Neuroprotective Mechanisms of Lithium in Murine Human Immunodeficiency Virus-1 Encephalitis

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Lithium (Li) has garnered considerable interest as a neuroprotective drug for a broad range of nervous system disorders. Its neuroprotective activities occur as a consequence of glycogen synthase kinase-3β (GSK-3β) inhibition leading to downstream blockade of β-catenin and Tau phosphorylation. In the present study, we investigated Li-mediated neuroprotective mechanisms in laboratory and murine human immunodeficiency virus-1 (HIV-1) encephalitis (HIVE) models. In laboratory tests, Li protected neurons from neurotoxic secretions of HIV-1-infected monocyte-derived macrophages (MDMs). This neuroprotection was mediated, in part, through the phosphatidylinositol 3-kinase/Akt and GSK-3β pathways. To examine the effects of Li treatment in vivo, MDMs were injected into the basal ganglia of severe combined immunodeficient mice and then Li was administered (60 mg/kg/d). Seven days after MDM injection, mice were killed and CNS tissue was collected and subjected to immunocytochemical and Western blot assays for leukocyte and neural antigens, GSK-3β, and key kinase substrates such as β-catenin and Tau. Numbers of HIV-1 p24 antigen-positive MDMs were unaltered by Li treatment of HIVE mice. Similarly, the greatly increased extent of astrocyte and microglia activation in HIV-1 mice was unaltered by Li. In contrast, Li restored HIVE-associated loss of microtubule-associated protein-2-positive neurites and synaptic density while reducing levels or activity of phospho-Tau Ser202, phospho-β-catenin, and GSK-3β. Electrophysiological recordings showed diminished long-term potentiation in hippocampal slices of HIVE mice that were restored by Li. Based on these data, the use of Li as an adjuvant for HIV-1-associated dementia is now being pursued.

Key words: HIV-1 encephalitis; lithium; neuroprotection; monocyte-derived macrophages; glycogen synthase kinase-3β; neurodegeneration

Introduction

Lithium (Li) is a neuroprotective agent with known antiapoptotic activities affecting phosphatidylinositol 3 (PI3)-kinase (PI3-K) and mitogen-activated protein kinase cell-signaling pathways (Chalecka-Franaszek and Chuang, 1999; Mora et al., 1999; Zorrilla Zubilete, 2003) and, importantly, potent inhibitory activity directed against glycogen synthase kinase-3β (GSK-3β) inhibition (Chalecka-Franaszek and Chuang, 1999; Harwood and Agam, 2003a,b). Collectively, these kinases stimulate the phosphorylation and aberrant assembly of neuronal microtubule-associated protein (MAP) Tau (Hernandez et al., 2003). Ultimately, Li-induced inhibition of GSK-3β affects a wide range of downstream effectors that mediate both antiapoptotic and proapoptotic pathways, including β-catenin, heat shock factor 1, activator protein 1, cAMP response element-binding protein, and Bcl-2 (Hokin et al., 1996; Jope and Bijur, 2002; Li et al., 2002; Tyson et al., 2002; Kim et al., 2003; Brunello, 2004). Although widely used in the treatment of bipolar mood disorder (Jope, 1999), interest in Li has now extended into its potential use for a wide range of environmental and neurodegenerative insults (Jope, 1999; Zorrilla Zubilete, 2003; Song et al., 2004). Other potential neuroprotective mechanisms induced by Li include induction of brain-derived neurotrophic factor (BDNF) and proapoptotic responses mediated through NMDA receptor engagement and calcium regulation (Chalecka-Franaszek and Chuang, 1999; Harwood and Agam, 2003; Berry et al., 2004). These observations support the idea that the neuroprotective activities of Li may be translated into effective treatments of a range of neurodegenerative diseases (Hashimoto et al., 2003a,b), including, perhaps, human immunodeficiency virus-1 (HIV-1)-associated dementia (HAD).

To this end, we tested whether Li could protect neurons in laboratory assays and in an animal model of HIV-1 encephalitis (HIVE), the pathological correlate of HAD. We raised the question of whether Li could ameliorate HIV-1-associated neurodegeneration. Because HIV-1-mediated neuronal injury stimulates GSK-3β, we assessed whether inhibition of this pathway by Li...
would be a potential mechanism for drug action. We show that Li treatment protects both rodent and human neurons from the neurotoxic effects of HIV-1-infected monocyte-derived macrophages (MDMs) in vitro. Also, Li treatment of severe combined immunodeficient disease (SCID) HIVE mice results in neuronal protection and induction of neurogenesis in the dentate gyrus (DG). We further demonstrate that Li protects against HIV-1 neurotoxicity by diminishing neuronal apoptosis and protecting synaptic density and dendritic arbor through GSK-3β inhibition and activating the PI3-kinase/Akt cell-signaling pathways. Because phosphorylation and assembly of tau are linked to HIV-1-associated neurodegeneration, the inhibition of GSK-3β and reduction in β-catenin and Tau phosphorylation provide a mechanism for how Li could elicit positive clinical outcomes and support its possible future use as an adjunctive therapy for HAD.

Materials and Methods
Isolation and HIV-1 infection of MDMs. Human monocytes were recovered from peripheral blood mononuclear cells of HIV-1, HIV-2, and hepatitis B virus seronegative donors after leukopheresis and purified by counter-current centrifugal elutriation (Gendelman et al., 1988). Monocytes were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated human serum, 2 mM L-glutamine, gentamicin (50 μg/ml), ciprofloxacin (10 μg/ml), and macrophage colony-stimulating factor (1000 U/ml; a generous gift from Genetics Institute, Cambridge, MA). Monocytes were cultivated for 7 d and then referred to as MDMs. MDMs were infected with HIV-1ADA (a macrophage tropic viral strain) at a multiplicity of infection (MOI) of 0.01.

