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Interleukin-1β-dependent Signaling between Astrocytes and Neurons Depends Critically on Astrocytic Calcineurin/NFAT Activity*

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Interleukin-1β (IL-1β) and the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin, have each been shown to play an important role in neuroinflammation. However, whether these signaling molecules interact to coordinate immune/inflammatory processes and neurodegeneration has not been investigated. Here, we show that exogenous application of IL-1β (10 ng/ml) recruited calcineurin/NFAT (nuclear factor of activated T cells) activation in primary astrocyte-enriched cultures within minutes, through a pathway involving IL-1 receptors and L-type Ca^{2+} channels. Adenovirus-mediated delivery of the NFAT inhibitor, VIVIT, suppressed the IL-1β-dependent induction of several inflammatory mediators and/or markers of astrocyte activation, including tumor necrosis factor α, granulocyte/macrophage colony-stimulating factor, and vimentin. Expression of an activated form of calcineurin in one set of astrocyte cultures also triggered the release of factors that, in turn, stimulated NFAT activity in a second set of "naive" astrocytes. This effect was prevented when calcineurin-expressing cultures co-expressed VIVIT, suggesting that the calcineurin/NFAT pathway coordinates positive feedback signaling between astrocytes. In the presence of astrocytes and neurons, 48-h delivery of IL-1β was associated with several excitotoxic effects, including NMDA receptor-dependent neuronal death, elevated extracellular glutamate, and hyperexcitable synaptic activity. Each of these effects were reversed or ameliorated by targeted delivery of VIVIT to astrocytes. IL-1β also caused an NFAT-dependent reduction in excitatory amino acid transporter levels, indicating a possible mechanism for IL-1β-mediated excitotoxicity. Taken together, the results have potentially important implications for the propagation and maintenance of neuroinflammatory signaling processes associated with many neurodegenerative conditions and diseases.

Astrocytes play a critical role in regulating the neuroimmune inflammatory processes that arise following acute brain injury and also during nearly every neurodegenerative disease (1–7). One of the most potent and best characterized signals for triggering astrocyte activation is the cytokine interleukin-1β (IL-1β),[3] which is released in large measure from activated microglia shortly after neural injury (8–11). IL-1 sets in motion a number of positive feedback cycles between astrocytes, microglia, and neurons underscored by the extensive interplay of multiple cytokine species (12–15). Activated astrocytes, in particular, release numerous inflammatory mediators, such as tumor necrosis factor α (TNFα), granulocyte macrophage colony-stimulating factor (GM-CSF), and S100B, to name a few (6, 16–18). These factors can cause further activation of local neuroglia and therefore perpetuate immune/inflammatory signaling cycles if not brought in check by endogenous or exogenous anti-inflammatory agents.

The intracellular cascades resulting in cytokine production in astrocytes may be similar to those in other immunocompetent cells, such as B and T lymphocytes. Astrocytes, for instance, express AP-1, the nuclear factor κB (NFκB), and several different MAPK pathway constituents, which have been shown to control transcriptional activity and to respond vigorously to increased IL-1β levels (15). Astrocytes also express the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (CN) (19–21), but unlike the MAPK- and NFκB-dependent pathways, CN-mediated immune/inflammatory signaling in astrocytes has received little attention until recently.

In neural tissue, CN is perhaps best known for its actions in neurons, where it regulates a diverse set of processes including cytoskeletal reorganization (22), synaptic plasticity and cognition (23), and apoptosis (24). Nonetheless, it is becoming increasingly clear that CN isoforms appear at high levels in astrocytes in response to brain injury, aging, and/or amyloidosis (20, 25). In astrocytes, the expression of activated CN (aCN)

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[3] The abbreviations used are: IL-1β, interleukin-1β; IL-1R, interleukin-1 receptor; IL-1RA, interleukin-1 receptor antagonist; aCN, activated calcineurin; CN, calcineurin; CsA, cyclosporin A; EAAT, excitatory amino acid transporter; sEPSC, spontaneous excitatory postsynaptic current; GM-CSF, granulocyte-macrophage colony-stimulating factor; LPS, lipopolysaccharide; VSCC, voltage-sensitive Ca^{2+} channel; L-VSCC, L-type voltage-sensitive Ca^{2+} channel; NMDA, *N*-methyl-D-aspartate; TNFα, tumor necrosis factor α; NFκB, nuclear factor κB; Ad, adenovirus; CMV, cytomegalovirus; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; RT, reverse transcription; MAPK, mitogen-activated protein kinase.

appears to initiate many of the same processes associated with elevated IL-1β levels, including astrocyte activation and the induction of numerous immune/inflammatory signaling molecules (25, 26). Moreover, blockade of IL-1 signaling and CN activity each helps to reduce glial activation and neuroinflammation following neural injury (27–30).

In lymphocytes and other cell types, CN regulates cytokine production primarily through the direct activation of a family of transcription factors called NFATs (nuclear factor of activated T cells) (31). In fact, most cytokines released by activated T cells are expressed from genes that encode multiple NFAT-binding elements (32). Typically, NFATs regulate cytokine gene expression by coupling with other transcription factors, such as AP-1 or NFκB (33), which, as mentioned, are present in astrocytes and responsive to IL-1β.

Although IL-1β stimulates Ca^{2+} elevations in astrocytes (34–36), a requisite for CN activation, no studies to our knowledge have examined whether these two key signaling molecules interact in the context of neuroinflammation. Here, we show that CN/NFAT activity in rat primary astrocyte cultures is strongly recruited by the exogenous application of IL-1β. Moreover, blockade of CN/NFAT activity in astrocytes inhibited IL-1β-dependent cytokine expression, interfered with positive feedback interactions between astrocytes, and ameliorated the deleterious effects of IL-1β on neuronal function and viability. The results may have important implications for the initiation and progression of neuroinflammation and neurodegenerative disease.

EXPERIMENTAL PROCEDURES

Primary Cell Cultures—All cell cultures were kept at 37 °C in a humidified CO_2 incubator until use. Primary astrocyte-enriched cultures (~95% pure) were prepared from cortical tissue of embryonic day 18 Sprague-Dawley rat pups, as described previously (40). In brief, tissue was harvested and suspended in Hanks' balanced salt solution before mechanical dissociation by trituration. Cells were then plated in culture flasks in minimal essential medium, buffered by $NaHCO_3$, and supplemented with KCl, L-glutamine, pyruvate, and 10% fetal bovine serum. At 80–90% confluence (typically 7–10 days), microglia were removed by vigorously shaking the flasks at room temperature (2–4 h) on an orbital shaker. Culture medium was aspirated and exchanged for fresh medium. When necessary, this step was repeated to remove essentially all microglia. Cells were then trypsinized and removed from flasks and plated on 35-mm culture dishes for experiments. Astrocyte cultures were grown to confluence (usually for 12–14 days) prior to use, and medium was changed every 7 days.

Primary microglial cultures were established from medium extracted from astrocyte cultures following the shaking step described above. Cells were plated in 35-mm dishes at a density of 2×10^5 cells/ml. After cell attachment (usually 24 h), culture medium was exchanged for Iscove's modified Dulbecco's medium with L-glutamine and 25 mM HEPES. Medium was supplemented with 10% fetal bovine serum and 1% antibiotic-antimitotic mix (Invitrogen). Medium was changed every 4–5 days until cells were used.

Mixed hippocampal cultures of neurons and astrocytes were prepared from embryonic day 18 Sprague-Dawley rat pups as described previously (41, 42). Dissociated neurons and astrocytes (~4×10^5/ml) were plated on poly-L-lysine-coated 35-mm dishes with 10% fetal bovine and 10% horse serum. At 3 days *in vitro*, culture medium was supplemented with 5-fluoro-2-deoxyuridine (15 μg/ml) to inhibit proliferation of nonneuronal cells.

Primary enriched cortical neuronal cultures were prepared from embryonic day 18 Sprague-Dawley rat pups in a similar manner as described previously (43). Dissociated cortical tissue was passed several times through a glass pipette and subjected to a 3-min incubation at 37 °C in a CO_2 incubator. Cells were then filtered through a 0.22-μm nylon mesh (Falcon, Franklin Lakes, NJ) and centrifuged at $272 \times g$ for 3 min at 4 °C. The pellet was washed three times in neurobasal medium supplemented with B27 (20 μl/ml), penicillin/streptomycin/neomycin (20 μl/ml) and glutamax-1 (10 μl/ml). Isolated cortical neurons were then diluted in 1 ml of neurobasal medium (2×10^6 cell/ml) and plated on poly-L-lysine-coated 35-mm dishes. After cells were attached, cell culture medium was exchanged for fresh neurobasal medium supplemented with B27, antibiotics, and Glutamax. Cells were used for viability assays at between 14 and 21 days *in vitro*.

