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Parallel high throughput neuronal toxicity assays demonstrate uncoupling between loss of mitochondrial membrane potential and neuronal damage in a model of HIV-induced neurodegeneration

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Abstract

Neurocognitive deficits seen in HIV-associated neurocognitive disorders (HAND) are attributed to the release of soluble factors from CNS-resident, HIV-infected and/or activated macrophages and microglia. To study HIV-associated neurotoxicity, we used our in vitro model in which primary rat neuronal/glial cultures are treated with supernatants from cultured human monocyte-derived macrophages, infected with a CNS-isolated HIV-1 strain (HIV-MDM). We found that neuronal damage, detected as a loss of microtubule-associated protein-2 (MAP2), begins as early as 2 hrs and is preceded by a loss of mitochondrial membrane potential ($\Delta\psi_m$). Interestingly, inhibitors of calpains, but not inhibitors of caspases, blocked MAP2 loss, however neither type of inhibitor prevented the loss of $\Delta\psi_m$. To facilitate throughput for these studies, we refined a MAP2 cell-based-ELISA whose data closely compare with our standardized method of hand counting neurons. In addition, we developed a tetramethyl rhodamine methyl ester (TMRM)-based multi-well fluorescent plate assay for the evaluation of whole culture $\Delta\psi_m$. Together, these findings indicate that calpain activation and loss of $\Delta\psi_m$ may be parallel pathways to death in HIV-MDM-treated neurons and also demonstrate the validity of plate assays for assessing multiple experimental parameters as is useful for screening neurotherapeutics for neuronal damage and death.

Keywords

neuroinflammation; neurotoxicity; excitotoxicity; mitochondria; calpain; neurovirology

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1. Introduction

It is estimated that HIV-associated neurocognitive disorders (HAND) continue to affect up to 50% of the HIV-positive population despite the introduction of combination antiretroviral therapies over a decade ago (Boisse et al., 2008; Brew et al., 2008; Ellis et al., 2007; Robertson et al., 2007; Tozzi et al., 2005). Although damage and loss of neurons are the primary pathologic correlates of neurocognitive deficits in HAD, little evidence supports productive viral infection of neurons in vivo (Gonzalez-Scarano and Martin-Garcia, 2005). Instead, two mechanisms of damage have been proposed, one or both of which may cause neurodegeneration in this disease: 1) damage is mediated by neurotoxic viral proteins and/or 2) damage is caused by neurotoxic factors (e.g. metabolites, cytokines, etc.) released as part of the concurrent perivascular inflammatory response that occurs near the regions of HIV entry into the brain.

We and others have previously described in vitro models to test the hypothesis that activated brain macrophages secrete soluble factors that can mediate neuronal damage (Jiang et al., 2001; O'Donnell et al., 2006). In our model, supernatants from HIV-infected, monocyte-derived macrophages (HIV-MDM) are collected at the time of peak infection and applied to 2-3-week-old primary rat hippocampal or cortical cultures for a 24 hr period. Treatment with HIV-MDM results in an NMDA receptor-dependent neuronal loss that is mediated by heat- and protease-resistant soluble excitotoxins under 3 kDa in size (O'Donnell et al., 2006). During classic NMDA receptor-mediated excitotoxic insults, cytoplasmic levels of Ca^{2+} are increased through activated ion channels (Choi, 1994). The influx of Ca^{2+} that occurs with these insults can result in depolarization of mitochondrial membrane potential ($\Delta\psi_m$) and mitochondrial dysfunction, ultimately contributing to neuronal damage and death (Brookes et al., 2004; Duchen, 2000; Gunter et al., 1994; Keelan et al., 1999; Khodorov et al., 1996; Schinder et al., 1996). Additionally, increased levels of Ca^{2+} can also directly activate cell-death proteases, such as the Ca^{2+} dependent protease, calpain, or caspases (Foos and Wu, 2002; Tian et al., 2008). Galicia and colleagues (2002) have suggested that increased cytoplasmic Ca^{2+} may play a role in neuronal death in HAD.

In this paper, we present data that further characterizes the neuronal death and damage that occurs in our in vitro model of HIV-associated neurotoxicity. Importantly, we have developed and employed two assays that have allowed us to quickly screen multiple variables and endpoints in order to evaluate the impact of HIV-mediated toxicity and potential therapeutics in our primary rat neural cultures. To develop these assays, we have built on and refined existing high-throughput multi-well techniques for evaluating the cellular components of our cultures, including 1) a cell-based-enzyme-linked immunosorbent assay (CB-ELISA) (Doherty et al., 1984; Sedgwick and Czerkinsky, 1992; Wang et al., 2007) that detects the neuronal marker, MAP2, thereby allowing assessment of neuronal death and damage, and 2) a tetramethyl rhodamine methyl ester (TMRM)-based multi-well fluorescent plate assay for assessing $\Delta\psi_m$ (White et al., 2007). Using these assays, we have carefully determined the time frame of neuronal damage following exposure of neuronal cultures to HIV-MDM. We have also determined that significant loss of $\Delta\psi_m$ occurs prior to any detectable decrease in MAP2 expression.

2. Materials and Methods

2.1 Primary rat mixed cortical cultures

Primary rat cortical cultures were prepared from embryonic day 17 Sprague Dawley rat pups, as described previously (Brewer, 1995). Cells were plated on surfaces coated with poly-L-lysine (Peninsula Laboratories, San Carlos, CA) and maintained in neurobasal media (Invitrogen) with B27 supplement (Invitrogen) at 37°C, 5% CO_2 . Half of the medium was

replenished every seven days and cultures were utilized at 14-28 days in vitro (DIV), unless otherwise indicated. These cultures contain both neurons and astrocytes.

2.2 Monocyte-derived macrophage (MDM) cultures and supernatants

Supernatants from HIV-infected, monocyte-derived macrophages (HIV-MDM) were derived from human MDMs infected with a primary, macrophage tropic, HIV-1 isolate (Jago) collected from cell-free CSF of a patient with confirmed HAD, as described previously (Chen et al., 2002). Only HIV-MDM supernatants exhibiting productive infection (e.g. p24 levels greater than 100 pg/ml of supernatant) were used to treat primary rat mixed cortical cultures. HIV-MDM or supernatants from mock-infected MDM (Mock-MDM) were collected at selected time points after infection and stored at -80°C .

Supernatants derived from MDM productively infected by HIV were consistently neurotoxic, and for each particular monocyte donor, the magnitude of MDM toxicity generally corresponded to p24 or HIV-RT levels. However, the degree of Mock- and HIV-MDM toxicity and the corresponding RT or p24 levels varied from donor to donor in a manner that could not be correlated between donors. Because different experiments utilizing Mock- and HIV-MDMs could not be normalized based on infectious criteria, we carefully screened Mock- and HIV-MDM toxicity at several dilutions and compared experimental results, and then used different HIV-MDMs only when they achieved degrees of neuronal toxicity within 10% of each other (O'Donnell et al., 2006; Wang et al., 2007). Pharmacologic compounds tested for their ability to impact MDM supernatant toxicities were added to the cultures ~20-30 min prior adding to the supernatants and remained in the cultures throughout the experiments.

2.3 Western blotting of primary cell cultures

Whole cell extracts were prepared from primary rat cortical cultures plated at a density of 8×10^5 cells per 35 mm dish and Western blotting was conducted, as described previously (Wang et al., 2007). Primary antibodies used: rabbit anti-cleaved caspase-3 Asp 175 (1:1000, Cell Signaling Technology, Beverly, MA), rabbit anti-caspase-cleaved spectrin A246 (1:5000, gift of Dr. Robert Siman, University of Pennsylvania, Philadelphia, PA) (Zhang et al., 2002), rabbit anti-calpain-cleaved spectrin A38 (1:15,000, gift of Dr. Robert Siman, Roberts-Lewis et al., 1994), or rabbit anti-actin (1:1,000, Sigma).