Human fetal neurons. Human fetal brain tissue (gestational age, 13–16 weeks) was obtained from elective abortions in full compliance with the University of Nebraska Medical Center and National Institutes of Health ethical guidelines. Cells were plated in poly-lysine-coated 24-well plates (BD Biosciences, San Diego, CA) at a density of 5 × 10^5 cells/well. Cells were cultured in Neurobasal media (Invitrogen, Rockville, MD) supplemented with B27 (Invitrogen). At 5 d after cell culture, 10 μg/ml 5-fluorodeoxyuridine was added to inhibit proliferation of dividing cells (astrocytes/fibroblasts). The purity of neural cell preparations was assayed by immunocytochemical methods as described below. At 2 weeks after cell cultivation, >70% of the neuronal-enriched preparations were MAP-2 immunopositive.

HIV-1-associated neurotoxicity and neuroprotection. Human fetal neurons were cultivated for 2 weeks and then exposed to culture fluids of uninfected (control) and HIV-1-infected MDM (25% v/v) for 5 d in the presence or absence of lithium chloride (Li; 10 mM; Sigma). 2-(4-morpholino)-8-phenyl-1(4H)-benzopyran-4-one [LY294002 (LY); 50 mM; EMD Biosciences, San Diego, CA], indirubin-3′-monoxamine (Ind; 10 μM; Sigma), K252a (100 nM; Calbiochem, La Jolla, CA), or myoinositol (Ino; 10 μM; EMD Biosciences) were added to HIV-1-infected cultures. Cells were fixed with 4% paraformaldehyde and subjected to assays of neuronal apoptosis, measures of neuronal dendrites, and synaptic processes.

Neuronal apoptosis. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining was performed using the in situ cell death detection kit, TMR Red (Roche Diagnostic, Indianapolis, IN), following the protocol of the manufacturer. Apoptotic cells were identified with TUNEL, which detects the DNA fragmentation characteristic of apoptotic cells. The cells were viewed under a fluorescence microscope, and the total numbers of bright green nuclei in each field were counted. Using the same field of view, 4′,6′-diamidino-2-phenylindole (DAPI) nuclei staining was used to normalize the data retrieved for neuronal apoptosis. This resulted in a neuronal apoptosis index that was calculated by dividing the number of counted TUNEL-positive green by total DAPI-positive cells.

Immunocytochemical analyses of neuronal injury. Primary human fetal neurons were cultivated for 2 weeks. Twenty-five percent (v/v) of culture fluids from MDMs infected with HIV-1ADA at an MOI of 0.01 for 7 d were prepared. These fluids contained 5 × 10^6 cpm/ml reverse transcriptase activity. The virus-infected MDM fluids were then added to neurons for an additional 5 d with Ind (10 μM) alone or LY (50 μM), K252a (100 nM), and Ino (10 μM) in the absence or presence of Li (10 mM). The culture media was removed, and the neuronal cells were fixed with methanol/acetic (1:1) for 10 min at ~20°C. The cells were treated with antibodies against MAP-2 and synaptophysin (SYP) (both from Chemicon, Temecula, CA) or gliad fibrillary acidic protein (GFAP) (Dako, High Wycombe, UK), respectively, overnight and then washed with PBS and incubated for 1 h with FITC-labeled secondary antibodies (Boehringer Mannheim, Indianapolis, IN). Histocytochemical tests were examined with a Nikon (Tokyo, Japan) Microphot-FXA microscope. For the quantification of immunoreactive cells, 15 randomly selected fields were analyzed.

SCID mouse model of HIVE. Four-week-old male C.B.-17 SCID mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in sterile microisolator cages. One day after infection, HIV-1ADA-infected MDMs (5 × 10^5 cells in 5 μl) were injected intracranially. Li was administered (60 mg/kg/d) 1 d after infection. Control animals were left untreated. All animals were killed at day 7 (peak of inflammation and neuronal injury).

Histopathology and image analysis. Brain tissue was collected at necropsy, fixed in 4% phosphate-buffered paraformaldehyde, and embedded in paraffin or frozen for later use. Blocks were cut to identify the injection site. For each mouse, 30–100 serial (5-μm-thick) sections were cut from the injection site and at the level of the hippocampus. Immunohistochemical staining followed a basic indirect protocol. Alternately, brains were frozen after fixation, and 30 μm sections were prepared for immunofluorescent staining. Antibodies to vimentin, intermediate filaments (clone 3B4; Dako) were used for detection of human cells in the mouse brain. Murine microglia were identified by Griffonia simplicifolia lectin I-isolate. Astrocytes were identified using antibodies specific for GFAP. Neuron-specific nuclear protein (NeuN), MAP-2, and SYP were used for neuronal detection. Antibodies to HIV-1 p24 antigen (Dako) were applied to determine the number of HIV-1-infected cells. Immature neurons were localized by antibodies to polysialylated neuronal cell adhesion molecules (PSA-NCAMs; mouse IgM; generously provided by Dr. T. Seki, Jutendo University School of Medicine, Tokyo, Japan). All paraffin-embedded sections were counterstained with Mayer’s hematoxylin. Deletion of primary antibody served as a control. Tissue examination was performed with an Eclipse E800 microscope (Nikon Instruments, Melville, NY). Images were obtained by an Optromics (Buffalo Grove, IL) digital camera with MagnaFire (Goleta, CA) 2.0 software and processed by Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA). Quantification of immunostaining was done using Image-Pro Plus (version 4.0) on an intracranial brain tissue section.