Replication-deficient Adenovirus—Adenovirus encoding an NFAT-dependent luciferase reporter construct (Ad-NFAT-Luc), which encodes nine copies of an NFAT binding site (from the IL-4 promoter) and an additional minimal promoter upstream of a luciferase sequence, was kindly provided by Dr. Jeff Molkentin (University of Cincinnati) and has been described in detail elsewhere (44). Ad-LacZ-GFP which encodes β-galactosidase driven by a cytomegalovirus (CMV) promoter as well as a green fluorescent protein (GFP) tag expressed from an internal ribosomal entry site was kindly provided by Dr. Rita Balice-Gordon (University of Pennsylvania). Where necessary, this virus was used as a control for nonspecific viral effects. A truncated, constitutively active CN A fragment was amplified from the pTJ66 vector (45), kindly provided by Dr. Grace Pavlath (Emory University), and subcloned into the polylinker site of a hybrid pCI vector containing IRES2-DsRed-Express (Clontech). The potent NFAT nuclear translocation inhibitor, VIVIT, was kindly provided by Dr. Anjana Rao (Harvard University) as a fusion construct (pCMV-VIVIT-EGFP), which has been described elsewhere (46). VIVIT-EGFP was extracted by restriction digestion and subcloned into a pCI vector. Both CMV-aCN-DsRed and CMV-VIVIT-EGFP were subcloned into adenoviral shuttle vectors (pAd-Link) and recombined with dl327 wild-type adenoviral cDNA in human embryonic kidney 293 cells via Ca^{2+}-phosphate transfection. The pAdLink vector and dl327 adenovirus were obtained from the Vector Core at the University of Pennsylvania. Adenoviruses were purified on CsCl gradients and titered using the Adeno-X Rapid Titer kit (Clontech). Viruses were added to cultures at a multiplicity of infection of ~100.

Drugs—Lipopolysaccharide (LPS; Sigma) was added to microglial cultures at a concentration of 100 ng/ml. Rat IL-1β (Pierce) was delivered to cultures at a final concentration of 10 ng/ml. Several pharmacological agents were added independ-

ently to cell cultures at ~1 h prior to administration of IL-1β. These included cyclosporin A to inhibit CN activity (Sigma), the IL-1 receptor antagonist (IL-1RA; R&D Systems, Minneapolis, MN), nifedipine (Sigma) to antagonize L-type voltage-sensitive Ca^{2+} channels (VSCCs), and ω-conotoxins GVIA and MVIIC (Sigma) to antagonize N- and P-/Q-type VSCCs, and MK-801 (Sigma) to inhibit NMDA receptor activity.

NFAT-luciferase Reporter Assays—At ~24 h before treatment with inflammatory mediators or active CN adenovirus, astrocyte culture dishes were infected with Ad-NFAT-Luc (see above). At a multiplicity of infection of 100, more than 90% of all astrocytes are infected within each dish, ensuring uniform distribution of the NFAT-reporter construct across treatment groups. After exposure to IL-1β (~3 h), each culture dish was washed three times in phosphate-buffered saline, and cells were scraped free and pelleted at 13,000 rpm. Supernatants from each 35-mm dish were removed and replaced with CAT buffer (250 mM Tris, pH 8.0, 1 mM EDTA), and pellets were stored at −20 °C until use. Samples were freeze/thawed twice, resuspended, and centrifuged at 13,000 rpm, and supernatants were collected. As a further control for potential between-group variability in NFAT-luciferase expression, all sample volumes were normalized to the same protein concentration using the Lowry method. Luciferase expression was quantified using a luciferase detection kit (luc Screen; Tropix, Bedford, MA) and a plate reader. Typically, six or more dishes were analyzed per treatment condition, resulting in a well powered experimental design.

NFAT Translocation—The pHA-NFAT1-(1–460)-GFP vector was kindly provided by Dr. Anjana Rao and has been described elsewhere (47). This vector encodes the first 460 amino acids of the mouse NFAT1 isoform fused to EGFP and driven by a CMV promoter. Astrocyte cultures were transfected with pHA-NFAT1-(1–460)-GFP using Lipofectamine LTX and PLUS transfection reagents (Invitrogen). At 24–48 h after transfection, culture dishes were transferred to the stage of a Nikon E600 microscope, and EGFP expression was detected with appropriate filters using a ×40 Fluor objective. Following treatment with inflammatory mediators, cells were imaged once every 5 min for 35 min using a Nikon CoolSnap ES digital camera.

Western Blot—Cells plated on 35-mm dishes were scraped free into ice-cold sucrose buffer (0.3 M sucrose, 0.75 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.02 M EDTA, 0.02 M EGTA), supplemented with protein phosphatase and protease inhibitor mixtures (Calbiochem). Within each treatment condition, material from six plates was combined and pelleted at 4 °C by spinning at 3500 rpm for 5 min. Approximately two-thirds of the supernatant was removed, and pellets were resuspended with a handheld homogenizer before the addition of SDS to a final concentration of 1%. Samples were then heated to 65 °C for 15 min and stored at −80 °C until use. The Lowry method was used to determine protein levels, and each sample was diluted 25% with running buffer. Samples were loaded into individual lanes of a gradient gel with protein concentrations held constant across lanes. Proteins were resolved by electrophoresis and transferred to polyvinylidene difluoride membranes for quantitative Western blot. Membranes were incubated at 4 °C overnight in I-Bloc (Tropix) along with primary antibodies, which included rabbit anti-CN Aα (1:1000; Millipore, Billerica, MA), rabbit anti-EAAT1 and rabbit anti-EAAT2 (both at 1:5000), and mouse glyceraldehyde-3-phosphate dehydrogenase (1:5000) (Abcam, Cambridge, MA). Primary antibodies were tagged with an appropriate horseradish peroxidase-conjugated secondary antibody, diluted in I-Block at 1:10,000, and detected using the ECL-plus Western kit (GE Healthcare). Protein levels were quantified using a Storm 860 molecular imager.

Real Time RT-PCR—Cells in 35-mm culture dishes were lysed in 800 μl of Trizol reagent (Sigma) and incubated at room temperature for 5 min before storage at −80 °C until use. RNA was chloroform-extracted from lysates and then further isolated and purified using the RNeasy kit (Qiagen, Valencia, CA). RNA concentration and quality was determined using a spectrophotometer and agarose gel electrophoresis. cDNA was made from RNA samples using a Superscript III kit from Invitrogen. All samples were equilibrated by adding 1 μg/ml of RNA as determined by pilot studies. cDNA samples were diluted by a factor of 10 and stored at −80 °C until use.

RT-PCR experiments were performed using the Superarray RT-2 SyBr Green system and were read on an ABI Mx3000 Multiplex RT-PCR instrument. All primer sets and the reaction mix were purchased from Superarray. Samples underwent 40 cycles of RT-PCR (95 °C, 10 min; 40 cycles at 95 °C for 15 s, 60 °C for 15 s). Results were analyzed using Multiplex software. The amount of target message in each reaction was determined from the detection threshold cycle number (C_t), which is inversely correlated with the abundance of the message's initial level. The C_t was then converted to relative quantity by normalizing to a standard curve.

Medium Exchange Experiments—For experiments involving the use of microglial conditioned medium (*i.e.* Fig. 1, *C* and *D*), primary microglia cultures were treated with or without the inflammatory factor LPS for 3–4 h, after which cells were washed three times in phosphate-buffered saline, and placed in serum-free medium and returned to the incubator. Twenty-four hours later, this conditioned microglia medium was transferred to primary astrocyte cultures preloaded with the NFAT-luciferase reporter construct. In other experiments (*i.e.* Fig. 3, *E* and *F*), astrocytes were infected with adenoviruses containing a control construct (*i.e.* LacZ (β-galactosidase)), aCN, or aCN plus VIVIT. Additional control cultures were left uninfected. After 4 h of viral incubation, cultures were washed three times, placed in serum-free medium, and returned to the incubator. Twenty-four hours later, conditioned medium from these cultures was applied to naive astrocytes preloaded with the NFAT-luc construct.

Neuronal Death Assay—For these experiments, sandwich cultures of neurons and astrocytes were used. Astrocyte-enriched cultures, prepared as described above, were plated on 25-mm diameter Thermanox plastic coverslips (Nunc, Naperville, IL). On day 1 of these experiments, astrocytes were incubated with adenovirus for 4 h and washed three times in culture medium and returned to the CO_2 incubator. On day 2, astrocyte coverslips were flipped, cell side down, onto 30-mm diameter permeable membranes (0.4-μm diameter pores) and placed into 35-mm dishes of cortical neurons, prepared as

described above. The permeable membrane was used to prevent any large cellular debris (*e.g.* membrane fragments) from contacting neurons. On day 3, sandwich cultures were treated with IL-1β at 10 ng/ml. Final conditions included Ad-LacZ-GFP with or without IL-1β, Ad-VIVIT-EGFP with or without IL-1β, and Ad-aCN-DsRed2 with or without IL-1β. On day 5, astrocyte-containing coverslips were removed from cortical neurons, and neuronal cultures were assayed for the presence of dead cells using the LIVE/DEAD cell toxicity assay (Invitrogen). In this assay, cells incorporating ethidium homodimer-1 (*i.e.* dead cells) are identified using fluorescence microscopy (E600 Nikon with appropriate filters) and a ×10 objective. Cells were counted and averaged within each of 10 ×10 fields, chosen at random, and compared across treatment conditions. Each of these experiments was repeated at least three times in separate cell cultures with similar results.

