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A Pivotal Role for Interleukin-4 in Atorvastatin-associated Neuroprotection in Rat Brain*

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Inflammatory changes, characterized by an increase in pro-inflammatory cytokine production and up-regulation of the corresponding signaling pathways, have been described in the brains of aged rats and rats treated with the potent immune modulatory molecule lipopolysaccharide (LPS). These changes have been coupled with a deficit in long-term potentiation (LTP) in hippocampus. The evidence suggests that anti-inflammatory agents, which attenuate the LPS-induced and age-associated increase in hippocampal interleukin-1β (IL-1β) concentration, lead to restoration of LTP. Here we report that atorvastatin, a member of the family of agents that act as inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, exerts powerful anti-inflammatory effects in brain and that these effects are mediated by IL-4 and independent of its cholesterol-lowering actions. Treatment of rats with atorvastatin increased IL-4 concentration in hippocampal tissue prepared from LPS-treated and aged rats and abrogated the age-related and LPS-induced increases in pro-inflammatory cytokines, interferon-γ (IFNγ) and IL-1β, and the accompanying deficit in LTP. The effect of atorvastatin on the LPS-induced increases in IFNγ and IL-1β was absent in tissue prepared from IL-4$^{-/-}$ mice. The increase in IL-1β in LPS-treated and aged rats is associated with increased microglial activation, assessed by analysis of major histocompatibility complex II expression, and the evidence suggests that IFNγ may trigger this activation. We propose that the primary effect of atorvastatin is to increase IL-4, which antagonizes the effects of IFNγ, the associated increase in microglial activation, and the subsequent cascade of events.

The adverse effects of inflammation in the brain have been shown to include down-regulation of synaptic function. One manifestation of this is a decrease in the ability of rats to sustain long-term potentiation (LTP),[3] a form of synaptic plasticity that is considered to be a potential biological substrate for learning and/or memory. Inflammatory changes, typified by an increase in concentration of the pro-inflammatory cytokine, interleukin-1β (IL-1β), have been observed in aged rats (1, 2) and in rats treated with lipopolysaccharide (LPS (3, 4)) or β-amyloid peptides (Aβ (5)). In each of these conditions, LTP is impaired and, in some cases, the impairment is abrogated by strategies that restore IL-1β concentration to the lower values observed in control conditions (4). A role for IL-4 in modulating IL-1β production has been demonstrated (6), and we have observed that two treatments with anti-inflammatory properties, the polyunsaturated fatty acid, eicosapentaenoic acid (6) and rosiglitazone,[4] abrogate age- and LPS-induced neuroinflammation by increasing IL-4 concentration in hippocampus. These data highlight the potential of anti-inflammatory treatments to restore functional deficits associated with inflammation and draw an interesting parallel with the observation that the incidence of Alzheimer disease is reduced in individuals undergoing anti-inflammatory treatments for other conditions (7).

It seems likely that the increase in IL-1β concentration in the brain in inflammatory conditions is derived from activated microglia, and therefore parallel changes in IL-1β concentration and microglial activation have been identified in brain of aged and LPS-treated rats (8). However, the trigger leading to activation of microglia has not been identified, although results from several studies have suggested that one of the most potent activators of microglia *in vitro* is interferon-γ (IFNγ (9–11)).

Recent work has identified anti-inflammatory properties of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (*i.e.* statins) that are distinct from their ability to lower cholesterol. It has been shown that statins reduce Aβ deposition and inflammatory changes in mouse models of Alzheimer disease (12–14), block paralysis in chronic and relapsing experimental autoimmune encephalomyelitis (15), reduce infarct size in ischemia, and improve neurologic outcome by directly up-regulating brain endothelial nitric-oxide synthase (16). Statins have also been shown to decrease symptoms and mortality in stroke-prone spontaneously hypertensive rats (17).

Because of their reported anti-inflammatory effects, it might be predicted that statins will counteract the inflammatory changes induced by age and by LPS treatment. To test this prediction, we investigated the effect of atorvastatin treatment on the age-related and LPS-induced changes in rat hippocampus. We report that the age- and LPS-associated microglial activation, increase in concentrations of IFNγ and IL-1β, and deficit

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[3] The abbreviations used are: LTP, long-term potentiation; IL-1, interleukin-1; Aβ, β-amyloid peptide; IFNγ, interferon-γ; BSA, bovine serum albumin; MHCII, major histocompatibility complex II; JNK, c-Jun NH_2-terminal kinase; TBS, Tris-buffered saline; ANOVA, analysis of variance; LPS, lipopolysaccharide; EPSP, excitatory postsynaptic potential.

[4] Loane, D. J., Deighan, B. F., Clarke, R. M., Griffin, R. J., Lynch, A. M., and Lynch, M. A. (2007) *Neurobiol. Aging*, in press.

in LTP were abrogated in atorvastatin-treated rats. Because atorvastatin failed to attenuate the LPS-induced increases in cytokine concentrations in tissue prepared from IL-$4^{-/-}$ mice, we propose that the primary action of atorvastatin may be to trigger production of IL-4 and thereby prevent the IFNγ-induced microglial activation.

EXPERIMENTAL PROCEDURES

Animals—Twenty-four, 2- to 3-month-old male Wistar rats (BioResources Unit, Trinity College, Dublin, Ireland), were used in the first study, and 18 aged (22–24 months) and 12 young (3–4 months) animals (Bantham and Kingman, UK) were used in the second study. We also used 24 2- to 5-month-old C57BL/6 (Harlan UK Ltd.) and C57BL/6 IL-4-defective (IL-$4^{-/-}$) mice (B&K Universal, UK). Rats were housed in groups of 2–6, and mice were housed singly, under a 12-h light schedule. Ambient temperature was controlled between 22 and 23 °C, and animals were maintained under veterinary supervision throughout the study. These experiments were performed under a license issued by the Department of Health (Ireland) and with ethical approval from the local ethical review group.

Atorvastatin Treatment Regimen—Food and water intake was assessed daily for 1 week, and animals were then randomly assigned to a control treatment or atorvastatin treatment group. Atorvastatin (5 and 10 mg/kg/day in the case of rats and mice, respectively; Lipitor, Pfizer-Parke Davis, Ireland) was given orally for 3 weeks in the laboratory chow. In the first and third studies, the control- and atorvastatin-treated groups of rats and mice were subsequently subdivided into those that received saline intraperitoneally and those that received LPS (100 μg/kg *Escherichia coli* serotype 0111.B4, Sigma-Aldrich, UK) intraperitoneally on the day of the experiment. In the second study, young and aged rats were subdivided into control- and atorvastatin-treated groups, and treatment continued for 8 weeks. Rats and mice were given their daily allowance of food and monitored to ensure that they received their full daily dose of atorvastatin. Animals were weighed at intervals throughout the study to ensure that similar weight changes were occurring in the different treatment groups. At the end of the treatment period, mice were killed by cervical dislocation, the brain was rapidly removed, dissected on ice, sliced (350 × 350μ m) using a McIlwain tissue chopper, and stored in Krebs buffer containing $CaCl_2$ (1.13 mM) and 10% Me_2SO at −80 °C as previously described (1) until required for analysis. Rats were assessed for their ability to sustain LTP.