2.4 MAP2 CB-ELISA assay

Primary rat cortical neuroglial cultures were plated in poly-L-lysine-coated 96-well plates at concentrations of 4×10^4 to 5×10^4 cells per well. Following treatments, cells were fixed for 30 min with 4% paraformaldehyde in 4% sucrose. After blocking for 1 h with 5% normal goat serum in phosphate buffered saline (PBS), plates were incubated with mouse anti-MAP2 antibody (SMI-52, 1:1000, Covance, Berkeley, CA) diluted in Normal Antibody Diluent (ScyTek Labs, Logan, UT, USA) overnight at 4°C , followed by washing with PBS with 0.1% Tween-20 (PBS-T). The plates were then incubated for 30 min with goat anti-mouse secondary antibody conjugated to beta-lactamase TEM-1 (1:500 or $2 \mu\text{g/mL}$, Molecular Probes, Eugene, OR), washed with PBS-T followed by a wash with PBS to remove suds. Plates were then incubated, while protected from light, with $50 \mu\text{L}$ of $9.0 \mu\text{M}$ ($0.5 \mu\text{g}$ total) Fluorocillin Green substrate (Molecular Probes) per well, on a rocker at room temperature for 60 min. Fluorocillin Green substrate was not removed from wells after incubation. Fluorescence intensity was measured using a Fluoroskan Ascent fluorometer plate reader (Thermo Electron, Waltham, MA) with excitation at 485 nm and emission at 527 nm. These incubation conditions maximize the value of the relative mean fluorescence (RMF) compared with background obtained within the linear range of Fluorocillin (Fig. 1a). Relative changes in the intensity of background-subtracted fluorescent MAP2 signal are

used to estimate neuronal damage and/or protection and corresponded closely to hand-counted changes in the numbers of MAP2-positive cells (Fig. 2).

2.5 Immunofluorescent imaging

In culture plates reprocessed for immunofluorescent imaging following the MAP2 CB-ELISA assay, Fluorocillin substrate was washed out of the culture wells with PBS. Plates were reblocked and then relabeled overnight at 4°C, as above, with mouse anti-MAP2 (1:500) with or without rabbit anti-GFAP (1:200, DAKO, Carpinteria, CA). Following wash, goat anti-mouse Alexia 488 (1:200, Molecular Probes) with or without goat anti-rabbit Cy5 (1:200, Jackson ImmunoResearch, West Grove, PA) and DAPI (3 μ M, Molecular Probes) were applied for 30 min, washed, and then hand counted and/or imaged using a Nikon Eclipse TE300 inverted microscope or a Nikon Eclipse E400 fluorescent microscope (Nikon Corp, Tokyo, Japan) equipped with Olympus DP70 digital camera (Olympus Corp, Tokyo, Japan). Hand counts of MAP2-positive somas were performed on one central field per well using 10X objectives. Automated post-acquisition analysis on captured images was performed using MetaMorph 6.0 image analysis software (Universal Imaging, Downingtown, PA). One or more random fields from each culture well were captured using a 4X objective and analyzed for total MAP2 by measurement of integrated pixel intensity, which is defined as total MAP2 pixel intensity per image times the area of pixels positive for the MAP2 signal. In some experiments, 96-well plate cultures were pre-incubated with PI (15 μ M Sigma, St Louis, MO) either prior to treatments or prior to fixation in order to selectively label nuclei of membrane compromised/dead cells. PI labeling did not alter cell viability (White et al., 2003) and was not detectable at the 488/527 nm wavelength used in the MAP2 CB-ELISA. Automated object counts, filtered for minimal and maximal size criteria, of 4X images for DAPI and PI labeled nuclei were expressed as percentage of total nuclei (DAPI) labeled for PI.

2.6 Laser confocal imaging

Cultures were plated on poly-L-lysine-coated glass coverslips grown in 24-well plates. Following specified treatment, cultures were fixed, blocked, and labeled as above and mounted on glass slides with Citifluor AF1 (Citifluor, London, UK) before imaging with a Bio-Rad Radiance 2100 confocal microscope equipped with Argon, Green He/Ne, Red Diode and Blue Diode lasers (Bio-Rad), as described previously (Strachan et al., 2005b). All images shown were captured with uniform threshold and intensity settings.

2.7 Mitochondrial membrane potential ($\Delta\psi_m$) assay

The TMRM-based assay described in here was built upon a similar assay described previously (White et al., 2007). Specifically, changes in total culture $\Delta\psi_m$ were measured using the fluorescent dye TMRM (Molecular Probes). Cultures in 96-well plates were incubated in 1 μ M TMRM for 30 min. Cultures were washed twice and then solubilized with 50% DMSO and 0.5% Triton in distilled water to disperse TMRM from the cells and to deactivate virus, respectively. This solubilization avoids the self-quenching effects of high [TMRM] within healthy mitochondria. Any bubbles in the plate wells were then removed via washing or popped using a syringe needle, if necessary, prior to the fluorescence being assayed using the Fluoroskan Ascent fluorometer plate reader (535 nm excitation, >590 nm emission). $\Delta\psi_m$ depolarization was detected as a decrease in TMRM fluorescence. Some culture wells were treated with a proton carrier carbonyl cyanide m-chloro phenyl hydrazone (CCCP, 2 μ M) plus 12 μ M oligomycin to thoroughly depolarize $\Delta\psi_m$, permitting measurement of background fluorescence.

2.8 Statistical analysis

Values are expressed as mean $\pm$ SEM. Data was analyzed using Prism 3.0 (GraphPad Software, San Diego, CA). Values of $p < 0.05$ were considered significant.

3. Results

3.1 The MAP2 CB-ELISA accurately reflects neuronal numbers in primary cortical cultures

We have previously employed manual hand counting techniques to quantify the number of neurons surviving experimental treatments in our culture system (O'Donnell et al., 2006; Wang et al., 2007). However, due to its time-consuming nature, hand counting is not conducive to quantitative evaluation of variable-intensive experiments (e.g. those with multiple doses and/or timepoints). To facilitate throughput, we have developed a fluorescent MAP2 CB-ELISA assay, similar to that described previously (Fuhrmann et al., 1997). Importantly, because of the non-destructive nature of this assay, cellular structure and antigenicity remain intact for verification of assay results by more traditional hand-counting methods, or for further exploration of additional antigens of interest using multiple fluorescent labeling.

To test the sensitivity of the fluorescent MAP2 CB-ELISA, we seeded 96-well plates at increasing concentrations of mixed primary rat cortical cells from 5,000 up to 80,000 cells/well. To permit enough time for the development of MAP2 expression and for the initiation of neurite extension, we allowed the cells to attach and differentiate for 3 days. Because the aim of this particular experiment was to test the sensitivity and to validate the accuracy of our assay through the use of a quantifiable variable (i.e. number of cells plated), this short timeframe was important because it avoids the development of elaborated dendritic networks or of substantial cell death, both of which could complicate comparisons of the measured MAP2 fluorescence to the original seeding number. The fluorescent signals from this and other experiments were well within the linear range of Fluorocillin detection by our plate reader, 5-200 relative mean fluorescent (RMF) units (Fig. 1a,b). We found that the assay could easily determine statistical differences ($p < 0.05$) in MAP2 expression between seeding densities differing by 10,000 plated cells (Fig. 1b). This result, using 3 DIV cultures, demonstrates that our MAP2 CB-ELISA can discriminate a 20% difference in MAP2 levels at the seeding densities used in all our experiments ($4-5 \times 10^4$ cells per well). While all further experiments presented in this study were conducted using cultures 14 DIV or older, by which time the neurons have more robust MAP2 expression and more extensively elaborated processes, the data presented here using cultures 3 DIV serve to validate the accuracy and sensitivity of this new assay.