Measurement of Extracellular Glutamate—A modified version of the UV method, described by Lund (48), was used to estimate extracellular glutamate levels. Sandwich cultures were treated with viruses and IL-1β as described for neuronal death assays. At 48 h after IL-1β treatment, conditioned medium was harvested from each culture and spun down at 13,000 rpm to remove cellular debris. 250 μl of each sample was transferred to a disposable cuvette and combined with reaction buffer (final volume of 2 ml) consisting of 1.6% hydrazine monohydrate, 50 mM Tris, 1 mM EDTA, 0.5 mM adenosine 5′-diphosphate, and 1.5 mM NAD (all chemicals from Sigma). L-Glutamate in each sample was converted to α-ketoglutarate via the addition of 30 μl of glutamate dehydrogenase (1200 units/ml from Calzyme Laboratories, San Luis Obispo, CA). The corresponding proportional conversion of NAD^+ to NADH was measured 80 min later using a spectrophotometer. For each sample, absorbence was read at 340 nM, and relative glutamate levels were determined from a standard curve of known glutamate concentrations.

Electrophysiological Analysis of Synaptic Activity in Mixed Cultures—Co-cultured hippocampal neurons were incubated with virus for 4 h prior to application of IL-1β (10 ng/ml) and 24 h prior to recording. Experimental groups included Ad-LacZ-GFP (control), Ad-LacZ-GFP with or without IL-1β, and Ad-VIVIT-EGFP with or without IL-1β. Immediately before experimentation, culture medium was replaced with recording solution, and dishes were transferred to the stage of a Nikon E600 physioscope.

External recording solution contained 145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 10 mM D-glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.01 mM glycine; pH was adjusted to 7.35 using NaOH. Osmolarity was adjusted to 310 mosM with sucrose. The recording pipette solution contained 150 mM KCH_3SO_4, 5 mM HEPES, 4 mM Tris-ATP, 0.3 mM Tris-GTP, 1.4 mM Tris-phosphocreatine, and 0.1 mM leupeptin; pH was adjusted to 7.35 using KOH, and osmolarity was adjusted to 290 mosM by dilution with distilled H_2O. All solutions were sterile-filtered.

Recording pipettes made from glass capillary tubes (Drummond Scientific, Broomall, PA) were pulled on a horizontal micropipette puller (model P-97; Sutter Instruments, Novato, CA). All pipettes were coated with polystyrene Q-dope and were fire-polished immediately before recording. The average tip resistance was 2.7 ± 0.06 megaohms. Spontaneous synaptic activity was monitored in individual neurons using whole cell voltage clamp. Membrane voltage was controlled, and current was recorded by a Multiclamp 700B amplifier and pClamp 9.0 software via a Digidata 1332A interface (Molecular Devices Corp., Sunnyvale, CA).

At the beginning of each experiment, junction potentials were nulled in the bath, and pipette capacitance was compensated. Whole cell membrane capacitance (C_m), access resistance (R_a), and holding current (I_h) were determined on-line using the membrane test feature of Clampex 9.0 and did not differ across treatment groups. Average values were as follows: $C_m = 68.80 \pm 4.12$ picofarads, $R_a = 8.17 \pm 0.50$ megaohms, and $I_h = -104.56 \pm 17.15$ pA.

Neurons were clamped at −80 mV throughout the experiment. Spontaneous excitatory postsynaptic currents (sEPSCs) were filtered at 2 kHz and digitized at 5 kHz. Properties of sEPSCs, including amplitude and total number of events, were analyzed using Minianalysis software (Synaptosoft, Inc., Decatur, GA). The number of sEPSCs was counted over a 3-min period after membrane parameters, such as R_a and I_h, stabilized (usually within 2 min). sEPSCs then were summed and binned offline according to amplitude (histograms for sEPSC amplitudes are provided in Fig. 5*E*). Similar to our previous work with this culture model (41), the majority of events in each treatment group fell between 15 and 20 pA (*i.e.* the peak of the distribution). After the peak, the number of events fell exponentially as the event amplitude increased to about 300 pA. Events of >300 pA were excluded from these histograms and analyzed separately, because their amplitudes were narrowly distributed and easily discriminated from small events. Electrophysiological recordings were performed on cells between 13 and 15 days *in vitro*.

Statistics—Analysis of variance was used for all statistical comparisons. Where necessary, Fisher's protected least significant difference was used for *post hoc* comparisons. For all statistical tests, significance was set at $p < 0.05$.

RESULTS

IL-1β Robustly Stimulates CN/NFAT Activity in Primary Astrocyte Cultures—To measure CN/NFAT activity in astrocytes, we preloaded primary astrocyte cultures with an NFAT-luciferase reporter construct via adenovirus, as described previously (44). IL-1β was applied alone at a concentration of 10 ng/ml or in the presence of the CN inhibitor cyclosporin A (5 μM) or VIVIT, a peptide that prevents CN from docking to and dephosphorylating NFAT transcription factors (46). VIVIT was expressed by adenovirus-mediated infection. As shown in Fig. 1*A*, IL-1β caused a marked and significant increase in NFAT-dependent luciferase activity ($p < 0.001$). Co-treatment with either CsA or VIVIT blocked these effects. IL-1β-mediated activation of CN/NFAT signaling was also inhibited in a concentration-dependent manner by the IL-1 receptor antagonist (IL-1RA) (Fig. 1*B*). Significant inhibition was observed at IL-1RA concentrations as low as 0.4 ng/ml (~43%, $p < 0.05$ *versus* 0 ng/ml IL-1RA), and nearly complete inhibition was observed with 40 ng/ml IL-1RA (~88%, $p < 0.0001$ *versus* 0