Western blot assays. Primary human fetal neuronal cultures were prepared as described above. Virus-infected MDM fluids were added to neurons with LY (50 μM), Ind (10 μM), and K252a (100 nM) with or without Li (10 mM) for 2 h. Protein lysates were prepared and electrophoretically separated on SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Membranes were incubated with primary antibodies to antibody β-catenin, phospho-β-catenin Ser33,37 (both from Sigma), GSK-3β (BD Biosciences), phospho-GSK-3β serine 9 (Ser9) (Affinity BioReagents, Golden, CO), and β-tubulin (Promega, Madison, WI). Two-millimeter SCID mice brain sections that included the site of injection were used for extraction of proteins. Tissue sections corresponding to the site of injection in the contralateral hemisphere served as controls. The brain was homogenized in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, aprotinin, bestatin, leupeptin, pepstatin A, aminooxybenzene sulfonylfluoride, and E-64. Proteins were electrophoretically separated on SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Membranes were incubated with primary antibodies to Tau5 (BD Biosciences), phospho-Tau Ser202 (Sigma), β-catenin, phospho-β-catenin Ser33,37, GSK-3β, MAP-2, and β-tubulin. Horseradish peroxidase-conjugated secondary antibodies were used, and membranes were treated with chemiluminescent substrate and then exposed to x-ray film. Images were digitized with a densitometer (Molecular Dynamics, Sunnyvale, CA), and protein levels
were induced by weak tetanic stimulation (10 events at 100 Hz) observed of the hippocampus was examined after a 20 min control recording. LTP was expressed as a ratio to untreated control cultures. In Li-treated cultures, the expression of SYP and MAP-2 was essentially restored (Fig. 1A). SYP expression was compared with untreated HIV-1 fluids compared with untreated control cultures. In Li-treated cultures, the expression of SYP and MAP-2 was essentially restored (p < 0.05 and p < 0.004, respectively) when compared with untreated HIV-1 fluids. LY added to HIV-1 fluid-exposed cultures in the presence of Li blocked the neuroprotective effects of Li on SYP and MAP-2 expression (p < 0.05 and p < 0.01, respectively). As a GSK-3 inhibitor, Ind also induced neuroprotective activities, parallel to what was observed when supplied to cells exposed to HIV-1 fluids (SYP; p < 0.05; MAP-2; p = 0.055). Additional 10 μM Ino added to Li-treated HIV-1-exposed neurons had no effect on Li neuroprotection. These results are representative of three individual experiments performed in quadruplicate determinations. Original magnification, 400 ×. The asterisk denotes a statistically significant difference when compared with controls that received HIV-1 fluids; the number sign denotes a statistically significant difference compared with cultures that were exposed to HIV-1 fluids in the presence of Li.

Figure 1. Li protects human fetal neurons against toxicity of culture fluids from HIV-1-infected MDMs. Human fetal neurons were cultivated for 2 weeks and then exposed to culture fluids of uninfected (control) and HIV-1-infected MDM (25% (v/v)) for 5 d in the presence or absence of Li (10 μM; as indicated), after which the cultures were analyzed by immunostaining. LY (30 μM), Ind (10 μM), and Ino (10 μM) were added at the time of neuronal exposure to the HIV-1 fluids. A, Fluorescent images show immunoreactivity for MAP-2 (left, green; right, red), SYP (left, red), and GFAP (right, green). This analysis revealed a high density of dendritic nodes and long neuritic processes with prominent cell bodies and synaptic staining in control neurons. Diminished dendritic nodes, short neurites, and loss of processes were apparent in neurons treated with HIV-1 fluids and were reversed by Li (right). In addition, extensive astrogliosis (reflected by prominent GFAP immunoreactivity) was detected in all cultures exposed to HIV-1 fluids. Quantitative analysis of these immunostaining results are presented for synaptophysin (B), MAP-2 (C), and GFAP expression (D). SYP expression (p < 0.01) and MAP-2 neurite length (p < 0.003) were reduced in neurons treated with HIV-1 fluids in the presence of Li.

Electrophysiological tests. Seven days after injection, brains were quickly removed from the cranial cavities. The ipsilateral and contralateral of hippocampi were separated and placed in ice-cold (4 °C) oxygenated artificial CSF before sectioning. The ability of high-frequency stimulation (HFS) to induce long-term potentiation (LTP) in the CA1 region of the hippocampus was examined after a 20 min control recording. LTP was induced by weak tetanic stimulation (10 events at 100 Hz) observed for 60 min as described previously (Anderson et al., 2003). Results from slices with large fluctuation (≥ 2 SDs) were rejected.

