Neuroscience 134 (2005) 671–676

CHEMOKINE RECEPTOR 5 ANTAGONIST D-ALA-PEPTIDE T-AMIDE REDUCES MICROGLIA AND ASTROCYTE ACTIVATION WITHIN THE HIPPOCAMPUS IN A NEUROINFLAMMATORY RAT MODEL OF ALZHEIMER'S DISEASE

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Abstract—Chronic neuroinflammation plays a prominent role in the progression of Alzheimer's disease. Reactive microglia and astrocytes are observed within the hippocampus during the early stages of the disease. Epidemiological findings suggest that anti-inflammatory therapies may slow the onset of Alzheimer's disease. Chemokine receptor 5 (CCR5) up-regulation may influence the recruitment and accumulation of glia near senile plaques; activated microglia express CCR5 and reactive astrocytes express chemokines. We have previously shown that neuroinflammation induced by chronic infusion of lipopolysaccharide into the 4th ventricle reproduces many of the behavioral, neurochemical, electrophysiological and neuropathological changes associated with Alzheimer's disease. The current study investigated the ability of D-Ala-peptide T-amide (DAPTA), a chemokine receptor 5 chemokine receptor antagonist of monocyte chemotaxis, to influence the consequences of chronic infusion of lipopolysaccharide. DAPTA (0.01 mg/kg, s.c., for 14 days) dramatically reduced the number of activated microglia and astrocytes, as compared with lipopolysaccharide-infused rats treated with vehicle. DAPTA treatment also reduced the number of immunoreactive cells expressing nuclear factor κ binding protein, a prominent component of the proinflammatory cytokine signaling pathway. The present study suggests that DAPTA and other CCR5 antagonists may attenuate critical aspects of the neuroinflammation associated with Alzheimer's disease.

Key words: anti-inflammatory, lipopolysaccharide, microglia, Alzheimer's disease.

Chemokines belong to a large family of chemotactic proteins that regulate leukocyte chemotaxis to sites of inflammation via interaction with chemokine receptors (Mueller and Strange, 2004). Chemokines and their receptors are present in the CNS where they play a critical role in neuronal development by guiding cellular migration and neuronal survival during development as well as in normal neurogenesis and pathological neuroinflammation (Xia et al., 1998). The chemokine receptor chemokine receptor 5 (CCR5) is up-regulated on reactive microglia associated with senile plaques in Alzheimer's disease (AD); stimulation of these receptors may drive the conversion of diffuse plaques into compact neuritic plaques and contribute to an amplification of the neuroinflammation processes (Xia et al., 1998). AD is associated with a condition of chronic neuroinflammation (Akiyama et al., 2000). Increased microglia activation occurs in brain regions that ultimately demonstrate the greatest concentration of senile plaques and brain atrophy in AD patients (Cagnin et al., 2001). Therefore, chemokine receptors may be a favorable target for drug development because of their potential role in chronic inflammation.

We tested the effect of D-Ala-peptide T-amide (DAPTA), a specific CCR5 chemokine receptor antagonist (Redwine et al., 1999; Polianova et al., in press) in an animal model of chronic neuroinflammation. Chronic infusion of the proinflammogen lipopolysaccharide (LPS) into the 4th ventricle of young rats reproduces many of the behavioral, neurochemical, electrophysiological and neuropathological changes associated with AD (Hauss-Wegrzyniak et al., 1998, 2000, 2002; Rosi et al., 2003, 2004, 2005). DAPTA blocks monocyte chemotaxis (Redwine et al., 1999) and improves memory and cognitive abilities in people with acquired immunodeficiency syndrome (AIDS, Wetterberg et al., 1987; Heseltine et al., 1998) a disease that is also characterized by glial activation and widespread brain inflammation. The results suggest that chemokine receptors may be an important therapeutic target for chronic neuroinflammation associated with neurodegenerative disease.