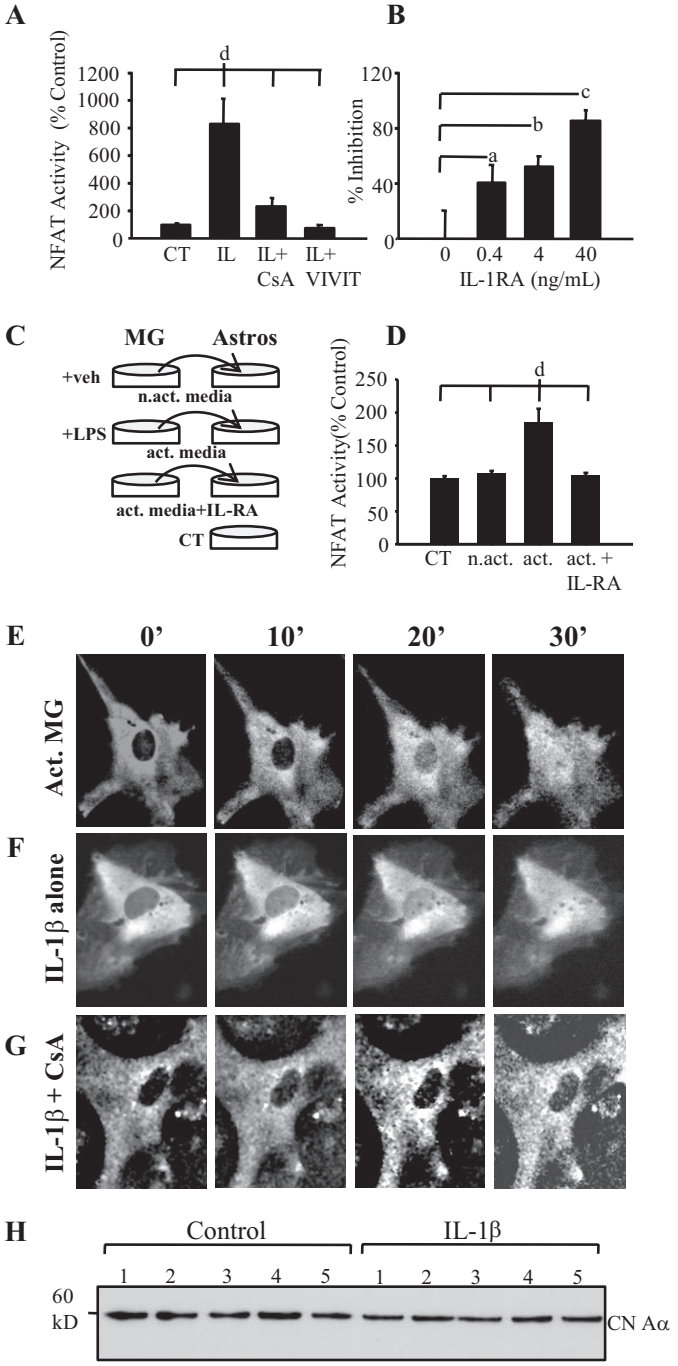

FIGURE 1. **Activation of NFAT activity in primary astrocyte cultures by IL-1β.** *A*, mean ± S.E. NFAT-luciferase activity in primary astrocyte cultures expressed as a percentage of the NFAT-luc alone control (*CT*) condition ($n = 6$ cultures/condition). Relative to control cultures, cultures treated with IL-1β (*IL*) showed a significant increase in NFAT-luc activity. Effects of IL-1β were blocked in cells co-treated with the CN inhibitor CsA (5 μM) or the NFAT inhibitor VIVIT. *B*, IL-1β was delivered in the presence of increasing concentrations of IL-1RA. The extent to which each concentration of IL-1RA inhibited IL-1β-mediated NFAT activation is expressed as a percentage of inhibition relative to 0 ng/ml ($n = 8$ cultures/condition). *C*, schematic diagram of medium exchange experiments between microglial cultures and astrocytes preloaded with the NFAT luciferase construct. Primary microglial cultures were either vehicle-treated (*i.e.* nonactivated (*n.act*)) or activated (*act.*) with LPS (100 ng/ml). Conditioned medium from microglial (*MG*) cultures was added 24 h later to astrocyte cultures preloaded with the NFAT-luc construct. As shown in *D*, NFAT-luc activity was significantly stimulated by conditioned medium from activated but not by nonactivated MG ($n = 7–8$ cultures/condition). Pretreatment of astrocyte cultures with IL-1RA (40 ng/ml) blocked the effects of activated MG medium on astrocytic NFAT activity. *E* and *F*, representative fluorescent images of NFAT-GFP expression in individual astrocytes at 0, 10, 20, and 30 min following treatment with either conditioned medium from activated microglia (*E*, *Act. MG*), IL-1β alone (*F*, *IL-1β alone*) or IL-1β with CsA (*G*, *IL-1β + CsA*). NFAT-GFP began to appear in the nucleus within minutes after treatment with activated microglia medium or IL-1β. By 30 min, the nucleus was largely indistinguishable from the cytosol in these conditions. Cytosol-to-nucleus translocation of NFAT-GFP was not observed in IL-1β-treated astrocytes in the presence of CsA. *H*, Western blot for expression of the CN-Aα isoform in astrocyte cultures 48 h after treatment with vehicle control or with IL-1β ($n = 5$ pooled samples/per group). IL-1β did not alter CN A protein levels. *a*, $p < 0.05$; *b*, $p < 0.01$; c, $p < 0.001$; d, $p < 0.0001$.

ng/ml IL-1RA). Thus, recruitment of CN most likely occurs through activation of the primary IL-1 receptor.

Activated microglia appear to be the primary source of IL-1β in the brain (10). To determine if endogenous IL-1 release from microglia stimulates the CN/NFAT pathway in astrocytes, primary microglial cultures were first activated for 3 h with LPS (100 ng/ml) or left untreated (Fig. 1*C*). Microglia were then washed three times and placed in serum-free medium for 24 h. This conditioned medium from activated and nonactivated microglia was then extracted and applied to naive astrocyte cultures preloaded with the NFAT-luc construct. For some cultures, the IL-1 receptor antagonist (IL-1RA; 40 ng/ml) was added to conditioned medium immediately prior to delivery to astrocytes. As shown in Fig. 1*D*, conditioned medium from activated, but not nonactivated, microglia significantly stimulated NFAT activity in astrocytes, and this effect was nearly completely inhibited by blockade of IL-1 receptors with IL-1RA ($p < 0.0001$). Together, the results suggest that IL-1 release from microglia plays a critical role in activating the CN/NFAT pathway in astrocytes.

Rapid Cytosolic-to-Nuclear Translocation of NFATs in Response to IL-1β—IL-1β may activate the CN/NFAT pathway in astrocytes directly and rapidly. Alternatively, recruitment of CN/NFAT activity may be secondary to, or a downstream consequence of, one or more immune/inflammatory cascades set in motion by IL-1β. To determine how rapidly IL-1β activates CN/NFAT activity, astrocytes were transfected with a plasmid encoding an NFAT-GFP fusion protein (pHA-NFAT1-(1–460)-GFP) and then visualized 24 h later using fluorescence microscopy.

As shown previously in other cell types (46, 49), NFAT-GFP was distributed widely throughout the cytosol in resting cells and largely excluded from presumptive nuclei (Fig. 1, *E–G*, 0 min time points). When conditioned medium from activated microglia (Fig. 1*E*) or exogenous IL-1β (Fig. 1*F*) was applied to astrocytes, the GFP tag showed a dramatic change in its distribution such that the nuclei became difficult to distinguish from the cytosolic compartments. Translocation of NFAT-GFP from the cytosol to the nucleus usually began within 10–20 min following treatment and was inhibited by co-treatment with the CN inhibitor, CsA (Fig. 1*G*). Rapid translocation of NFATs in response to IL-1β is consistent with a direct route of activation and suggests that the CN/NFAT pathway is well suited as an upstream regulator of IL-1β-mediated immune/inflammatory cascades in astrocytes.

IL-1β Does Not Up-regulate CN Protein Levels in Astrocytes—We next investigated whether increased IL-1β levels are capa-

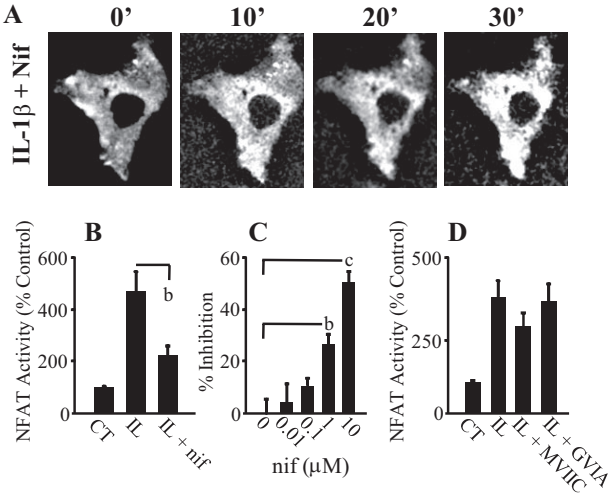

FIGURE 2. **CN/NFAT activation in astrocytes selectively depends on L-VSCCs.** *A*, representative fluorescent images of NFAT-GFP expression in individual astrocytes at 0, 10, 20, and 30 min following treatment with IL-1β in the presence of the L-VSCC blocker nifedipine (*nif*; 10 μM). Very little cytosol-to-nucleus translocation of NFAT-GFP was observed following IL-1β treatment when L-VSCCs were blocked. *B*, pretreatment of astrocyte cultures with nifedipine (10 μM) also blocked the actions of IL-1β on NFAT-dependent luciferase activity (mean ± S.E.) in astrocytes. *C*, inhibition of IL-dependent NFAT activity increased with increasing concentrations of nifedipine. Maximal inhibition (~50%) was observed at 10 μM. *D*, inhibitors of N- and P-/Q-type VSCCs with ω-conotoxins MVIIC and GVIA did not significantly alter IL-1β-mediated activation of NFAT-luc activity. For *B–D*, $n = 8$ cultures/group. *b*, $p < 0.01$; *c*, $p < 0.001$. *CT*, control.

ble of driving the expression of CN in astrocytes. Indeed, under certain pathological conditions, such as cardiac hypertrophy, increased activation of CN precipitates elevated CN protein levels (49, 50). Moreover, activated astrocytes associated with injury, aging, and/or amyloidosis exhibit increased CN expression levels, similar to hypertrophic cardiomyocytes (20, 25). As shown in Fig. 1*H*, however, Western blots for astrocyte cultures treated with or without IL-1β for 48 h showed no differences in the expression of the CN Aα isoform. The other major CN A isoform, CN Aβ, was nearly undetectable in this culture model (data not shown). Thus, IL-1β does not appear sufficient to drive CN expression in astrocytes.

Role of VSCCs in CN/NFAT Activation—In many cell types, CN is directly, and sometimes selectively, activated by Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels (51), which are expressed and functional in primary cortical astrocytes (52–54). As shown in Fig. 2, the cytosol-to-nuclear translocation of NFAT-GFP (Fig. 2A) and the increase in NFAT-luciferase activity (Fig. 2*B*), normally observed in astrocytes following IL-1β delivery (see Fig. 1), were markedly inhibited by pretreatment with the L-VSCC antagonist, nifedipine (10 μM). Indeed, nifedipine inhibited CN/NFAT activity in a concentration-dependent manner (Fig. 2*C*), with significant inhibition observed at 1 and 10 μM (~27 and ~51%, respectively; $p < 0.001$). In contrast, conotoxin-mediated blockade of N- and P-/Q-type VSCCs, which are also expressed in astrocytes (53), did little to impair the effects of IL-1β on CN/NFAT activity (Fig. 2*D*). L-VSCCs therefore provide a relatively selective source for CN/NFAT activation in astrocytes, at least in response to IL-1β.