Results

Li protects neurons after exposure to HIV-1-infected MDM fluids

We investigated whether Li protects primary human fetal neurons after exposure to culture fluids from HIV-1-infected MDM (Fig. 1). In these experiments, cells were treated with fluids from uninfected (control) and HIV-1-infected MDM [25% (v/v) (HIV-1-)] in the absence or presence of 10 mM Li for 5 d. Li effects on synapse formation were then examined, and representative results are shown in photomicrographs of cultured cells stained with antibodies to SYP (Fig. 1, red) and MAP-2 (Fig. 1, green) (Fig. 1A, left column). SYP, a major integral membrane glycoprotein, is expressed in abundance in presynaptic vesicles of neurons; SYP immunostaining was found to be punctuated in MAP-2+ dendrites. Quantitative analysis of SYP expression in 12 random fields of view (Fig. 1B) showed diminished staining in neurons treated with HIV-1 culture fluids (p < 0.01). However, in Li-treated neurons that were exposed to HIV-1 culture fluids, the distribution and staining "intensity" of SYP was essentially restored (Fig. 1A, B) (p < 0.05).

We compared SYP immunostaining data to those obtained for MAP-2. MAP-2+ dendrites (Fig. 1A) were readily detected in primary human neuronal cells. These were measured by quantitative image analysis to quantitate neurite length (Fig. 1C). HIV-1+ culture fluids induced significant decreases in MAP-2 immunostaining and MAP-2+ neurite length in primary neurons when compared with untreated controls (p < 0.003); these changes were mostly reversed in the presence of Li, which increased the numbers and density of MAP-2+ neurites, compared with untreated controls (p < 0.004). Moreover, connections among neurites were also increased when staining results were compared with cultures treated with HIV-1+ fluids alone (Fig. 1A, C). Importantly, the neuroprotective effects of Li in this neuronal culture system occurred in the absence of effects on astrocyte activation. HIV-1+ fluids induced extensive astrogliosis, as reflected by a strong increase in GFAP immunostaining (Fig. 1A, D), and this was unaltered by the addition of Li. Thus, Li exerted its neuroprotective effects through direct action(s) on neurons.

To investigate the mechanism(s) whereby Li might be exerting its effects, we performed additional studies designed to examine the role of specific signaling pathways that have been linked to the
effects of Li in other experimental systems. We first focused on the GSK-3β pathway and examined the neuroprotective efficacy of a highly specific GSK-3β inhibitor, Ind. As shown in Figure 1, Ind elicited a very similar neuroprotective response to Li. Next, we explored the role of inositol monophosphatase in Li-mediated neuronal protection. Because Li inhibits inositol monophosphatase (Klein and Melton, 1996; Maggirwar et al., 1999) and could deplete cells of an endogenous source of inositol, replicate experiments were performed in which we treated cells with Li in the presence of an exogenous source of inositol (myo-inositol; denoted as Ino in Fig. 1) to ascertain its effects on neuronal protection. Such experiments demonstrated no significant changes in Li-mediated neuroprotection after addition of 10 μM Ino, and neuroprotection was seen at levels equivalent to what was observed with Li alone (Fig. 1A–C).

We also examined whether the PI3-K/Akt signaling pathway might contribute to Li-mediated neuroprotection. To do this, cells were exposed to HIV-1+ culture fluids in the presence of either Li alone or Li plus the potent PI3-K inhibitor LY294002. This revealed that the neuroprotective effects of Li were blocked by LY, resulting in diminished SYP staining and MAP-2+ neurites (p < 0.05 and p < 0.01) when compared with HIV-1+ fluid-exposed cultures that were treated with Li alone. Together, we demonstrate that Li protects the neuronal cytoskeleton (MAP-2) and promotes neurite outgrowth through inhibition of GSK-3 and activation of PI3-K/Akt pathways. Li also stabilizes SYP and may engage PI3-related mechanisms, but at reduced levels.

**Li-mediated protection of HIV-1-mediated apoptotic cell death**

We next analyzed whether Li affects neuronal apoptosis induced by HIV-1+ fluids in human fetal neurons. To do this, neuronal apoptosis was quantitated by combined TUNEL/DAPI staining (Fig. 2) in cultures exposed to HIV-1+ fluids for 5 d in the presence or absence of Li. In replicate experiments, specific inhibitors of key pathways that are regulated by Li were added (Fig. 2). We focused on tyrosine receptor kinase (Trk) signaling (inhibited by K252a) (Fig. 2A), PI3-K/Akt activation (blocked by LY) (Fig. 2B), GSK-3β activation (prevented by Ino) (Fig. 2C), and inositol monophosphatase (IMPase) inhibition (functionally reversed by the addition of exogenous myo-inositol) (Fig. 2D). The percentage of TUNEL-positive cells from three independent experiments is shown in Figure 2. Vehicle (control) cultures showed few TUNEL-positive neurons, whereas cultures treated with HIV-1+ fluids showed large numbers of neurons undergoing apoptosis (p < 0.001). In contrast, neurons that were exposed to culture fluids from HIV-1-infected MDMs in the presence of 10 μM Li showed a dramatic decrease in the numbers of apoptotic cells (p < 0.003).

To investigate the mechanisms of Li-mediated neuroprotection, we used specific inhibitors of four key pathways known to be impacted by Li. K252a, a Trk inhibitor, reversed in part the neuroprotective effect of Li treatment, resulting in an increase in TUNEL staining similar to what was found in Li-treated neurons (p = 0.063). Treatment with K252a alone did not affect neuronal TUNEL staining (data not shown). These results suggested that Li neuroprotection could be independent of the TrkB signaling pathway. We next tested whether the PI3-kinase/Akt pathway was operative in Li neuroprotective activities. The PI3-kinase-specific inhibitor LY was added to Li-treated neurons exposed to HIV-1+ fluids. LY attenuated the neuroprotective effectiveness of Li (Fig. 2B) (p < 0.02). To determine whether GSK-3β inhibition might contribute to Li-mediated neuroprotection, a specific GSK-3β inhibitor (Ind) was added to neurons exposed to HIV-1+ fluids. Ind diminished neuronal apoptosis induced by HIV-1+ fluids when administered in the absence of Li (Fig. 2C) (p < 0.05). These findings indicate that specific inhibition of GSK-3β can protect neurons from HIV-1 MDM-mediated apoptosis and that Li and Ind both affect neuronal survival, implying a shared mechanism of action. These data are consistent with a model in which the neuroprotective activity of Li is mediated, at least in part, through inhibition of GSK-3β.