Induction of LTP in Vivo—Young rats were anesthetized by intraperitoneal injection of urethane (1.5 g/kg), and aged rats were initially given 1.2 g/kg urethane with further increments to a maximum of 2.0 g/kg when required. The absence of a pedal reflex was considered to be an indicator of deep anesthesia. In the case of the experiments in which the effect of atorvastatin was assessed on LPS-induced changes, LPS (100 μg/kg, Sigma, UK) or saline was injected intraperitoneally and 3 h later animals were assessed for their ability to sustain LTP. In some experiments rats were injected intracerebroventricularly (2.5 mm posterior, and 0.5 mm lateral, to Bregma) with IFNγ (50 ng/ml, 5 μl, Chemicon International, Inc.), IL-4 (20 ng/ml, 5 μl, R&D Systems, UK) or both, and 30 min later these rats were assessed for their ability to sustain LTP in perforant path-granule cell synapses as described previously (1). Briefly, a bipolar-stimulating electrode and a unipolar-recording electrode were stereotaxically positioned in the perforant path (4.4 mm lateral to lambda) and dorsal cell body region of the dentate gyrus (3.9 mm posterior, and 2.5 mm lateral, to Bregma), respectively. Test shocks were delivered at 30-s intervals, and recorded for 10 min before and 45 min after tetanic stimulation. The stimulation paradigm used involved delivery of 3 trains of stimuli (250 Hz for 200 ms) with an intertrain interval of 30 s, which has been shown to induce saturable LTP in perforant path-granule cell synapses in young adult rats (18); this stimulation paradigm has revealed a deficit in LTP in aged rats.

At the end of the recording period, rats were killed by decapitation, the brains were rapidly removed, and the hippocampus was dissected. One-third of the hippocampus was flash-frozen in liquid N_2 for later analysis of mRNA (see below), and slices (350 × 350 μm) were prepared from the rest of the tissue using a McIlwain tissue chopper. These slices were stored in Krebs buffer containing $CaCl_2$ (1.13 mM) and 10% Me_2SO at −80 °C as previously described (1) until required for analysis. Cortical tissue was similarly prepared and stored for later analysis of cholesterol.

Analysis of IL-1β, IFN-γ, and IL-4—The concentrations of IL-1β, IFNγ, and IL-4 were assessed by enzyme-linked immunosorbent assay (R&D Systems, UK) in the stored hippocampal slices prepared from rats (2). IFNγ and IL-1β concentrations were also assessed in tissue prepared from wild-type and IL-$4^{-/-}$ mice. Slices were thawed, rinsed, homogenized, and equalized for protein (19). For analysis of rat tissue the following antibodies were used to coat 96-well plates; 1.0 μg/ml goat anti-rat IL-1β antibody (R&D Systems, UK), or 2.0 μg/ml mouse anti-rat IFNγ antibody, or 2.0 μg/ml mouse anti-rat IL-4 antibody (diluted in phosphate-buffered saline, pH 7.3). For analysis of tissue prepared from mice, 1.25 μg/ml goat anti-mouse IFNγ antibody (BIOSOURCE, UK) or 4 μg/ml goat anti-mouse IL-1β antibody (R&D Systems, UK) were used. Plates were incubated overnight at room temperature, washed, blocked, and incubated with standards (0–1000 pg/ml) or samples for 2 h at room temperature. Wells were washed and incubated with the following antibodies: 350 ng/ml biotinylated goat anti-rat antibody for IL-1β, 150 ng/ml biotinylated goat anti-rat antibody for IFNγ, or 50 ng/ml biotinylated goat anti-rat antibody for IL-4, each diluted in phosphate-buffered saline containing 1% BSA and 2% normal goat serum, or 100 ng/ml biotinylated goat anti-mouse IL-1β antibody, and 125 ng/ml biotinylated goat anti-mouse IFNγ antibody (R&D Systems, UK). Horseradish peroxidase-conjugated streptavidin (1:200) and substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) were added, incubation continued in the dark for 20–30 min, and the reaction was stopped using 1 M H_2SO_4. Absorbance was read at 450 nm, and values were corrected for protein in the case of homogenates and expressed as picograms/mg of protein.

Analysis of Expression of MHCII—We assessed OX6 mRNA expression as an indicator of MHCII. Total RNA was extracted from hippocampal tissue using TRI reagent (Sigma), reverse transcription-PCR was undertaken (5), and cDNA synthesis

was performed on 1 μg of total RNA using oligo(dT) primers (Superscript reverse transcriptase; Invitrogen Ltd., UK). Equal amounts of cDNA were used for PCR amplification for a total of 30 cycles, and the following sequences of primers were used: upstream 5′-CAG TCA CAG AAG GCG TTT ATG-3′; downstream, 5′-TGC AGC ATC TGA CAG CAG GA-3′; and for rat β-actin mRNA expression: upstream, 5′-AGA AGA GCT ATG AGC TGC CTG AGG-3′; downstream, 5′-CTT CTG CAT CCT GTC AGC GAT GC-3′. The cycling conditions were 95 °C for 300 s, 65 °C for 60 s, and 72 °C for 120 s. The reaction was stopped by a final extension at 72 °C for 10 min. These primers generated OX-6 PCR products at 245 bp and β-actin PCR products at 250 bp. Equal volumes of PCR product from each sample were loaded onto 1.5% agarose gels, and bands were separated by application of 90 V, photographed, and quantified using densitometry. The target genes were normalized to β-actin mRNA expression (*i.e.* the housekeeping gene). No change in β-actin mRNA was observed with treatment.

Western Immunoblot Analysis of JNK, c-Jun, p38, and NFκB—Phosphorylation of JNK, c-Jun, p38, and NFκB was analyzed in samples prepared from hippocampal tissue as described for analysis of JNK phosphorylation (20); JNK phosphorylation was assessed in nuclear and cytosolic fractions and c-Jun phosphorylation, p38, and NFκB activation in nuclear fractions only. The cytosolic fraction was prepared by homogenizing hippocampal slices in lysis buffer (composition in mM: 20 HEPES, pH 7.4, 10 KCl, 1.5 $MgCl_2$, 1 EDTA, 1 EGTA, 1 dithiothreitol, 0.1 phenylmethylsulfonyl fluoride, containing pepstatin A (5 μg/ml), leupeptin (2 μg/ml), and aprotinin (2 μg/ml)), incubating for 20 min on ice, and centrifuging (15,000 × *g* for 10 min at 4 °C). The supernatant (*i.e.* cytosolic fraction) was suspended in sample buffer (Tris-HCl, 150 mM, pH 6.8; glycerol 10% v/v; SDS, 4% w/v; β-mercaptoethanol, 5% v/v; bromphenol blue, 0.002% w/v) to a final concentration of 300 μg/ml, boiled for 3 min, and loaded (10 μg/lane) onto 10% SDS gels. The nuclear fraction was prepared by homogenizing hippocampal slices in Krebs solution containing 2 mM $CaCl_2$, incubating for 15 min on ice in permeabilization buffer (composition: 70 mM KCl, 250 mM sucrose, 137 mM NaCl, 4.5 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 200 μg/ml digitonin) and centrifuging (600 × *g* for 15 min at 4 °C). The pellet (*i.e.* nuclear fraction) was resuspended in sample buffer (Tris-HCl, 150 mM, pH 6.8; glycerol 10% v/v; SDS, 4% w/v; β-mercaptoethanol, 5% v/v; bromphenol blue, 0.002% w/v) to a final concentration of 10 μg/ml, boiled for 3 min, and loaded onto 10% SDS gels. All tissue samples were equalized for protein concentration, and 10-μl aliquots (1 mg/ml) were added to sample buffer (5 μl; Tris-HCl, 0.5 mM, pH 6.8; glycerol, 10%; SDS, 10%; β-mercaptoethanol, 5%; bromphenol blue, 0.05% w/v), boiled for 5 min, and loaded onto 10% SDS gels. Proteins were separated by application of a 30-mA constant current for 25–30 min, transferred onto nitrocellulose strips (225 mA for 75 min), and immunoblotted with the appropriate antibody.