IL-1β-mediated Expression of Inflammatory Mediators Requires CN/NFAT Activity—Increased IL-1β levels are associated with increased expression of several inflammatory mediators in astrocytes, including the cytokines TNF-α and GM-CSF, as well as the trophic factor S100B (9, 55–57), among others. Production of these factors may be critical to the continued activation of neuroglia and also for the maintenance of immune/inflammatory signaling in the brain. Genes for TNFα and GM-CSF encode NFAT binding elements (32), and the expression of these factors in Jurkat T cells is robustly stimulated by NFAT activation (58). Additionally, expression of activated CN is associated with elevated S100B levels in mixed hippocampal cultures of astrocytes and neurons (25).

To determine whether the IL-1β-mediated induction of these inflammatory mediators in astrocytes requires NFAT activation, cultures were treated for 3 h with or without IL-1β in the presence or absence of the NFAT inhibitor VIVIT. mRNA levels then were quantified using real time RT-PCR. We also analyzed mRNA levels for the intermediate filament protein vimentin, since our work and work by others have found vimentin to be an excellent biomarker for neuroinflammation, aging, Alzheimer disease, and increased CN activity (25, 59, 60). As shown in Fig. 3, *A–D*, VIVIT suppressed the IL-1β-mediated increase in mRNA levels for TNFα, GM-CSF, and vimentin but did not appreciably limit the expression of S100B. Together, the results suggest that the CN/NFAT pathway in astrocytes is essential to the expression of several neuroinflammatory biomarkers in response to elevated IL-1β levels. The insensitivity of S100B expression to VIVIT suggests that IL-1β regulates this trophic factor through an NFAT-independent pathway.

CN/NFAT as a Coordinator for Positive Feedback Cycles between Astrocytes—Cytokines derived from astrocytes and microglia have both autocrine and paracrine actions that may contribute to local cytokine cycles and chronic neuroinflammation (13). As illustrated in Fig. 3, *A–D*, CN/NFAT activation in astrocytes leads to increased expression of multiple cytokine species. One of these (*i.e.* TNF-α) was recently shown to stimulate astrocyte-based CN/NFAT activity (26). In fact, we have found that the exogenous application of multiple cytokine species, including TNFα and interferon γ, causes robust activation of the CN/NFAT pathway in astrocytes (data not shown). These results suggest that the CN/NFAT pathway in astrocytes is poised for positive feedback control over local cytokine cycles and neuroinflammatory signaling.

If the CN/NFAT pathway is involved in positive feedback signaling, then activation of CN/NFAT activity in astrocytes should stimulate CN/NFAT activity in adjacent astrocytes via paracrine signaling. To test this possibility, we treated astrocytes with Ad-aCN-DsRed2 to activate CN signaling (Fig. 3*E*). Untreated cultures and cultures infected with control virus (Ad-LacZ-GFP), served as controls for CN activation. For some aCN-treated cultures, cells were co-infected with Ad-VIVIT-EGFP to inhibit CN-NFAT interactions. Four hours after infection, cultures were washed three times to remove extracellular viral particles, and serum-free medium was applied to cells. Twenty-four hours later, conditioned medium from each of these cultures was applied to naive astrocyte cultures preloaded with the NFAT-luc reporter construct.

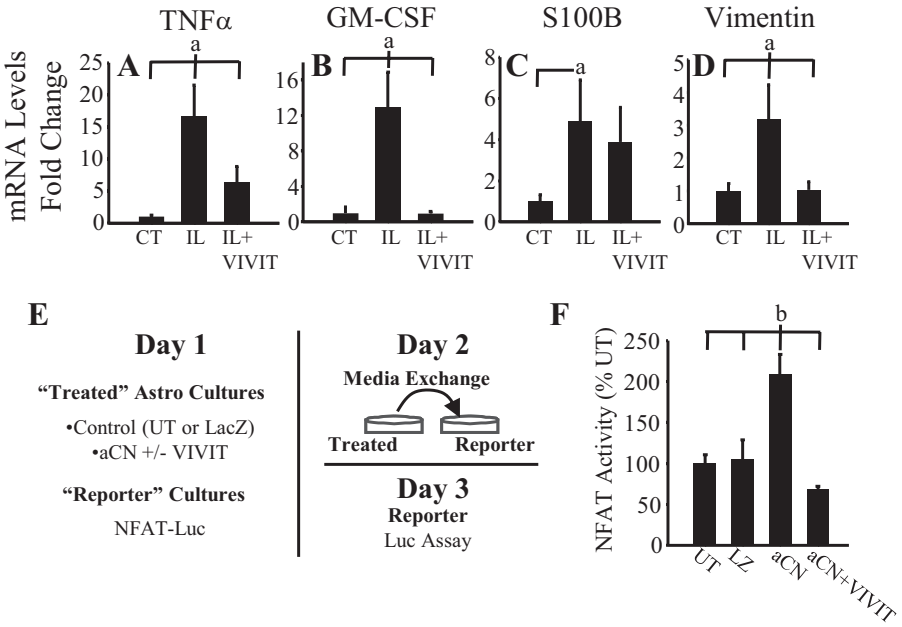

FIGURE 3. **CN/NFAT activity is required for IL-1β-mediated induction of inflammatory markers and governs positive feedback interactions among astrocytes.** *A–D*, real time RT-PCR was used to analyze mRNA levels for TNFα (*A*), GM-CSF (*B*), S100B (*C*), and vimentin (*D*) in primary astrocyte cultures treated for 3 h with IL-1β (*IL*) in the presence or absence of VIVIT ($n = 5–6$ cultures/condition). mRNA levels are expressed as -fold change (mean ± S.E.) relative to control (*CT*). The actions of IL-1β on each mRNA species, except S100B, were inhibited by VIVIT, suggesting the critical involvement of NFAT-dependent transcriptional regulation. *E*, experimental protocol for medium exchange experiments. On day 1, astrocyte cultures were incubated for 4 h with control adenovirus (*LacZ*) or adenovirus encoding activated CN (*aCN*) with or without (±) VIVIT. Cultures were then washed (three times) and incubated in serum-free medium for 24 h. Some cultures were left untreated (*UT*). In parallel, separate cultures (*i.e.* reporter cultures) were preloaded with NFAT-luc ($n = 6–8$ cultures/condition). On day 2, conditioned medium from untreated and LacZ- and aCN-treated cultures (±*VIVIT*) were transferred to reporter cultures. NFAT-luciferase activity was quantified on day 3 and is shown in *F* as the mean ± S.E., relative to the untreated condition. Conditioned medium from aCN-expressing cultures, but not from LacZ-expressing cultures, stimulated NFAT-luc activity in reporter cultures. Medium from aCN-expressing cultures co-treated with VIVIT did not stimulate NFAT activity in reporter cultures. *a*, $p < 0.05$; *b*, $p < 0.01$.

As shown in Fig. 3*F*, conditioned medium from aCN-treated cultures stimulated CN/NFAT activity in naive astrocyte cultures ($p < 0.01$, $n = 6–8$ dishes/condition). NFAT activity was not stimulated by medium extracted from cultures infected with control adenovirus, thus ruling out possible viral confounds. Moreover, conditioned medium from aCN-treated cultures, co-treated with VIVIT, also failed to stimulate CN/NFAT activity in naive astrocytes. The results demonstrate that CN/NFAT activity in astrocytes is capable of driving CN/NFAT activity in adjacent astrocytes through paracrine signaling.

Astrocytic NFAT Activation Is Required for IL-1β-mediated Neuronal Toxicity—Mounting evidence suggests that IL-1β impairs the protective properties of astrocytes (11), leading to alterations in neuronal function, plasticity, and viability. When IL-1β (10 ng/ml) was applied for 48 h to neuron/astrocyte sandwich cultures (see "Experimental Procedures"), the appearance of dead neurons was increased by ~50% (Fig. 4*A*, *1*; $p < 0.0001$). Consistent with previous work (61), IL-1β did not kill neurons in the absence of astrocytes (Fig. 4*A*, *2*). The results demonstrate that IL-1β can modulate neuronal viability via an astrocyte-specific mechanism of action.

The sandwich culture model was next used to determine the extent to which astrocytic CN/NFAT activity is required for IL-1β-mediated neurotoxicity (see Fig. 4*B*). For this study, astrocyte-enriched cultures grown on 25-mm inserts were infected with adenovirus encoding LacZ (viral control) or VIVIT. At 24 h postinfection, astrocyte inserts were transferred to pure neuronal cultures, where they were treated with IL-1β and remained for an additional 48 h. Astrocyte inserts were then removed, and the number of dead neurons was counted. Similar to the experiment shown in Fig. 4*A*, *1*, IL-1β application resulted in a significant increase in the number of dead neurons ($p < 0.0001$) in sandwich cultures containing control LacZ-treated astrocytes (Fig. 4, *C* (*2*) and *F*). In contrast, IL-1β did not kill neurons coupled to VIVIT-expressing astrocytes (Fig. 4, *D* (*2*) and *F*). The results demonstrate a necessary role for astrocytic CN/NFAT activity in IL-1β-mediated neurotoxicity.