We next evaluated the efficacy of the assay in measuring neuronal loss in our primary rat cortical cultures treated with various dilutions of HIV-MDM supernatants. As demonstrated using laser confocal imaging of cells immunostained for MAP2 (Fig. 2a), mixed cortical cultures treated with HIV-MDM at a dilution of 1:10 begin to show dendritic beading and an accompanying loss of MAP2 by 2 hrs when compared with cultures treated with mock-MDM, which appear healthy. By 4hrs the vast majority of MAP2 has been lost in HIV-MDM-treated cultures. To determine the efficacy of our MAP2 CB-ELISA for measuring this HIV-MDM-induced neuronal loss, we used it in parallel with traditional hand counting to quantify loss induced by various dilutions of HIV-MDM. When evaluating the numbers of MAP2 positive cells in the wells of the 96-well plate utilizing the blinded manual hand counting technique, we found no statistical differences between the changes in neuronal number and the changes in MAP2 fluorescence for each experimental group (Fig. 2b). Additionally, because toxicity in our model is mediated by the NMDA glutamate receptor (O'Donnell, et al., 2006), we also determined that our MAP2 CB-ELISA can detect

changes in MAP2 fluorescence at titrated concentration of glutamate (FIG. 2c). These findings demonstrate that our assay is able to detect MAP2 loss due to our in vitro model itself, as well as that due to a well-characterized, quantifiable, toxic factor known to, at least partially, mediate neurotoxicity in this model.

3.2 HIV-MDM supernatants induce neuronal damage and death as measured by MAP2 CB-ELISA

To further characterize the neurotoxicity that occurs in our in vitro model of HIV-associated neuronal damage, we employed the MAP2 CB-ELISA to determine the toxicity, at multiple doses and time points, of supernatants from a single macrophage donor (Fig. 3). By 3 hr, cultures treated with a 1:10 dilution of HIV-MDM showed a greater than 60% loss of MAP2 relative mean fluorescence (RMF) as compared with untreated cultures. This loss was also significantly greater than the loss of MAP2 RMF in cultures treated with a 1:40 dilution of HIV-MDM ($p<0.001$) at 3 hr. However, by 12 hr, the 1:40 dilution of HIV-MDM also showed significant MAP2 RMF loss (~50%) compared with untreated cultures ($p<0.001$). Interestingly, by 60 hr, cultures treated with a 1:40 dilution of HIV-MDM showed significant recovery of MAP2 RMF levels compared with the 24 hr ($p<0.05$) and 36 hr time points ($*p<0.01$), whereas the MAP2 RMF values in cultures treated with the 1:10 dilution of HIV-MDM continued to decline over time and never recovered. These data suggest that, following exposure to the 1:40 dilution of HIV-MDM, a recovery or regrowth of dendrites may occur, which is a process that has been observed following other sub-lethal excitotoxic insults (Bellizzi et al., 2005). These data also support the ability of this assay to monitor recovery of dendritic damage following sublethal insults in order to distinguish between reversible and irreversible neuronal damage.

3.3 Calpain, not caspase, activation, contributes to HIV-MDM-induced neuronal damage and death

We have previously implicated a family of Ca^{2+} -activated proteases, the calpains, as cellular mediators of neuronal damage in cultures following treatment with HIV-MDM (O'Donnell et al., 2006; Wang et al., 2007). Here, via immunoblotting, we show a more than 9-fold increase in levels of calpain-cleaved spectrin following treatment with HIV-MDM over that in untreated cultures (Fig. 4a,b). The rise in calpain activity closely paralleled the decreases in MAP2 RMF in cultures, as evaluated by the MAP2 CB-ELISA (Fig. 3), even though the HIV-MDMs used in these two experiments were prepared from different macrophage donors. The decrease in the level of calpain-cleaved spectrin at 48 hr likely reflects the robust loss of neurons in HIV-MDM-treated cultures at that time point. It has also been suggested that caspase activation may play a role in HIV-mediated neuronal death (Adle-Biassette et al., 1995; Petito and Roberts, 1995; Ullrich et al., 2000). We therefore evaluated caspase activation in the same cultures in which we evaluated the activation of calpains. Compared with increases observed in levels of calpain-cleaved spectrin, we detected noticeably smaller increases in caspase-cleaved spectrin and in cleaved caspase-3 (Fig. 4a,b).

Since these results suggest that calpain activation is a component of the mechanism of neurotoxicity in our model, we evaluated the ability of the calpain inhibitor, MDL28170 (Mehdi et al., 1988), to protect neurons from HIV-MDM (Fig. 5a). We also tested whether the pan-caspase inhibitor, qVD-OPH (White et al., 2003), could protect our cultures from HIV-MDM-induced toxicity and, since previous studies have implicated NMDA receptors as the major mediator of neuronal death in cultures treated with HIV-MDM (Chen et al., 2002; Lipton, 1993; O'Donnell et al., 2006; Wang et al., 2007), we also examined whether the NMDA antagonist, MK801, could protect against MAP2 loss in our model. Further, because the severity of an excitotoxic insult can determine the mechanisms of cell death

(Ayata et al., 1997; Bonfoco et al., 1995; Lieberthal et al., 1998), we evaluated the ability of these pharmacologic inhibitors to protect from neurotoxic injury resulting from more (1:10 dilution) and less (1:40 dilution) severe insults of HIV-MDM treatment. As expected, treatment of cultures with MK801 prevented loss of MAP2 RMF when cultures were exposed to 1:10 and 1:40 dilutions of HIV-MDM (Figure 5a). Similar to the results above, treatment of cultures with MDL28170 prevented more than 50% of the HIV-MDM-induced MAP2 RMF loss seen in cultures exposed to 1:10 (* $p < 0.001$) and 1:40 ($p < 0.05$) dilutions of HIV-MDM. However, qVD-OPH was unable to prevent loss of MAP2 RMF in cultures exposed to either concentration of HIV-MDM. Further, no additional effect was seen when calpain inhibitors were used in combination with caspase inhibitors (data not shown). Additionally, to ensure that the lack of protection we found with the caspase inhibitor was not simply due to incorrect dosage of this pharmacologic inhibitor, we also tested differing doses of both the caspase inhibitor, qVD-OPH (Fig. 5b), and the calpain inhibitor, MDL28170 (Fig. 5c). While MDL28170 provided protection at several doses, none of the tested concentrations of qVD-OPH protected against MAP2 loss due to HIV-MDM treatment. Finally, as an additional experimental paradigm, we also tested an HIV-MDM insult that resulted in a less severe loss of MAP2 RMF (32%), and similarly, MDL28170, but not qVD-OPH, was protective (data not shown). These results confirm that calpain activation occurs following treatment with HIV-MDM and that this activation contributes to the resultant neurotoxicity, whereas the role of caspase activation in our model of HIV-induced neuronal damage appears to be far less prominent. Further, these studies demonstrate the efficacy of the MAP2 CB-ELISA in the evaluation of pharmacological agents as potential ameliorators of the neurotoxicity of HIV-MDMs in our model.