To assess JNK activity, proteins were immunoblotted with an antibody that specifically targets phosphorylated JNK (1:400 in Tris-buffered saline (TBS)-Tween (0.05% Tween-20) containing 0.1% BSA; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. Immunoreactive bands were detected using peroxidase-conjugated anti-mouse IgG (Sigma) and Super Signal chemiluminescence (Pierce). To assess phosphorylation of c-Jun, nitrocellulose membranes were blocked in TBS containing 5% BSA overnight at 4 °C and immunoblotted with a mouse monoclonal antibody (1:300 dilution in phosphate-buffered saline-Tween containing 2% nonfat dried milk, Cell Signaling) for 2 h at room temperature. In the case of p38, nitrocellulose membranes were blocked in 2% BSA in TBS overnight at 4 °C and immunoblotted with a mouse monoclonal antibody (1:200 dilution in TBS-Tween containing 0.1% BSA, Sigma) for 2 h at room temperature. Immunoreactive bands were detected using peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich) and Super Signal chemiluminescence (Pierce). In the case of NFκB, nitrocellulose membranes were blocked in 5% BSA in TBS overnight at 4 °C and immunoblotted with a rabbit monoclonal antibody (1:300 dilution in TBS-Tween containing 0.1% BSA, Cell Signaling Technology) for 2 h at room temperature. Immunoreactive bands were detected using peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich) and Super Signal chemiluminescence (Pierce). Blots were stripped (Reblot Plus, Chemicon) and reprobed with an anti-actin antibody (Santa Cruz Biotechnology); in the case of JNK, c-Jun, and NFκB, antibodies raised against the unphosphorylated form were used to confirm equal loading.

Cholesterol Analysis—NMR spectra were recorded at room temperature on a JEOL ECA600 NMR spectrometer operating at 600-Hz proton frequency. The spectra were acquired in the Fourier transformation mode with 32 K data points, using a 45° pulse width. The residual monodeuterated water signal at ~4.7 ppm was suppressed by the application of a continuous and selective secondary irradiation during the relaxation delay. Chemical shifts were referenced to the residual methanol peak at 3.31 ppm. Spectral assignments were made by reference to data already in the literature (21, 22). Cholesterol was identified and quantified by its characteristic C-18 methyl singlet at 0.68 ppm.

Statistical Analysis—Data were analyzed, as appropriate, using either Student's *t* test for independent means or a two-way analysis of variance (ANOVA) followed by post hoc Student Newman-Keuls test to determine which conditions were significantly different from each other. Data are expressed as means with standard errors and deemed statistically significant when $p < 0.05$.

RESULTS

Atorvastatin Attenuates LPS-induced Inflammatory Changes—MHCII mRNA expression and IL-1β concentration were significantly increased in hippocampal tissue prepared from LPS-injected rats, compared with controls (*, $p < 0.05$, ANOVA, Fig. 1, *a* and *b*); both changes were attenuated in hippocampal tissue prepared from atorvastatin-treated rats so that mean values were similar in tissue prepared from control-treated rats and LPS-treated rats, which received atorvastatin. Interaction of IL-1β with its receptor IL-1RI has been shown to trigger sequential activation of JNK and c-Jun (4, 5), and activation of p38 and NFκB (23), therefore expression of the phosphorylated forms of these proteins was assessed in hippocampal tissue.

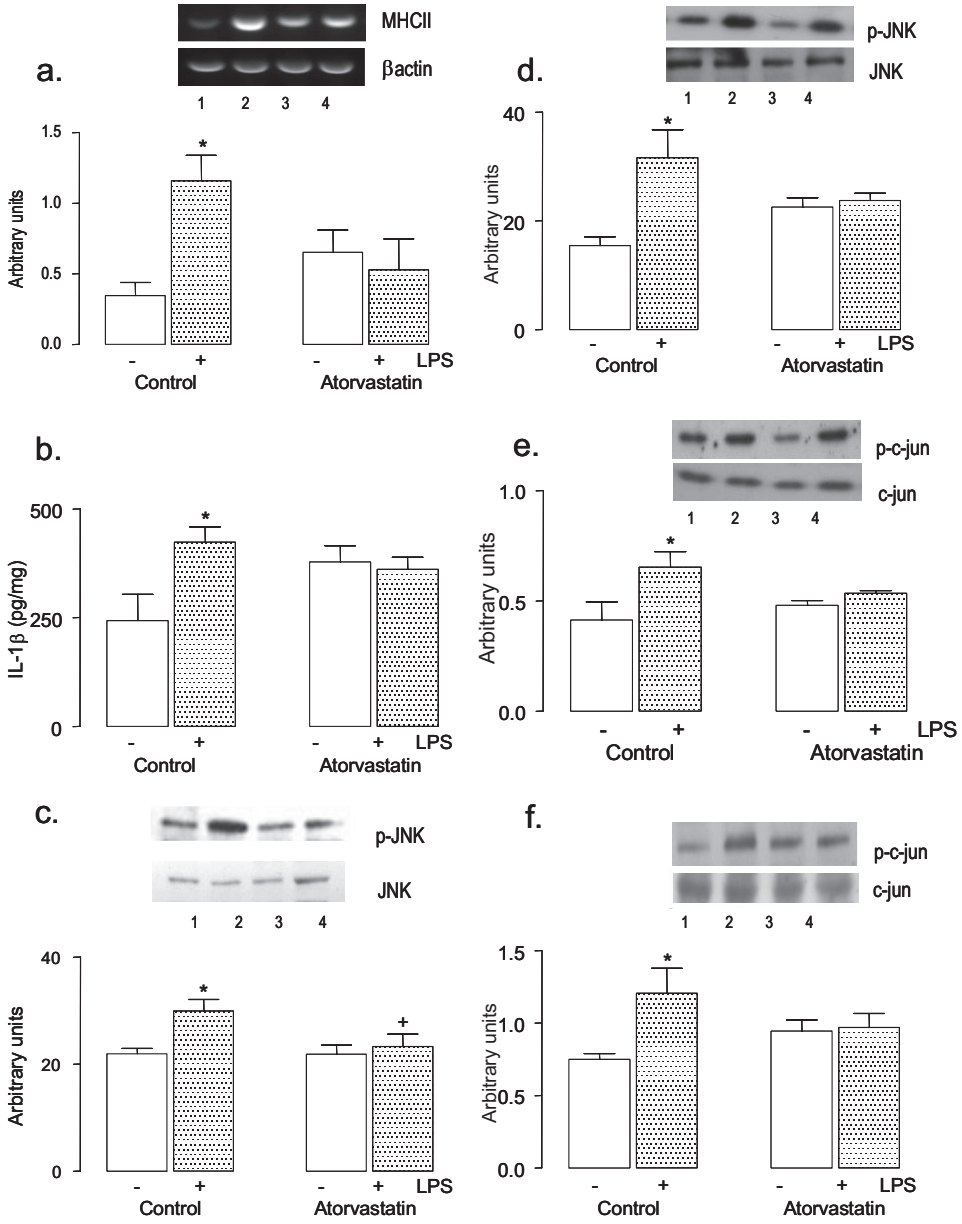

FIGURE 1. **Atorvastatin blocks the LPS-induced changes in hippocampus.** LPS injection (100 μg/kg, intraperitoneally) significantly increased expression of MHCII mRNA (*a*), IL-1β concentration (*b*), JNK activation in cytosolic (*c*), and nuclear (*d*) fractions, and phosphorylation of c-Jun on Serine-63 (*e*) and Serine-73 (*f*, *, $p < 0.05$, ANOVA). These effects were blocked in tissue prepared from animals treated orally with atorvastatin (5 mg/kg/day) for 3 weeks ($^+$, $p < 0.05$, ANOVA, *versus* LPS alone). Atorvastatin exerted no significant effect in saline-treated rats. Data in all cases are expressed as means of five or six observations (±S.E.). For *c–f*, mean arbitrary values (±S.E.) obtained from densitometric analysis are presented, and sample blots are shown (for control-treated (*lane 1*), LPS-treated (*lane 2*), atorvastatin-treated (*lane 3*), and LPS plus atorvastatin-treated (*lane 4*) rats; in all cases blots were stripped and reprobed with total JNK or total c-Jun to confirm equal loading of proteins.