Because astrocytic CN activity alone was sufficient to drive paracrine signaling among astrocytes (see Fig. 3, *E* and *F*), we predicted that CN activity in astrocytes would also be sufficient to precipitate neuronal death. Contrary to this prediction, however, astrocytic expression of aCN by itself was not associated with an increase in the appearance of dead neurons (Fig. 4, *E* (*1*) and *F*); nor did the presence of aCN exacerbate the lethal effects of IL-1β (Fig. 4, compare *C* (*2*) and *E* (*2*); also see Fig. 4*F*). Thus, although CN/NFAT activity is necessary for the toxic effects of IL-1β (as indicated by the inhibitory actions of VIVIT), this pathway alone does not appear sufficient for driving lethal astrocyte-neuron interactions.

IL-1β Causes an NFAT-dependent Reduction in Astrocytic Excitatory Amino Acid Transporter (EAAT) Protein Levels—One of the primary neuroprotective functions of astrocytes is the removal of extracellular glutamate from the extracellular milieu using high affinity EAATs. These transporters are highly sensitive to neuroinflammatory signaling and undergo down-regulation in response to both IL-1β and TNFα (62, 63). Moreover, the major astrocytic EAATs (EAAT1 and EAAT2) contain multiple putative NFAT binding elements in their promoter regions (64–66), suggesting a possible point of interaction between IL-1β, NFATs, and glutamate homeostasis.

Based on this evidence, we investigated whether astrocytic EAAT1 and -2 are regulated by IL-1β-mediated NFAT activity. As shown in Fig. 5, *A* and *B*, a 48-h application of IL-1β to primary astrocyte cultures caused a significant reduction in protein levels for both EAAT subtypes, especially for EAAT2, which was decreased by ~50% relative to control cultures ($p < 0.001$). EAAT1 levels were reduced by ~25% ($p < 0.05$). Pretreatment of cultures with the VIVIT adenovirus partially inhibited these IL-1β-mediated effects. In the presence of VIVIT, IL-1β reduced EAAT2 levels only by about 16% ($p >$

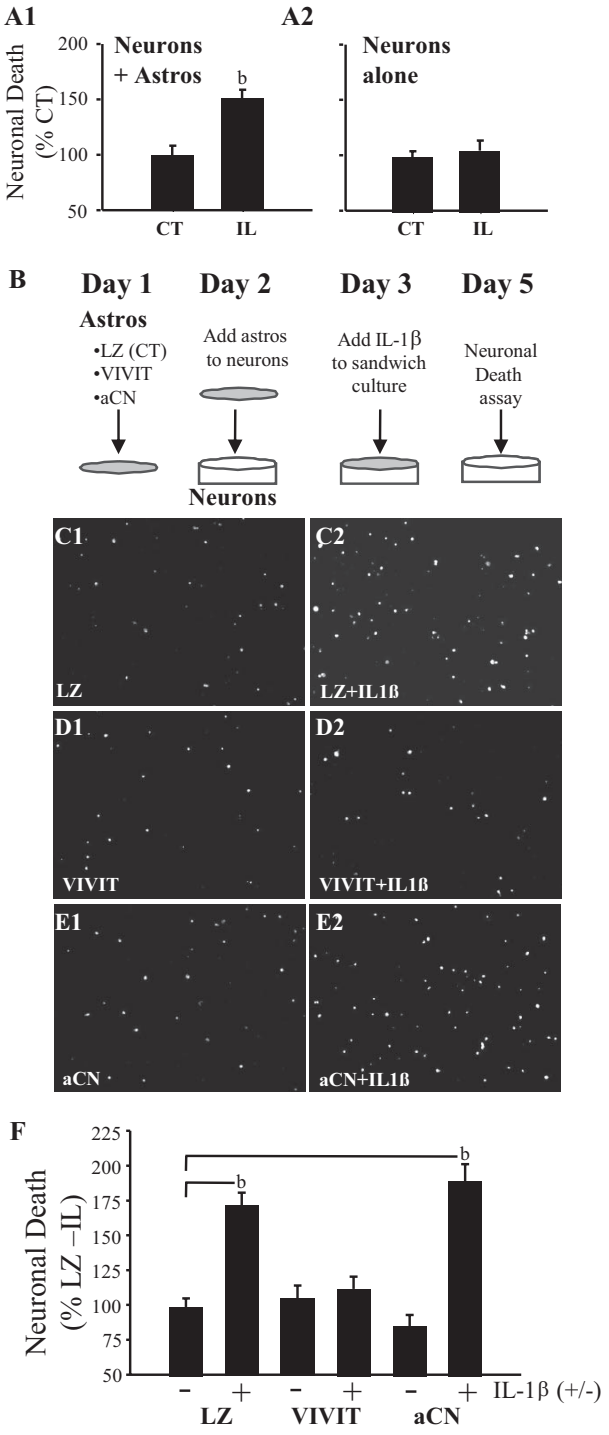

FIGURE 4. **Effects of IL-1β on neuronal viability require astrocytic CN/NFAT activity.** The presence of dead neurons was assayed 48 h after IL-1β treatment (*IL*) of pure neuronal cultures plated with pure astrocyte cultures in a "sandwich" configuration (*A*, *1*) or neuronal cultures alone (*A*, *2*). The average ± S.E. number of dead neurons in each condition is expressed as a percentage of vehicle-treated control (*CT*) cultures ($n = 10$ fields/condition). In *B*, the protocol for selectively targeting astrocytic CN/NFAT activity in astrocyte/neuron sandwich cultures is shown. On day 1, astrocyte-enriched cultures seeded on plastic inserts were infected with control Ad-LacZ adenovirus (*LZ*) or adenovirus encoding VIVIT or active CN (*aCN*). On day 2, astrocyte inserts were transferred (cell side-down) to pure neuronal cultures. Sandwich cultures were treated with vehicle or IL-1β on day 3, and on day 5, astrocytes were removed, and neuronal viability was assayed. *C–E*, representative fluorescent images (at ×10) of dead neurons in cultures treated without IL-1β (*C* (*1*) to *E* (*1*)) or with IL-1β (*C2–E2*). *F* shows the mean ± S.E. number of dead cells in each condition ($n = 10$ fields/condition) expressed as a percentage of the 0.05 *versus* control cultures, $p < 0.001$ *versus* IL-1β alone). Although VIVIT slightly inhibited the actions of IL-1β on EAAT1 as well, these effects did not reach significance ($p > 0.05$ *versus* IL-1β alone).

Consistent with a reduction in astrocytic EAAT expression, IL-1β also caused an ~33% increase in extracellular glutamate levels in astrocyte/neuron sandwich cultures ($p < 0.0001$; Fig. 5*C*). As with neuronal death and reduced EAAT expression, increased glutamate levels were not observed in cultures containing VIVIT-expressing astrocytes ($p < 0.01$). To determine if glutamate toxicity plays a role in IL-1β-mediated, NFAT-dependent neuronal death, sandwich cultures were co-treated with IL-1β and the NMDA-type glutamate receptor antagonist, MK-801 (10 μM). As shown in Fig. 5*D*, neuronal death due to IL-1β was nearly completely prevented by MK-801 ($p < 0.01$ *versus* IL-1β alone).

Neuronal Network Hyperexcitability after IL-1β Exposure Requires NFAT Activation—Hyperexcitability in neuronal networks may be a key process in the amplification and maintenance of excitotoxicity (67). Moreover, reduction of EAAT expression/function typically leads to aberrantly high levels of neuronal network activity characterized, in part, by increased neuronal firing rates (68–70). To determine if IL-1β has similar effects on network activity, we applied IL-1β (24 h) to mixed neuronal and astrocyte cultures (13–15 days *in vitro*) and recorded sEPSCs from individual neurons using whole-cell voltage clamp techniques. Cultured neurons at this age show extensive synaptic connectivity (42) and robust sEPSC activity (Fig. 5*D*) that can be separated roughly into two categories based on amplitude: small events (the vast majority of which are ≤50 pA; Fig. 5*D* (*1*)) and compound events (≥300 pA; Fig. 5*D* (*2*)). Although both kinds of activity depend critically on the activation of AMPA-type glutamate receptors, only the compound events require synchronous, action-potential-driven activity in the surrounding neuronal network (41). For these studies, cultures were also infected with control adenovirus encoding LacZ or virus encoding VIVIT to inhibit CN/NFAT activation.