3.4 Mitochondrial Membrane potential is lost in neurons prior to loss of MAP2

To determine cellular events that may precede MAP2 loss in HIV-MDM-mediated injury, we first expanded our time points in the early hours of treatment using the MAP2 CB-ELISA, to pinpoint more precisely the onset of MAP2 loss following HIV-MDM treatment. A 1:20 dilution of HIV-MDM revealed no statistical loss of MAP2 RMF by 1 hr compared with untreated cultures (Fig. 6, closed symbols and solid lines). By 90 min, greater than 25% of MAP2 RMF was lost in HIV-MDM-treated cultures compared both with untreated cultures (* $p < 0.001$) and with those receiving a 1:20 dilution of Mock-MDM for the same length of time ($p < 0.01$). Then, in addition to examining MAP2 loss at these early time points, we used our TMRM-based multi-well fluorescent plate assay to examine the integrity of the mitochondrial potential ($\Delta\psi_m$) in parallel rat cortical cultures following treatment with HIV-MDM, because mitochondrial dysfunction is often associated with excitotoxic insults (Budd and Nicholls, 1996; Nicholls et al., 2007; Wang and Thayer, 1996), such as those incurred following HIV-MDM treatment in this model. TMRM is membrane-permeable and positively charged, and thus accumulates within the negatively charged mitochondrial matrix. As early as 15 min following treatment with HIV-MDM, significant loss (* $p < 0.001$) of TMRM fluorescence, as compared with untreated cultures, was evident (Fig. 6, open symbols and dotted lines). These data indicate that alterations in $\Delta\psi_m$ precede detectable loss of MAP2 or cell death. Mock-MDM-treated cultures also showed significantly reduced TMRM fluorescence by 60 min ($p < 0.05$), although it was a smaller loss than that seen in HIV-MDM-treated cultures. However, Mock-MDM had no effect on MAP2 RMF, suggesting that changes in $\Delta\psi_m$ may not be connected to MAP2 loss, or that a minimal threshold of $\Delta\psi_m$ loss is required for subsequent MAP2 loss and that this threshold is not achieved with Mock-MDM treatment. Therefore, the TMRM assay may be more sensitive than the MAP2 CB-ELISA in its ability to detect dysfunction or damage. However, it should be noted that the TMRM assay is unable to dissect out changes in particular cellular phenotypes, unlike the neuronal specificity inherent to the MAP2 CB-ELISA.

Next, we further evaluated $\Delta\psi_m$ at additional time points, and found significant loss ($*p<0.001$) of TMRM fluorescence in HIV-MDM-treated cultures as early as 2 min, as compared with cultures that did not receive HIV-MDM (Fig. 7). After 30 min, the loss of TMRM fluorescence steadily became more pronounced. Except at 2 min and 180 min, HIV-MDM-treated cultures had significantly lower TMRM fluorescence than Mock-MDM-treated cultures at each time point ($@p<0.05$, $^{\dagger}p<0.01$). Only at the 12 hr time point did the Mock-MDM-treated cultures show significantly reduced TMRM fluorescence compared with untreated cultures ($^{\wedge}p<0.05$).

3.5 Loss of mitochondrial membrane potential is independent of calpain activation

To determine whether calpain activation mediates loss of $\Delta\psi_m$ in cultures exposed to HIV- and Mock-MDM, we tested the calpain inhibitor, MDL28170, using our TMRM assay. Additionally, we conducted our cell damage and death assays in parallel with the TMRM assay to ensure consistency of the assays and continuity across the experimental paradigms. A greater than 60% loss of MAP2 was detected by the MAP2 CB-ELISA at 4 hr ($^{\wedge}p<0.05$) that was completely prevented by 10 μ M MDL28170 (Fig. 8a). Following the completion of the CB-ELISA assay, substrate was washed out of the wells, and cultures were relabeled with MAP2, GFAP, and DAPI. These cultures were then evaluated for morphology and using the more traditional assay of cell damage and death, PI-exclusion. By conventional epifluorescence microscopy and subsequent quantification of MAP2 staining by automated image processing, loss of MAP2-labeled dendrites and somas was apparent by the 4 hr time point (Fig. 8a,b, left column, green), and a loss of over 50% of integrated MAP2 pixel intensity occurred by 60 min, compared with untreated cultures ($*p<0.001$, Fig. 8a). These cultures also displayed an enhanced brightness in DAPI labeling suggesting increased condensation and fragmentation of nuclei and, further, showed an increase in nuclei labeling for PI, added prior to fixation, suggesting increased loss of nuclear membrane integrity and/or increased cell death by 4 hr (Fig. 8b, right column, red). When quantified, we observed a decrease in PI-exclusion (i.e. an increase in nuclei labeling for PI) (Fig. 8a), indicating a decrease in cell survival of over 60% at 4 hr in HIV-MDM-exposed cultures compared with untreated cultures ($*p<0.001$). As expected, based on our results with the MAP2 CB-ELISA, HIV-MDM-exposed cultures that had received MDL28170 treatment showed a much smaller loss of MAP2 integrated pixel intensity compared with HIV-MDM-exposed cultures that did not receive MDL28170 (Fig. 8a). Likewise, the decrease in cell survival as determined by PI-exclusion was prevented by treatment with MDL28170 (Fig. 8a). For any particular condition, there was no statistical difference between any of the cell death/damage assays in the % change from untreated at 4 hrs (Fig. 8a). In contrast, when we examined earlier time points (e.g. 1 hr) (Fig. 8c), PI labeling lagged behind loss of MAP2 RMF and MAP2 integrated pixel intensity. These results indicate that loss of nuclear membrane integrity is a later step in neuronal damage and demonstrate the increased sensitivity of the TMRM and MAP2 CB-ELISA assays in detecting early neuronal dysfunction or damage compared with methods such as PI-exclusion.

Evaluation of parallel cultures by the TMRM assay revealed that 10 μ M MDL28170 did not prevent loss of TMRM fluorescence at 1 or 4 hr after HIV-MDM treatment (Fig. 8d), despite the ability of MDL28170 to reduce MAP2 loss in parallel experiments (Fig. 8a,b). Loss of TMRM fluorescence seen in Mock-MDM-treated cultures was less severe than in HIV-MDM-treated cultures, and was also not prevented by MDL28170. Based on previous reports (O'Donnell et al., 2006; Wang et al., 2007), MDL28170 at the concentration used is expected to inhibit calpains completely, which suggests that the loss of $\Delta\psi_m$ seen here may be upstream or independent of calpain activation.

4. Discussion

Progression of HIV-associated neurocognitive disorder has been linked to factors released from HIV-infected and/or activated macrophages that have infiltrated the central nervous system (Gonzalez-Scarano and Martin-Garcia, 2005; Kaul et al., 2001; Lipton, 1993). To model this disease in vitro, we have used primary rat cortical cultures as a substrate to determine the mechanisms of neurotoxicity induced by supernatants from HIV-infected human macrophages. Using this model, we have employed a novel MAP2 CB-ELISA to show that sub-lethal excitotoxic HIV-MDM-induced neuronal damage may be reversible (Fig. 3). Our MAP2 CB-ELISA, through the use of Fluorocillin, demonstrates an increased sensitivity over previous CB-ELISA assays (Qin et al., 2006; Thery et al., 1991), and it, therefore, may be sensitive enough to detect dendritic damage prior to, or in absence of, cell death. Dendritic damage has been implicated as an important neuropathological correlate of HIV-related cognitive changes (Masliah et al., 1997). In previous in vitro studies of excitotoxicity, exposure to sub-lethal excitotoxic stimuli caused dendritic beading (Al-Noori and Swann, 2000; Hasbani et al., 1998; Park et al., 1996), retraction of spines (Hasbani et al., 2001), and loss of synaptic transmission (Ikegaya et al., 2001), all of which recover after washout of the excitotoxin (Bellizzi et al., 2005; Ellis et al., 2007). Our results suggest this could also be occurring in our model when HIV-MDM-mediated damage is sub-lethal.