EXPERIMENTAL PROCEDURES

Subjects

Eighteen male, three month old, F-344 (Harlan Sprague–Dawley, Indianapolis, IN, USA) were assigned to three groups: 1) artificial cerebrospinal fluid (aCSF)-infused (n=6); 2) LPS-infused, vehicle-treated (n=6); 3) LPS-infused, DAPTA-treated (0.01 mg/kg/day, s.c, n=6). LPS (1.0 μg/μl) or was chronically infused (0.25 μl/h for 14 days) through a cannula implanted into the 4th ventricle of the brain that was attached to an osmotic minipump as previously

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Abbreviations: aCSF, artificial cerebrospinal fluid; AD, Alzheimer's disease; AIDS, acquired immunodeficiency syndrome; CCR5, chemokine receptor 5; DAPTA, D-Ala-peptide T-amide; GFAP, glial fibrillary acidic protein; LPS, lipopolysaccharide; MHC II, major histocompatibility complex, class II; NFκB, nuclear factor κ binding protein; PBS, phosphate-buffer saline.

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doi:10.1016/j.neuroscience.2005.04.029

described (Hauss-Wegrzyniak et al., 1998, 2000, 2002; Rosi et al., 2003, 2004, 2005). DAPTA was freshly dissolved in the vehicle (0.01 mg/ml in distilled water) and administered every morning for 14 days beginning the day after the surgery.

Materials

LPS (*E. coli*, serotype 055:B5) was obtained from Sigma Chem. (St. Louis, MO, USA). DAPTA, D-ASTTTNYT-NH_2, Pert et al., 1986) was obtained from Bachem (Torrence, CA, USA) and was >95% pure as determined by tandem mass spectroscopy. Solutions were prepared fresh daily from powder.

Histological procedures

Two weeks after surgery each rat was anesthetized with isoflurane and prepared for histological analysis by *in situ* perfusion of the brain with cold saline containing 1 U/ml heparin, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brain was removed and the location of the cannula in the 4th ventricle was confirmed. The brains were then post-fixed 1 h in the same fixative and then stored (4 °C) in phosphate buffered saline.

Immunohistochemistry

Single fluorescence staining was run for activated microglia, astrocytes and NFkB. Free-floating, serial coronal sections (30 μm) were preparing using a vibratome, six sections from each animal were selected from the medial portion of the dorsal hippocampus (ca 3.0–3.5 mm posterior to Bregma).and washed in phosphate-buffer saline (PBS) (pH 7.4). The mouse monoclonal antibody OX-6 (final dilution 1:400, Pharmingen, San Diego, CA, USA), directed against class II major histocompatibility complex (MHC II) antigen, was used to visualize activated microglial cells. A glial fibrillary acidic protein (GFAP) rabbit polyclonal antibody (1:5000, Pharmingen) was used to visualize astrocytes. Nuclear factor κ binding protein (NFkB) was visualized using anti-rabbit polyclonal antibodies raised against the amino terminal domain of NFkB p65. NFkB is a heterodimer composed of p50 and p65 subunits. The p65 subunit has a nuclear translocation signal sequence which is masked by a specific inhibitor of NFkB holds in the cytosol. Release of the inhibitor is initiated when specific kinases phosphorylate the inhibitor and the released p65 subunit is then translocated to the nucleus. Considering that the proinflammatory transcriptional factor NFkB induces transcription of proinflammatory cytokines (Baeuerle and Henkel, 1994) and chemokines (Ueda et al., 1997); and since DAPTA is an inhibitor of interleukin-1 and tumor necrosis factor-α release in HIV patients (Ruff et al., 2003) as well as an antagonist of the CCR5 chemokine receptor, it was of interest to determine whether LPS infusion and the subsequent treatment with DAPTA involved the activation of NKkB. The inducible transcription factor NFkB was visualized using rabbit polyclonal or goat polyclonal antibody raised against the amino terminal domain of NFkB p65 (1:1000 and 1:500, Santa Cruz Biotech., Santa Cruz, CA, USA). These antibodies allow the investigation of the activation state of NFkB, because they detect p65. The sections were incubated overnight at 4 °C, in 5% normal goat serum with primary antibodies directed against the specific epitopes. After washing in PBS, the sections were incubated for 1 h with the secondary antibodies for OX-6, monoclonal anti-mouse (Vector, Burlingame, CA, USA), for GFAP and NKkB, biotinylated anti-rabbit (Vector). After additional treatment with an Avidin Biotin amplification system (Vector), the staining was visualized with a TSA fluorescence system CY3 (PerkinElmer Life Sciences, Boston, MA, USA) and the nuclei were counterstained with SYTOX Green (Molecular Probes, Eugene, OR, USA). No staining was detected in the absence of the primary or secondary antibodies.