Finally, neurons were treated with myo-inositol in the absence or presence of Li to maintain cytosolic Ino levels. Ino failed to affect Li neuroprotection (Fig. 2D). These data support the notion that IMPases are not the targets of Li neuroprotective responses. Importantly, all of these results using pharmacological modifiers of Li-targeted signaling pathways were repeated and confirmed by substituting HIV-1 gp120 for infected MDM culture fluids and by substituting rat for human neurons as indicator cells (data not shown). Together, these findings demonstrate potential mechanisms for Li-mediated neuroprotection against HIV-1 and macrophage neurotoxins.
Effects of Li on HIV-1 replication and neuroinflammation in murine HIVE

We next determined whether Li provides neuroprotection in a rodent model of HIVE. In our initial experiments, we analyzed the effects of Li on levels of viral infection and on neuroinflammation. Human HIV-1-infected MDMs were stereotactically injected into the basal ganglia (BG) of SCID mice. Histopathological changes observed in murine brain tissue paralleled those observed for human HIVE and included HIV-1 infection in perivascular and parenchymal human MDMs, formation of multinucleated giant cells, astrocytosis, and neuronal dropout (Persidsky et al., 1996). Numbers of human uninfected and HIV-1-infected MDMs were determined by immunostaining with vimentin and HIV-1 p24 antibodies in serial 5 μm brain slices that encompassed the injection site (total cells per section). Numbers of virus-infected and total cell numbers were assessed by examination of 30 fields obtained from 15 separate histological sections. Three sections per mouse and five mice per group (Fig. 3) were examined. Day 7 after injection, the total mean number of MDM was 157.79 ± 19.79/section and 141 ± 24.94/section in HIVE (vehicle) mice and Li-treated HIVE mice, respectively. Of these cells, 26 and 34% were HIV-1 p24 positive in untreated and Li-treated HIVE brains, respectively. Li treatment, thus, had no statistically significant effect on HIV-1 replication or MDM engraftment in the HIVE mice. Similarly, astrocyte and microglial reactivity were equivalent in the Li- and vehicle-treated mice. This was revealed by immunostaining of serial brain sections for GFAP (for astrocytes) and G. simplicifolia lectin-isolectin B4 (lectin for activated microglia) (Fig. 3). Microglial activation (as reflected by the presence of large ramified lectin-positive cells) was detected in and around human MDMs in HIVE mice. The GFAP index (percentage of the microscope field that was positive for GFAP immunoreactivity) was 10.03 ± 1.67 and 6.23 ± 1.13 in HIVE and sham-operated mice, respectively. The GFAP index of Li-treated HIVE mice was 9.33 ± 1.28. No differences were observed between Li- and vehicle-treated HIVE mice in the GFAP index and in microglial reactions. All together, the data demonstrated that Li had no effect on HIV-1 infection, MDM engraftment, or astrocyte and microglial responses.

Neuroprotective activities of Li in HIVE mice

The next series of experiments examined possible neuroprotective activities of Li in the HIVE mice. To assess changes in neuritic processes in the encephalitic mice, double immunostaining with antibodies to MAP-2 and vimentin was used to determine the spatial relationship between neurites and human MDM (Fig. 4). In HIVE animals, MAP-2 (green)-positive neuronal perikarya and process loss after injection of HIV-1-infected MDMs (Fig. 4). Analysis of immunofluorescent-stained sections showed that Li enhanced neuronal survival and process formation in affected brain tissues with pathological evidence of HIVE.

To examine and quantitate the extent of cell, nuclei, dendrite, and process loss after injection of HIV-1-infected MDM, quantitative immunostaining was performed using antibodies to MAP-2 and SYP (Fig. 5). Minimal neuronal degeneration was observed in sham-operated animals. Significant neuronal loss was detected throughout the BG and cerebral cortex of the HIVE mice when compared with sham-operated animals. This was mostly reversed by Li treatment. Quantitative analysis of digitized microscope images revealed a reduced neuronal index (ratio of MAP-2/NeuN) in HIVE compared with sham-operated animals. This was revealed by immunofluorescent-stained sections showing that Li enhanced neuronal survival and process formation in affected brain tissues with pathological evidence of HIVE.

**Figure 3.** Neuropathological analysis of Li-treated HIVE mice. Serial 5 μm brain sections, cut through the needle track, were immunostained for vimentin, HIV-1 p24, GFAP, and lectin. In all cases, the chromagen used to reveal the immunostained cells is brown. Thirty fields in 15 sections were collected from the injection site through the BG and cerebral cortex and subjected to quantitative morphometric analysis. No differences were detected in numbers of human cells (vimentin staining), HIV-1 p24 antigen, astrocyte reactivity (GFAP staining), or microglial activation (lectin staining) in the HIVE mice versus the HIVE mice that were exposed to Li (as shown by the representative microscope fields; immunostaining data not shown). Original magnification, 200×.
correlated with MAP-2⁺ dendrites. SYP expression was also significantly decreased in HIVE mice relative to sham-operated animals (p < 0.03). In contrast, Li-treated HIVE mice showed SYP levels that were statistically indistinguishable from sham controls (p = 0.2). Overall, HIVE mice showed neuronal nuclei, dendrite, and synaptic cleft loss that was reversed by Li.