Besides the increased sensitivity demonstrated by our MAP2 CB-ELISA, this assay also allowed for a quick determination of the neuronal protection provided by a range of doses of several pharmacologies against treatments of multiple concentrations of HIV-MDM. Specifically, we found that inhibition of calpains by MDL28170, but not of caspases by qVD-OPH, was effective in protecting neurons from HIV-MDM (Fig. 5). These findings suggest that a delayed necrotic-type death may be responsible for neuronal loss following treatment with HIV-MDM, which corresponds well to the conditions of intracellular Ca^{2+} dysregulation that have been reported in other models of excitotoxicity (Budd and Nicholls, 1996; Nicholls et al., 2007; Wang and Thayer, 1996). Interestingly, loss of MAP2 RMF detected at early time points (i.e. 4hr, Fig. 8a) is completely prevented by calpain inhibitors, suggesting that this early damage may be primarily mediated by calpain activation. However, by later time points (20-25 hrs, Fig. 5a,c), inhibition of calpains only prevents $53.2 \pm 14.5\%$ to $63.2 \pm 6.4\%$ of the loss of MAP2 RMF. Because the calpain inhibitor is present in the cultures throughout the experiment, it is possible that the calpain inhibitor may eventually become overwhelmed. However, it is also possible that additional mechanisms of neuronal damage may become involved, such as cell cycle machinery dysfunction, endoplasmic reticulum stress, or cytoskeletal disruption (Bezprozvanny and Mattson, 2008; Byun et al., 2008; Caron et al., 2008; Harris and DeLorenzo, 1987; Jordan-Sciutto et al., 2002; Lindholm et al., 2006; Lindl et al., 2007; Macleod, 2008; Mattson, 2007; Paschen and Mengesdorf, 2005; Strachan et al., 2005a)

In addition to our MAP2 CB-ELISA, in this study we have also implemented the use of a multi-well, TMRM-based assay to evaluate $\Delta\psi_m$ in our cultures. Using this assay, we have found that loss of $\Delta\psi_m$ occurs prior to any detectable loss of MAP2 (Fig. 6) and as early as 2 min following treatment with HIV-MDM (Fig. 7). This loss of $\Delta\psi_m$ is not prevented by calpain inhibition (Fig. 8d), suggesting the possibility that loss of $\Delta\psi_m$ may be upstream or disconnected from the subsequent loss of MAP2 in our HIV-MDM-treated cultures. Disruption of $\Delta\psi_m$ may result in an energy crisis or, perhaps indirectly, in an increased production of reactive oxygen species (Sengpiel et al., 1998; Vesce et al., 2004; White and Reynolds, 1996). These consequences could contribute to neuronal damage, as well as to delayed necrosis resulting from an uncontrolled rise in cytoplasmic free Ca^{2+} (Jekabsons and Nicholls, 2004; Ward et al., 2000). It is possible that loss of cytoplasmic free Ca^{2+} regulation could result in the activation of Ca^{2+} -activated proteases (Markowitz et al., 2007),

thereby contributing to some portion of the neuronal damage that is seemingly due to calpains in our model.

In conclusion, the data present here suggest that the neuronal loss and damage in our model may be reversible early in disease pathogenesis, suggesting the possibility for ameliorative intervention of excitotoxic damage in the early stages of HAND. Further, neuronal damage is preceded by mitochondrial dysfunction, and calpains, possibly activated as a downstream consequence of loss of $\Delta\psi_m$, seem to play a primary role in this neuronal damage. Finally, our MAP2 CB-ELISA and TMRM-based assays are efficient and effective tools for ascertaining parameters of damage or viability in neuronal cultures.

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References

- Adle-Biassette H, Levy Y, Colombel M, Poron F, Natchev S, Keohane C, Gray F. Neuronal apoptosis in HIV infection in adults. *Neuropathol Appl Neurobiol*. 1995; 21:218–227. [PubMed: 7477730]
- Al-Noori S, Swann JW. A role for sodium and chloride in kainic acid-induced beading of inhibitory interneuron dendrites. *Neuroscience*. 2000; 101:337–348. [PubMed: 11074157]
- Ayata C, Ayata G, Hara H, Matthews RT, Beal MF, Ferrante RJ, Endres M, Kim A, Christie RH, Waeber C, Huang PL, Hyman BT, Moskowitz MA. Mechanisms of reduced striatal NMDA excitotoxicity in type I nitric oxide synthase knock-out mice. *J Neurosci*. 1997; 17:6908–6917. [PubMed: 9278526]
- Bellizzi MJ, Lu SM, Masliah E, Gelbard HA. Synaptic activity becomes excitotoxic in neurons exposed to elevated levels of platelet-activating factor. *J Clin Invest*. 2005; 115:3185–3192. [PubMed: 16276420]
- Bezprozvanny I, Mattson MP. Neuronal calcium mishandling and the pathogenesis of Alzheimer's disease. *Trends Neurosci*. 2008; 31:454–463. [PubMed: 18675468]
- Boisse L, Gill MJ, Power C. HIV infection of the central nervous system: clinical features and neuropathogenesis. *Neurol Clin*. 2008; 26:799–819. [PubMed: 18657727]
- Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci USA*. 1995; 92:7162–7166. [PubMed: 7638161]
- Brew BJ, Crowe SM, Landay A, Cysique LA, Guillemin G. Neurodegeneration and Ageing in the HAART Era. *J Neuroimmune Pharmacol*. 2008 Epub Dec 6.
- Brewer GJ. Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. *J Neurosci Res*. 1995; 42:674–683. [PubMed: 8600300]
- Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol*. 2004; 287:C817–833. [PubMed: 15355853]
- Budd SL, Nicholls DG. Mitochondria, calcium regulation, and acute glutamate excitotoxicity in cultured cerebellar granule cells. *J Neurochem*. 1996; 67:2282–2291. [PubMed: 8931459]
- Byun HO, Kim HY, Lim JJ, Seo YH, Yoon G. Mitochondrial dysfunction by complex II inhibition delays overall cell cycle progression via reactive oxygen species production. *J Cell Biochem*. 2008; 104:1747–1759. [PubMed: 18395845]