Double immunofluorescence staining for NFkB/GFAP, NFkB/OX-6 and NFkB/NeuN. The double staining followed the immunohistochemistry for NKkB as described above; following several washes in PBS the slides were quenched and blocked, as described above and then incubated either with anti-NeuN antibody (1:1000, Chemicon, San Diego, USA) or with the OX-6 monoclonal antibody (1:400, Pharmingen). Before applying the biotinylated monoclonal secondary rat-adsorbed antibody (Vector) for 1 h, the tissue was incubated with Avidin Biotin Blocking Kit (Vector) for 30 min to block cross reaction with the primary antibody. After additional treatment with an Avidin Biotin amplification system (Vector), the staining was then visualized with Cy5 TSA fluorescence system (PerkinElmer) and the nuclei counterstained with SYTOX Green (1:10,000, Molecular Probes). No staining was detected in the absence of the primary or secondary antibodies. For co-localization with the rabbit polyclonal GFAP antibody the goat polyclonal antibody against NFkB p65 was used in order to avoid any cross-reaction with the rabbit GFAP.

Image acquisition (confocal and light microscopy). In order to define the anatomic boundaries and degree of microglial activation within the hippocampus, sections from the entire hippocampus of an LPS-infused rat were imaged (Fig. 1A) by overlapping 10× Z-stacks (1.0 μm optical thickness/plane) using a Zeiss LSM 510 NLO-meta multiphoton/confocal microscope equipped with a 488 nm argon laser and a 543 nm and 633 nm helium/neon laser. The images were collected with a small overlap between each image using the shape of cell groups as landmarks. The parameters were kept constant across sections using the 488 nm (for SYTOX Green) and the 543 nm (for CY3) laser.

Image analysis. The entire hippocampus was reconstructed using the middle plain from each 10× image stacks and two flat hippocampal images were obtained for each rat for each staining (OX6, GFAP, NFkB). The quantification of activated microglia was performed as previously described (Rosi et al., 2005); the hippocampus was divided into two regions of interest (DG and CA3) and the analysis performed using MetaMorph imaging software (Universal Image Corporation, West Chester, PA, USA).

RESULTS

Chronic infusion of LPS into the 4th ventricle of young rats for 2 weeks was well tolerated by all rats. Initially after surgery, all LPS-infused rats lost a few grams of weight. Within a few days, however, most rats had regained weight and continued to gain weight normally for the duration of the study (Rosi et al., 2005).

DAPTA treatment dramatically reduced activated microglia during LPS-infusion

Brain inflammation was determined using standard immunocytochemical biomarkers for activated microglia, i.e. the presence of the MHC II antigen. Immunofluorescence staining for MHC II found numerous and highly activated microglia distributed throughout the DG and CA3 hippocampal areas following chronic infusion of LPS into the 4th ventricle (Fig. 1A). CA1 areas did not show immunopositive microglial cells, consistent with previous study (Rosi et al., 2005). Activated microglia were characterized by a contraction of their highly ramified processes that appeared bushy in morphology (Fig. 1B). In contrast, rats infused with aCSF had few mildly activated microglia scat-