Li effects on neural progenitor cells in HIVE mice

We next evaluated the effects of Li on neural progenitor cells in the DG of HIVE mice. The approach first evaluated expression of MAP-2 to assess alterations in hippocampal neuronal morphology in HIVE mice and its effects by Li (Fig. 6A). Reductions in MAP-2 staining were observed in the CA1 and DG in HIVE mice compared with sham-operated controls (Fig. 6B). In contrast, Li-treated HIVE mice showed increased hippocampal MAP-2 expression similar to that of sham controls. Moreover, the length of MAP-2⁺ dendrites was reduced in the CA1 (p < 0.003) and DG (p < 0.03) regions of HIVE mice compared with sham-operated controls. Similarly, in Li-treated HIVE mice, a significant increase in the length of MAP-2⁺ dendrites in the CA1 (p < 0.005) and DG (p < 0.03) regions was seen compared with HIVE animals. We next evaluated expression of PSA-NCAM as a marker of hippocampal neurogenesis. This is a cell surface marker that is associated with newly generated neurons within the inner border of the granule cell layer (GCL). Small, round, PSA-NCAM⁺ cells with dark nuclei were found bordering the polymorph layer (hilus) and the DG GCL but not the CA1 region. Cells with apical dendrites or with marked dendritic processes extending from the GCL to molecular layer were observed. HIVE mice showed reduced numbers of PSA-NCAM⁺ cells (number of PSA-NCAM⁺ cell/millimeter of length of the hippocampus) in the DG when compared with sham-operated mice (Fig. 6C) (p < 0.0002). Li significantly increased PSA-NCAM⁺ cell numbers compared with the untreated mice (p < 0.002). Quantitative analysis of dendritic length on PSA-NCAM⁺ neurons further revealed the protective effects of Li treatment (Fig. 6D,E). The average numbers of dendrites expressed on the cells (numbers of PSA-NCAM⁺ dendrites/millimeter of length of the hippocampus) were also reduced in HIVE mice relative to sham controls (p < 0.001), as was the average length of dendrites (p < 0.01). Li increased both PSA-NCAM⁺ dendrite numbers (p < 0.01) and length (p < 0.03) in the HIVE mice.

Physiology of Li-induced neuroprotection

In our previous studies, we demonstrated that synaptic dysfunction peaked in HIVE animals 7 d after HIV-1-infected MDM injection and that these functional deficits persisted for >15 d (Zink et al., 2002; Anderson et al., 2003). To assess the physiological basis of Li-induced neuroprotection, we analyzed LTP activity using hippocampal slides that were prepared from sham-operated (controls) and from HIV mice with and without Li treatment. As shown in Figure 7, field EPSP traces from the CA1 region were recorded at 60 min after HFS. Average LTP magnitudes recorded in control animals were 263.2 ± 32.5% of basal levels (n = 7) and from animals injected with HIV-1-infected MDM; LTP magnitudes were 106.6 ± 29.2% of basal levels (n = 7; p < 0.01). Notably, administration of Li in mice injected with HIV-1-infected MDM led to LTP enhancement. The average LTP magnitude was 192.2 ± 29.4% relative to basal activity (n = 5; p < 0.05). These results demonstrate that Li protects neuronal synaptic function in HIVE mice.

Li neuroprotection and GSK-3β activity in HIV mice

Li-mediated neuroprotection in HIVE mice was analyzed in lysates of brain tissue protein by Western blot assays (Fig. 8A). In Figure 8B, a modest decrease in total β-catenin and GSK-3β, with a concomitant rise in p-β-catenin, is illustrated in HIVE mice (p < 0.01) when compared with sham-operated animals. These changes were mostly reversed by Li (Fig. 8B). To evaluate the potential role of phosphorylated Tau in HIV-1-mediated neuronal injury, immunoblot analysis of tissue extracts was performed using antibodies specific for phosphorylated Tau (Ser202, Thr181) or total Tau (Tau5 antibody) (Fig. 8C). Quantitation of total Tau and phosphorylated Tau Ser202 was performed in HIVE mice. All groups showed similar Tau5 immunoreactivity (total Tau). In contrast, the levels of Tau phosphorylation increased in HIVE animals compared with sham-operated controls (p < 0.01). Li-treated HIVE mice showed a significant reduction in phosho-Tau Ser202 compared with vehicle-treated HIVE ani-
in HIV-1 encephalitis. Li treatment can increase BDNF production leading to activation of the TrkB receptor (Hashimoto et al., 2002). Our studies showed that K252a, an inhibitor of Trk receptor tyrosine kinase activity, did not block the anti-apoptosis effects of Li. Thus, the neuroprotective effects of Li are mediated independently of TrkB signaling.