Expression of phosphorylated JNK (pJNK, expressed as a ratio of pJNK to total JNK in cytosolic and nuclear fractions) and p38 (expressed in a cytosolic fraction as a ratio of phosphorylated p38 (p-p38) to actin) were significantly increased in tissue prepared from LPS-treated, compared with control-treated, rats (*, $p < 0.05$, ANOVA, Fig. 1, *c* and *d*, and Fig. 2*a*). These increases were attenuated in LPS-treated rats that received atorvastatin so that the mean values in these groups were similar to those in controls and, in the case of cytosolic pJNK, significantly reduced compared with the value in tissue prepared from LPS-treated rats ($^+$, $p < 0.05$, ANOVA). Expression of total JNK and actin was unchanged with treatment. In parallel with these changes, activation of c-Jun (expressed as a ratio of c-Jun phosphorylated on serine 63 or serine 73 to c-Jun) and NFκB (expressed as a ratio of phosphorylated NFκ B to total NFκB) in nuclear fractions obtained from the same tissue, were significantly increased in LPS-treated, compared with control, rats (*, $p < 0.05$; **, $p < 0.01$, ANOVA, Figs. 1*e*, 1*f*, and 2*b*). These increases were attenuated in LPS-treated rats that received atorvastatin so that the mean values in these groups were similar to those in controls, and, in the case of NFkB, significantly decreased compared with the value in tissue prepared from LPS-treated rats ($^{++}$, $p < 0.01$, ANOVA). Expression of unphosphorylated NFκB was unchanged with treatment.

Atorvastatin Attenuates the LPS-induced Inhibition of LTP—It has been consistently shown that, when hippocampal IL-1β concentration is increased, LTP is impaired and that inhibition of JNK (3) and p38 (23) antagonizes the LPS-induced inhibition of LTP. The present results support these observations and show that, in parallel with the increases in IL-1β concentration and JNK and p38 activation, LTP is impaired in LPS-treated animals. Thus, high frequency stimulation of the perforant path led to an immediate and sustained increase in population EPSP slope in control rats, whereas LTP was markedly attenuated in rats injected with LPS ($p < 0.001$, ANOVA, Fig. 3*a*). There was a significant decrease in the mean percentage change in population EPSP slope in the last 10 min of the experiment compared with the mean value in the 5 min prior to stimulation in the LPS-treated group (84.46 ± 0.62), compared with the controls (123.3 ± 1.16; **, $p < 0.01$, ANOVA, Fig. 3*b*). Mean population EPSP slope was significantly decreased in rats that received atorvastatin (109.6 ± 0.86; *, $p < 0.05$, ANOVA, Fig. 3*b*) compared with controls, and atorvastatin blocked the inhibitory effect of LPS on LTP so that the mean percentage change in EPSP slope in the last 10 min of the experiment was significantly greater in LPS-treated animals that received atorvastatin (137.0 ± 1.09) compared with controls (**, $p < 0.01$, ANOVA) or animals that received LPS ($^{++}$, $p < 0.01$, ANOVA).

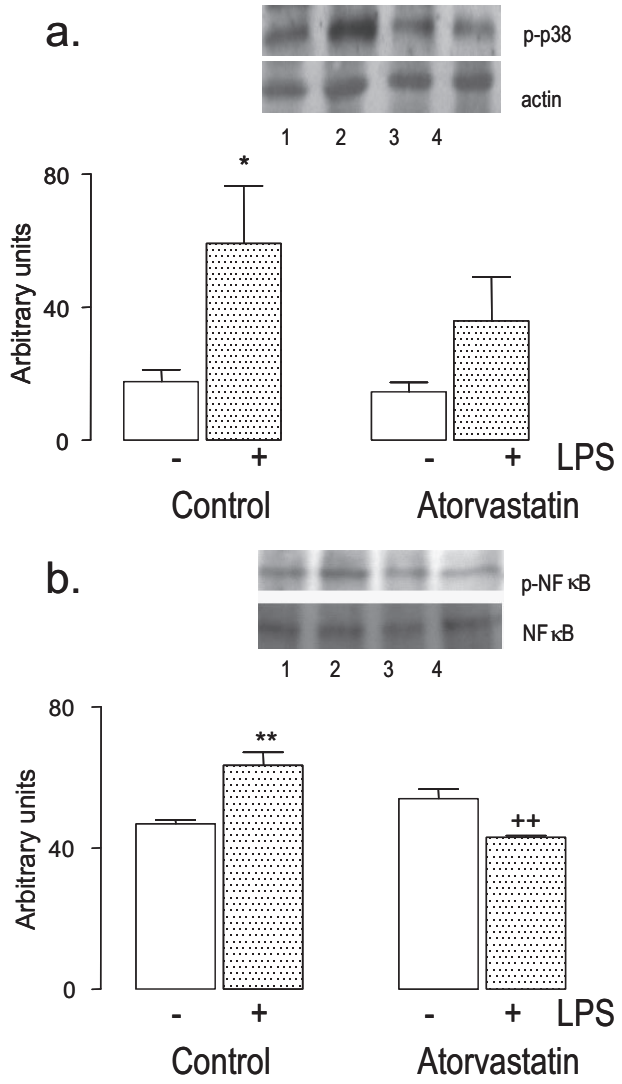

FIGURE 2. **Atorvastatin abrogates the LPS-induced activation of p38 and NFκB.** LPS injection (100 μg/kg, intraperitoneally) significantly increased activation of p38 (*a*) and NFκ B (*b*) in hippocampal tissue (* $p < 0.05$, ** $p < 0.01$, ANOVA, $n = 5$ or 6). These effects were blocked in tissue prepared from animals treated orally with atorvastatin (5 mg/kg/day) for 3 weeks ($^{++}$, $p < 0.01$, ANOVA, *versus* LPS alone). Atorvastatin exerted no significant effect in saline-treated rats. Mean arbitrary values (±S.E.) obtained from densitometric analysis are presented, and sample blots are shown; in all cases blots were stripped and reprobed with actin or total NFκB to confirm equal loading of proteins.