IL-1β application, with or without VIVIT, had virtually no impact on several membrane and recording parameters, including membrane capacitance, membrane resistance, input resistance, and holding current (see "Experimental Procedures"). Similarly, IL-1β, with or without VIVIT, did not reveal any effects on the appearance of small sEPSCs, as shown in the sEPSC amplitude histograms (Fig. 5, *E* and *G*). In contrast, IL-1β significantly increased the occurrence of compound sEPSCs (Fig. 5, *F* and *H*; $p < 0.05$), and this effect was prevented in VIVIT-expressing cultures (Fig. 5, *F* and *H*). The results are consistent with the actions of VIVIT on neuronal viability and EAAT protein levels and provide further evidence for a role of CN/NFAT activity in IL-1β-mediated excitotoxicity.

Ad-LacZ alone condition (*i.e.* LZ – interleukin). Note that IL-1β did not kill neurons when NFAT activity was blocked in astrocytes. *b*, $p < 0.01$.

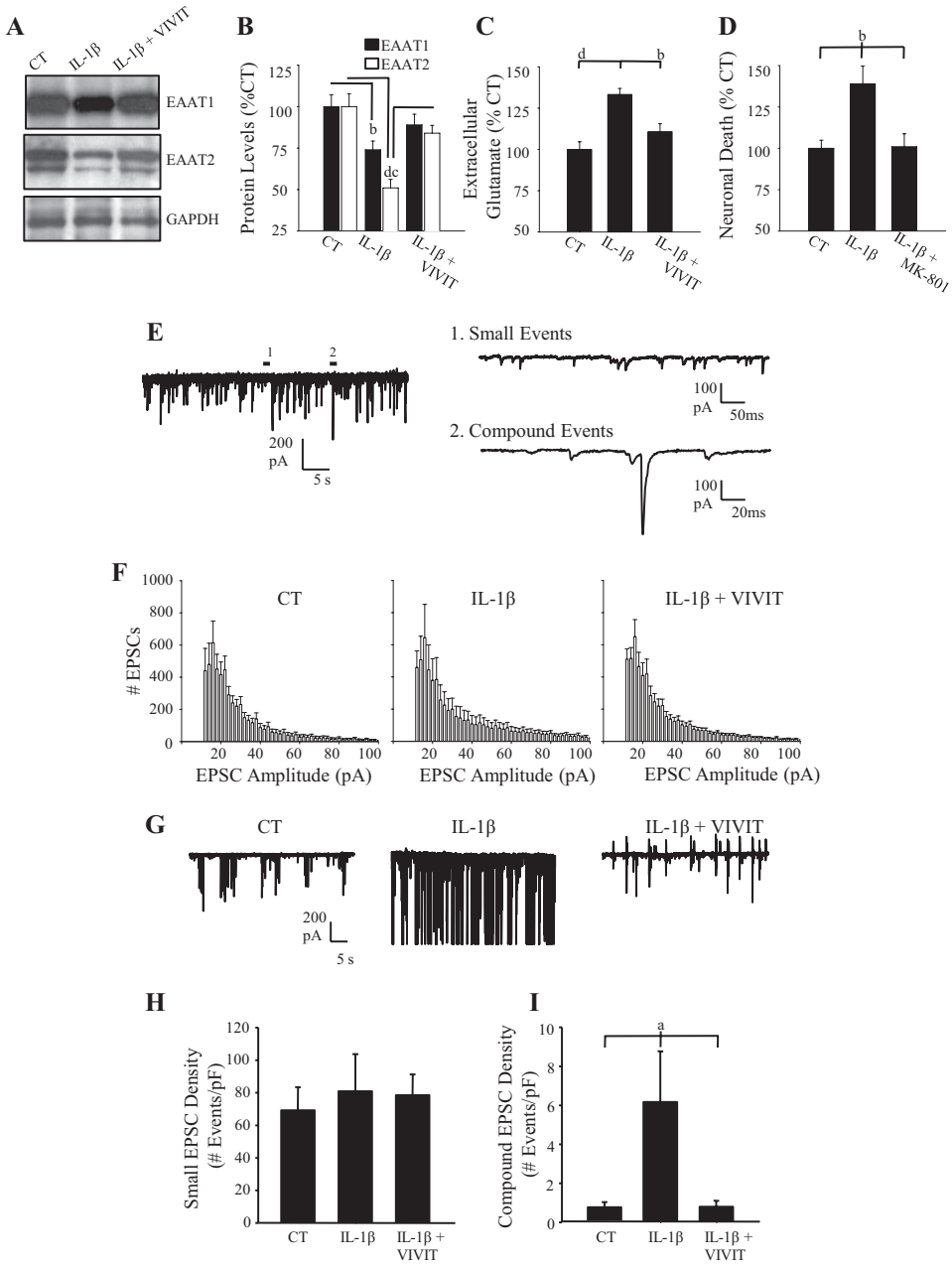

FIGURE 5. **Effects of IL-1β on glutamate regulation and excitotoxicity.** *A*, representative Western blots for EAAT1 and -2 and glyceraldehyde-3-phosphate dehydrogenase (loading control) in control cultures infected with Ad-LacZ-EGFP without IL-1β (control; *CT*), cultures infected with Ad-LacZ-GFP and treated with IL-1β (*IL-1β*), and cultures treated with IL-1β and infected with Ad-VIVIT-EGFP (*IL-1β + VIVIT*). *B*, mean ± S.E. protein levels for EAAT1 and -2 expressed as percentage of control ($n = 5–6$ pooled samples/condition). *C*, mean ± S.E. change in extracellular glutamate levels in astrocyte/neuron sandwich cultures treated for 48 h with IL-1β. Treatment conditions were the same as in *A* ($n = 8–10$ cultures/condition). *D*, mean ± S.E. number of dead neurons in sandwich cultures left untreated (*CT*) or cultures treated with IL-1β with or without MK-801 ($n = 10$ fields/condition). *E*, representative record of ongoing sEPSC activity in a voltage-clamped neuron from a mixed primary culture. Areas marked *1* and *2* are shown at a higher gain and temporal resolution on the *right* and highlight the occurrence of small sEPSCs (1) and larger, compound sEPSCs (2). *F*, sEPSC amplitude histograms (mean ± S.E.) for events of <100 pA are shown for cultures treated with Ad-LacZ-GFP control (*CT*), IL-1β + Ad-LacZ-GFP (*IL-1β*), and IL-1β + Ad-VIVIT-EGFP (*IL-1β + VIVIT*) ($n = 6–9$ cultures/condition). IL-1β was applied for 24 h. These distributions were highly similar across treatment groups. *G*, representative spontaneous activity recorded in individual neurons from each treatment condition. *H* and *I*, mean ± S.E. sEPSC density (number of events/picofarads) for small (<50 pA) (*H*) and compound (*I*) events show that IL-1β caused a significant increase in the occurrence of compound sEPSCs when administered to control cultures but not when given to VIVIT-treated cultures. *a*, $p < 0.05$; *b*, $p < 0.01$; *d*, $p < 0.0001$.

DISCUSSION

Previously, we found that CN levels were up-regulated in activated astrocytes in aging mice and mice with severe amyloid pathology (25). In that same study, we also found that activation of CN in mixed cultures of neurons and astrocytes recapitulated much of the neuroinflammatory transcriptional profile associated with aging and the earliest stages of Alzheimer disease. Here, we show that the CN/NFAT pathway in astrocytes is a major target of IL-1β, which is perhaps the primary extracellular coordinator of immune inflammatory signaling in the brain. More importantly, however, the CN/NFAT pathway in astrocytes appears to play a critical role in mediating the degenerative, excitotoxic effects of IL-1β on neurons. The results could have important implications for the propagation and maintenance of neuroinflammatory signaling processes associated with many neurodegenerative conditions and diseases.

IL-mediated Immune/Inflammatory Signaling and the CN/NFAT Pathway—IL-1β expression and release from microglia is among the earliest events in neuroinflammatory signaling following neural injury (11). Autocrine and paracrine activation of microglia and astrocytes by IL-1β causes the further release of numerous cytokines, growth factors, reactive oxygen species, and other factors that, in turn, potentiate or maintain glial activation. Over time, these positive feedback cycles may cause or hasten neurodegeneration (4, 13).

Effects of IL-1β in the brain and in other tissues are often ascribed to activation of NFκB and/or activation of MAPK-dependent AP1 transcription factors (15, 71–73). Although IL-1β or downstream targets of the IL-1R, such as TRAF6, have also been shown to activate NFAT isoforms in liver cells and in osteoclasts (74, 75), a possible linkage between IL-1β and the CN/NFAT pathway has yet to be established in nervous tissue until now.

The results of the present study show for the first time that IL-1β also recruits CN/NFAT signaling in astrocytes. Our results do not, however, minimize the importance of other IL-1β-regulated pathways. In fact, NFATs

usually direct transcriptional activity in synergy with AP-1 and/or NFκB factors (33). Thus, it is likely that IL-1β coordinates immune/inflammatory signaling in astrocytes and perhaps other cell types through the combined activation of CN and MAPK cascades. Further work will be necessary to determine how NFATs and other transcription factors work together to fine tune cytokine expression in astrocytes.