- Caron M, Auclair M, Vissian A, Vigouroux C, Capeau J. Contribution of mitochondrial dysfunction and oxidative stress to cellular premature senescence induced by antiretroviral thymidine analogues. *Antivir Ther.* 2008; 13:27–38. [PubMed: 18389896]
- Chen W, Sulcove J, Frank I, Jaffer S, Ozdener H, Kolson DL. Development of a human neuronal cell model for human immunodeficiency virus (HIV)-infected macrophage-induced neurotoxicity: apoptosis induced by HIV type 1 primary isolates and evidence for involvement of the Bcl-2/Bcl-xL-sensitive intrinsic apoptosis pathway. *J Virol.* 2002; 76:9407–9419. [PubMed: 12186923]
- Choi DW. Calcium and excitotoxic neuronal injury. *Ann N Y Acad Sci.* 1994; 747:162–171. [PubMed: 7847669]
- Doherty P, Dickson JG, Flanigan TP, Walsh FS. Quantitative evaluation of neurite outgrowth in cultures of human foetal brain and dorsal root ganglion cells using an enzyme-linked immunoadsorbent assay for human neurofilament protein. *J Neurochem.* 1984; 42:1116–1122. [PubMed: 6421998]
- Duchen MR. Mitochondria and calcium: from cell signalling to cell death. *J Physiol.* 2000; 529(Pt 1): 57–68. [PubMed: 11080251]
- Ellis R, Langford D, Masliah E. HIV and antiretroviral therapy in the brain: neuronal injury and repair. *Nat Rev Neurosci.* 2007; 8:33–44. [PubMed: 17180161]
- Foos TM, Wu JY. The role of taurine in the central nervous system and the modulation of intracellular calcium homeostasis. *Neurochem Res.* 2002; 27:21–26. [PubMed: 11926272]
- Fuhrmann S, Kirsch M, Wewetzer K, Hofmann HD. Use of cell ELISA for the screening of neurotrophic activities on minor cell populations in retinal monolayer cultures. *J Neurosci Methods.* 1997; 75:199–205. [PubMed: 9288653]
- Galicía O, Sanchez-Alavez M, Mendez Diaz M, Navarro L, Prospero-Garcia O. HIV glycoprotein 120: possible etiological agent of AIDS-associated dementia. *Rev Invest Clin.* 2002; 54:437–452. [PubMed: 12587419]
- Gonzalez-Scarano F, Martin-Garcia J. The neuropathogenesis of AIDS. *Nat Rev Immunol.* 2005; 5:69–81. [PubMed: 15630430]
- Gunter TE, Gunter KK, Sheu SS, Gavin CE. Mitochondrial calcium transport: physiological and pathological relevance. *Am J Physiol.* 1994; 267:C313–339. [PubMed: 8074170]
- Harris JK, DeLorenzo RJ. Calcium and neuronal cytoskeletal proteins: alterations with aging. *Neurobiol Aging.* 1987; 8:359–361. [PubMed: 2819753]
- Hasbani MJ, Hyrc KL, Faddis BT, Romano C, Goldberg MP. Distinct roles for sodium, chloride, and calcium in excitotoxic dendritic injury and recovery. *Exp Neurol.* 1998; 154:241–258. [PubMed: 9875285]
- Hasbani MJ, Schlieff ML, Fisher DA, Goldberg MP. Dendritic spines lost during glutamate receptor activation reemerge at original sites of synaptic contact. *J Neurosci.* 2001; 21:2393–2403. [PubMed: 11264313]
- Ikegaya Y, Kim JA, Baba M, Iwatsubo T, Nishiyama N, Matsuki N. Rapid and reversible changes in dendrite morphology and synaptic efficacy following NMDA receptor activation: implication for a cellular defense against excitotoxicity. *J Cell Sci.* 2001; 114:4083–4093. [PubMed: 11739640]
- Jekabsons MB, Nicholls DG. In situ respiration and bioenergetic status of mitochondria in primary cerebellar granule neuronal cultures exposed continuously to glutamate. *J Biol Chem.* 2004; 279:32989–33000. [PubMed: 15166243]
- Jiang ZG, Piggee C, Heyes MP, Murphy C, Quearry B, Bauer M, Zheng J, Gendelman HE, Markey SP. Glutamate is a mediator of neurotoxicity in secretions of activated HIV-1-infected macrophages. *J Neuroimmunol.* 2001; 117:97–107. [PubMed: 11431009]
- Jordan-Sciutto KL, Wang G, Murphey-Corb M, Wiley CA. Cell cycle proteins exhibit altered expression patterns in lentiviral-associated encephalitis. *J Neurosci.* 2002; 22:2185–2195. [PubMed: 11896158]
- Kaul M, Garden GA, Lipton SA. Pathways to neuronal injury and apoptosis in HIV-associated dementia. *Nature.* 2001; 410:988–994. [PubMed: 11309629]
- Keelan J, Vergun O, Duchen MR. Excitotoxic mitochondrial depolarisation requires both calcium and nitric oxide in rat hippocampal neurons. *J Physiol.* 1999; 520(Pt 3):797–813. [PubMed: 10545145]

- Khodorov B, Pinelis V, Vergun O, Storozhevych T, Vinskaya N. Mitochondrial deenergization underlies neuronal calcium overload following a prolonged glutamate challenge. *FEBS Lett.* 1996; 397:230–234. [PubMed: 8955353]
- Lieberthal W, Menza SA, Levine JS. Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells. *Am J Physiol.* 1998; 274:F315–327. [PubMed: 9486226]
- Lindholm D, Wootz H, Korhonen L. ER stress and neurodegenerative diseases. *Cell Death Differ.* 2006; 13:385–392. [PubMed: 16397584]
- Lindl KA, Akay C, Wang Y, White MG, Jordan-Sciutto KL. Expression of the endoplasmic reticulum stress response marker, BiP, in the central nervous system of HIV-positive individuals. *Neuropathol Appl Neurobiol.* 2007; 33:658–669. [PubMed: 17931354]
- Lipton SA. Human immunodeficiency virus-infected macrophages, gp120, and N-methyl-D-aspartate receptor-mediated neurotoxicity. *Ann Neurol.* 1993; 33:227–228. [PubMed: 8434889]
- Macleod KF. The role of the RB tumour suppressor pathway in oxidative stress responses in the haematopoietic system. *Nat Rev Cancer.* 2008; 8:769–781. [PubMed: 18800074]
- Markowitz AJ, White MG, Kolson DL, Jordan-Sciutto KL. Cellular interplay between neurons and glia: toward a comprehensive mechanism for excitotoxic neuronal loss in neurodegeneration. *Cellscience.* 2007; 4:111–146. [PubMed: 19122795]
- Masliah E, Heaton RK, Marcotte TD, Ellis RJ, Wiley CA, Mallory M, Achim CL, McCutchan JA, Nelson JA, Atkinson JH, Grant I, HNRC Group. The HIV Neurobehavioral Research Center. Dendritic injury is a pathological substrate for human immunodeficiency virus-related cognitive disorders. *Ann Neurol.* 1997; 42:963–972. [PubMed: 9403489]
- Mattson MP. Calcium and neurodegeneration. *Aging Cell.* 2007; 6:337–350. [PubMed: 17328689]
- Mehdi S, Angelastro MR, Wiseman JS, Bey P. Inhibition of the proteolysis of rat erythrocyte membrane proteins by a synthetic inhibitor of calpain. *Biochem Biophys Res Commun.* 1988; 157:1117–1123. [PubMed: 2849930]
- Nicholls DG, Johnson-Cadwell L, Vesce S, Jekabsons M, Yadava N. Bioenergetics of mitochondria in cultured neurons and their role in glutamate excitotoxicity. *J Neurosci Res.* 2007; 85:3206–3212. [PubMed: 17455297]
- O'Donnell LA, Agrawal A, Jordan-Sciutto KL, Dichter MA, Lynch DR, Kolson DL. Human immunodeficiency virus (HIV)-induced neurotoxicity: roles for the NMDA receptor subtypes. *J Neurosci.* 2006; 26:981–990. [PubMed: 16421318]
- Park JS, Bateman MC, Goldberg MP. Rapid alterations in dendrite morphology during sublethal hypoxia or glutamate receptor activation. *Neurobiol Dis.* 1996; 3:215–227. [PubMed: 8980022]
- Paschen W, Mengesdorf T. Endoplasmic reticulum stress response and neurodegeneration. *Cell Calcium.* 2005; 38:409–415. [PubMed: 16087231]
- Petito CK, Roberts B. Evidence of apoptotic cell death in HIV encephalitis. *Am J Pathol.* 1995; 146:1121–1130. [PubMed: 7747806]
- Qin S, Colin C, Hinners I, Gervais A, Cheret C, Mallat M. System Xc- and apolipoprotein E expressed by microglia have opposite effects on the neurotoxicity of amyloid-beta peptide 1-40. *J Neurosci.* 2006; 26:3345–3356. [PubMed: 16554485]
- Roberts-Lewis JM, Savage MJ, Marcy VR, Pinsker LR, Siman R. Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain. *J Neurosci.* 1994; 14:3934–3944. [PubMed: 8207497]
- Robertson KR, Smurzynski M, Parsons TD, Wu K, Bosch RJ, Wu J, McArthur JC, Collier AC, Evans SR, Ellis RJ. The prevalence and incidence of neurocognitive impairment in the HAART era. *AIDS.* 2007; 21:1915–1921. [PubMed: 17721099]
- Schinder AF, Olson EC, Spitzer NC, Montal M. Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. *J Neurosci.* 1996; 16:6125–6133. [PubMed: 8815895]
- Sedgwick JD, Czerkinsky C. Detection of cell-surface molecules, secreted products of single cells and cellular proliferation by enzyme immunoassay. *J Immunol Methods.* 1992; 150:159–175. [PubMed: 1613252]
- Sengpiel B, Preis E, Kriegstein J, Prehn JH. NMDA-induced superoxide production and neurotoxicity in cultured rat hippocampal neurons: role of mitochondria. *Eur J Neurosci.* 1998; 10:1903–1910. [PubMed: 9751160]