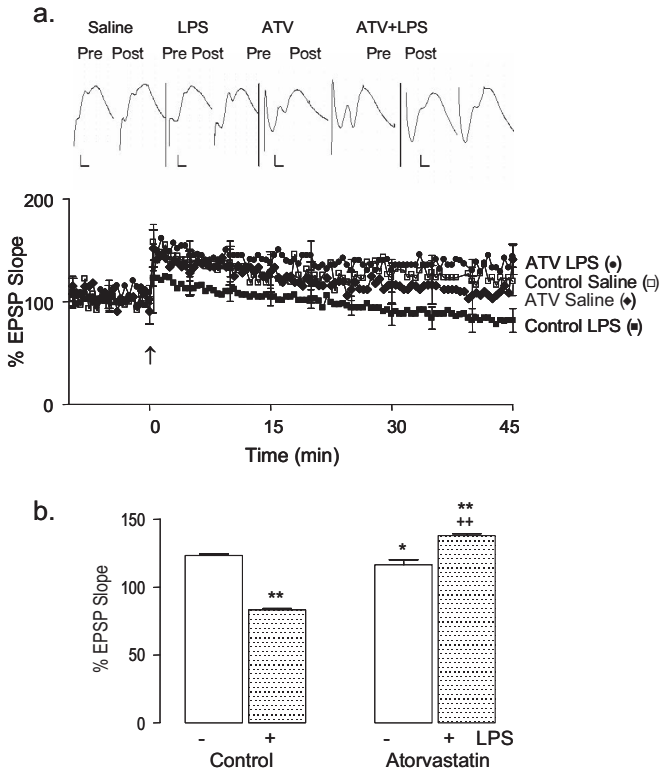

FIGURE 3. **Atorvastatin reverses the LPS-induced impairment in LTP.** *a*, intraperitoneal injection of LPS (100 μg/kg, intraperitoneally) blocked tetanus-induced LTP in perforant path-granule cell synapses, but this effect was suppressed by atorvastatin (*ATV*: 5 mg/kg/day) treatment. Data are expressed as the mean percentage change in population EPSP slope (relative to the mean population EPSP slope in the 5 min immediately prior to tetanic stimulation). Values are means and bars (denoting ± S.E.) are included at 5-min intervals. Sample recordings in the 5 min prior to tetanic stimulation (*pre*) and in the last 5 min of the experiment (*post*) are given for rats that received saline (*Saline*), LPS, ATV, or atorvastatin and LPS (*ATV+LPS*). *b*, analysis of the mean percentage changes in population EPSP slope in the last 10 min of the experiment compared with the mean value in the 5 min prior to tetanic stimulation are shown; data reveal a significant decrease in LPS-treated rats compared with control rats (**, $p < 0.01$, ANOVA); atorvastatin treatment significantly attenuated the LPS-induced effect ($^{++}$, $p < 0.01$, ANOVA, *versus* LPS alone). Mean EPSP slope was significantly reduced in saline-treated rats that received atorvastatin (*, $p < 0.05$, ANOVA, *versus* saline). The mean value in the last 10 min of the experiment was significantly reduced in atorvastatin-treated, compared with control-treated, rats (*, $p < 0.05$, ANOVA). Values are presented as means of between five and six observations (±S.E.).

Atorvastatin Also Attenuates the LPS-induced Increase in IFNγ—These data suggest that LPS-induced microglial activation may trigger the cascade of events leading to inhibition of LTP and that atorvastatin exerts its effects, because it down-regulates microglial activation or the trigger leading to activation of microglia. Because previous evidence indicated that IFNγ triggers activation of microglia *in vitro* (24) we considered that it might also trigger the response observed here *in vivo*. Mean IFNγ concentration was significantly increased in tissue prepared from LPS-treated rats (*, $p < 0.05$, ANOVA, Fig. 4*a*), and this was attenuated by atorvastatin so that IFNγ concentration was similar in tissue prepared from LPS-treated rats that received atorvastatin and in tissue prepared from control-treated rats.

IL-4 Mediates the Effects of Atorvastatin and Blocks IFNγ-induced Changes—It has been reported that atorvastatin can induce a Th2 response characterized by increased secretion of anti-inflammatory cytokines like IL-4 (15); because of this and because we have previously shown that IL-4 inhibits LPS-induced changes in hippocampus (3), we considered that the action of atorvastatin might be mediated by IL-4. We report that IL-4 concentration was significantly enhanced in hippocampal tissue prepared from atorvastatin-treated compared with control-treated rats (*, $p < 0.05$, ANOVA, Fig. 4*b*) but that IL-4 concentration was significantly decreased in tissue prepared from LPS-treated rats that did not receive atorvastatin compared with those that did ($^{+}$, $p < 0.05$, ANOVA, Fig. 4*b*). If IL-4 mediates the effects of atorvastatin, then it must be predicted that atorvastatin will not exert any effect in tissue prepared from IL-$4^{-/-}$ mice, and to check this we compared its effect on LPS-induced changes in these and wild-type mice. In

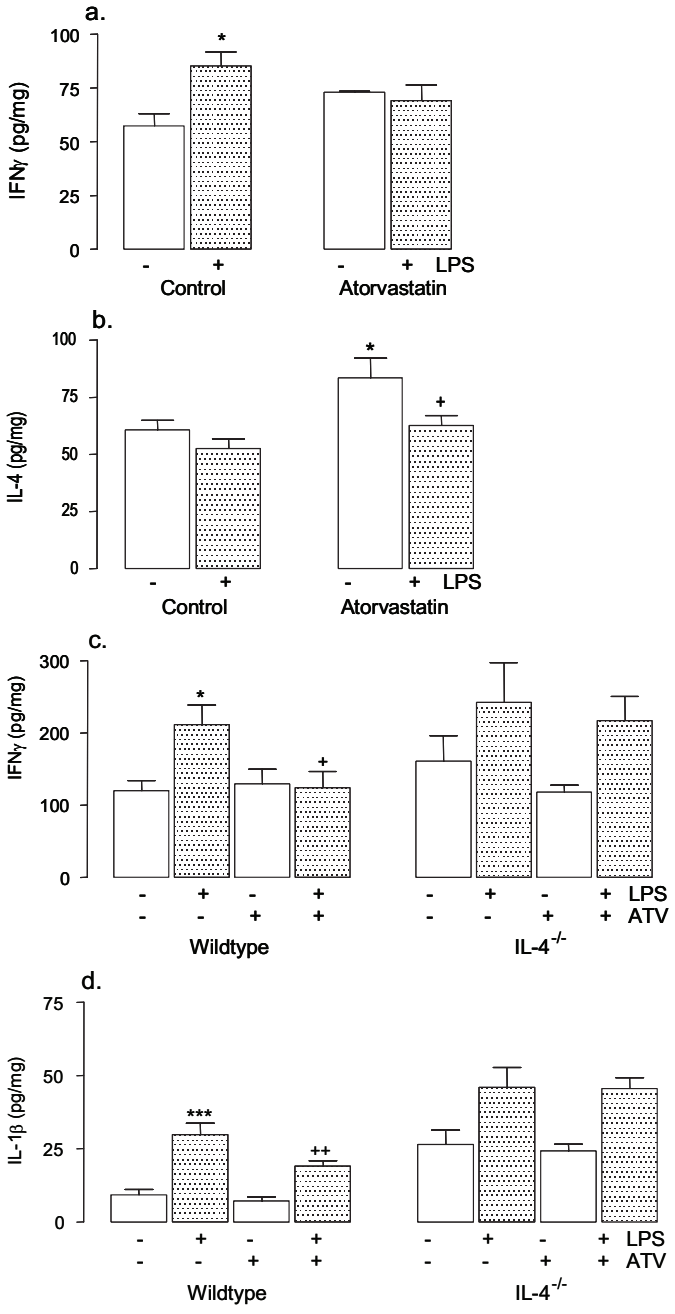

FIGURE 4. **Atorvastatin increases hippocampal IL-4 concentration and attenuates the LPS-induced changes in IFNγ hippocampal concentrations.** *a*, mean IFNγ concentration was increased in tissue prepared from LPS-treated, compared with control-treated, rats (*, $p < 0.05$, ANOVA, $n = 8/9$); this effect was attenuated in tissue prepared from atorvastatin-treated rats. *b*, mean IL-4 concentration was significantly increased with tissue prepared from atorvastatin-treated compared with control-treated, rats (*, $p < 0.05$, ANOVA); this effect was significantly reduced in tissue prepared from atorvastatin-treated animals that received LPS ($^{+}$, $p < 0.05$, ANOVA, $n = 5$). *c* and *d*, IFNγ and IL-1β concentrations were significantly increased in tissue prepared from LPS-treated wild-type mice (*, $p < 0.05$; ***, $p < 0.001$, ANOVA), and atorvastatin significantly attenuated these changes ($^{+}$, $p < 0.05$; $^{++}$, $p < 0.01$, ANOVA, *versus* LPS alone). In contrast, the effect of atorvastatin (*ATV*) was absent in tissue prepared from IL-4$^{-/-}$ mice.

parallel with the findings in rats, IFNγ and IL-1β were significantly increased in tissue prepared from LPS-treated wild-type mice (*, $p < 0.05$; ***, $p < 0.001$, ANOVA, Fig. 4, *c* and *d*), and atorvastatin significantly attenuated these changes ($^{+}$, $p < 0.05$; $^{++}$, $p < 0.01$, ANOVA), so that mean cytokine values were similar in tissue prepared from LPS-treated animals that received atorvastatin and in control-treated animals. In contrast, whereas LPS increased IFNγ and IL-1β, albeit insignificantly, in samples prepared from IL-4$^{-/-}$ mice, there was no evidence of an effect of atorvastatin in these animals.