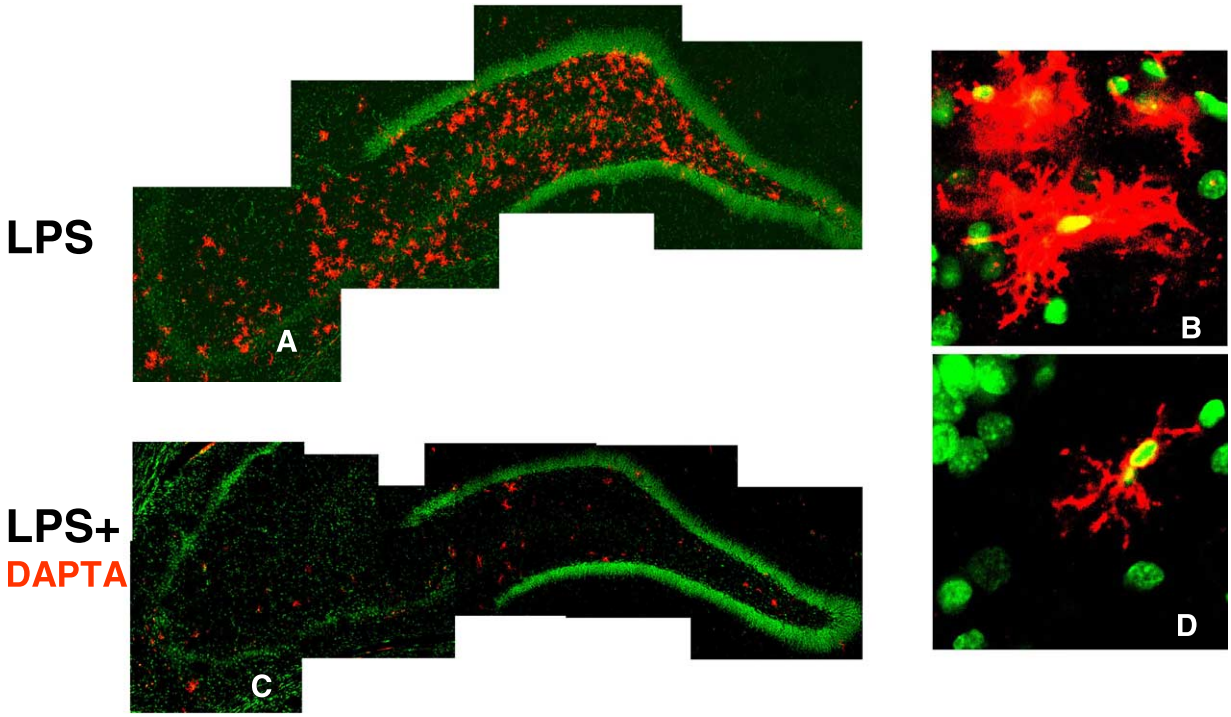

Fig. 1. Reconstruction of a 20 μm coronal section from the dorsal hippocampus (approx. 3.6 mm posterior from Bregma), from a young rat with chronic LPS-infusion into the 4th ventricle. (A) Activated microglia (OX-6 immunoreactive cells- in red) were numerous within the granule cell layer and the hilar region of the dentate gyrus (DG) as well as inside and nearby the CA3 molecular layer. No immunopositive staining was found in CA1. (B) Activated microglia showed the characteristic bushy morphology with contracted and ramified processes. Nuclei are counterstained green. (C) DAPTA (0.01 mg/ml/kg, s.c.) reduced the number of activated microglia within the DG and CA3; (D) the morphology of these microglia suggests a less activated condition than those from LPS-infused, saline-treated rats. To view this figure in color, please see the version of this paper published online. Scale bars=100 μm (A); (B) 2.5 μm.

tered throughout the brain (not shown) similar to our previous reports (Rosi et al., 2003, 2004, 2005). Daily injections of DAPTA (0.01 mg/ml/kg, s.c.) reduced the number of activated microglia within the DG and CA3 hippocampal areas (Fig. 1C). Most importantly, these microglia were characterized by a morphology that suggest they were less activated than those from LPS-infused rats (Fig. 1B), i.e. the MCH II positive cells showed long and thin ramified processes typical of the resting state (Fig. 1D).

The quantitative cell count analysis demonstrated that DAPTA treatment was associated with a statistically significant ($P<0.001$, by paired *t*-tests) reduction in the number of OX-6 immunopositive cells in the DG (LPS: 157±17 vs. LPS+DAPTA: 33±22, mean±standard deviation) and CA3 region (LPS: 133±22 vs. LPS+DAPTA: 18±3). Consistent with our previous report (Rosi et al., 2005), the DG had the highest number of OX6 immunopositive-cells per mm^2 (mean=157), followed by the CA3 (mean=133) and CA1 (mean=2) LPS-infused rats.

DAPTA treatment reduced astrocytes hypertrophy during LPS-infusion

Immunofluorescence staining for GFAP found mildly activated astrocytes throughout the brain of aCSF infused rats (not shown). Chronic LPS infusion into the 4th ventricle was associated with astrocyte hypertrophy (Fig. 2A, B) that was consistent with previous studies (Hauss-Wegrzyniak et al., 1998). DAPTA significantly reduced the number of hypertrophic astrocytes (Fig. 2D, E).

Reduced fluorescence immunostaining for NFkB

Chronic LPS infusion increased NFkB immunostaining throughout the hippocampus (Fig. 2C). Following treatment with DAPTA, NFkB immunofluorescence staining was dramatically reduced (Fig. 2F). Double immunofluorescence staining demonstrated that the NFkB was associated primarily with hypertrophic astrocytes (Fig. 3A–C). LPS infused animals had numerous NFkB immunopositive cells within the hilar region of the dentate gyrus (Fig. 2C); the enhanced staining was found prominently within the cytoplasm and the nuclei. Reduction of NFkB staining was observed following treatment with the chemokine receptor antagonist DAPTA (Fig. 2F).