The CN/NFAT pathway in astrocytes appears to be an attractive mechanism for coordinating and maintaining autocrine and paracrine feedback loops in local glial networks. In a previous study using gene microarrays and mixed neuronal/astrocyte cultures, we found that many of the transcripts upregulated by CN were also known to stimulate CN activity (25). In the present study, we showed that CN activation in astrocytes caused the secretion of factors that, in turn, recruited further CN/NFAT activity in resting astrocytes (Fig. 3*F*). Blockade of CN/NFAT activity also prevented the IL-1β-mediated increase in a number of critical cytokines, such as TNFα and GM-CSF, which are also implicated in neuroinflammation and neurodegeneration. In the brain, TNFα is a potent signal for the activation of both microglia and astroglia (76), and like IL-1β, TNFα also robustly activates CN/NFAT signaling in astrocytes (26). GM-CSF appears to play a crucial role in amplifying the local microglial response in areas of neuronal damage and/or protein aggregation (77). Indeed, GM-CSF and other colony-stimulating factors are powerful signals for microglial activation and subsequent release of IL-1β (78). Of course, IL-1β and CN activity stimulate the production of numerous other cytokines that may also play an important role in positive feedback signaling.

L-VSCCs and CN Activation in Astrocytes—Astrocytes express a wide array of Ca^{2+}-dependent molecules and are capable of generating sophisticated Ca^{2+} oscillations and signals (79). In the present work, IL-1β-mediated activation of the CN/NFAT pathway was highly dependent on L-type VSCCs but not on several other VSCC subtypes, including N-/P- and Q-type channels. This finding is consistent with several other reports that also found a selective coupling between CN and L-VSCCs in other cell types (51, 80–82).

Like CN, L-VSCC expression is also increased in activated astrocytes following brain injury (83). In fact, CN and L-VSCCs often change in similar ways during pathophysiological conditions. This coupling may be best illustrated during cardiomyocyte hypertrophy and neuronal aging, in which the activity or expression of both CN and L-VSCCs are increased (84–86). In neurons, we have found that CN stabilizes or enhances L-VSCC activity in an aging-dependent manner, both in culture and *in vivo* (87, 88). Thus, in addition to maintaining its own activity in astrocytes through the coordination of cytokine cycles, CN may also sustain itself by amplifying the expression and/or activity of Ca^{2+} sources, like L-type VSCCs.

It is worthy of note, however, that although L-VSCC blockade strongly inhibited CN/NFAT activity, this inhibition was incomplete (~50%), suggesting that other Ca^{2+} sources not identified in these experiments were also involved in CN/NFAT activation. In addition to expressing VSCCs, astrocytes also express functional store-operated channels (35, 89, 90) as well as IP_3 and ryanodine receptors (91–93), which gate Ca^{2+} release from intracellular stores. Similar Ca^{2+} sources are critical for CN activation in T-cells and other cell types and may well contribute significantly to CN activation in astrocytes. Further research will be necessary to identify the multiple Ca^{2+} sources that recruit CN signaling in astrocytes and whether these sources are specifically linked to immune/inflammatory processes.

Role of Astrocytic CN/NFAT Activity in Neuronal Function and Viability—In cell cultures or intact animals, elevated IL-1β levels are often linked to altered neuronal function and/or neuronal death (11). However, because IL-1Rs are expressed in multiple cell types, it is not always clear whether IL-1β affects neurons directly or indirectly. Similar to other recent reports (61, 94), however, we found that IL-1β only killed neurons in the presence of astrocytes (Fig. 4*A*). Perhaps most importantly, IL-1β-mediated neurotoxicity was virtually prevented when the potent NFAT inhibitor, VIVIT, was selectively expressed in astrocytes. This novel observation suggests that CN/NFAT signaling in astrocytes is key to neuronal viability.

Astrocytes regulate neuronal function and survival through multiple mechanisms, one of which is the removal of excess glutamate from the extracellular milieu. Glutamate toxicity (*i.e.* excitoxicity), due in large measure to altered astrocyte function, may be one of the primary causes of neuronal deterioration and death associated with numerous neuroinflammatory conditions, including Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, stroke, and head/spinal cord trauma, to name a few (7). IL-1β and other inflammatory mediators can precipitate elevations in extracellular glutamate and/or exacerbate excitotoxic insults to nervous tissue (10, 94, 95). Consistent with an excitotoxic mechanism of action, IL-1β did not kill neurons when astrocyte/neuron sandwich cultures were co-treated with the NMDA type glutamate receptor antagonist, MK-801 (Fig. 5*C*).

Several inflammatory mediators appear to disrupt glutamate homeostasis, in part, by down-regulating the expression or function of high affinity EAATs. Similar to a recent study on spinal cord tissue (62), we found that IL-1β suppressed protein levels for EAAT1 and -2 (Fig. 5, *A* and *B*). Effects were especially pronounced for EAAT2, which is the major glutamate transporter expressed in astrocytes and is responsible for the bulk of glutamate uptake (96). In parallel to reduced EAAT levels, IL-1β also caused an increase in extracellular glutamate levels and triggered an increase in the frequency of large, compound sEPSCs. Interestingly, the high level of spontaneous synaptic activity observed in IL-1β-treated cultures is reminiscent of older neuronal cultures (*i.e.* >21 days *in vitro*) that show increased vulnerability to glutamate toxicity (41). Similarly, aberrant synaptic activity has also been noted in cell cultures and acute slices treated with EAAT inhibitors (68–70).

The effects of IL-1β on EAAT protein expression (again, particularly for EAAT2) and extracellular glutamate levels were blunted by the delivery of VIVIT to astrocytes, indicating a novel role for the CN/NFAT pathway in glutamate homeostasis. Although not specifically investigated here, the presence of multiple putative NFAT-binding elements (*i.e.* GGAAAA) in the promoter regions of EAAT1 and -2 (64–66) suggests that CN/NFAT activity probably regulates these transporters at the

transcriptional level. However, additional work will be required to fully characterize the interaction of the CN/NFAT pathway with EAAT expression.

Consistent with its stabilizing effects on EAATs, VIVIT also quelled hyperexcitable neuronal network activity (Fig. 5, *F* and *H*). Together, the results suggest that astrocytic CN/NFAT signaling exerts powerful modulation over the balance of excitatory activity across neuronal ensembles. Overactivation of the CN/NFAT pathway, in combination with other signaling pathways in astrocytes (see below), seems to tip the balance in favor of increased neuronal excitability, which helps to perpetuate the excitotoxic cycle leading to neuronal deterioration and death. In turn, the breakdown products derived from deteriorating neurons contribute to chronic neuroinflammation by sustaining the activation of astrocytes and microglia (4).

Contribution of Other Signaling Pathways in Astrocytes—Although CN/NFAT activity was necessary for multiple IL-1β-mediated actions in this study, CN activity in astrocytes alone was not sufficient to drive neuronal death (Fig. 4*E*). It may be that expression of aCN by itself is unable to replicate the full spectrum of signaling events mediated by the endogenous CN holoenzyme. Consistent with this idea, a recent study found that aCN required co-expression with the regulatory CN B subunit to produce maximum toxicity (97). Alternatively, the CN/NFAT pathway in astrocytes may only modulate neuronal viability when it is recruited in conjunction with other IL-1β-regulated pathways (*e.g.* MAPK and NFκB pathways). As discussed above, expression of a variety of immune/inflammatory signaling molecules are coordinated through the combined activity of multiple transcription factors, including AP1 and/or NFκB, as well as NFATs. As shown in T cells and cardiomyocytes, inhibition of any of these components may be sufficient to severely limit transcription of target genes, although the activity of each is insufficient to direct transcription on its own (33). Interestingly, EAAT2 levels were recently shown to exhibit down-regulation in response to TNFα through a mechanism involving NFκB (98). Note then that the CN/NFAT pathway could regulate EAAT2 at multiple levels: by increasing levels of TNFα, as shown in Fig. 3*A*, and/or by synergizing with NFκB to limit the transcription of EAAT2. Clearly, additional work will be required to determine the extent to which these multiple pathways interact in astrocytes and whether targeted disruption of these interactions can eliminate the specific components of immune/inflammatory signaling that are detrimental to neuronal function.

Conclusions—The results of the present study demonstrate that activation of the CN/NFAT pathway in astrocytes is a major outcome of elevated IL-1β levels and may coordinate immune/inflammatory signaling cascades in nervous tissue. Recently, the CN inhibitor, FK506, was shown to exhibit nootropic (99) and anti-inflammatory properties (27) in mouse models of Alzheimer disease and Tau pathology, respectively. NFAT inhibitors, such as VIVIT, may be an attractive alternative to CN inhibitors, since they impart specific and potent immunosuppression without many of the severe adverse effects associated with blanket inhibition of CN (100). However, the therapeutic potential of NFAT inhibitors in treating neuroinflammatory conditions remains relatively unexplored. Although promising, the neuroprotective properties of VIVIT, as shown here, will require further verification in appropriate animal models.

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