If IL-4 mediates the effect of atorvastatin, and if the key LPS-induced action leading to degenerative change is an increase in IFNγ, then it must be predicted that IL-4, like atorvastatin, will antagonize the effects of IFNγ. Intracerebroventricular injection of IFNγ significantly increased hippocampal MHCII mRNA expression, IL-1β concentration, and JNK activation (**, $p < 0.01$, ANOVA, Fig. 5, *a–c*), and these effects were attenuated in tissue prepared from rats treated with IFNγ and IL-4 ($^{+}$, $p < 0.05$ in the case of MHCII expression and $^{++}$, $p < 0.01$ in the case of JNK activation, ANOVA). Consistent with our previous findings, the increases in microglial activation, IL-1β concentration, and JNK activation were coupled with a deficit in LTP. Thus LTP was significantly attenuated in IFNγ-treated rats (Fig. 6, *a* and *b*); the mean percentage changes in population EPSP slope in the last 10 min of the experiment (compared with the mean value in the 5 min prior to stimulation), were 129.9 ± 0.72 in control-treated rats and 86.36 ± 0.73 in IFNγ-treated animals (***, $p < 0.001$, ANOVA, Fig. 6*b*). IL-4 treatment completely abrogated the effect of IFNγ; the mean percentage change in population EPSP slope was 135.1 ± 0.81, which was not significantly different from that in control-treated rats, but was significantly greater than the value observed in rats that received IFNγ alone ($^{+++}$, $p < 0.001$, ANOVA, Fig. 6*b*).

Atorvastatin Attenuates the Age-related Inflammatory Changes and the Deficit in LTP—Previous studies from this laboratory have identified similarities in signaling events between LPS-treated rats and aged rats, in particular signaling events triggered by increased IL-1β concentration (1, 3, 4). Here we report that there were significant age-related increases in concentrations of IFNγ and IL-1β and in MHCII mRNA expression in hippocampal tissue prepared from aged, compared with young rats (*, $p < 0.05$; **, $p < 0.01$, ANOVA, Fig. 7, *a–c*) and that treatment with atorvastatin blocked these age-related changes so that the values in tissue prepared from hippocampus of aged atorvastatin-treated rats were significantly decreased compared with those in tissue prepared from aged rats that did not receive atorvastatin ($^{+}$, $p < 0.05$; $^{++}$, $p < 0.01$, ANOVA). Although atorvastatin completely reversed the IFNγ-induced increases in cytokine concentration, its effect on MHCII was partial, and, therefore, the mean value in tissue prepared from aged rats that received atorvastatin was significantly greater than in both groups of young rats (*, $p < 0.05$, ANOVA).

The data show that there was a significant age-related decrease in IL-4 concentration (*, $p < 0.05$, ANOVA, Fig. 7*d*) and that this was significantly attenuated by atorvastatin so that mean hippocampal IL-4 concentration was significantly greater in tissue prepared from aged rats that received atorvastatin compared with aged control rats ($^{+}$, $p < 0.05$, ANOVA, Fig. 7*d*). Importantly the atorvastatin-induced changes were associated with rescue of the age-related deficit in LTP; LTP was signifi-

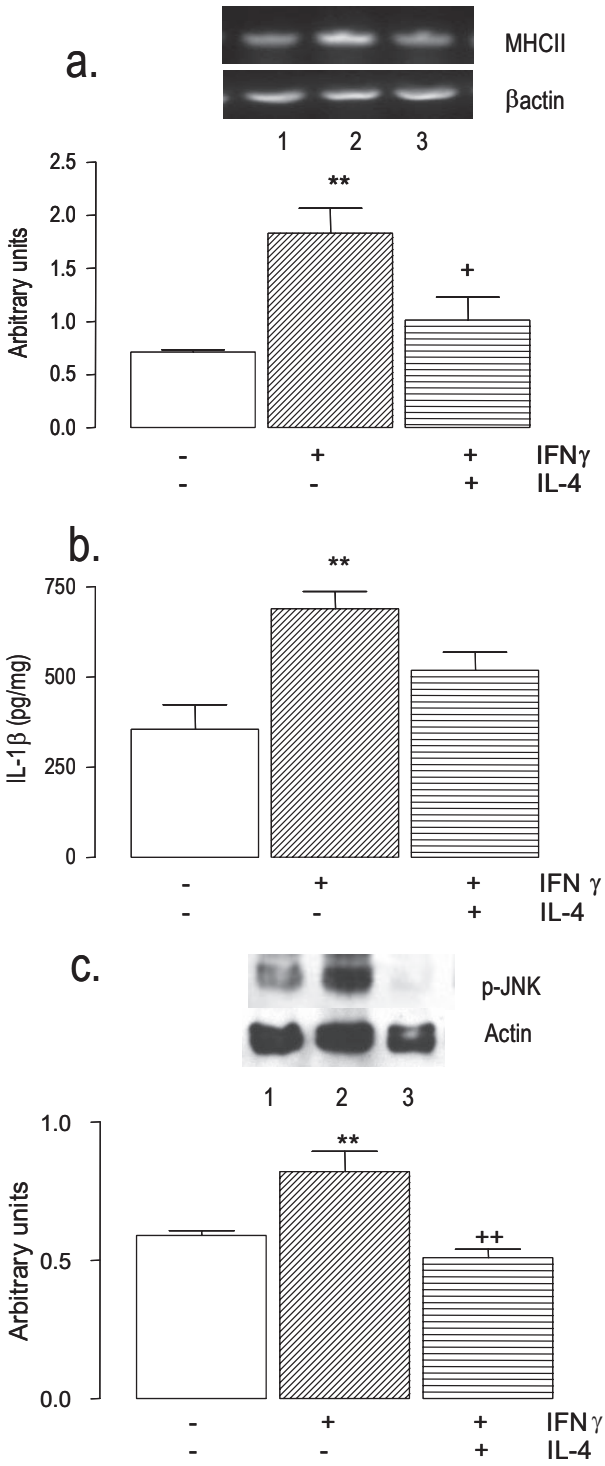

FIGURE 5. **IL-4 attenuates IFNγ-induced changes in hippocampus.** *a*, intracerebroventricular injection of IFNγ (5 μl; 50 ng/ml) significantly increased expression of MHCII mRNA (**, $p < 0.01$, ANOVA, $n = 5$), and this effect was blocked by co-injection with IL-4 (5 μl; 20 ng/ml; $^{+}$, $p < 0.05$, ANOVA). *b* and *c*, the IFNγ-induced increases in IL-1β concentration (*b*) and JNK activation (*c*) in hippocampal tissue prepared from IFNγ-treated rats were significantly greater than in saline-treated rats (**, $p < 0.01$, ANOVA), whereas the values in hippocampal tissue prepared from IFNγ and IL-4 co-injected rats were similar to those in saline-treated controls and, in the case of JNK activation, significantly decreased compared with the value observed in tissue prepared from rats treated with IFNγ alone ($^{++}$, $p < 0.01$, ANOVA). Sample blots indicating changes in phosphorylated JNK in hippocampal tissue prepared from control (*lane 1*), IFNγ- (*lane 2*), and IFNγ+IL-4- (*lane 3*)-treated rats are presented. Values are presented as means of between five and six observations (±S.E.).