Co-localization of NFkB with the NeuN selective neuronal marker revealed only scattered double-labeled cells within the hippocampus (Fig. 3D). In addition, while activated microglia were found near NFkB positive cells OX-6 did not co-localization with NFkB (Fig. 3E–F). Double immunohistochemistry for NFkB and GFAP demonstrated considerable co-localization (Fig. 3A–C). The NFkB immunopositive staining was found in the cytoplasm within the cell body and inside the astrocyte nuclei (see Fig. 3A, B). The remaining NFkB positive cells that were not co-local-

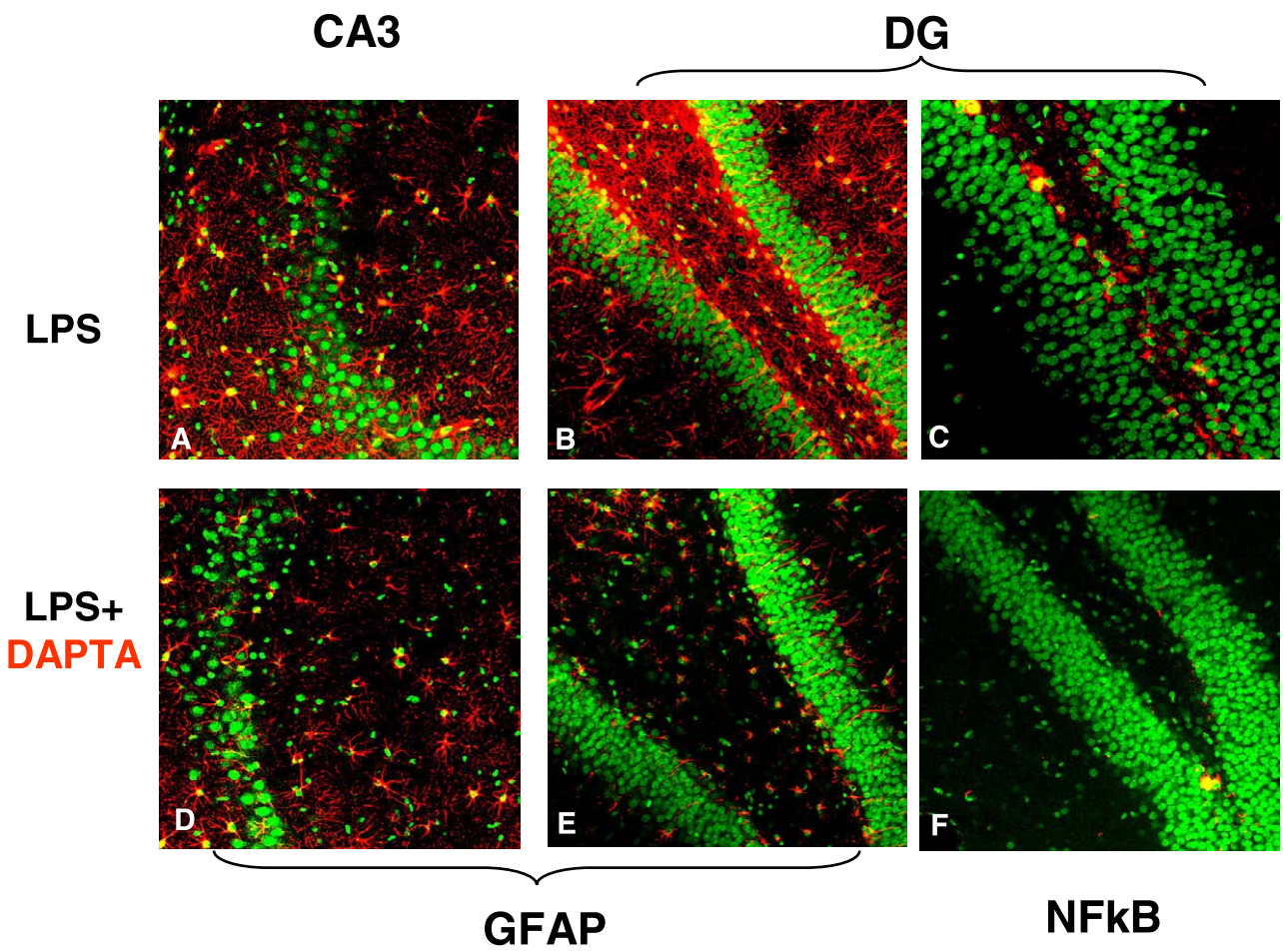

Fig. 2. Immunohistochemical staining for either GFAP-positive astrocytes (A, B, D, E) and NFkB (C, F) (both in red) within the CA3 (A, D) and DG (B, C, E, F) area of the hippocampus of LPS-infused, saline-treated (top row) or LPS-infused, DAPTA-treated, rats (bottom row). Nuclei are counterstained green. To view this figure in color, please see the version of this paper published online. Scale bars=100 μm.

ized with GFAP were characteristic of vascular endothelial cells (arrows in Fig. 3D, E).

DISCUSSION

Chronic infusion of LPS into the 4th ventricle produced an extensive inflammatory reaction throughout the brain, particularly within the hippocampus and temporal lobe regions. The inflammatory response was characterized by a significant increase in the number of reactive microglial cells, a marked hypertrophy of numerous astrocytes and an elevation in the NFkB staining. The effects of LPS infusion upon microglial and astrocyte activation were consistent with our previous reports (Hauss-Wegrzyniak et al., 1998, 2000; Rosi et al., 2003, 2004, 