Mechanisms of Li neuroprotection

To further examine the potential mechanisms by which Li treatment may protect neurons against candidate HIV-1 neurotoxins, we performed in vitro experiments using human fetal neurons exposed to HIV-1 fluids. The effects of Li on the expression levels and phosphorylation status of GSK-3β and β-catenin were analyzed by Western blot assays using isoform-specific antibodies (Fig. 9A). Quantitative analysis (Fig. 9B–E) of these experiments, as expected, revealed that neuron exposure to HIV-1 fluids resulted in an increase in β-catenin phosphorylation (p < 0.01) (Fig. 9E) and a decrease in the levels of Ser9-phosphorylated GSK-3β (p < 0.01) (Fig. 9E). Changes in β-catenin and GSK-3β phosphorylation were reversed by Li (p < 0.01) for both p-β-catenin and Ser9-p-GSK-3β (Fig. 9C,E). This was observed in the absence of changes in the overall expression levels of either protein. The highly specific GSK-3β inhibitor, Ind, had essentially identical effects on β-catenin phosphorylation and levels of Ser9-phosphorylated GSK-3β.

Because GSK-3β is a downstream target for PI3-K, we also investigated whether the Li neuroprotective effects involved activation of the PI3-kinase/AKT pathway, using the PI3-kinase-specific inhibitor LY. Treatment with LY reversed the effects of Li on both β-catenin phosphorylation and levels of Ser9-phosphorylated GSK-3β. Neurons exposed to HIV-1 fluids treated with Li induced GSK-3β phosphorylation. Together, our results suggest that the neuroprotective effects of Li involve activation of the PI3-kinase/AKT pathway, inhibition of GSK-3β, and accumulation of β-catenin. These data further support the involvement of the GSK-3β and PI3-kinase/Akt pathways in Li neuroprotective activities.

Discussion

We demonstrate that Li protects neurons against HIV-1-infected macrophage neurotoxins in both a laboratory and an animal model system of HIVE and HAD. In recent years, Li was shown to possess neuroprotective activities in addition to its known abilities to positively affect the treatment of bipolar disorders. Moreover, it is now well established that Li elicits neuroprotection, in part, through its ability to inhibit GSK-3β (Hong et al., 1997; Everall et al., 2002; Facci et al., 2003; Hongisto et al., 2003; Kirshenboim et al., 2004).

One mechanism whereby Li can inhibit GSK-3β is through increasing the phosphorylation of a key inhibitory site of GSK-3β, Ser9 (Zhang et al., 2003); this process is reduced by inhibition of protein kinase C (PKC) (Lenox et al., 1996; Kirshenboim et al., 2004). Because PI3-kinase is a potential upstream regulator of PKC, its inhibition by LY294002 might be expected to affect Li-induced phosphorylation of the Ser9 residue of GSK-3β in neurons. Our data support this idea and show that Li-mediated neuroprotective activities involve activation of PI3-kinase/AKT but not depletion of inositol. Thus, Li may interfere with the proapoptotic effects of candidate HIV-1 neurotoxins by affecting phosphatidylinositol 3 kinase-dependent activation of PKC. The inhibition of Li-mediated neuronal protection by LY294002 strongly supports this notion. Li may target not only the PI3K/Akt survival pathway (Stambolic et al., 1996; Mora et al., 2001; De Sarno et al., 2002, Sinha et al., 2005) but also GSK-3β-mediated signaling to accomplish its protective effects in neurons exposed to candidate HIV-1 neurotoxins.

A previous study demonstrated that Li treatment can increase BDNF and the phosphorylation of TrkB at Tyr490, suggesting that Li affects BDNF production leading to activation of the TrkB receptor (Hashimoto et al., 2002). Our in vitro studies showed that K252a, an inhibitor of Trk receptor tyrosine kinase activity, did not block the anti-apoptosis effects of Li. Thus, the neuroprotective effects of Li are mediated independently of TrkB signaling pathways.

This study is multifaceted and complex in both design and
analysis. Laboratory studies and those in a rodent model of human HAD show changes in structure, function, and development of neurons. We demonstrated previously that alterations in neuronal physiology can occur as a consequence of the introduction of HIV-1-infected macrophages into the CNS (Xiong et al., 1999; Anderson et al., 2003). Impairment in hippocampal function was also shown as reflected by deficits in LTP, which may underlie the cognitive dysfunction seen in a patient with autoimmune deficiency syndrome. HIV-1-mediated neuronal damage may contribute to these changes in neuronal physiology by reducing the complexity of the dendritic architecture, thereby causing an intrinsically lower capacity for LTP induction in the HIVE mice. This is supported by morphological changes in MAP-2 staining and increases in Li-induced dendritic lengths in HIVE animals (changes that we have shown previously to occur not only in brain regions proximal to the site of injection of HIV-1-infected MDM but also at distant anatomic sites, including the ipsilateral cortex and hippocampus).

HAD is a metabolic encephalopathy fueled by HIV-1-infected and immune-activated mononuclear phagocytes (MPs; perivascular and parenchymal macrophages and microglia) that secrete a plethora of viral and cellular neurotoxins. At the clinical level, it is characterized by significant cognitive, motor, and behavioral abnormalities (Ivanisevic, 1987; Lipton and Gendelman, 1995; Gendelman et al., 1997) and is pathologically linked to a giant cell encephalitis referred to commonly as HIV-1 encephalitis (Gelbard et al., 1994; Navia, 1997; Zink et al., 1999). Virus infection and immune activation of MPs, astroglia, myelin pallor, and neuronal dropout are prominent neuropathological features of disease (Navia et al., 1986). MPs may also affect the pruning of neuronal dendrites and changes in synaptic processes linked to disease (Masliah et al., 1996). The mechanisms underlying neuronal injury for HAD are mirrored in our laboratory and in HIV SCID mouse models of human disease. These two model systems demonstrate the importance of HIV-infected or activated MPs in inducing neuronal dysfunction through the secretion of cellular and viral toxins including proinflammatory cytokines, glutamate, and a variety of excitotoxins (Genis et al., 1992; Nottet et al., 1995; Persidsky et al., 1996, 1997; Anderson et al., 2003). Previous studies performed in our laboratories also demonstrated that sodium valproate induces neuroprotective effects by downregulation of Tau phosphorylation and GSK-3β in HIV-1 SCID mouse models (Dou et al., 2003). Although both valproic acid and Li affect GSK-3β and Tau phosphorylation, the mechanisms of neuronal protection were found, in part, to be distinct.