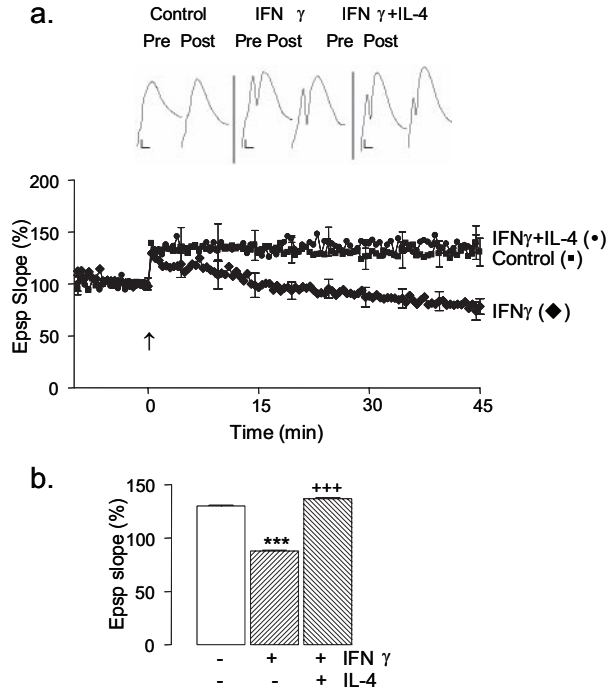

FIGURE 6. **IL-4 abrogates the IFNγ-induced inhibition of LTP.** *a*, intracerebroventricular injection of IL-4 (5 μl; 20 ng/ml) abrogated the deficit in LTP induced by IFNγ (5 μl; 50 ng/ml) so that the population EPSP slope in response to tetanic stimulation in the rats treated with IFNγ was significantly reduced compared with that in rats treated with saline, or IL-4 and IFNγ. Sample recordings in the 5 min prior to tetanic stimulation (*pre*) and in the last 5 min of the experiment (*post*) are given for control-treated, IFNγ-treated, and IFNγ+IL-4-treated rats. *b*, analysis of the mean percentage changes in population EPSP slope in the last 10 min of the experiment compared with the mean value in the 5 min prior to tetanic stimulation revealed a significant decrease in IFNγ-treated rats compared with control-treated rats (***, $p < 0.001$, ANOVA) and a significant attenuation of the IFNγ-induced change in rats treated with IFNγ and IL-4 ($^{+++}$, $p < 0.001$, ANOVA). Values are presented as means of between four and seven observations (±S.E.).

cantly decreased in aged, compared with young, rats, whereas LTP in aged rats that received atorvastatin was similar to that in young animals. The mean percentage changes in population EPSP slope in the last 10 min of the experiment (compared with the mean value in the 5 min prior to stimulation) were 118.1 ± 0.77, 94.36 ± 0.58, and 133.3 ± 0.38 in young rats, aged control-treated rats, and aged rats that received atorvastatin, respectively. These values represent a significant difference in aged control-treated rats compared with young rats (**, $p < 0.01$, ANOVA, Fig. 7*f*) and between aged control-treated and aged atorvastatin-treated rats ($^{++}$, $p < 0.01$, ANOVA, Fig. 7*f*). EPSP slope in young, atorvastatin-treated rats was also decreased (123.7 ± 0.39) compared with the value in young, control-treated rats (**, $p < 0.01$, ANOVA, Fig. 7*f*).

Atorvastatin Does Not Exert Any Effect on Cholesterol Concentration in Brain—We assessed cholesterol concentration in samples of cortical tissue prepared from control- and LPS-treated young rats that did/did not receive atorvastatin and in young and aged, control-treated, and atorvastatin-treated, rats. Cholesterol was similar in control-treated and LPS-treated rats (37.16 ± 0.3 and 36.37 ± 0.67 mol%), and atorvastatin exerted no significant effect on cholesterol (37.16 ± 0.2 and 36.57 ± 0.41 in control-treated and LPS-treated rats, respectively). In contrast cholesterol was significantly increased in tissue pre-

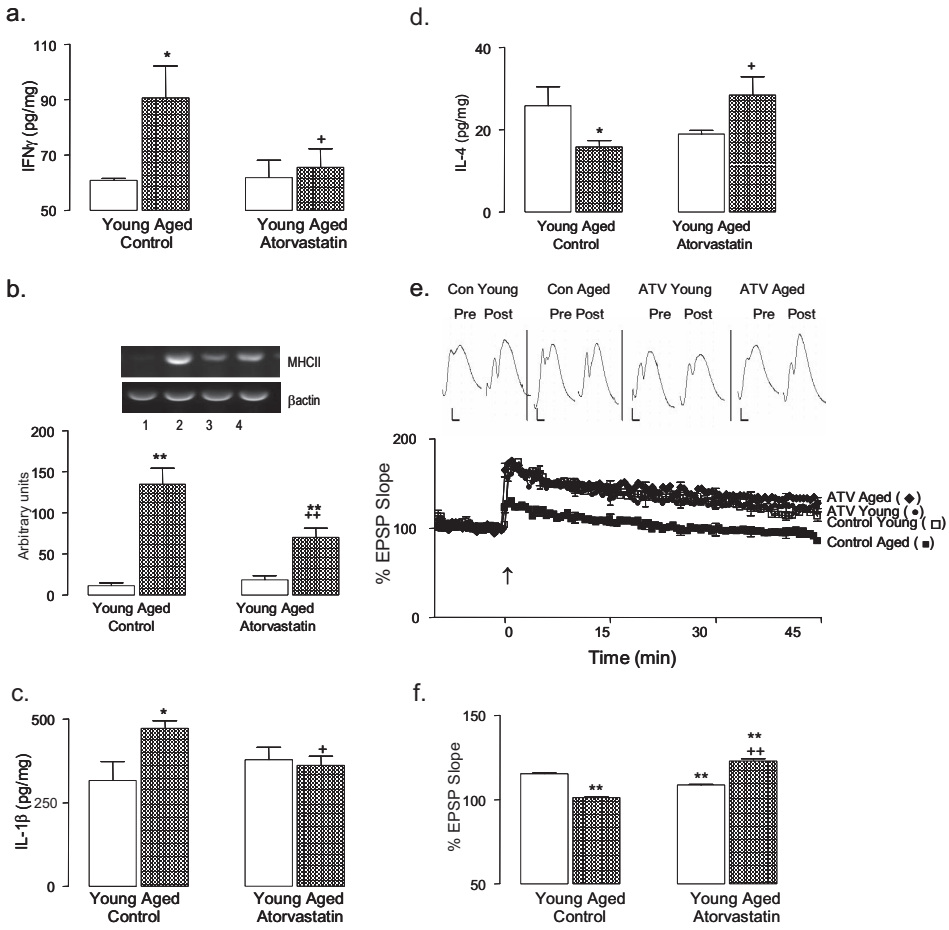

FIGURE 7. **Atorvastatin abrogates age-related changes in the rat hippocampus.** Mean IFNγ concentration (*a*), MHCII expression (*b*), and IL-1β concentration (*c*) were significantly increased and mean IL-4 concentration (*d*) was significantly decreased in hippocampal tissue prepared from aged, compared with young, rats (*, $p < 0.05$, **, $p < 0.01$, ANOVA). Atorvastatin treatment (*ATV*; 5 mg/kg/day) for 8 weeks abrogated these changes ($^{+}$, $p < 0.05$; $^{++}$, $p < 0.01$, ANOVA, *versus* LPS alone). *e*, a marked deficit in LTP was observed in aged, compared with young, rats, and treatment with ATV attenuated the age-related change. Sample recordings in the 5 min prior to tetanic stimulation (*pre*) and in the last 5 min of the experiment (*post*) are given for control-treated young and aged rats (*Con young* and *Con aged*, respectively), and atorvastatin-treated young and aged rats (*ATV young* and *ATV aged*, respectively). *f*, the mean percentage change in population EPSP slope in the last 10 min of the experiment compared with the mean value in the 5 min prior to tetanic stimulation revealed a significant age-related change (**, $p < 0.01$, ANOVA), which was significantly reversed by atorvastatin ($^{++}$, $p < 0.01$, ANOVA). Mean EPSP slope was significantly reduced in rats that received atorvastatin (*, $p < 0.05$, ANOVA, *versus* saline). Data are expressed as means of five or six observations (±S.E.).

pared from aged (39.97 ± 0.34), compared with young (37.89 ± 0.41), rats ($p < 0.01$, ANOVA), but there was no evidence of any effect of atorvastatin treatment in either age group (38.19 ± 0.20 and 40.48 ± 0.43 in young and aged atorvastatin-treated rats, respectively).