2005). Previous findings show that DAPTA completely prevents NBM lesion-induced cortical atrophy in aged rats (Socci et al., 1996). Here we demonstrate that daily peripheral administration of DAPTA dramatically attenuated the inflammatory response induced by LPS as demonstrated by a decrease in both the number and reactive state of microglial and astrocytes.

The effects of LPS are mediated primarily through the release of cytokines from activated microglial cells (Wenk and Hauss-Wegrzyniak, 2003). DAPTA targets the CCR5 chemokine receptor expressed by activated microglia and this may block the release of the proinflammatory cytokines tumor necrosis factor-α and interleukin-1 (Ruff et al., 2003). In turn, pro-inflammatory stimuli can activate NFkB expression (Sparacio et al., 1992), which can further induce specific genes that regulate the expression of inflammation and acute phase genes leading to the continued elevation of inflammatory proteins. This cycle may exist for many years in the brains of AD patients and contribute to the degeneration and vulnerability of selected brain regions (Akiyama et al., 2000; Cagnin et al., 2001; Griffin et al., 1998; Wenk and Hauss-Wegrzyniak, 2001, 2003). In the present study, chronic infusion of LPS elevated intracellular expression of NFkB immunoreactivity within astrocytes but not within neurons or microglia. DAPTA treatment significantly reduced the level of NFkB expression within astrocytes in the hippocampus. Astrocytosis is a typical morphological feature of the AD brain and chemokines are released from activated astrocytes that are near senile plaques (Griffin et al., 1998; Akiyama et al., 2000).