- Strachan GD, Koike MA, Siman R, Hall DJ, Jordan-Sciutto KL. E2F1 induces cell death, calpain activation, and MDMX degradation in a transcription independent manner implicating a novel role for E2F1 in neuronal loss in SIV encephalitis. *J Cell Biochem.* 2005a; 96:728–740. [PubMed: 16088944]
- Strachan GD, Kopp AS, Koike MA, Morgan KL, Jordan-Sciutto KL. Chemokine- and neurotrophic factor-induced changes in E2F1 localization and phosphorylation of the retinoblastoma susceptibility gene product (pRb) occur by distinct mechanisms in murine cortical cultures. *Exp Neurol.* 2005b; 193:455–468. [PubMed: 15869948]
- Thery C, Chamak B, Mallat M. Cytotoxic Effect of Brain Macrophages on Developing. *Eur J Neurosci.* 1991; 3:1155–1164. [PubMed: 12106245]
- Tian C, Erdmann N, Zhao J, Cao Z, Peng H, Zheng J. HIV-infected macrophages mediate neuronal apoptosis through mitochondrial glutaminase. *J Neurochem.* 2008; 105:994–1005. [PubMed: 18088378]
- Tozzi V, Balestra P, Lorenzini P, Bellagamba R, Galgani S, Corpolongo A, Vlassi C, Larussa D, Zaccarelli M, Noto P, Visco-Comandini U, Giulianelli M, Ippolito G, Antinori A, Narciso P. Prevalence and risk factors for human immunodeficiency virus-associated neurocognitive impairment, 1996 to 2002: results from an urban observational cohort. *J Neurovirol.* 2005; 11:265–273. [PubMed: 16036806]
- Ullrich CK, Groopman JE, Ganju RK. HIV-1 gp120- and gp160-induced apoptosis in cultured endothelial cells is mediated by caspases. *Blood.* 2000; 96:1438–1442. [PubMed: 10942389]
- Vesce S, Kirk L, Nicholls DG. Relationships between superoxide levels and delayed calcium deregulation in cultured cerebellar granule cells exposed continuously to glutamate. *J Neurochem.* 2004; 90:683–693. [PubMed: 15255947]
- Wang GJ, Thayer SA. Sequestration of glutamate-induced Ca²⁺ loads by mitochondria in cultured rat hippocampal neurons. *J Neurophysiol.* 1996; 76:1611–1621. [PubMed: 8890280]
- Wang Y, White MG, Akay C, Chodroff RA, Robinson J, Lindl KA, Dichter MA, Qian Y, Mao Z, Kolson DL, Jordan-Sciutto KL. Activation of cyclin-dependent kinase 5 by calpains contributes to human immunodeficiency virus-induced neurotoxicity. *J Neurochem.* 2007; 103:439–455. [PubMed: 17897354]
- Ward MW, Rego AC, Frenguelli BG, Nicholls DG. Mitochondrial membrane potential and glutamate excitotoxicity in cultured cerebellar granule cells. *J Neurosci.* 2000; 20:7208–7219. [PubMed: 11007877]
- White MG, Emery M, Nonner D, Barrett JN. Caspase activation contributes to delayed death of heat-stressed striatal neurons. *J Neurochem.* 2003; 87:958–968. [PubMed: 14622126]
- White MG, Luca LE, Nonner D, Saleh O, Hu B, Barrett EF, Barrett JN. Cellular mechanisms of neuronal damage from hyperthermia. *Prog Brain Res.* 2007; 162:347–371. [PubMed: 17645927]
- White RJ, Reynolds IJ. Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure. *J Neurosci.* 1996; 16:5688–5697. [PubMed: 8795624]
- Zhang C, Siman R, Xu YA, Mills AM, Frederick JR, Neumar RW. Comparison of calpain and caspase activities in the adult rat brain after transient forebrain ischemia. *Neurobiol Dis.* 2002; 10:289–205. [PubMed: 12270691]

Fig. 1. A MAP2 CB-ELISA using Fluorocillin Green substrate can detect variability in neuronal numbers in wells of a 96-well plate

(a) MAP2 RMF in the linear range of fluorescence is maximal (Brewer, 1995) compared with no primary control background when $0.5\mu\text{g}$ of Fluorocillin substrate is incubated for 60 min. (b) Background-subtracted MAP2 RMF values increase linearly with increased plating density of primary rat cortical neurons evaluated after 3 days in culture. $n=2-3$ wells (a), $n=14-16$ wells (b) one-way ANOVA, Newman-Keuls, $p<0.05$ for all differences in cell densities $\geq 10 \times 10^3$; in some cases the SEM was smaller than the plotting symbol.

Fig. 2. The MAP2 CB-ELISA accurately replicates results obtained from hand-counting
 (a) Cultures were labeled for MAP2 (red) and DAPI (blue) and imaged by immunofluorescent laser confocal microscopy at various timepoints (2, 4, and 20 hr) following the application of HIV- or Mock-MDM (1:10 dilutions). In HIV-MDM-treated cultures, dendritic beading is evident by 2hr, and widespread loss of MAP2-positive neurons and dendrites, as well as condensed nuclei, is pronounced by 20h. (b) 96-well plate cortical cultures were treated with serial dilutions of HIV-MDM for 40 hr and were assayed for changes in MAP2-positive neurons by MAP2 CB-ELISA (left) or by hand-counting MAP2-positive cells (right). Each dilution of HIV-MDM resulted in a significant decrease in % of MAP2 RMF (left) or % of neuron number (right) when compared with untreated. Each dilution of HIV-MDM was also significantly different from all other HIV-MDM treatments evaluated by the same assay ($p < 0.001$ one-way ANOVA, Newman-Keuls). For any particular dilution of HIV-MDM, there was no statistical difference in the % changes from untreated obtained from the MAP-CB ELISA (MAP2 RMF values) compared with the % change from untreated obtained from traditional hand-counting assay (number of MAP-2 positive cells) in the same plate ($p < 0.05$, one-way ANOVA, Bonferroni), $n = 6-12$ wells. (c) 96-well plate cortical cultures were treated with serial dilutions of glutamate for 24 hr. Values are shown normalized to vehicle control. Each increase concentration of glutamate resulted in a statistically significant ($p < 0.001$, one-way ANOVA, Newman-Keuls) observed increase in MAP2 RMF compared with all other dilutions, with two exceptions: for 1 μM glutamate compared with vehicle $p < 0.05$, and 10 μM compared with 5 μM is not statistically different.