DISCUSSION

We set out to establish whether atorvastatin, which is known to possess anti-inflammatory properties, might modulate the neuro-inflammatory changes induced by LPS and age in the rat hippocampus that lead to impairment of LTP. We report that microglial activation, IFNγ, and IL-1β were increased in hippocampal tissue prepared from aged and LPS-treated rats and that these changes were accompanied by inhibition of LTP. We hypothesize that the primary action of atorvastatin is to increase IL-4 and that this prevents the IFNγ-induced microglial activation, which triggers the cascade of events. This hypothesis is supported by the finding that atorvastatin fails to attenuate the LPS-induced increases in IFNγ and IL-1β in tissue prepared from IL-$4^{-/-}$ mice.

Our data describe an age-related, as well as an LPS-induced, increase in IL-1β concentration in the hippocampus paralleled by increased microglial activation, which is consistent with the view that these cells are the likely source of IL-1β (6, 25, 26). We considered that IFNγ might trigger the activation of microglia in the hippocampus of LPS-treated and aged rats, because it a potent activator of microglia *in vitro* (24, 27). We found that hippocampal concentration of IFNγ was increased in parallel with the increase in microglial activation in the hippocampal tissue prepared from LPS-treated and aged rats, whereas these changes were also observed following intracerebroventricular injection of IFNγ. Recent evidence from studies in aged rats has indicated that the impairment in LTP is associated, not only with increased IL-1β, but with an array of changes in pro-inflammatory and anti-inflammatory cytokines, (26, 27); specifically, an inverse correlation between hippocampal concentrations of IL-1β and IL-4 has been identified as being critical in determining the ability of aged rats to sustain LTP (28). Significantly, we have previously reported that increasing hippocampal IL-4 concentration reverses the age-related and LPS-induced deficits in LTP and that intracerebroventricular injection of IL-4 partly attenuates the age-related deficit in LTP (6, 29).

We addressed the possible role of IL-4 here by asking whether the atorvastatin-induced modulation of IL-1β was coupled with a change in IL-4, and our data show that IL-4 concentration was indeed increased in atorvastatin-treated rats that received LPS and that atorvastatin treatment was associated with a reversal of the age-related decrease in IL-4. We demonstrate that IL-4 attenuates the IFNγ-triggered increases in MHCII expression and IL-1β concentration and, in parallel, antagonizes the IFNγ-induced inhibition of LTP. This antagonistic effect of IL-4 on IFNγ-induced changes indicates that the atorvastatin-induced increase in IL-4 inhibits the cascade of events triggered by the age- and LPS-induced increase in the hippocampal concentration of IFNγ and the subsequent microglial activation. Moreover it is in agreement with the previous

compelling evidence indicating that IL-4 potently blocks the effects of IFNγ, including the IFNγ-induced activation of CD40 in a microglial cell line (24).

If the action of atorvastatin is mediated through IL-4, then it follows that atorvastatin will not have the same anti-inflammatory effects in IL-$4^{-/-}$ mice, and therefore we compared its ability to attenuate the LPS-induced changes in wild-type and IL-$4^{-/-}$ mice. Intraperitoneal injection of LPS increased both IFNγ and IL-1β in brain tissue prepared from wild-type mice, and these effects were attenuated in the atorvastatin-treated mice. In contrast, atorvastatin exerted no effect on LPS-induced changes in tissue prepared from IL-$4^{-/-}$ mice, demonstrating that the effect of atorvastatin is IL-4-dependent. Consistent with these data, Youssef and colleagues (15) suggested that the beneficial effects of atorvastatin in attenuating the detrimental changes associated with experimental autoimmune encephalomyelitis were achieved by up-regulating release of Th2-derived cytokines, which include IL-4, from splenocytes.

Although the data presented here suggest that the atorvastatin-induced increase in IL-4 is the key factor in decreasing microglial activation, there is a lack of concordance in the literature regarding the effect of statins on microglial activation. On the one hand certain statins have been reported to induce inflammation, for instance lovastatin has been shown to increase IL-1β concentration in brain tissue of transgenic mice, which overexpress human amyloid precursor protein (30), and TNFα concentration in cultured hippocampal slices (13). Similarly, cerivastatin and fluvastatin have been reported to up-regulate IL-1β- and IFNγ-induced changes in smooth muscle (31, 32) and hepatoma cells (32). In contrast, simvastatin and lovastatin block the Aβ-induced increase in IL-1β production in monocytes and BV2 cells (33) and the IFNγ-induced increase in release of TNFα, IL-1β and IL-6 in cultured microglia (34). We have recently reported a beneficial effect of atorvastatin treatment *in vivo* against Aβ-induced increases in microglial activation and IL-1β concentration (35). This anti-inflammatory role is supported by a large body of evidence indicating that certain statins are protective in ischemia (36–39) and experimental autoimmune encephalomyelitis (see Ref. 40) in which the degenerative effects are attributed, at least in part, to inflammation and microglial activation.

We demonstrate that the effects of LPS and atorvastatin on microglial activation and IL-1β concentration were mirrored by changes in activation of JNK and c-Jun and in activation of p38 and NFκB. The importance of these signaling cascades in modulating LTP has been highlighted by the demonstration that the LPS-induced inhibition of LTP was blocked by inhibitors of JNK, p38, and NFκB (3, 23). To our knowledge there are no data showing that statins can attenuate LPS-induced and/or IL-1β-mediated signaling cascades in brain *in vivo* and few data documenting a modulatory effect *in vitro* (41). In contrast, several studies have revealed that statins block stimulus-induced activation of JNK and/or c-Jun in myocytes (42), Chinese hamster ovary cells (43), and macrophages (44), whereas a similar antagonistic effect on stimulus-induced activation of p38 and NFκB in monocytes (45), vascular endothelial cells (46), and macrophages (47, 48) has been reported. A significant finding of this study is that atorvastatin treatment abrogates the inflammatory and signaling changes in rat hippocampus which we believe are mediated by increased IL-1β concentration. The evidence suggests that a key action relies on its ability to modulate the IFNγ-induced microglial activation in LPS-treated and in aged rats. One downstream consequence of this is that atorvastatin restores LTP in both groups of rats. Results from epidemiological studies indicate a beneficial effect of statin therapy on cognition (49–53), which is consistent with the present data. However, it has also been suggested that statins may exert a negative effect on cognitive function (54), although a recent report indicated that atorvastatin (10 mg/day) had no such effect (55). We have previously reported a small but statistically significant atorvastatin-induced decrease in LTP in young rats following a 3-week treatment period (35), and the data presented here concur with this, but treatment for 8 weeks did not modulate LTP.

One of the key issues raised regarding the potential effects of statins in the central nervous system relates to their ability to cross the blood-brain barrier (56). Some researchers have asserted that atorvastatin is lipophilic (57, 58), whereas others suggest it is not (59). We detected small amounts of atorvastatin (~30 pg) in the brains of atorvastatin-treated animals (data not shown), which suggests that it can enter the brain, but whether this is a result of blood-brain barrier breakdown following LPS challenge, facilitated transport via atorvastatin transporters, or simple diffusion remains to be determined. Whether a statin that can cross the blood-brain barrier is advantageous or not is a question that remains, and it must be concluded that the effect of statins is dependent on the specific statin, the tissue under investigation, and the stimulus upon which the modulatory effects are analyzed.

Importantly, although we observed an age-related increase in cholesterol concentration in brain tissue (though LPS exerted no effect), atorvastatin failed to modulate brain cholesterol concentration. We conclude that the effects of atorvastatin described here are unrelated to its well described cholesterol-lowering action. The data suggest that its beneficial effects derive from its ability to maintain microglia in a quiescent state, and in particular, from its ability to increase IL-4.

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