown). Similar to a previous study, in which LPV induced astrocyte and monocyte/microglia activation [16], we found that LPV treatment also caused gliosis. One of the major responsibilities of astrocytes is the removal of extracellular glutamate from glutamatergic synapses via the EAAT2 [30]. The level of glutamate in neurons is maintained by the glutamate-glutamine cycle between astrocytes and neurons. The substrates for glutamate *de novo* synthesis are α -ketoglutarate from the citric-acid cycle and the amino acids aspartate or alanine. The levels of glutamine in ritonavir boosted APV and LPV-treated mice were comparable to vehicle-treated mice (Supp. Fig. 5A, <http://links.lww.com/QAD/A827>). Our studies suggested LPV and APV (to a lesser extent) exerted direct suppressive actions on EAAT2 expression, making astrocytes more responsive to glutamate as signaled by increased calcium activation; indeed, this finding is in agreement with the reduced EAAT2 expression resulting in diminished glutamate uptake and as a consequence depleted intracellular glutamate levels. The in-vivo corollary to this finding was the reduced total glutamate and aspartate levels in cortex, which might reflect a reduction in the intracellular pool of amino acids, as only a small fraction of total brain glutamate or aspartate is located in the extracellular space. Of interest, previous reports from our group showed that amino-acid levels in the cortex of feline immunodeficiency virus-infected cats revealed a trend toward reduced glutamate levels and reduced glutamate receptor expression although there were no concentration differences compared with uninfected

animals for other amino acids (e.g. glutamine, GABA, aspartate, L-serine, and alanine) except for a reduction in D-serine [24].

The present studies represent experimental efforts to model the events arising from chronic exposure to contemporary antiretroviral therapies, which have garnered increasing attention in the literature because of the high prevalence of neurocognitive impairment among HIV-infected persons receiving cART [31]. Nonetheless, there are several issues that remain unclear from the present studies. Single drugs were investigated herein but in the clinical setting, three or more drugs are utilized; we considered adding in other antiretroviral drugs but the delivery or multiple drugs together with the interpretation of the findings would be complicated by potential drug interactions that might be specific to the model being used. Another issue was the use of the Vpr transgenic animals, which express Vpr protein in microglia and exhibit a neurocognitive impairment phenotype but without substantial neuroinflammation; however, among HIV-infected individuals, other viral proteins as well as both systemic and CNS inflammation are integral factors that might exert other unrecognized effects on antiretroviral therapy bioavailability and actions. Finally, the implementation of multiple models and assays herein led to some divergent findings including the relative lack of neurotoxic effects of APV compared with LPV in some studies; these dichotomous results likely reflect the limitations of the present models and may be resolved by larger clinical studies in the future.

In the current studies, cell death did not appear to be a feature of protease inhibitor-induced astrocyte perturbation. In-vitro studies showed overall astrocyte viability was unaffected by exposure to both protease inhibitors based on β -III-tubulin expression. Similarly, gliosis was evident in the LPV-treated animals whereas neuronal density in cortex (Fig. 5) was unaffected by either protease inhibitor. PCNA transcript levels in cultured astrocytes and PCNA protein expression in white matter glial cell nuclei in brain were repressed by LPV treatment in keeping with its role in cell proliferation. EAAT2 expression was similarly reduced by LPV treatment in both in-vitro and in-vivo studies. These latter observations point to functional disruptions of LPV-treated astrocytes including selective transcriptional repression. In contrast, Iba-1 and to a lesser extent GFAP expression *in vivo* were increased likely reflecting these proteins function in cell structure and capacity to respond cell injury. The immediate consequence of these changes in astrocytes is a reduced ability to handle excess extracellular glutamate potentially leading to eventual excitotoxicity, perhaps resulting in neuronal dysfunction with associated neurobehavioral effects. As astrocytes represent the principal cell type in the brain, the capacity of protease inhibitors to wield wide ranging effects in the brain is substantial and might underlie the present

neurobehavioral observations of protease inhibitor-induced actions. Future studies are warranted to delineate the precise mechanism(s) by which protease inhibitors exert their neurotoxicity effects.

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Conflicts of interest

None of the authors have commercial interests in the present studies.

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