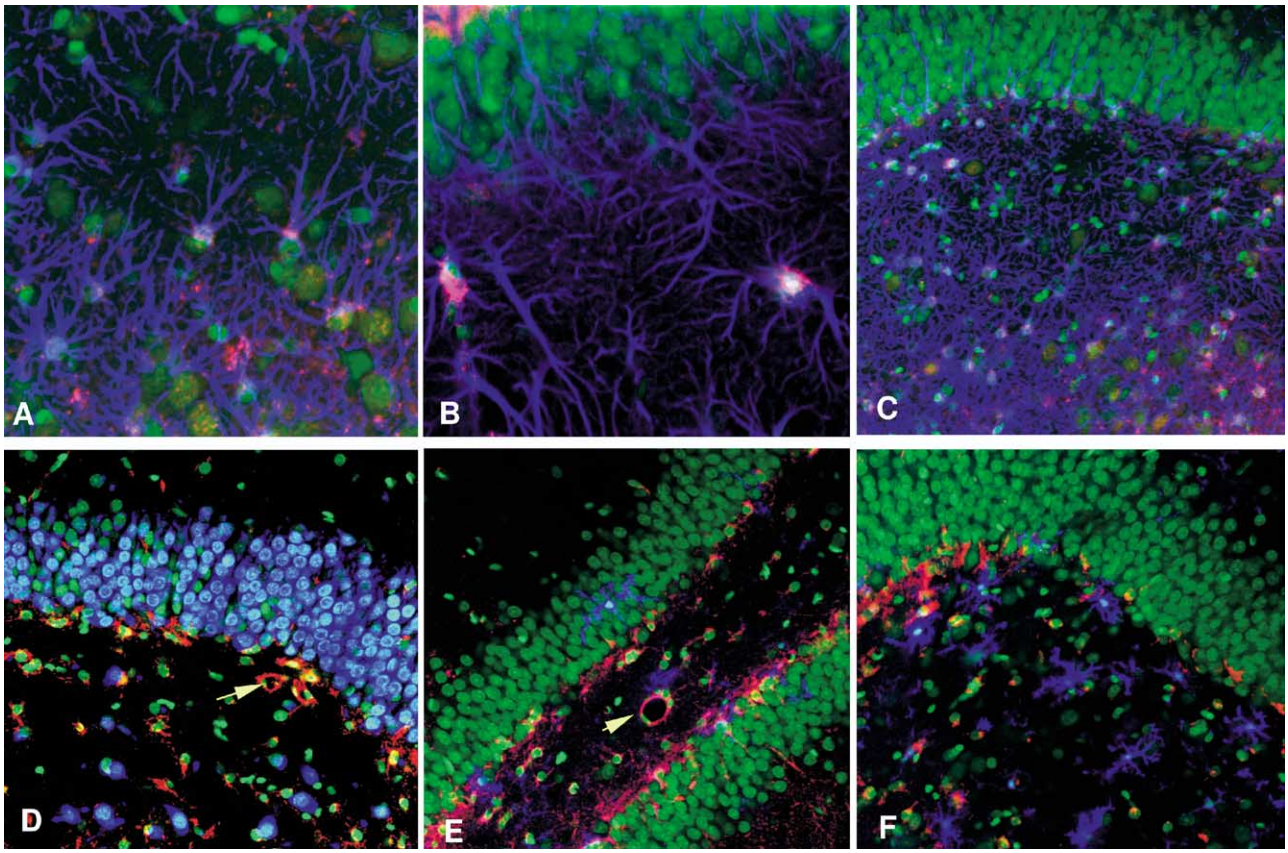

Fig. 3. Double immunohistochemistry for NFkB (red) and GFAP (blue) on sections from LPS-infused rats showed considerable co-localization (A–C). The NFkB immunopositive staining was found in the cytoplasm within the soma as well as inside the astrocyte nuclei (A, B). Co-localization of NFkB with the NeuN selective neuronal marker revealed only scattered double-labeled cells within the hippocampus (D). Activated microglia (OX-6 immunoreactive) were not co-localized with NFkB (E, F). The remaining NFkB positive cells that did not co-localize with GFAP were characteristic of vascular endothelial cells; arrows in D, E. To view this figure in color, please see the version of this paper published online. Scale bars=100 μm

We speculate that the beneficial effects of DAPTA treatment in the current study were due to its antagonism at the CCR5 receptor which attenuates the cascade of biochemical and immunological changes that were induced by the chronic infusion of LPS. The AD brain is characterized by chronic neuroinflammation (Akiyama et al., 2000) and long term treatments with anti-inflammatory drugs lessen the risk of AD (Aisen and Davis, 1994). Previous studies using this animal model of chronic brain inflammation have demonstrated that treatment with non-steroidal anti-inflammatory drugs reduces the level of activation of microglia but not astrocytes (Hauss-Wegrzyniak et al., 1998 and unpublished findings); in contrast to the actions of these anti-inflammatory drugs, DAPTA was effective against both important inflammatory cells in the brain. This dual action of DAPTA may also explain its cognitive benefits in patients with AIDS, a disease characterized by microglial and astrocyte activation, as well as enhanced inflammatory cytokines. Our results suggest that DAPTA, currently in phase II trials for HIV disease, should be tested for efficacy in AD, and that other chemokine receptor antagonists may reduce the consequences of chronic neuroinflammation that appear to drive the pathology associated with AD.

Acknowledgments—Supported by the U.S. Public Health Service, Contract grant number AG10546 and the Alzheimer's Association, IIRG-01-2654 to G.L.W.

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(Accepted 15 April 2005)
(Available online 27 June 2005)