All together, we demonstrate that Li induces neuroprotective activities in laboratory and animal models of HIVE. Although a range of cell-signaling pathways affect neuronal function in HAD...
treated with control fluids or HIV-1 control cells that did not receive HIV-1 were significantly upregulated in the HIVE mice (Figure 9).

Engagement of GSK-3

Figure 9. Pathways for Li-mediated neuroprotection in HIVE mice. A, Western blot assays were performed on tissue extracts using antibodies specific for GSK-3β, phosphorylated β-catenin, β-catenin, Tau (all isoforms; Tau5 antibody), and two phosphorylated isoforms of Tau (Ser33,37 and Thr181). B, Data were quantitated densitometrically. As expected, expression levels of GSK-3β were unaltered in all groups of mice. Total levels of β-catenin were slightly reduced in the HIVE mice relative to sham controls, whereas levels of phosphorylated β-catenin were significantly upregulated in the HIVE mice (p < 0.05). This is consistent with a rise in the enzymatic activity of GSK-3β in the HIVE mice. Li treatment essentially reversed these effects on β-catenin. C, Quantitative analysis of the Tau phosphorylation at residues 202 and 181 was performed by measuring immunoreactivity with phospho-specific antisera directed against the Tau202 and Tau181 residues and then normalizing the resulting densitometric data in terms of total Tau immunoreactivity (determined using the Tau5 antibody, which recognizes all forms of the protein). This analysis revealed increases in both Ser33,37 (p < 0.05) and Thr181 (p < 0.01) phosphorylated isoforms of Tau in HIVE mice (HIV-1), compared with control animals (sham). These changes were reversed by Li treatment of HIVE mice. The asterisk denotes a statistically significant difference when compared with sham mice; the number sign denotes a statistically significant difference when compared with Li-treated HIVE mice.

Figure 8. Pathways for Li-mediated neuroprotection in HIVE mice. A, Western blot assays were performed on tissue extracts using antibodies specific for GSK-3β, phosphorylated β-catenin, β-catenin, Tau (all isoforms; Tau5 antibody), and two phosphorylated isoforms of Tau (Ser33,37 and Thr181). B, C, Data were quantitated densitometrically. As expected, expression levels of GSK-3β were unaltered in all groups of mice. Total levels of β-catenin were slightly reduced in the HIVE mice relative to sham controls, whereas levels of phosphorylated β-catenin were significantly upregulated in the HIVE mice (p < 0.05). This is consistent with a rise in the enzymatic activity of GSK-3β in the HIVE mice. Li treatment essentially reversed these effects on β-catenin. C, Quantitative analysis of the Tau phosphorylation at residues 202 and 181 was performed by measuring immunoreactivity with phospho-specific antisera directed against the Tau202 and Tau181 residues and then normalizing the resulting densitometric data in terms of total Tau immunoreactivity (determined using the Tau5 antibody, which recognizes all forms of the protein). This analysis revealed increases in both Ser33,37 (p < 0.05) and Thr181 (p < 0.01) phosphorylated isoforms of Tau in HIVE mice (HIV-1), compared with control animals (sham). These changes were reversed by Li treatment of HIVE mice. The asterisk denotes a statistically significant difference when compared with sham mice; the number sign denotes a statistically significant difference when compared with Li-treated HIVE mice.

Neuronal and synaptic impairments are attenuated by Li. LTP promotes stabilization and growth of synaptic connections, primarily through the induction of new protein synthesis. A primary clinical manifestation of HAD is memory and behavioral impairments associated with virus-mediated neuronal dysfunction and injury (Wilkie et al., 1992; Heaton et al., 2004). In support of this, we demonstrated previously that losses in LTP correlate with behavioral and cognitive impairments in HIVE mice (Xiong et al., 1999; Anderson et al., 2003, 2004; Xiong et al., 2003). Our electrophysiological findings suggest that Li can restore hippocampal synaptic transmission during HIVE. This may be related to protection of dendritic and synaptic architecture, because Li treatment was associated with preservation of MAP-2+ dendrites and SYP expression. In this context, it is noteworthy that Li has been shown previously to affect neuronal SYP expression in other experimental paradigms through effects on both inositol metabolism (O’Donnell et al., 2000; Williams et al., 2002) and PI3-K/Akt signaling (Chaleycka-Franaszek and Chuang, 1999; Mora et al., 1999). Our results suggest that only the latter pathway may be relevant to the protective effects of Li on neurons exposed to HIV-1 and macrophage neurotoxins.

In conclusion, our results show that Li exerts powerful neuroprotective effects that include neuronal survival, architecture, and function in a murine model of HIVE. At the same time, Li had no appreciable effects on HIV-1 replication or on microglial and astrocyte activation. Thus, the protective effects of Li appear to be chiefly attributable to its effects on neurons. The results suggest that Li may be used as adjunctive therapy for HAD. Combinations of Li, together with potent anti-retroviral therapies and anti-inflammatory therapies, may be particularly effective in the clinic.
References