Fig. 3. Cultures treated with HIV-MDM showed delayed loss of MAP2-positive dendrites and somas over time

Loss of MAP2 RMF as measured by MAP2 CB-ELISA in 2-week old cortical cultures either untreated or following treatment with 1:10 or 1:40 dilutions of HIV-MDM at 3, 6, 12, 24, 36, and 60 hr. By 12 hr, both dilutions of HIV-MDM showed significant loss of MAP2 RMF compared with untreated cultures. MAP2 RMF loss following treatment with a 1:40 dilution of HIV-MDM was significantly reversed at 60 hr compared with 36 hr. $n=12-16$ wells; one-way ANOVA, Newman-Keuls, * $p<0.01$ compared with 36 hr; in some cases the SEM was smaller than the plotting symbol.

Fig. 4. Calpain is robustly activated following HIV-MDM treatment

a) Western blots for calpain activity (calpain-cleaved spectrin) and caspase activity (caspase-cleaved spectrin and cleaved caspase-3) in untreated cultures or in those receiving 1:40 dilutions of HIV- or Mock-MDM for 4, 8, 20, or 48 hr. Blot for actin serves as a loading control. b) Western blots were quantified and normalized to actin. Changes in protein expression are expressed as a fold change from Mock-MDM-treated cultures, which is represented as a dotted line at 1. HIV-MDM-treated cultures show a 9-fold increase in calpain activity (boxes, solid line) compared with Mock-MDM-treated cultures. A less robust increase in caspase activity (0-2.4 fold increase; triangles, dashed lines) is seen following HIV-MDM treatment.

Fig. 5. An NMDA antagonist and a calpain inhibitor, but not a caspase inhibitor, prevent loss of MAP2 following exposure to HIV-MDM

(a) 5-week-old cultures were left unexposed or exposed for 20 hr to 1:40 or 1:10 dilutions of HIV- or Mock-MDM with or without treatment with 20 μM qVD-OPH, 10 μM MDL28170, or 10 μM MK801. There were no differences between any conditions in cultures exposed to Mock-MDM. At both dilutions of HIV-MDM, MDL28170 and MK801 significantly reduced the loss of MAP2 RMF. Cultures treated with qVD-OPH did not show statistical differences between treatments. $^{\wedge}p < 0.05$, $^{*}p < 0.001$ compared with vehicle-treated cultures receiving matching dilution of HIV-MDM (1:10 – black bars; 1:40 – hatched bars), or the vehicle-treated control cultures not receiving HIV-MDM (white bar) compared with vehicle-treated cultures receiving either 1:10 or 1:40 dilutions of HIV-MDM, one-way ANOVA, Newman-Keuls, $n = 13$ -30 wells. (b) MAP2 RMF loss in cultures exposed to a 1:10 dilution of HIV-MDM for 24 hr was not reduced when treated with various concentrations of qVD-OPH compared with vehicle-treated (0.5% DMSO) cultures. (c) In a separate experiment, cultures receiving a 1:10 dilution of HIV-MDM for 25 hr had reduced loss of MAP2 RMF when they were treated with 1, 10, and 100 μM MDL28170 compared with vehicle (1% DMSO) treated cultures. For b and c, v=vehicle; $^{\wedge}p < 0.05$, $^{*}p < 0.001$ compared with vehicle in HIV-MDM-treated cultures, one-way ANOVA, Dunnett's, $n = 6$ -24 wells. All values are presented as a percentage of vehicle-treated control cultures (dash line, open circle). Maximum MAP2 RMF loss is indicated with a dashed line with closed circle. In some cases the SEM was smaller than the plotting symbol.

Fig. 6. Loss of MAP2 and neuronal damage occurs within the first hrs following HIV-MDM exposure

Cortical cultures exposed to a 1:20 dilution of HIV-MDM showed decreased TMRM fluorescence (open symbols and dotted lines) by 15 min and decreased MAP2 RMF by 90 min (closed symbols and solid lines). Cccp+oligomycin was used to uncouple mitochondrial electron chain transport and thoroughly depolarize cultures in order to assess background TMRM fluorescence (2.34 ± 0.90) and is indicated by a dotted line. For visual purposes, untreated control cultures are placed near the 0 min time point at slightly offset (-0.8, 0, and 0.8) x-axis positions in order to avoid symbol overlap. [^] $p < 0.05$, ^{*} $p < 0.001$ compared with untreated, one-way ANOVA, Dunnett's; [†] $p < 0.01$, [#] $p < 0.001$ compared with Mock-MDM at the same time point, unpaired two-tailed t-test, $n = 6-12$ wells; in some cases the SEM was smaller than the plotting symbol.

Fig. 7. Reduction of culture $\Delta\psi_m$ occurs within 2 min following exposure to HIV-MDM
 One-month-old cultures were exposed to a 1:10 dilution of HIV- or Mock-MDM and assayed for $\Delta\psi_m$ by TMRM fluorescence. Cccp+oligomycin was used to depolarize cultures in order to assess background TMRM fluorescence (6.38 ± 0.37) and is indicated by a dotted line. $n=6-8$ wells; $^{\wedge}p<0.05$, $*p<0.001$ compared with untreated, one-way ANOVA, Dunnet's; $@p<0.05$, $^{\dagger}p<0.01$ compared with Mock-MDM at the same time point, unpaired two-tailed t-test.

Fig. 8. MDL28170 protects from neuronal damage, but does not protect from loss of $\Delta\psi_m$

Two-week-old cortical cultures were exposed to a 1:20 dilution of HIV- or Mock-MDM. a) Neuronal damage, as determined by loss of MAP2 RMF by the MAP2 CB-ELISA and by automated image analysis expressed as integrated pixel intensity of MAP2, is significant by 4 hr and is prevented by MDL28170. In addition, exclusion of PI by cells in the cultures is decreased at 4 hr and is also prevented by MDL28170. b) Wells used in the MAP2 assay were reused for immunofluorescent imaging. Images in the left column show MAP2 (green), GFAP (red), and DAPI (blue). Images on the right column show PI labeling (red) in the same field. PI was added prior to fixation of the cultures in order to label only cells with compromised membranes. Little damage is seen by 1 hr (not shown), but by 4 hr loss of MAP2 and increased PI labeling are evident. c) MAP2 loss (solid symbols, solid lines) is detectable prior to loss of PI-exclusion (open symbols, dotted lines) following treatment with HIV-MDM. d) MDL28170 fails to prevent loss of TMRM fluorescence following HIV- or Mock-MDM treatment at 1 or 4 hr. Cccp+oligomycin was used to depolarize cultures in order to assess background TMRM fluorescence (4.43 ± 0.82) and is indicated by a dotted line. $^{\dagger}p < 0.05$, $^*p < 0.01$, $^{***}p < 0.001$ compared with assay specific b) HIV-MDM treated or c) untreated cultures, one-way ANOVA, Newman-Keuls, $n = 4-16$ wells.